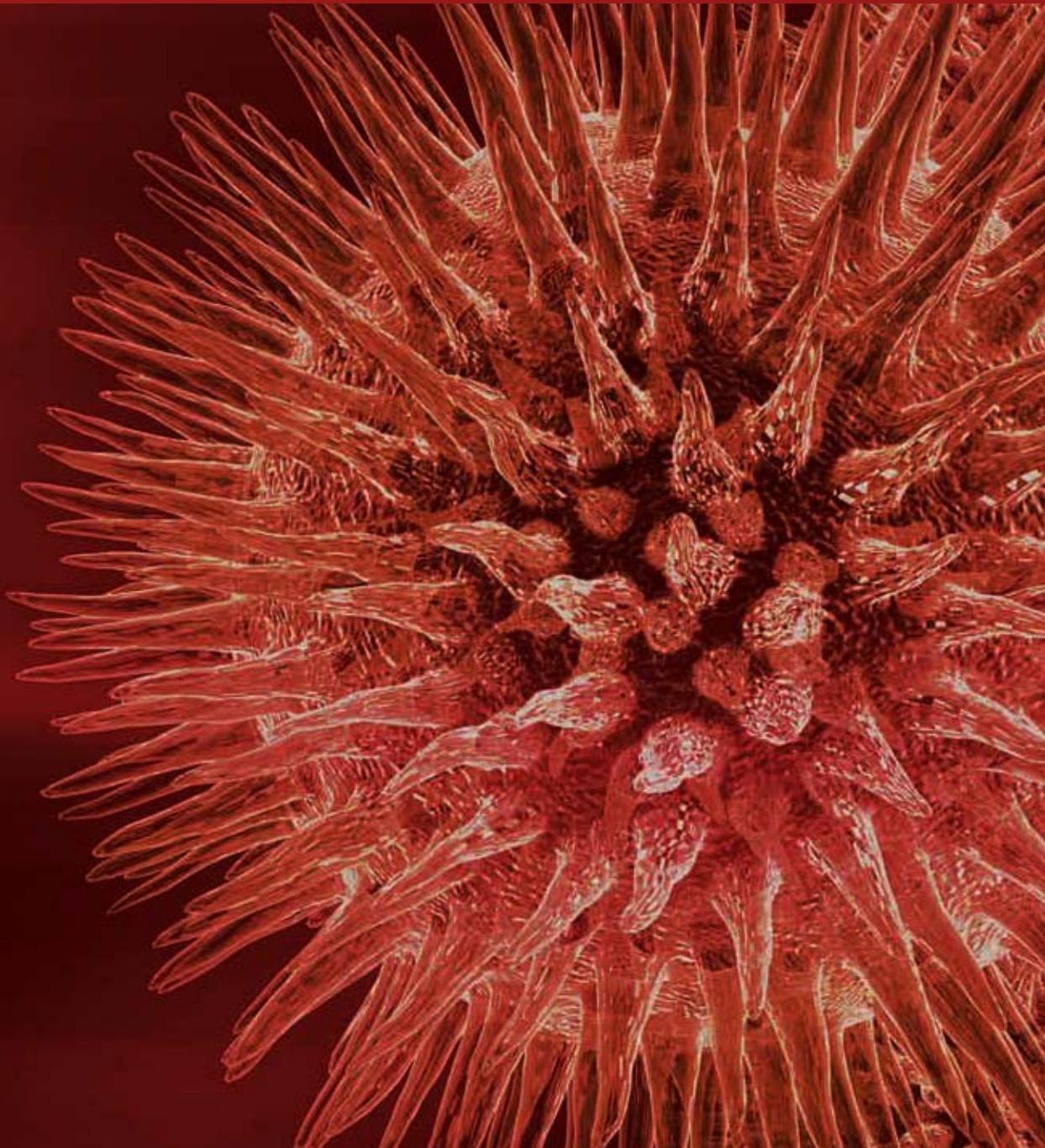


Immunology and Cell Biology of Parasitic Diseases

Guest Editors: Luis I. Terrazas, Abhay R. Satoskar,
and Jorge Morales-Montor





Immunology and Cell Biology of Parasitic Diseases

Journal of Biomedicine and Biotechnology

**Immunology and Cell Biology of
Parasitic Diseases**

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Contents

Immunology and Cell Biology of Parasitic Diseases, Luis I. Terrazas, Abhay R. Satoskar, and Jorge Morales-Montor
Volume 2010, Article ID 419849, 5 pages

The Role of Lipopeptidophosphoglycan in the Immune Response to *Entamoeba histolytica*, Isabel Wong-Baeza, Marcela Alcántara-Hernández, Ismael Mancilla-Herrera, Itzmel Ramírez-Saldivar, Lourdes Arriaga-Pizano, Eduardo Ferat-Osorio, Constantino López-Macías, and Armando Isibasi
Volume 2010, Article ID 254521, 12 pages

Detection of the Endosomal Sorting Complex Required for Transport in *Entamoeba histolytica* and Characterization of the EhVps4 Protein, Israel López-Reyes, Guillermina García-Rivera, Cecilia Banuelos, Silvia Herranz, Olivier Vincent, César López-Camarillo, Laurence A. Marchat, and Esther Orozco
Volume 2010, Article ID 890674, 15 pages

Toll-Like Receptor Initiated Host Defense against *Toxoplasma gondii*, Eric Y. Denkers
Volume 2010, Article ID 737125, 7 pages

Reduction of Foxp3⁺ Cells by Depletion with the PC61 mAb Induces Mortality in Resistant BALB/c Mice Infected with *Toxoplasma gondii*, Eda Patricia Tenorio, Jonadab Efraín Olgún, Jacqueline Fernández, Pablo Vieyra, and Rafael Saavedra
Volume 2010, Article ID 786078, 9 pages

The Unexpected Role for the Aryl Hydrocarbon Receptor on Susceptibility to Experimental Toxoplasmosis, Yuriko Sanchez, Juan de Dios Rosado, Libia Vega, Guillermo Elizondo, Elizabeth Estrada-Muñiz, Rafael Saavedra, Imelda Juárez, and Miriam Rodríguez-Sosa
Volume 2010, Article ID 505694, 15 pages

The Prominent Role of Neutrophils during the Initial Phase of Infection by *Leishmania Parasites*, Mélanie Charmoy, Floriane Auderset, Cindy Allenbach, and Fabienne Tacchini-Cottier
Volume 2010, Article ID 719361, 8 pages

Cytokines and Their STATs in Cutaneous and Visceral Leishmaniasis, Hannah E. Cummings, Rashmi Tuladhar, and Abhay R. Satoskar
Volume 2010, Article ID 294389, 6 pages

***Leishmania* Interferes with Host Cell Signaling to Devise a Survival Strategy**, Suvercha Bhardwaj, Neetu Srivastava, Raki Sudan, and Bhaskar Saha
Volume 2010, Article ID 109189, 13 pages

BALB/c Mice Vaccinated with *Leishmania major* Ribosomal Proteins Extracts Combined with CpG Oligodeoxynucleotides Become Resistant to Disease Caused by a Secondary Parasite Challenge, Laura Ramírez, Salvador Iborra, Jimena Corts, Pedro Bonay, Carlos Alonso, Manoel Barral-Netto, and Manuel Soto
Volume 2010, Article ID 181690, 9 pages

Drug Resistance in Visceral Leishmaniasis, Helena C. Maltezou
Volume 2010, Article ID 617521, 8 pages

A Multifactorial Mechanism in the Superior Antimalarial Activity of α -C-GalCer, John Schmiege, Guangli Yang, Richard W. Franck, and Moriya Tsuji
Volume 2010, Article ID 283612, 11 pages

The Coming-Out of Malaria Gametocytes, Andrea Kuehn and Gabriele Pradel
Volume 2010, Article ID 976827, 11 pages

Telomeric Heterochromatin in *Plasmodium falciparum*, Rosaura Hernandez-Rivas, Karla Pérez-Toledo, Abril-Marcela Herrera Solorio, Dulce María Delgadillo, and Miguel Vargas
Volume 2010, Article ID 290501, 11 pages

The Lipid Moiety of Haemozoin (Malaria Pigment) and *P. falciparum* Parasitised Red Blood Cells Bind Synthetic and Native Endothelin-1, Nicoletta Basilico, Silvia Parapini, Francesca Sisto, Fausta Omodeo-Salè, Paolo Coghi, Fernando Ravagnani, Piero Olliaro, and Donatella Taramelli
Volume 2010, Article ID 854927, 9 pages

Tomatine Adjuvantation of Protective Immunity to a Major Pre-erythrocytic Vaccine Candidate of Malaria is Mediated via CD8⁺ T Cell Release of IFN- γ , Karen G. Heal and Andrew W. Taylor-Robinson
Volume 2010, Article ID 834326, 7 pages

Pretreatment with Cry1Ac Protoxin Modulates the Immune Response, and Increases the Survival of *Plasmodium*-Infected CBA/Ca Mice, Martha Legorreta-Herrera, Rodrigo Oviedo Meza, and Leticia Moreno-Fierros
Volume 2010, Article ID 198921, 11 pages

Excreted/Secreted Proteins from Trypanosome Procyclic Strains, Celestine Michelle Atyame Nten, Nicolas Sommerer, Valerie Rofidal, Christophe Hirtz, Michel Rossignol, Gerard Cuny, Jean-Benoit Peltier, and Anne Geiger
Volume 2010, Article ID 212817, 8 pages

An Experimental Approach for the Identification of Conserved Secreted Proteins in Trypanosomatids, Rosa M. Corrales, Françoise Mathieu-Daudé, Déborah Garcia, Simone F. Brenière, and Denis Sereno
Volume 2010, Article ID 752698, 13 pages

Characterization of Major Surface Protease Homologues of *Trypanosoma congolense*, Veronica Marcoux, Guojian Wei, Henry Tabel, and Harold J. Bull
Volume 2010, Article ID 418157, 14 pages

Gene Expression in Trypanosomatid Parasites, Santiago Martínez-Calvillo, Juan C. Vizuet-de-Rueda, Luis E. Florencio-Martínez, Rebeca G. Manning-Cela, and Elisa E. Figueroa-Angulo
Volume 2010, Article ID 525241, 15 pages

Regulation of Gene Expression in Protozoa Parasites, Consuelo Gomez, M. Esther Ramirez, Mercedes Calixto-Galvez, Olivia Medel, and Mario A. Rodríguez
Volume 2010, Article ID 726045, 24 pages

Improved Method for In Vitro Secondary Amastigogenesis of *Trypanosoma cruzi*: Morphometrical and Molecular Analysis of Intermediate Developmental Forms, L. A. Hernández-Osorio, C. Márquez-Dueñas, L. E. Florencio-Martínez, G. Ballesteros-Rodea, S. Martínez-Calvillo, and R. G. Manning-Cela
Volume 2010, Article ID 283842, 10 pages

Immunobiology of African Trypanosomes: Need of Alternative Interventions, Toya Nath Baral
Volume 2010, Article ID 389153, 24 pages

Mexican *Trypanosoma cruzi* TCI Strains with Different Degrees of Virulence Induce Diverse Humoral and Cellular Immune Responses in a Murine Experimental Infection Model, B. Espinoza, T. Rico, S. Sosa, E. Oaxaca, A. Vizcaino-Castillo, M. L. Caballero, and I. Martínez
Volume 2010, Article ID 890672, 10 pages

Cysteine-Free Proteins in the Immunobiology of Arthropod-Borne Diseases, J. Santiago Mejia, Erik N. Arthun, and Richard G. Titus
Volume 2010, Article ID 171537, 10 pages

Differential Midgut Attachment of *Leishmania (Viannia) braziliensis* in the Sand Flies *Lutzomyia (Nyssomyia) whitmani* and *Lutzomyia (Nyssomyia) intermedia*, Rodrigo P. Soares, Carina Margonari, Nágila C. Secundino, Maria E. Macêdo, Simone M. da Costa, Elizabeth F. Rangel, Paulo F. Pimenta, and Salvatore J. Turco
Volume 2010, Article ID 439174, 7 pages

The Vectorial Potential of *Lutzomyia (Nyssomyia) intermedia* and *Lutzomyia (N.) whitmani* in the Transmission of *Leishmania (V.) braziliensis* Can Also Be Related to Proteins Attaching, Carlos R. Alves, Luzia M. C. Côrtes, and Reginaldo P. Brazil
Volume 2010, Article ID 827851, 2 pages

***Echinococcus multilocularis* and Its Intermediate Host: A Model of Parasite-Host Interplay,** Dominique Angèle Vuitton and Bruno Gottstein
Volume 2010, Article ID 923193, 14 pages

Release of Glycoprotein (GP1) from the Tegumental Surface of *Taenia solium* by Phospholipase C from *Clostridium perfringens* Suggests a Novel Protein-Anchor to Membranes, Abraham Landa, Kaethe Willms, and Juan Pedro Laclette
Volume 2010, Article ID 910375, 6 pages

Progesterone Induces Scolex Evagination of the Human Parasite *Taenia solium*: Evolutionary Implications to the Host-Parasite Relationship, Galileo Escobedo, Ignacio Camacho-Arroyo, Olivia Tania Hernández-Hernández, Pedro Ostoa-Saloma, Martín García-Varela, and Jorge Morales-Montor
Volume 2010, Article ID 591079, 10 pages

Budding of *Taenia crassiceps* Cysticerci In Vitro Is Promoted by Crowding in Addition to Hormonal, Stress, and Energy-Related Signals, Pedro Ostoa-Saloma, Pedro Ostoa-Jacobo, Marcela Esquivel-Velázquez, Silvana Bazúa, and Carlos Larralde
Volume 2010, Article ID 510978, 5 pages

A New MAP Kinase Protein Involved in Estradiol-Stimulated Reproduction of the Helminth Parasite *Taenia crassiceps*, Galileo Escobedo, Gloria Soldevila, Guadalupe Ortega-Pierres, Jesús Ramsés Chávez-Ríos, Karen Nava, Rocío Fonseca-Liñán, Lorena López-Griego, Claudia Hallal-Calleros, Pedro Ostoa-Saloma, and Jorge Morales-Montor
Volume 2010, Article ID 747121, 11 pages

Substance P Signaling Contributes to Granuloma Formation in *Taenia crassiceps* Infection, a Murine Model of Cysticercosis, Armandina Garza, David J. Twardy, Joel Weinstock, Balaji Viswanathan, and Prema Robinson
Volume 2010, Article ID 597086, 6 pages

Activation-Induced T Helper Cell Death Contributes to Th1/Th2 Polarization following Murine *Schistosoma japonicum* Infection, Xinyu Xu, Xiaoyun Wen, Ying Chi, Lei He, Sha Zhou, Xuefeng Wang, Jiaqing Zhao, Feng Liu, and Chuan Su
Volume 2010, Article ID 202397, 12 pages

Anti-Inflammatory Protein of *Schistosoma japonicum* Directs the Differentiation of the WEHI-3B JCS Cells and Mouse Bone Marrow Cells to Macrophages, Shaomin Hu, Linlin Yang, Zhongdao Wu, Nai Ki Mak, Kwok Nam Leung, and Ming Chiu Fung
Volume 2010, Article ID 867368, 13 pages

Immunity against Helminths: Interactions with the Host and the Intercurrent Infections, Emmanuelle Moreau and Alain Chauvin
Volume 2010, Article ID 428593, 9 pages

Are the Immunocompetence and the Presence of Metazoan Parasites in Cyprinid Fish Affected by Reproductive Efforts of Cyprinid Fish?, Karolína Rohlenová and Andrea Šimková
Volume 2010, Article ID 418382, 14 pages

Immunological and Therapeutic Strategies against Salmonid Cryptobiosis, Patrick T. K. Woo
Volume 2010, Article ID 341783, 9 pages

Dendritic Cells in the Gut: Interaction with Intestinal Helminths, Fela Mendlovic and Ana Flisser
Volume 2010, Article ID 250563, 10 pages

Modulation of Dendritic Cell Responses by Parasites: A Common Strategy to Survive, César A. Terrazas, Luis I. Terrazas, and Lorena Gómez-García
Volume 2010, Article ID 357106, 19 pages

Similarity and Diversity in Macrophage Activation by Nematodes, Trematodes, and Cestodes, Stephen J. Jenkins and Judith E. Allen
Volume 2010, Article ID 262609, 14 pages

Nitric Oxide and Respiratory Helminthic Diseases, Antonio Muro and José-Luis Pérez-Arellano
Volume 2010, Article ID 958108, 8 pages

Peroxisome Proliferator-Activated Receptor (PPAR): Balance for Survival in Parasitic Infections, Marion M. Chan, Kyle W. Evans, Andrea R. Moore, and Dunne Fong
Volume 2010, Article ID 828951, 9 pages

Arginase in Parasitic Infections: Macrophage Activation, Immunosuppression, and Intracellular Signals, Cinthia C. Stempin, Laura R. Dulgerian, Vanina V. Garrido, and Fabio M. Cerban
Volume 2010, Article ID 683485, 10 pages

Parasitic Helminths: New Weapons against Immunological Disorders, Yoshio Osada and Tamotsu Kanazawa
Volume 2010, Article ID 743758, 9 pages

Infection with *Hymenolepis diminuta* Is More Effective than Daily Corticosteroids in Blocking Chemically Induced Colitis in Mice, Alexandra Melon, Arthur Wang, Van Phan, and Derek M. McKay
Volume 2010, Article ID 384523, 7 pages

***Taenia crassiceps* Infection Attenuates Multiple Low-Dose Streptozotocin-Induced Diabetes,** Arlett Espinoza-Jiménez, Irma Rivera-Montoya, Roberto Cárdenas-Arreola, Liborio Morán, and Luis I. Terrazas
Volume 2010, Article ID 850541, 11 pages

Immune Modulation by *Schistosoma mansoni* Antigens in NOD Mice: Effects on Both Innate and Adaptive Immune Systems, Paola Zaccone, Oliver T. Burton, Sarah Gibbs, Nigel Miller, Frances M. Jones, David W. Dunne, and Anne Cooke
Volume 2010, Article ID 795210, 11 pages

Editorial

Immunology and Cell Biology of Parasitic Diseases

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This special issue of Journal of Biomedicine and Biotechnology focuses on research in immunology and cell biology of parasitic diseases carried out worldwide. In this issue, you will find contributions by world leaders and also have the opportunity to read some of the hottest topics in immunoparasitology and cell biology of parasites, with emphasis on parasites that are responsible for significant morbidity and mortality in the world. The question is: why this special issue was devoted to present these works? There are three main reasons for this; the first is related to the fact that this research field has made an impressive progress during the past decade, supported by numbers showing that the proportion of the world's scientific publications increased almost 100% in the past two decades. The number of Ph.D. scientists awarded in the world, and particularly in underdeveloped countries, has also risen significantly in recent years, particularly in the field of parasitology. Notably, albeit at different rates, a growing number of scientists are dedicated to this field in developing as well as developed countries. Indeed, in underdeveloped countries, general efforts have been made to promote the development of this science during the last decade, mainly due to the fact that parasites are indeed a significant health problem. The second reason is that a quarter of world population is at risk or exposed to different parasites, that endanger and compromise health of these individuals. Particularly, underdeveloped countries are places where ecology, climate, economic, and social status allow establishment of a wide variety of parasitic diseases. Moreover, globalization with

increased migration between developed and underdeveloped countries favor the spread of many parasitic diseases worldwide. In addition to the migration, our unquestionable global climate change is also contributing to emergence of parasitic diseases in previously nonendemic areas. Thus, a great number of scientists and institutions in the world are really interested and involved in parasitology research. The third reason is that we have passed the first decade of the 21st century without development of a confident vaccine against any parasitic disease that affects humans, even though great efforts have been made for many institutions. This fact propels us to do more in-depth research in order to know closer our enemies and, in some moment, reach the adequate solution for people that are suffering some of such infections and of course prevent the infection of our next generations, our children. However, nothing of these will be possible without research, and of course, without funding to develop more in-depth knowledge that can be applied to find new drugs or vaccines.

Here, we selected a series of papers including reviews as well as original research on different topics of distinct important parasitic diseases affecting the world; they range since basic biology, genetics, and pathology until vaccine development as well as immune response to protozoa and helminths. Finally, it is important to say that approximately 35% of the papers received for this special issue were rejected in order to keep the quality of JBB; it is also noteworthy that more than a half of the papers published in this special issue have been already cited at least once during 2010.

Protozoan Parasites

In this special issue, you can find studies on some of the most important protozoan infections such as malaria, amebiasis, Chagas' disease, African tripanosomiasis, toxoplasmosis, and leishmaniasis.

Amebiasis caused by *Entamoeba histolytica* is still a health problem in Asia and Latin America, and travelers worldwide are also affected; in their review, I. Wong-Baeza et al. "*The role of lipopeptidophosphoglycan in the immune response to Entamoeba histolytica*" describe the importance of lipopeptidophosphoglycan, which is recognized through TLR2 and TLR4 and leads to the release of cytokines from human monocytes, in the immune control of this infection that mainly cause diarrhea and hepatic abscess; thus lipopeptidophosphoglycan is a possible candidate molecule to develop a vaccine against amebiasis. Understanding this parasite also includes the knowledge of its genes and their function. In their original paper, I. López-Reyes et al. "*Detection of the endosomal sorting complex required for transport in Entamoeba histolytica and characterization of the EhVps4 protein*" found the presence and expression of endosomal sorting complexes required for intracellular transport, named EhVps4, which were related to increased phagocytosis and virulence in *E. histolytica*.

Another widely spread parasitic infection is toxoplasmosis, caused by the protozoa *Toxoplasma gondii*. Infections caused by these parasites are common and fatal in immunocompromised patients such as those infected with HIV. E. Y. Denkers "*Toll-like receptor initiated host defense against Toxoplasma gondii*" review recent advances in innate immunity to this parasite with special emphasis on the toll-like receptors (TLRs) which can be triggered by the parasite itself and emphasizes the complex interactions with the intestinal microbiota worsening the disease during enteric infection. Immune regulation is also a key factor during toxoplasmosis, and the research performed by E. P. Tenorio et al. "*Reduction of Foxp3+ cells by depletion with the PC61 mAb induces mortality in resistant BALB/c mice infected with Toxoplasma gondii*" shows that ablation of Tregs in otherwise resistant mice, induce high mortality. On the other hand, Y. Sanchez et al. "*The unexpected role for the aryl hydrocarbon receptor on susceptibility to experimental toxoplasmosis*" demonstrated for the first time a role for the aryl hydrocarbon receptor (AHR) in a parasitic disease. They report that genetically deficient mice in AHR succumbed faster to *T. gondii* infection which was associated with an increased inflammatory cytokine response, thus revealing a role for AHR in immune regulation in toxoplasmosis.

Leishmaniasis is another wide spread parasitic infection in the world. These parasites need a vector; thus, they are transmitted by the bite of a sand fly. Several species contribute to its prevalence in all the continents. Here, we selected a series of papers that clearly show the very different sides that these infections display; the initial phase of the infection is critical where neutrophils appear to have a prominent role contributing to determine the type and magnitude of the *L. major* specific immune response that will develop "Charmoy et al.: *The prominent role of neutrophils*

during the initial phase of infection by Leishmania parasites." In line with this, M. Cummings et al. "*Cytokines and their STATs in cutaneous and visceral leishmaniasis*" review the key importance of the signal transducer and activator of transcription (STATs) during cutaneous and visceral leishmaniasis. Interestingly, *Leishmania* infection is able to alter the host cell signaling in order to survive according to the original report by S. Bhardwaj et al. "*Leishmania interferes with host cell signaling to devise a survival strategy.*" Notwithstanding, more efforts are being directed to develop new strategies to get useful vaccines "Ramírez et al.: *BALB/c mice vaccinated with Leishmania major ribosomal proteins extracts combined with CpG oligodeoxynucleotides become resistant to disease caused by a secondary parasite challenge*" as well as development of new drugs after detecting high resistance to the old drugs used against leishmaniasis as in "Maltezou: *Drug resistance in visceral leishmaniasis.*"

Malaria is a tropical disease, which results in more than one million deaths annually and is caused by protozoan parasites of the genus *Plasmodium* and transmitted by blood-feeding Anopheline mosquitoes; effective vaccines are not available. The work by J. Schmiege et al. "*A multifactorial mechanism in the superior antimalarial activity of α -C-GalCer*" demonstrates that α -C-GalCer, that has an antimalarial activity, has a multifactorial mechanism. The in vivo administration of α -C-GalCer induces prolonged maturation of dendritic cells (DCs), as well as an enhanced proliferative response of mouse invariant V α 14 (V α 14i) NKT cells, both of which may also contribute to some degree to the superior activity of α -C-GalCer in vivo. On the same line, A. Kuehn and G. Pradel "*The coming-out of malaria gametocytes*" reviewed the recent findings on the role of gametocytes during transmission to the mosquito and pay particular focus on the molecular mechanisms underlying gametocyte activation and emergence from the host erythrocyte during gametogenesis. Until very recently, little was known about the chromatin structure of the telomeres and subtelomeric regions in *Plasmodium falciparum*; that is why R. Hernandez-Rivas and collaborators "*Telomeric heterochromatin in Plasmodium falciparum*" discuss the telomeric heterochromatin in *P. falciparum* and argue that such structure may be important for telomere functions such as the silencing of the *var* gene family implicated in the cytoadherence and antigenic variation of these parasites. N. Basilio et al.'s "*The lipid moiety of haemozoin (Malaria Pigment) and P. falciparum parasitised red blood cells bind synthetic and native endothelin-1*" worked on the binding of a malaria pigment of *P. falciparum* parasitized red blood cells to a synthetic and native endothelin-1. Their findings may help understanding the consequences of parasite sequestration in severe malaria. Tomatine adjuvantation of protective immunity to a major pre-erythrocytic vaccine candidate of malaria is mediated via CD8⁺ T-cell release of IFN- γ , according to K. G. Heal and Taylor-Robinson "*Tomatine adjuvantation of protective immunity to a major pre-erythrocytic vaccine candidate of malaria is mediated via CD8+ T Cell release of IFN- γ .*" They conclude that further characterization of tomatine as an adjuvant in malaria vaccine development is indicated. Finally, in the same line of

adjuvant activity, M. Legorreta-Herrera et al.'s "Pretreatment with Cry1Ac protoxin modulates the immune response, and increases the survival of plasmodium-infected CBA/Ca mice" demonstrated that the pretreatment with Cry1Ac protoxin modulates the immune response of the host and increases the survival of *Plasmodium*-infected mice, and pointed out that by understanding how to boost innate immunity to *Plasmodium* infection should lead to immunological based intervention strategies.

Trypanosomiasis is caused by the protozoan *Trypanosoma cruzi* and *T. brucei* which are transmitted by insect vectors. Here, we present a series of original works as well as reviews covering both American and African trypanosomiasis also called Chagas' disease and sleeping sickness, respectively. Many people as well as domestic and wild animals are infected or at a risk of getting these diseases by both migration and global warming. C. M. Atyame Nten et al. "Excreted/Secreted proteins from trypanosome procyclic strains" using mass spectrometry characterized almost 500 proteins secreted by *T. brucei* and suggested a critical role for such secretome in the virulence of this parasite. In a similar way, R. M. Corrales et al. "An experimental approach for the identification of conserved secreted proteins in Trypanosomatids" developed an experimental approach to identify conserved secreted proteins in the same parasite. In African trypanosomiasis, V. Marcoux et al. "Characterization of major surface protease homologues of *Trypanosoma congolense*" have characterized six major surface proteases encoded within the partially sequenced *T. congolense* genome. As the regulation and expression of such important proteins for the virulence of these parasites appears to be a critical field, we have two interesting reviews dealing with expression and regulation of genes in these protozoans: "S. Martínez-Calvillo et al.: Gene expression in trypanosomatid parasites" and "C. Gomez et al.: Regulation of gene expression in protozoa parasites." In line with this, Hernández-Osorio et al.'s "Improved method for in vitro secondary amastigogenesis of *Trypanosoma cruzi*: morphometrical and molecular analysis of intermediate developmental forms" developed a method to analyze morphometrically and molecularly the intermediate forms of *T. cruzi* in vitro. Finally, regarding to immune regulation during trypanosomiasis, we have one review and one original work. T. N. Baral "Immunobiology of African trypanosomes: need of alternative interventions" wrote a very complete review regarding immunobiology of African trypanosomiasis suggesting alternate focus to improve approaches in both treatment and vaccination against trypanosomiasis. B. Espinoza et al.'s "Mexican *Trypanosoma cruzi* I strains with different degrees of virulence induce diverse humoral and cellular immune responses in a murine experimental infection model," on the other hand, show us how different Mexican strains of *T. cruzi* display distinct degrees of virulence as well as induce very different cellular and humoral immune response, thus explaining part of the complexity to get successful treatment or vaccine development for this parasitic disease.

Many of the protozoan infections are transmitted by hematophagous vectors; here, these important biological

agents are analyzed in three papers. The role of cysteine-free proteins in the immunobiology of arthropod-borne diseases is afforded by S. Mejia et al. "Cysteine-Free proteins in the immunobiology of arthropod-borne diseases." They discuss their findings in the context of protein structure and function, antigenicity and immunogenicity, and host-parasite relationships. In another work, R. P. Soares et al. "Differential midgut attachment of *Leishmania (Viannia) braziliensis* in the sand flies *Lutzomyia (Nyssomyia) whitmani* and *Lutzomyia (Nyssomyia) intermedia*" demonstrate how *L. braziliensis* display a differential attachment to the midgut of different species of its vector, the sand fly *Lutzomyia*. In the same context and in a letter specifically for this report, C. R. Alves et al. "The vectorial potential of *Lutzomyia (Nyssomyia) intermedia* and *Lutzomyia (N.) whitmani* in the transmission of *Leishmania (V.) braziliensis* can also be related to proteins attaching" suggest that also the specific proteins involved in the attaching to the midgut of the vector may play a critical role for this infection.

Helminth Infections

Helminths are multicellular organisms which can colonize almost any tissue in their hosts. They are classified according to their adult form in nematodes (roundworms), trematodes, and cestodes (flatworms). Given the big size they can reach, the immune response against these infections is very complex. Worms infect millions of people worldwide, and they can go since inoffensive until highly dangerous and, in some cases, threatening for humans. In this special issue, we have selected research papers as well as state-of-the-art reviews covering all the three main classes of helminths.

Echinococcus multilocularis is the causative agent of alveolar echinococcosis. In their review, D. A. Vuitton and B. Gottstein "Echinococcus multilocularis and its intermediate host: a model of parasite-host interplay" discuss the immunomodulatory mechanisms involved in the pathology and protection against the larval stage (metacestodes) of this parasite and suggest the use of cytokines such as interferon- α , as well as specific antigens to treat patients in the future in order to reduce the immunopathology associated with alveolar echinococcosis and/or to prevent this very severe parasitic disease.

In the case of *Taenia solium*, a contribution by A. Landa et al. "Release of glycoprotein (GP1) from the tegumental surface of *Taenia solium* by phospholipase C from *Clostridium perfringens* suggests a novel protein-anchor to membranes" demonstrates that the release of glycoprotein (GP1) from the tegumental surface of its metacestode by Phospholipase C from *Clostridium perfringens* is a novel protein-anchor to membranes. Another aspect researched on *T. solium*, is the role that sex steroids plays during the development of the metacestode stage of the parasite. Particularly, progesterone in vitro treatment of the parasite induces scolex evagination and growth of the same. These effects are mediated by what looks to be a progesterone receptor of the parasite. Authors point out that this use of the hormone by the parasite

could have strong evolutionary implications to the host-parasite relationship “Escobeda et al.: *Progesterone induces scolex evagination of the human parasite Taenia solium: evolutionary implications to the host-parasite relationship.*” *Taenia crassiceps* is a parasite of rodents, and their intermediate hosts are canids. However, this parasite for a long time has been accepted to be a good alternative to develop research to give more insights of the infection produced by *T. solium*. In this issue, readers will find several papers dealing with different aspects of the infection by *T. crassiceps*. Firstly, P. Ostoa-Saloma et al. “*Budding of Taenia crassiceps cysticerci in vitro is promoted by crowding in addition to hormonal, stress, and energy-related signals,*” demonstrate that budding (the asexual form of reproduction of *T. crassiceps*) of *T. crassiceps* cysticerci in vitro is promoted by crowding, in addition to hormonal, stress, and energy-related signals. On the other hand, G. Escobedo et al. “*A new MAP kinase protein involved in estradiol-stimulated reproduction of the helminth parasite Taenia crassiceps*” by using different approaches, including flow cytometry, confocal microscopy, and proteomics, show the process of discovery of a new protein from parasite origin, a parasite MAP kinase, that may be involved in the estradiol-stimulated reproduction of this helminth parasite. Finally, A. Garza and coworkers “*Substance P signaling contributes to granuloma formation in Taenia crassiceps infection, a murine model of cysticercosis*” suggest that substance P signaling may contribute to granuloma formation and proinflammatory cytokine production in this infection.

Regarding *Schistosoma japonicum*, X. Xu et al. “*Activation-induced T helper cell death contributes to Th1/Th2 polarization following murine Schistosoma japonicum infection,*” demonstrate that activation-induced T helper cell death contributes to Th1/Th2 polarization in this infection, and suggest that *S. japonicum* antigen-induced Th1 and Th2 cell apoptosis involves the Th1/Th2 shift and favor both hosts and parasites. Finally, S. Hu et al. “*Anti-inflammatory protein of Schistosoma japonicum directs the differentiation of the WEHI-3B JCS cells and mouse bone marrow cells to macrophages*” developed a study that found an anti-inflammatory protein of *S. japonicum* (rSj16) which directs the differentiation of the WEHI-3B JCS cells and mouse bone marrow cells to macrophages. Their results revealed that rSj16 biased the colony formation of mouse bone marrow cells towards macrophage lineage.

Immunity against helminths and their interactions with the host and the intercurrent infections that they cause is presented by E. Moreau and A. Chauvin’s “*Immunity against helminths: interactions with the host and the intercurrent infections.*” They argue that studies of the immune response against helminths are of great interest in understanding interactions between the host immune system and parasites. Finally, another type of parasites and hosts that are discussed here is represented in the work by K. Rohlenová and A. Šimková “*Are the immunocompetence and the presence of metazoan parasites in cyprinid fish affected by reproductive efforts of cyprinid fish?*”, about the immunocompetence and the presence of metazoan parasites in cyprinid fish, that could be affected by their reproductive efforts. On the same line of fish and parasites, the work by P. T. K. Woo

“*Immunological and therapeutic strategies against Salmonid cryptobiosis*” deals with immunological and therapeutic strategies against Salmonid cryptobiosis, caused by the haemoflagellate, *Cryptobia salmositica*. The disease-causing factor is a metalloprotease and a monoclonal antibody against its therapeutic. Though vaccine has been developed, only isometamidium chloride is therapeutic against the pathogen and its effectiveness is increased after conjugation to antibodies.

The early interactions between the immune system and either helminth and protozoan parasites or their derivatives have started to be a hot topic in the immunoparasitology field. In this issue, we offer four different reviews which critically show the key role played by innate cells such as dendritic cells “F. Mendlovic and A. Flisser: *Dendritic cells in the gut: interaction with intestinal helminthes,*” “C. A. Terrazas et al.: *Modulation of dendritic cell responses by parasites: a common strategy to survive,*” macrophages “S. J. Jenkins and J. E. Allen: *Similarity and diversity in macrophage activation by nematodes, trematodes, and cestodes,*” and molecules such as nitric oxide against different helminth infections “A. Muro and J. L. Pérez-Arellano: *Nitric oxide and respiratory helminthic diseases,*”; they explain how DCs did not mature after exposure to helminth antigens, as well as how macrophages are shifted towards an alternate state of activation, and pointed out how important is the induction of nitric oxide production during different helminth infections. another 2 reviews are focused on the role of these cells in both helminth and protozoan infections, for example the role of Peroxisome Proliferator-Activated Receptors (PPARs-) mediating suppression of parasitidal response is analyzed in detail by M. M. Chan et al. “*Peroxisome proliferator-activated receptor (PPAR): balance for survival in parasitic infections,*”; on the other hand, C. C. Stempin et al. “*Arginase in parasitic infections: macrophage activation, immunosuppression, and intracellular signals,*” resume the role that arginase plays in promote susceptibility to protozoan and helminth infections.

Using Helminths to Fight Inflammation and Autoimmunity

The more in-depth knowledge generated in the last few years on the understanding of the immune regulation induced by helminth infections or their antigens has generated a new positive point of view regarding immune modulation by helminthes; thus, most of the helminth infections studied in more detail have showed a general mechanism that include inhibition of the immune response, low proliferative range of T cells and induction of regulatory cells such as Tregs, alternative activated macrophages, and “immature” DCs, which in turn may inhibited dangerous immune-reactions. Here, we offer a nice review by Y. Osada and T. Kanazawa “*Parasitic helminths: new weapons against immunological disorders*” suggesting that helminths can be used in a near future as weapons against autoimmune diseases. Reinforcement or support for this point of view is given by three original works using different helminth infections as regulators for

three different pathologies, thus A. Melon's group "*Infection with Hymenolepis diminuta is more effective than daily corticosteroids in blocking chemically induced colitis in mice*" show how a concurrent infection with *Hymenolepis diminuta* is much better in reducing the pathology associated with chemically-induced colitis than classical treatment using corticosteroids. Using another cestode, *Taenia crassiceps*, A. Espinoza-Jiménez et al. "*Taenia crassiceps infection attenuates multiple low-dose streptozotocin-induced diabetes*" demonstrated for the first time how this parasite, restricted to the peritoneal cavity, was able to avoid the development of Type 1 diabetes (T1D) induced by multiple low-doses of streptozotocin. Finally, in the same order of ideas the work by P. Zaccone et al. "*Immune modulation by Schistosoma mansoni antigens in NOD mice: effects on both innate and adaptive immune systems*" shows how injection of soluble egg antigens from *S. mansoni* was able to induce TGF- β from T cells which is important not just for Treg expansion but also for the successful Th2 response needed to reduce T1D in nonobese diabetic mice.

We hope our readers to find this first special issue of Immunobiology of Parasitic Diseases enticing and enjoy reading contributions by all authors.

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Review Article

The Role of Lipopeptidophosphoglycan in the Immune Response to *Entamoeba histolytica*

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The sensing of Pathogen Associated Molecular Patterns (PAMPs) by innate immune receptors, such as Toll-like receptors (TLRs), is the first step in the inflammatory response to pathogens. *Entamoeba histolytica*, the etiological agent of amebiasis, has a surface molecule with the characteristics of a PAMP. This molecule, which was termed lipopeptidophosphoglycan (LPPG), is recognized through TLR2 and TLR4 and leads to the release of cytokines from human monocytes, macrophages, and dendritic cells; LPPG-activated dendritic cells have increased expression of costimulatory molecules. LPPG activates NKT cells in a CD1d-dependent manner, and this interaction limits amebic liver abscess development. LPPG also induces antibody production, and anti-LPPG antibodies prevent disease development in animal models of amebiasis. Because LPPG is recognized by both the innate and the adaptive immune system (it is a “Pamptigen”), it may be a good candidate to develop a vaccine against *E. histolytica* infection and an effective adjuvant.

1. Introduction

Amebiasis is a disease caused by *Entamoeba histolytica*, a parasite protozoan that infects humans and is responsible for 40,000 to 110,000 deaths per year [1]. Ten percent of infected persons exhibit clinical symptoms; 80% to 98% of these are intestinal, and 2% to 20% are extraintestinal. The clinical symptoms can range from a mild and nonspecific presentation (constipation alternated with diarrhea, pain in the lower abdomen, mild nausea during or after meals, and mild abdominal distension with pain in the right iliac fossa) to dysentery, fulminating colitis, and toxic megacolon. Less frequently, amebiasis can cause appendicitis and ameboma.

Amebic liver abscess is the most frequent presentation of invasive extraintestinal amebiasis, but the lungs, heart, brain, skin, and genitals can also be affected [2].

Approximately 500 million people in the world are currently infected with *E. histolytica* [1]. The incidence of amebiasis has decreased significantly in recent years because of increased sanitation in many countries and the use of effective therapeutic agents. The World Health Organization and the Pan-American Health Organization recommend the treatment of all patients with confirmed *E. histolytica* infection, regardless of the presence of symptoms. The treatments of choice for asymptomatic intestinal amebiasis are the luminal amebicides paromomycin sulfate and diloxanide

furoate. Symptomatic intestinal or extraintestinal infection is treated with metronidazole in combination with a luminal amebicide. Nitazoxanide is an effective luminal amebicide, and it is also effective for invasive amebiasis. Gastrointestinal complications, such as perforation, intestinal obstruction, and toxic megacolon, are treated with surgery. Most hepatic abscesses respond to metronidazole, but if they do not, they can be aspirated by puncture or treated with open surgery [3–5].

In spite of the effective therapeutic agents that are available for the treatment of amebiasis, it still constitutes a global health problem [6]. The prevalence of amebiasis varies from 1% in industrialized countries to 50%–80% in tropical countries [7–10].

2. Identification of Lipopeptidophosphoglycan

In the 1970s, amebiasis was the fourth most frequent infectious disease in Mexico, with an incidence of 118.9 per 10,000 inhabitants (almost 1500 times higher than the incidence in the United States in the same year) [11]. This situation prompted many researchers to study several aspects of this parasitic disease, including comparative studies of drugs for the treatment of acute amebic liver abscess [12] and various studies of seroepidemiology of amebiasis in adults [13–17].

Several genes from *E. histolytica* were cloned, sequenced and expressed in an effort to identify new drug targets for this parasite, including the alcohol dehydrogenase gene (Ehadh3) [18], the ferredoxin oxidoreductase gene [19], the EhDEAD1 RNA helicase gene [20], and the Ehvma2 gene (which encodes the B subunit of the vacuolar ATPase) [21]. Mechanisms of drug resistance in *E. histolytica* were also studied, and it was determined that the multidrug-resistant phenotype is regulated at the transcriptional level by the P-glycoprotein-like genes (EhPgp) 1 and 5 [22]. A protein complex (EhCPADH) was identified on the surface of *E. histolytica*. This complex is formed by a cysteine proteinase that digests gelatin, collagen type I, fibronectin and hemoglobin (EhCP112), and an adhesin (EhADH112), and is involved in adherence, phagocytosis, and cytolysis [23, 24]. Polypeptides derived from this complex were assessed as vaccine candidates, and it was demonstrated that they confer partial protection from amebic liver abscess in hamsters (*Mesocricetus auratus*) [25].

The role of the immune response in the pathogenesis of amebiasis was also studied; the early approaches demonstrated that serum from infected patients could neutralize the virulence of *E. histolytica* cultures [26] and that this serum could confer antiameba passive immunity in hamsters [27]. The importance of cellular immunity in the control of amebiasis was addressed in several studies, which demonstrated the ability of activated eosinophils to kill the parasite in vitro [28] and to protect from amebic liver abscess in vivo [29], and the killing of trophozoites by peritoneal macrophages in hamsters [30] and by activated T lymphocytes and macrophages in humans [31]. It was shown that patients cured from amebic liver abscess had specific T lymphocytes that killed trophozoites in vitro [31].

It was also demonstrated that molecules from *E. histolytica* were able to modulate the host immune response. The supernatant fluid of axenically grown *E. histolytica* could inhibit chemotaxis and random mobility of human monocytes, without affecting the locomotion of neutrophils [32]. The effect was attributed to a monocyte locomotion inhibitory factor (MLIF), and physicochemical analysis revealed that MLIF is a heat-stable pentapeptide (Met-Gln-Cys-Asn-Ser) that inhibits locomotion of monocytes, respiratory burst of monocytes and neutrophils, and delayed hypersensitivity skin reactions to dinitrochlorobenzene in guinea pigs (*Cavia porcellus*) [33]. MLIF decreased the expression of macrophage inflammatory protein-1 (MIP-1) alpha, MIP-1beta, and chemokine receptor CCR1 in a phorbol myristate acetate- (PMA-) stimulated human monocyte cell line, which suggests that the inhibition of monocyte locomotion could be attributed to downregulation of chemokines and chemokine receptors [34]. MLIF also decreased interleukin-1 (IL-1) beta and increased IL-10 production by PMA-stimulated human CD4 T lymphocytes [35]. Immunization with a tetramer of MLIF around a lysine core completely protected gerbils (*Meriones unguiculatus*) against amebic liver abscess [36].

In 1969, Galanos et al. developed a new method for the extraction of bacterial lipopolysaccharide (LPS, Figure 1(a)) [37] and, in the following years, many of the chemical, biological, and immunological properties of the so-called endotoxin were determined [38–44]. LPS is a major structural component of the outer membrane of Gram-negative bacteria; it activates many cell types, induces inflammation, and produces fever and shock. We decided to determine if *E. histolytica* had a surface molecule with chemical and immunological properties similar to those of bacterial LPS. The use of a modified phenol-water extraction procedure on *E. histolytica* trophozoites yielded a molecule with 85% carbohydrate, 8% peptide, 2.5% lipid, and 1% phosphate, which was termed lipopeptidophosphoglycan (LPPG, Figure 1(c)) [45, 46]. The isolation and structural characterization of microbial molecules can lead to the identification of new drug targets and new antigens that are recognized by the immune system; some antigens are good candidates for vaccine development. LPPG was first identified as an antigen; antiameba IgG antibodies were detected in rats after intracecal inoculation of trophozoites [47], anti-LPPG IgA antibodies were found in colostrum of healthy volunteers [48], and antiameba plasma cells were found in peripheral blood of patients with amebic liver abscess [49]. Monoclonal antiproteophosphoglycan antibodies were described by several groups [50–53]. However, as research in immunology progressed, LPPG was studied as a molecule that could be sensed not only by the adaptive immune system but also by the innate immune system.

3. Sensing of Parasites by the Immune System

The relevance of adaptive immunity (whose main effectors are T and B lymphocytes) in protection against infections was well recognized in the last decades of the past century, while the role of neutrophils, monocytes, macrophages, and

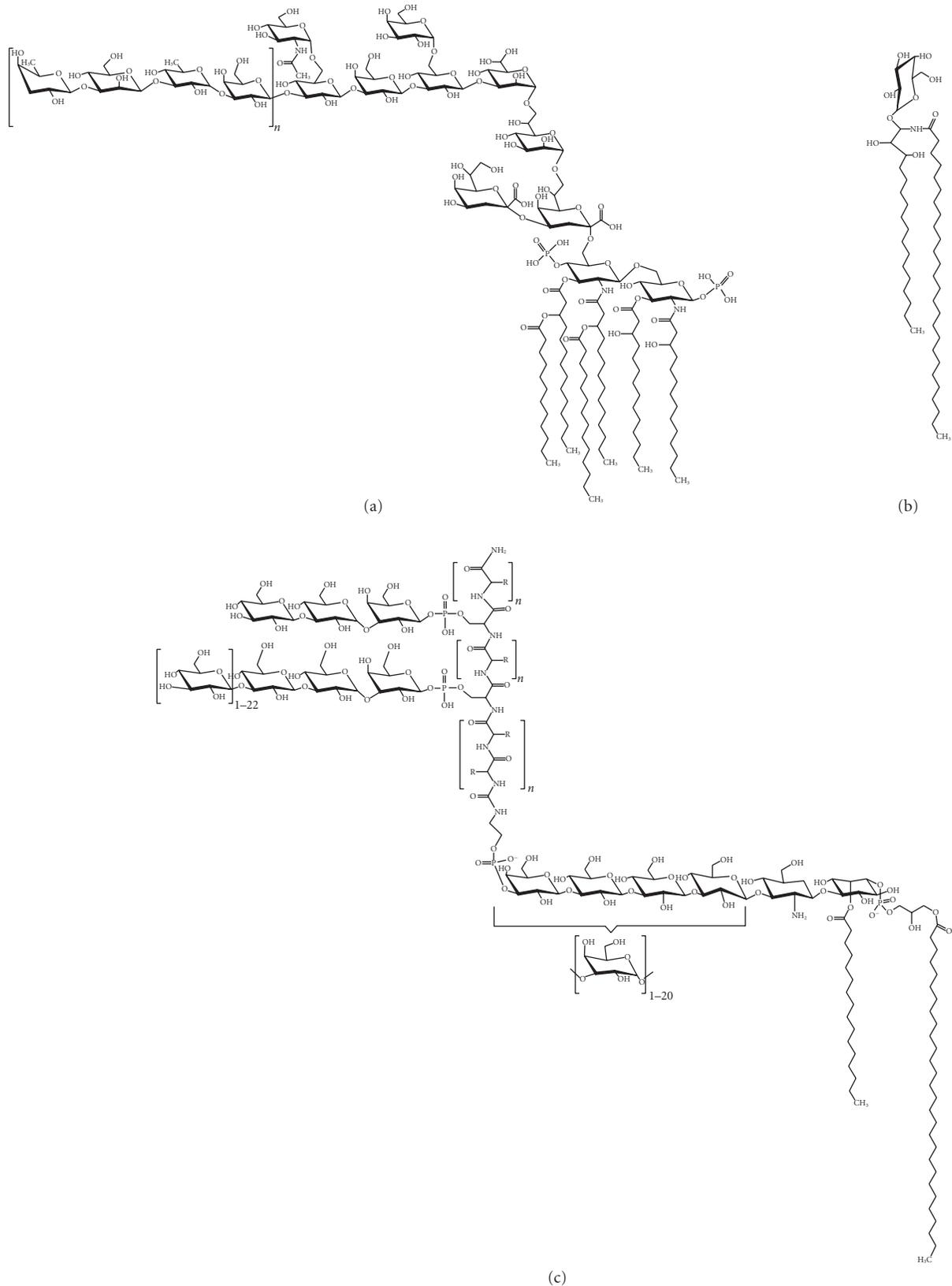


FIGURE 1: (a) Lipopolysaccharide (LPS) from the Gram-negative bacterium *Escherichia coli* is the most potent activator of TLR4 [54]. (b) Alpha-galactosyl ceramide from the marine sponge *Agelas mauritanicus* is presented via CD1d and activates NKT cells [55]. (c) Partial structure of lipopeptidophosphoglycan (LPPG) from *Entamoeba histolytica* of the HM1:IMSS strain, which was originally isolated from a patient with liver abscess [56, 57]. The structure of the active phosphoinositol moiety of LPPG was characterized in [58].

other cells of the innate immune system was seen as a that of a “first line of defense” that contained infections until adaptive immunity was fully activated. It was known that LPS, a component of the outer membrane of Gram-negative bacteria, caused fever and shock in animal models and that it induced the secretion of proinflammatory cytokines by monocytes, macrophages, and epithelial cells. However, the receptor that sensed LPS remained elusive. Many phagocytic receptors on macrophages had been described, but none of these was responsible for the biological properties of LPS [59].

Two strains of mice, C3H/HeJ and C57BL/10ScCr, which were resistant to endotoxic shock, were identified [60, 61]. In 1998, the positional cloning of the affected locus in C3H/HeJ mice showed a point mutation in Toll-like receptor (TLR) 4, a previously orphan receptor, and C57BL/10ScCr mouse were found to lack TLR4 [62]. TLR4 is a member of a family of proteins that share a signaling domain (TIR) with IL-1 receptor and are related to the Toll proteins of the fruit fly, *Drosophila*. Toll was described in 1988 as a transmembrane protein that is required for establishing the embryonic dorsal-ventral pattern in flies [63]. In 1996, Toll was shown to be critical for the antifungal response in *Drosophila* [64], and it was suspected that the human homologues of Toll, which were described in 1998 [65], would be relevant for the immune response in humans. Indeed, it was found that a constitutively active mutant of human TLR4, transfected into a monocytic cell line, could induce the activation of NF-kappaB and the expression of the proinflammatory cytokines IL-1, IL-6, and IL-8 as well as the expression of the costimulatory molecule CD80 (B7.1), which is required for the activation of naïve T lymphocytes [66]. After the establishment of TLR4 as the main sensor for LPS in mice, it was immediately suggested that other members of the Toll family in mammals could also serve as sensors for microbial molecules. Many molecules of bacteria, viruses, and fungi, and others that are found during viral replication, have been identified as agonists of mammalian TLRs (Table 1) [67, 68]. The study of TLRs and other innate receptors has established innate immunity not only as a first line of defense against infections but also as a critical component of the immune system that induces and regulates the adaptive response [69].

The molecules that are sensed by TLRs are widely distributed among groups of microorganisms, and they are essential for the metabolism or the structural integrity of the microbe, so they are highly conserved in evolution. These molecules were termed pathogen-associated molecular patterns (PAMPs) [70], although their expression is not restricted to pathogens, and this term is used widely to this day. Several molecules from protozoan and helminth parasites were also identified as PAMPs [71] (Table 2). In protozoan parasites, many surface molecules are linked to glycosylphosphatidylinositol (GPI), which is inserted in the plasma membrane. GPI-anchored molecules include lipophosphoglycan (LPG) and LPPG; they are essential for survival and virulence of the parasite, and they are likely the major macromolecules on the trophozoite surface [72]. In *Leishmania*, LPG is involved in intestinal adhesion and

TABLE 1: Some TLR agonists from bacteria, viruses, and fungi (modified from [67, 68]).

TLR	Microbial ligand	Source
TLR1/TLR2	Triacyl lipopeptides	Bacteria
	Lipoarabinomannan	Mycobacteria
TLR2	Peptidoglycan	Bacteria
	Porins	Gram-negative bacteria
	Lipoteichoic acid	Gram-positive bacteria
	Zymosan	Fungi
TLR2/TLR6	Diacyl lipopeptides	Mycoplasma
TLR3	dsRNA	Virus
TLR4	LPS	Gram-negative bacteria
	Porins	Gram-negative bacteria
	Respiratory syncytial virus fusion protein	Respiratory syncytial virus
TLR5	Flagellin	Bacteria
TLR7	ssRNA	Virus
TLR8	ssRNA	Virus
TLR9	CpG DNA	Bacteria, virus
TLR11	—	Uropathogenic bacteria

resistance to insect hydrolases; LPG-deficient strains are unable to survive in their vector. *Leishmania* LPG gains phosphosaccharide domains as procyclic promastigotes in the vector midgut differentiate to infectious metacyclic promastigotes; this structural change in LPG mediates detachment from vector midgut and acquisition of complement resistance [73]. LPG also induces the production of nitric oxide and proinflammatory cytokines by macrophages in the host [74]. LPG from metacyclic promastigotes is a more effective activator of TLR2 in NK cells than LPG from procyclic promastigotes [75].

Plasmodium GPI-anchored molecules are required for the induction of proinflammatory responses, which promote pathogenesis [76]. However, activation of innate and adaptive immune responses is necessary to control parasite growth and frequent *tlr4* polymorphisms predispose African children to severe malaria [77]. Therefore, it is proposed that overactivation or deregulation of the inflammatory response is the cause of the pathological condition [78, 79]. Several mucin-like GPI-anchored glycoproteins have been isolated from the *Trypanosoma cruzi* surface. A *T. cruzi* trans-sialidase adds sialic acid residues to these molecules, which are required for survival and infectivity [72, 80]. GPI-anchored molecules purified from *T. cruzi* trypomastigotes signal through TLR2 and induce the production of IL-12, tumor necrosis factor (TNF)-alpha and nitric oxide by murine macrophages [81]; signaling through TLR2 synergizes with TLR9 and is crucial to control the infection [82]. Tc52 is a soluble molecule that is released by *T. cruzi* during parasitemia, and it activates macrophages and dendritic cells via TLR2 [83]. GPI-anchored molecules isolated from *Toxoplasma* activate TLR4, while glycan cores and phospholipid moieties from these molecules activate both TLR2 and TLR4 [84]. Lysophosphatidylserine-containing lipids from

TABLE 2: TLR agonists from protozoan and helminth parasites.

TLR	Parasite ligand	Source	Reference
TLR2	Lipopeptidophosphoglycan	<i>Entamoeba histolytica</i> (trophozoite)	[85]
	Glycosylphosphatidylinositol	<i>Plasmodium falciparum</i> (merozoite)	[76]
		<i>Toxoplasma gondii</i> (tachyzoite)	[84]
	Glycoinositol phospholipid	<i>Plasmodium falciparum</i> (merozoite)	[76]
		<i>Toxoplasma gondii</i> (tachyzoite)	[86]
	Lysophosphatidylserine	<i>Schistosoma mansoni</i> (egg and adult worm)	[87]
	Lipophosphoglycan	<i>Leishmania major</i> (promastigote)	[74]
TLR2/TLR6	Glycosylphosphatidylinositol	<i>Plasmodium falciparum</i> (schizont)	[78]
	Lipopeptidophosphoglycan	<i>Entamoeba histolytica</i> (trophozoite)	[85]
	Glycoinositol phospholipid with ceramides	<i>Trypanosoma cruzi</i> (epimastigote)	[88]
TLR4	Glycosylphosphatidylinositol	<i>Plasmodium falciparum</i> (merozoite)	[76]
		<i>Toxoplasma gondii</i> (tachyzoite)	[84]
	Glycoinositol phospholipid	<i>Plasmodium falciparum</i> (merozoite)	[76]
		<i>Toxoplasma gondii</i> (tachyzoite)	[86]
	Phosphorylcholine	Filarial nematode	[89]
	Lacto- <i>N</i> -fucopentaose III	<i>Schistosoma mansoni</i>	[90]
TLR9	Hemozoin	<i>Plasmodium falciparum</i>	[91]
	DNA	<i>Trypanosoma brucei</i>	[92]
		<i>Trypanosoma cruzi</i>	[92]
		<i>Leishmania major</i>	[93]
		<i>Entamoeba histolytica</i>	[94]
TLR11	Profilin-like molecule	<i>Toxoplasma gondii</i>	[95]

Schistosoma mansoni induce the maturation of dendritic cells that prime Th2 and regulatory T cell responses, which favor the establishment of chronic infections with little tissue damage [87].

4. Function of LPPG as a PAMP and Role of Inflammation in the Pathogenesis of Amebiasis

The similarities in chemical structure between LPS and LPPG (Figures 1(a) and 1(c)), and the presence of a GPI anchor in LPPG, suggested that LPPG might be a PAMP. This would explain how the innate immune system senses the presence of *E. histolytica*, an event that is necessary for the orchestration of the inflammatory response in amebiasis. Studies from our laboratory demonstrated that LPPG is recognized through TLR2 and TLR4. Human embryonic kidney- (HEK-) 293 cells were rendered LPPG responsive through overexpression of TLR2 or TLR4/MD2. Coexpression of CD14 enhanced LPPG signal transmission through TLR2 and TLR4. The interaction of LPPG with TLR2 and TLR4 resulted in activation of NF- κ B and release of IL-8, IL-10, IL-12p40, and TNF- α from human monocytes [85, 96]. Human macrophages and dendritic cells internalize LPPG. As shown by colocalization of LPPG with late endosomes marked

with fluorescein isothiocyanate-dextran and LAMP-1, the internalization process involves intracellular traffic from the cell membrane to late endosomes. LPPG-activated dendritic cells have increased expression of costimulatory molecules CD80, CD86, and CD40 and produce TNF- α , IL-8, and IL-12 [97]. These results show that LPPG activates antigen-presenting cells and reaches intracellular compartments that are involved in antigen presentation. Responsiveness of mouse macrophages lacking TLR2 expression (TLR2 $-/-$) or functional TLR4 (C3H/HeJ) to LPPG challenge was impaired, while macrophages from C3H/HeJ/TLR2 $-/-$ mice were unresponsive. In contrast to wild-type and TLR2 $-/-$ mice, which succumbed to LPPG-induced shock, C3H/HeJ mice were resistant [85]. All these results clearly establish that LPPG is a PAMP from *E. histolytica* that induces the activation of innate immunity.

In humans, the pathogenesis of *E. histolytica* requires adhesion of trophozoites to the host cells, phagocytosis of host cells and bacteria by trophozoites, and tissue destruction by amebic enzymes and by enzymes released from lysed neutrophils. The adhesion of trophozoites to host cells is required for tissue invasion; this adhesion is mediated, in part, by a galactose/N-acetylgalactosamine- (Gal/GalNAc-) binding lectin, which is also cytotoxic and confers protection from complement. Other important adhesins are a 220 kDa cell surface protein, a 112 kDa adhesin (EhADH112), and

a surface LPG [23, 98–100]. Phagocytosis is regulated by adhesins and by signaling pathways that control cytoskeleton structure and vesicular traffic. A phagosome-associated transmembrane kinase (PATMK) binds to phosphatidylserine on host cells and initiates their phagocytosis by trophozoites [101]. Trophozoites cause damage of host cells and extracellular matrix through the action of amebapore, amebic phospholipases, and proteolytic enzymes (cysteine endopeptidases, cysteine proteinase, acid and neutral proteinases, collagenases, histolysin, amebapain, cathepsin B) [102–108]. Neutrophils are the first cells that infiltrate the necrotic lesions caused by *E. histolytica* in the intestine and liver [109–113], where they are killed by trophozoites. The enzymes and reactive oxygen species released from these neutrophils increase tissue damage, and in this context, LPPG could be seen as a virulence factor that promotes tissue invasion by causing inflammatory damage to host cells. However, the role of inflammation in amebiasis is still controversial [114]: in susceptible animals (hamsters and gerbils) inflammation is related to host cell lysis and facilitates the spreading of trophozoites [115], while in resistant animals (mice, guinea pigs), inflammatory cells protect the host by killing trophozoites [116, 117].

E. histolytica genomic DNA is recognized by TLR9 and induces the production of TNF- α by a macrophage cell line [94], and Gal/GalNAc-binding lectin activates NF- κ B and mitogen-activated protein (MAP) kinases in macrophages. These transcription factors increase the expression of several genes, including TLR2 [118]. It is likely that genomic DNA and Gal/GalNAc-binding lectin, along with LPPG, contribute to the initiation of inflammation in response to *E. histolytica*.

Silencing of the expression of *E. histolytica* GPI-anchored molecules by antisense RNA-mediated inhibition of their biosynthetic pathways suppresses endocytosis, adhesion, and proliferation of the trophozoites [119]. Specific blockade of LPG and LPPG by monoclonal antibody EH5 reduces intestinal inflammation and tissue damage in a severe combined immunodeficient (SCID) mouse model of intestinal amebiasis with human intestine xenograft [120]. EH5 also prevents liver abscess development in an SCID mouse model [52] and *E. histolytica* adhesion and cytotoxicity to a hamster cell line [101]. These results suggest that LPPG is a virulence factor of *E. histolytica*. Mirelman and colleagues found that a nonvirulent *E. histolytica* strain had reduced expression of LPG and LPPG; they also found no LPG and a modified LPPG (with a higher negative charge and different lengths of oligosaccharide chains) in the low-virulence strain Rahman and in the nonpathogenic *Entamoeba dispar* [121].

Recently, a role for LPPG in protection against invasive amebiasis was shown. The chemical structure of LPPG has some similarities with alpha-galactosyl ceramide, a known activator of NKT cells [55] (Figures 1(b) and 1(c)). NKT cells share many surface receptors with natural killer (NK) cells and, like conventional T cells, express T cell receptors that are generated by somatic DNA rearrangement. However, most NKT cells express semi-invariant T cell receptors, consisting of $V\alpha 14$ - $J\alpha 18$ / $V\beta 8.2$ chains in mouse and $V\alpha 24$ - $J\alpha 18$ / $V\beta 11$ chains in humans [122]. This limited repertoire, conserved

between individuals and presumably selected by evolution, is more closely related to the pattern-recognition receptors of innate immunity than to the highly diverse receptors of adaptive immunity. NKT cells recognize glycolipid antigens presented by nonpolymorphic CD1d molecules; these glycolipids can be endogenous, like lysosomal isoglobotrihexosyl ceramide [123], and exogenous, like glycosyl ceramides from Gram-negative, LPS-negative *Sphingomonas capsulata* [124]. In a mouse model, it was demonstrated that NKT cells play a central role in the control of amebic liver abscess caused by *E. histolytica*. Specific activation of NKT cells by alpha-galactosyl ceramide or LPPG induced significant protection, while CD1d $^{-/-}$ mice suffered from severe abscess development [58]. The phosphoinositol moiety of LPPG was shown to induce interferon- (IFN-) gamma but not IL-4 secretion in NKT cells. NKT cell activation was dependent on the presence of CD1d and simultaneous TLR receptor signaling, as indicated by the absence of IFN-gamma secretion in antigen-presenting cells from TLR2- or TLR6-deficient mice [58]. These results suggest that NKT cell activation by LPPG is important to limit amebic liver abscess development and may help to explain why the vast majority of *E. histolytica*-infected individuals do not develop invasive amebiasis.

5. LPPG as a Molecule That Is Sensed by Both the Innate and the Adaptive Immune Systems

Molecules that are recognized by receptors of both innate and adaptive immune systems are, in general, highly immunogenic; we have referred to these molecules as “Pamptigens” [125, 126]. Some examples of molecules that present this dual recognition include porins, profilin, polysaccharide A, yellow fever vaccine, and respiratory syncytial virus vaccine. *Salmonella typhi* porins are recognized by TLR2 and TLR4 [126], and they induce high antibody titers that persist during the whole lifetime of mice [127]. *Toxoplasma gondii* profilin, a TLR11 agonist, is an immunodominant antigen in the CD4 $^{+}$ T cell response to the pathogen [128]. *Bacteroides fragilis* polysaccharide A activates CD4 $^{+}$ T cells by a mechanism that depends on TLR2 signaling and antigen presentation by the MHCII pathway [129]. Live attenuated yellow fever vaccine 17D, one of the most effective vaccines available, activates TLR2, 7, 8, and 9 and induces antigen-specific CD8 $^{+}$ T cells [130]. Poor TLR signaling by a formalin-inactivated respiratory syncytial virus vaccine led to the induction of low-affinity antibodies and to the failure of the vaccine to protect immunized children [131].

Molecules that are recognized by innate and adaptive receptors of the immune system are also effective adjuvants. Innate immunity participates in the induction and regulation of adaptive responses; without adjuvants, molecules that are recognized by adaptive receptors but not by innate receptors fail to elicit antibody or T cell responses. Antigen recognition alone is not sufficient to activate adaptive immune responses, and innate signals are required to indicate the microbial origin of the antigen; adjuvants provide this signal by activating innate immune receptors [132, 133].

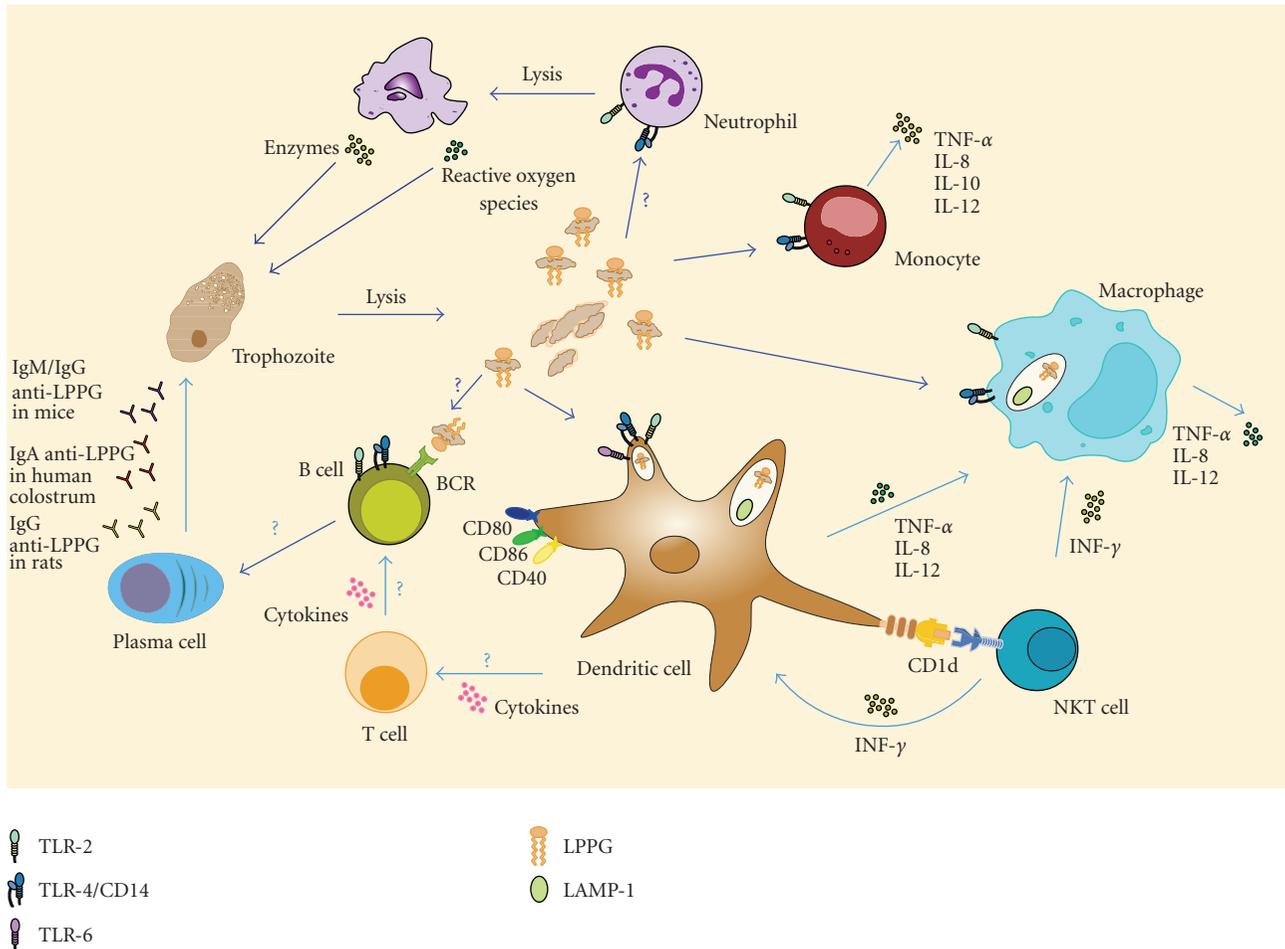


FIGURE 2: The role of lipopeptidophosphoglycan (LPPG) in the immune response to *Entamoeba histolytica*. During *E. histolytica* infection, amebic enzymes and enzymes and reactive oxygen species from neutrophils cause tissue damage. LPPG released from lysed trophozoites is recognized through TLR2 and TLR4/CD14 and induces the production of IL-8, IL-10, IL-12p40, and TNF-alpha by monocytes [85, 96]. Macrophages and dendritic cells internalize LPPG into LAMP-1+ endosomes, and LPPG-activated dendritic cells have increased expression of costimulatory molecules CD80, CD86, and CD40 and produce TNF-alpha, IL-8, and IL-12 [97]. NKT cells are also activated by LPPG, and this depends on the presence of CD1d on dendritic cells and simultaneous TLR2 and TLR6 signaling [58]. Anti-LPPG antibodies have been described in humans and in animal models [47–53]. The mechanism that leads to the production of these antibodies has not been determined, but it is probably influenced by the innate signaling of LPPG on dendritic cells and B cells.

LPPG signals through TLR2 and TLR4, and it induces the production of IFN-gamma (a cytokine that activates macrophages and increases cytotoxic T cell responses) by NKT cells. LPPG is also an antigen, because anti-LPPG antibodies have been detected in animal models and in patients with amebiasis. The mechanism that leads to the production of these antibodies has not been determined, but it is probably influenced by the innate signaling of LPPG (Figure 2). The intrinsic immunogenicity of LPPG and the fact that it is a virulence factor of *E. histolytica* make LPPG an attractive candidate for vaccine development. Its properties as an adjuvant also deserve further study.

6. Concluding Remarks

The study of *E. histolytica* was initially motivated by the high morbidity and mortality of amebiasis, and in our

group, this research led to the identification of LPPG, one of the first PAMPs described in parasites, and a promising vaccine candidate and potential adjuvant. The incidence and severity of amebiasis has declined, because of improved sanitation and effective treatments, but this disease is still a health problem in many parts of the world. The development of a vaccine that effectively protects against *E. histolytica* infection would have a positive impact on global health.

Abbreviations

Gal/GalNAc:	Galactose/N-acetylgalactosamine
GPI:	Glycosylphosphatidylinositol
IFN:	Interferon
IL:	Interleukin
MAP kinases:	Mitogen-activated protein kinases

LAMP: Lysosome-associated membrane glycoprotein
 LPG: Lipophosphoglycan
 LPPG: Lipopeptidophosphoglycan
 LPS: Lipopolysaccharide
 MHCII: Major histocompatibility complex class II
 MIP: Macrophage inflammatory protein
 MLIF: Monocyte locomotion inhibitory factor
 NK: Natural killer
 PAMP: Pathogen associated molecular pattern
 PATMK: Phagosome-associated transmembrane kinase
 PMA: Phorbol myristate acetate
 SCID: Severe combined immunodeficiency
 TLR: Toll-like receptor
 TNF: Tumor necrosis factor.

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Research Article

Detection of the Endosomal Sorting Complex Required for Transport in *Entamoeba histolytica* and Characterization of the EhVps4 Protein

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Eukaryotic endocytosis involves multivesicular bodies formation, which is driven by endosomal sorting complexes required for transport (ESCRT). Here, we showed the presence and expression of homologous ESCRT genes in *Entamoeba histolytica*. We cloned and expressed the *Ehvps4* gene, an ESCRT member, to obtain the recombinant EhVps4 and generate specific antibodies, which immunodetected EhVps4 in cytoplasm of trophozoites. Bioinformatics and biochemical studies evidenced that rEhVps4 is an ATPase, whose activity depends on the conserved E211 residue. Next, we generated trophozoites overexpressing EhVps4 and mutant EhVps4-E211Q FLAG-tagged proteins. The EhVps4-FLAG was located in cytosol and at plasma membrane, whereas the EhVps4-E211Q-FLAG was detected as abundant cytoplasmic dots in trophozoites. Erythrophagocytosis, cytopathic activity, and hepatic damage in hamsters were not improved in trophozoites overexpressing EhVps4-FLAG. In contrast, EhVps4-E211Q-FLAG protein overexpression impaired these properties. The localization of EhVps4-FLAG around ingested erythrocytes, together with our previous results, strengthens the role for EhVps4 in *E. histolytica* phagocytosis and virulence.

1. Introduction

Entamoeba histolytica is the enteric protozoan parasite responsible for human amoebiasis that affects 50 million people around the world, causing colitis and liver abscesses [1]. In this organism, phagocytosis and vesicular trafficking play a critical role in ingestion and degradation of host cells and microorganisms. Vacuolar protein sorting (Vps) factors and some proteins that participate in vesicle transport in eukaryotes have been identified in *E. histolytica* [2–4].

In eukaryotic cells, endocytosis consists in phagocytosis, micropinocytosis and pinocytosis. Particularly, phagocytosis involves the ingestion of particles of varying size into

phagosomes, which sequentially fuse with early and late endosomes forming multivesicular bodies (MVB), as well as with lysosomes to form phagolysosomes [5]. Additionally, MVB are critical for cell receptors down-regulation, retroviral budding and other processes [6–8]. MVB formation is driven by the assembly of endosomal sorting complexes required for transport (ESCRT), which result from the interaction of different class E Vps proteins [9, 10].

The MVB-sorting process initiates with the association of Vps27 and Hse1 proteins to form the ESCRT-0 complex, which is targeted to endosomal membrane domains that bind ubiquitinated cargo proteins (reviewed in [6, 7]). Then, ESCRT-0 recruits ESCRT-I formed by Vps23, Vps28, and

Vps37 proteins, as well as an additional subunit called Mvb12 [11, 12]. Later, ESCRT-II, composed by Vps22, Vps25, and Vps36 proteins, is activated by ESCRT-I [13]. Ubiquitinated cargo proteins are recognized by ESCRT-I via Vps23 and by ESCRT-II via Vps36. Then, ESCRT-III, formed by Vps20, Vps32, Vps2, and Vps24, interacts with ESCRT-II components to complete ESCRT formation [14]. ESCRT-III is required for cargo molecules concentration into MVB vesicles and it coordinates the association of Bro1 protein and Doa4-deubiquitinating enzyme [15–17]. Finally, Vps4 protein catalyzes the ATP-dependent dissociation of ESCRT complexes from endosomes to initiate new rounds of vesicle formation and cargo molecules transport [6, 7]. Particularly, it has been reported that substitution of the conserved E amino acid residue by a Q residue in the Vps4 ATPase motif impairs ATP hydrolysis activity [18, 19], resulting in an inefficient protein transport from endosomal compartments to vacuoles or lysosomes [20–22], which evidenced the critical role of Vps4 in protein transport and vesicle trafficking.

In *E. histolytica*, experimental evidence suggests that MVB-like structures are formed by fusion of inward budding membranes with phagosomes [23]. Ubiquitin, deubiquitinating enzymes and a SNF7 homologue (vesicular trafficking protein) have been identified in isolated phagosomes by proteomic analysis, suggesting that a mechanism similar to MVB formation could be present in *E. histolytica* [23–25]. Additionally, the Bro1 domain-containing EhADH112 protein, which forms part of the *E. histolytica* EhCPADH complex involved in parasite virulence, is located in MVB-like structures in trophozoites [26, 27]. However, molecular mechanisms regulating MVB formation in *E. histolytica* remain poorly understood. Here, by *in silico* analysis from parasite genome databases, we identified 20 ESCRT protein-encoding genes in *E. histolytica* and showed that most of them were transcribed in trophozoites. Since Vps4 has been described as a key molecule to complete the disassembly of ESCRT and associated factors in other systems, we initiated the study of ESCRT machinery in *E. histolytica* by cloning and characterizing the EhVps4 protein. Biochemical assays showed that EhVps4 exhibits ATPase activity *in vitro*. Interestingly, by using trophozoites overexpressing the wild type and a mutant version of EhVps4, we provide data supporting a role for this protein in phagocytosis and virulence.

2. Material and Methods

2.1. In Silico Identification of Putative ESCRT Genes in *E. histolytica*. Sequence similarity searches for ESCRT genes were performed in *E. histolytica* genome database (<http://pathema.jcvi.org/cgi-bin/Entamoeba/PathemaHomePage.cgi>) by BLAST using human and yeast ESCRT protein sequences as queries. Putative *E. histolytica* ESCRT homologous proteins were selected using the following criteria: (i) at least 20% identity and 35% similarity to the query sequence; (ii) *e*-value lower than 0.002; and (iii) absence of stop codons in the coding sequence. Predicted

amino acids sequences were aligned by ClustalW software (<http://www.ebi.ac.uk/clustalw/>). Functional and structural domains were predicted using PROSITE (<http://www.expasy.org/tools/scanPROSITE/>) and Pfam (<http://www.sanger.ac.uk/Software/Pfam/>) databases. Phylo-genetic relationships among putative ESCRT proteins from *E. histolytica* and other organisms were analyzed using the Neighbor-Joining distance method [28] as implemented in the MEGA package version 3.1 [29]. Phylogenetic trees were generated for each putative *E. histolytica* ESCRT protein aligned with homologues from different species. Robustness of phylogenetic inferences was tested by bootstrapping method, involving 1000 replications of the data based on the criteria of 50% majority-rule consensus.

For 3D modeling of MIT and AAA domains of *E. histolytica* EhVps4, predicted tertiary structures were obtained with the Phyre server (<http://www.sbg.bio.ic.ac.uk/phyre/>), using crystal data from yeast Vps4 MIT (2v6xA) and AAA (2qpaB) domains as templates.

2.2. *E. histolytica* Cultures. Trophozoites of *E. histolytica* clone A (strain HM1: IMSS) were axenically cultured in TYI-S-33 medium at 37°C and harvested during exponential growth phase [30]. Medium for transfected trophozoites was supplemented with 40 µg/mL G418 (Gibco). Cell viability was monitored by microscopy using Trypan blue dye exclusion test.

2.3. Semi-Quantitative RT-PCR Assays. Using Trizol reagent (Invitrogen), total RNA was extracted from 10⁶ trophozoites grown in TYI-S-33 medium or 5 minutes after red blood cells (RBC) ingestion. Semiquantitative RT-PCR was performed using 1 µg of DNase I-treated total RNA that was reverse transcribed using Superscript II (Invitrogen) for 2 hours at 42°C. Control samples without Superscript-II were included in all experiments. cDNA samples were subjected to PCR amplification using specific internal primers for distinct *E. histolytica* putative ESCRT genes (see Table T1 in Supplementary material available on line at doi: 10.1155/2010/890674). Briefly, PCR consisted in an initial denaturation step at 94°C for 5 minutes followed by 25 cycles of 35 s at 94°C, 30 s at T_m calculated for each gene (Supplementary data Table T1), 1 minute 30 s at 72°C and a final extension step at 72°C for 7 minutes. As a control, we amplified a *Eh25S rRNA* gene internal sequence which was used to normalize densitometric data. Products were separated by 1% agarose gel electrophoresis, stained with ethidium bromide and visualized by UV light in a Gel Doc 1000 apparatus (BioRad). Densitometric analysis was performed using the Quantity One software. Three independent experiments were done by duplicate. Statistical significance was determined by *T* Student test [31].

2.4. Cloning and Sequencing of *EhVps4* and *EhVps4-E211Q* Genes. The full-length *EhVps4* gene (1260 bp) reported at locus EHI_118900 in *E. histolytica* Pathema database was PCR amplified from *E. histolytica* genomic DNA using the

EhVps4-forward (5'-CCCCGGATCCATGACATCGTTACTTGATAAAGG-3') and *EhVps4*-reverse (5'-CCCCCTCGAGTTATCCATCTTGTCCAAATTGTTC-3') primers in the presence of 0.2 U Pfx DNA polymerase (Invitrogen). Briefly, PCR consisted in an initial denaturation step at 94°C for 5 minutes followed by 28 cycles (30 s at 94°C, 35 s at 55°C, and 1 minute 30 s at 72°C) and a final extension step at 72°C for 7 minutes. The PCR product was cloned into TOPO vector (Invitrogen) yielding the TOPO-*EhVps4* plasmid. Using the QuikChange mutagenesis kit (Stratagene), we generated a point mutation in the EhVps4 ATPase domain at amino acid 211 to replace glutamic acid (E) by glutamine (Q) to obtain the TOPO *EhVps4*-E211Q plasmid. Both constructions were confirmed by automated DNA sequencing. Then, *EhVps4* and *EhVps4*-E211Q genes were PCR amplified from TOPO plasmids and subcloned in the pGEX-6P1 expression vector (Amersham Biosciences) to generate the recombinant pGEX-6P1-*EhVps4* and pGEX-6P1-*EhVps4*-E211Q plasmids, respectively. Constructions were confirmed by automated DNA sequencing.

2.5. Expression and Purification of Recombinant EhVps4-GST and EhVps4-E211Q-GST Proteins. *Escherichia coli* BL21 (DE3) pLysS (Invitrogen) bacteria were transformed with pGEX-6P1-*EhVps4* or pGEX-6P1-*EhVps4*-E211Q plasmids to produce the GST-tagged rEhVps4 and rEhVps4E211Q proteins. Bacteria were grown at 37°C in 2-TY medium containing 100 µg/mL ampicillin and 34 µg/mL chloramphenicol. rEhVps4-GST expression was induced by 1 mM isopropyl beta-D-thiogalactopyranoside (IPTG) for 3 hours at 37°C. Cells were harvested, resuspended in ice cold PBS buffer (140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, and 1.8 mM KH₂PO₄ pH 7.3) and lysed by sonication at 4°C in the presence of lysozyme (1 mg/mL). rEhVps4-GST and rEhVps4-E211Q-GST polypeptides were purified near to homogeneity through glutathione affinity chromatography, according to manufacturer recommendations (Amersham Biosciences). To further use the recombinant protein as antigen, the GST tag (25 kDa) was removed from rEhVps4-GST protein by incubation with 2 U PreScission Protease (Amersham Biosciences) during 12 hours at 4°C. Cleaved rEhVps4 protein was dialyzed into PBS pH 7.4. Identity and integrity of purified rEhVps4-GST, rEhVps4-E211Q-GST and rEhVps4 proteins were confirmed by 10% SDS-PAGE and Western blot assays using anti-GST, (GE-Healthcare, 1 : 5000 dilution) and anti-rEhVps4 (1 : 15000 dilution) antibodies, respectively, and the ECL-Plus Western blotting detection system (Amersham Biosciences).

2.6. Generation of Polyclonal Antibodies against EhVps4. Purified rEhVps4 without GST tag was submitted to preparative 10% SDS-PAGE and electroeluted from Coomassie stained gels. rEhVps4 (200 µg) in complete Freund's adjuvant (Sigma) was subcutaneously inoculated into a New Zealand male rabbit, and then, three doses of 100 µg rEhVps4 in incomplete Freund's adjuvant were injected at 15 days intervals. Rabbit was bled to obtain polyclonal serum.

2.7. ATPase Activity Assay. The ATPase activity assay procedure was based on that previously described [32] using the PiPer Phosphate Assay kit (Invitrogen), with minimal modifications. Briefly, Amplex red was diluted in DMSO to a 10 mM final concentration. Maltose phosphorylase, maltose, glucose oxidase, and horseradish peroxidase were diluted in enzyme buffer (0.1 M Tris-HCl, pH 7.5) to a final concentration of 200 U/mL, 40 mM, 200 U/mL, and 100 U/mL, respectively. Assay buffer (100 mM Tris, 20 mM KCl, and 6 mM MgCl₂, pH 7.4) was added to a 96-well plate. The volume corresponding to 3.5 µg and 5 µg of purified and dialyzed rEhVps4-GST, rEhVps4-E211Q-GST, and rGST proteins was added to wells and enzyme buffer was added up to 27 µL. 50 µL of working solution (2 U/mL glucose oxidase, 4 U/mL maltose phosphorylase, 0.4 mM maltose, 100 µM amplex red, 0.4 U/mL HRP) were added to wells. Controls consisting in enzyme buffer or enzyme buffer with working solution were included. Fresh ATP was diluted in assay buffer to a concentration of 2.5 mM and added to each well to a final volume of 100 µL. Plates were mixed by pipetting and shaken for 30 s to ensure homogeneity. Then, reactions were kept at 25°C for 30 minutes and protected from the light. The absorbance was measured at 562 nm at 30 minutes and data were documented. The purified rGST protein was used as negative control in ATPase assays. Assays were performed twice by triplicate.

2.8. Plasmids Construction for Transfection Assays. The mutant *EhVps4*-E211Q gene and the wild type *EhVps4* gene were PCR amplified from TOPO *EhVps4*-E211Q and TOPO-*EhVps4*, respectively, using the *EhVps4*-S-*KpnI* (5'-CCCCGGTACCATGACATCGTTACTTGATAAAGG-3') and *EhVps4*-AS-FLAG-*BamHI* (5'-CCCCGGATCCTTACTTATCGTCGTCATCCTTGTAATCTCCATCTTGTCCAAATTGTTC-3') primers and cloned into pNEO vector for further transfection assays [33]. The underlined nucleotide sequence corresponds to the FLAG epitope (1 kDa) [34] that was added to the carboxy terminus of recombinant proteins to allow their specific immunodetection in transfected trophozoites. The resulting plasmids named p*EhVps4*-E211Q and p*EhVps4* were confirmed by automated DNA sequencing.

2.9. In Vitro and In Vivo Virulence of Transfected Cells. Trophozoites were transfected with 200 µg of p*EhVps4*-E211Q, p*EhVps4* and control pNEO plasmids by electroporation as described [33]. Briefly, amoebae (10⁷) were washed twice in cold PBS and once in cold complete cytomix buffer. Electroporation was performed with the Bio-Rad Gene Pulser using 1200 V/cm and 25 µF, with a constant time of 0.4 ms. Electroporated cells were transferred into fresh culture medium for 48 hours before selecting them with 10 µg/mL G418.

In vitro virulence of nontransfected and transfected trophozoites (10⁵) was measured on MDCK cells (6 × 10⁴) as described [35, 36]. Rate of erythrophagocytosis was evaluated using trophozoites and RBC in a 1 : 100 relation, at 5, 10, and 15 minutes [37].

In vivo virulence of transfected trophozoites was evaluated as described [38]. Briefly, under sterile conditions and intraperitoneal anaesthesia with 0.2 mg Anesthasal (Pfizer), three groups of six hamsters each were laparotomized and intraperitoneally challenged with p*EhVps4*-E211Q, p*EhVps4*, or p*NEO* transfected trophozoites (2.5×10^6) in a volume of 0.1 mL PBS using a 29-gauge needle. Seven days after challenge, animals were anaesthetized and livers were removed to evaluate hepatic damage.

2.10. Immunodetection of *EhVps4* in Trophozoites. Western blot assays were performed using 30 μ g of total proteins from nontransfected or p*NEO*, p*EhVps4*, and p*EhVps4*-E211Q transfected trophozoites. We used anti-FLAG monoclonal (1 : 700) or anti-r*EhVps4* polyclonal (1 : 15000) antibodies and rabbit anti-mouse and goat anti-rabbit IgG horseradish peroxidase-labeled secondary antibodies (Zymed; 1 : 10000), respectively. Immunodetected proteins were revealed with the ECL Plus Western blotting system (Amersham Biosciences). As an internal control, we used monoclonal antibodies raised against *E. histolytica* actin (1 : 1000) and goat anti-mouse IgG horseradish peroxidase-labeled secondary antibodies (Zymed; 1 : 10000).

For confocal microscopy assays, nontransfected and transfected trophozoites were grown on coverslips, fixed with 4% paraformaldehyde at 37°C for 1 hour, permeabilized with Triton X-100 and blocked with 1% bovine serum albumin in PBS. Then, nontransfected and transfected cells were incubated with polyclonal anti-r*EhVps4* (1 : 5000) or monoclonal anti-FLAG primary antibodies (Sigma; 1 : 500), respectively, at 37°C for 2 hours, followed by incubation with anti-rabbit or anti-mouse fluoresceinated (Zymed; 1 : 100) monoclonal antibodies, at 37°C for 1 hour. Next, trophozoites were washed three times with PBS 1X at room temperature and DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI) (5 μ g/mL) for 5 minutes. To immunolocalize *EhVps4* during erythrophagocytosis, p*EhVps4* transfected cells were incubated with RBC (1 : 20) for 10 minutes. Ingested RBC were stained with diaminobenzidine (0.84 mM 3,3'-diaminobenzidine, 0.048% H₂O₂ and 50 mM Tris-HCl, pH 9.5) for 5 minutes and cells were incubated with polyclonal anti-r*EhVps4* (1 : 5000) to be processed for immunofluorescence as described above. Light optical sections (1.5 μ m) were obtained through a Leica inverted microscope attached to a laser confocal scanning system TCS-SP2 (Leica).

3. Results

3.1. *E. histolytica* Genome Contains Homologous ESCRT Genes That Are Expressed in Trophozoites. To investigate the presence of ESCRT-related genes in *E. histolytica*, we surveyed the parasite genome sequence at Pathema database using the amino acid sequences of yeast and human ESCRT proteins as queries. We detected a set of 20 putative ESCRT protein-encoding genes, which exhibited significant *e*-values ($1.1e - 114$ to 0.00032) and high similarity (20 to 62%) to yeast and human ESCRT (ESCRT 0-III and associated factors) orthologues (see Table 1). However, we

did not find homologues for yeast Mvb12, and Bullp protein involved in protein ubiquitination, neither human Vps37-B and Vps37-C proteins. Phylogenetic inference revealed that *E. histolytica* ESCRT predicted proteins are closely related to homologous proteins from other protozoa, such as *Giardia lamblia*, *Tetrahymena thermophila*, *Trichomonas vaginalis*, *Plasmodium vivax*, *Cryptosporidium homini*, and *Trypanosoma cruzi* (data not shown). Bioinformatics analysis showed that *E. histolytica* ESCRT predicted proteins have the most characteristic functional domains (see Supplementary data Figure S1) and tertiary structure folding described for yeast and human ESCRT factors (data not shown).

To determine whether the putative *E. histolytica* ESCRT genes were expressed, we performed RT-PCR assays for the 16 most conserved genes. In basal culture conditions, 15 genes were transcribed, but the *EhVps22* transcript was not detected in these experiments (see Figures 1(a) and 1(b)). We also performed RT-PCR assays using RNA obtained from trophozoites incubated with RBC for 5 minutes to investigate whether erythrophagocytosis has an effect on ESCRT gene expression (see Figures 1(a) and 1(b)). In these assays, we found that all genes were expressed during erythrophagocytosis at similar levels as in basal conditions. Again, we did not detect the *EhVps22* gene expression. Interestingly, *EhVps23* and *Ehadh112* were 2- and 3-fold up-regulated, respectively. By densitometric analysis, the band given by the amplified *Eh25S rRNA* product in both conditions was taken as 100% and used to normalize ESCRT genes data.

3.2. The Predicted *EhVps4* Protein Conserves the Typical Architecture of *Vps4* Homologues. Since *Vps4* orthologues have been described as key molecules for the dissociation of ESCRT, we first focused on the characterization of the *E. histolytica* *EhVps4* protein. The predicted *EhVps4* contains the N-terminal MIT (microtubule interacting and transport), AAA (ATPase associated with a variety of activities) and *Vps4* C-terminal domains (see Figure 2(a)) that are characteristic and essential for *Vps4* proteins biological functions [39, 40]. Phylogenetic trees revealed that *EhVps4* is more related to protozoan *Vps4* than orthologous proteins from higher eukaryotes (see Figure 2(b)). Interestingly, 3D modeling of *EhVps4*, using the crystal structure of MIT and AAA domains from yeast *Vps4p* and human *Vps4B* as templates (see Figure 2(c)), showed that *EhVps4* region spanning the N-terminal MIT and AAA ATPase domains exhibited a similar folding with the conserved *Vps4* α -helices.

3.3. Expression and Immunodetection of *EhVps4* Protein in Trophozoites. We produced and purified the recombinant *EhVps4* protein as a GST-tagged fusion polypeptide (r*EhVps4*-GST) in *E. coli* (see Figure 3(a), lanes 3, 4). By Western blot assays using commercial anti-GST monoclonal antibodies, we immunodetected the purified r*EhVps4*-GST as a 72 kDa protein in IPTG-induced bacteria (see Figure 3(b), lane 2) but not in protein extracts from noninduced bacteria (see Figure 3(b), lane 1). The r*EhVps4*-GST was digested with the PreScission Protease to obtain the r*EhVps4*

TABLE 1: Comparison of *Entamoeba histolytica*, *Homo sapiens*, and *Saccharomyces cerevisiae* ESCRT machineries.

Putative complex	Predicted protein	<i>Entamoeba histolytica</i>					<i>Homo sapiens</i>					<i>Saccharomyces cerevisiae</i>						
		Accession number ^a	Protein	Accession number ^b	e-value	S (%)	I (%)	Protein	Accession number ^a	e-value	S (%)	I (%)	Protein	Accession number ^a	e-value	S (%)	I (%)	
ESCRT-0 complex	EhHse1	EHL_091530	STAM1	Q92783	9e-08	72	50	Hse1p	P38753	2e-10	70	62						
	EhVps27	EHL_117910	HRS	O14964	—	—	—	Vps27p	P40343	1e-11	61	49						
	EhVps23	EHL_135460	TSG101	Q99816	1.4e-10	51	33	Vps23p	P25604	0.0052	31	25						
ESCRT-I complex	EhVps28	EHL_108630	hVps28	Q9UK41	—	—	—	Vps28p	Q02767	0.00057	45	28						
	EhVps37A	EHL_077870	hVps37A	Q6NWX7	0.00022	55	24	Vps37p	Q99176	0.00084	46	21						
	EhVps37D	EHL_060400	hVps37D	Q6P2C3	3.8e-06	50	35	nd	—	—	—	—						
	nd	—	nd	—	—	—	—	Mvb12	P42939	—	—	—						
ESCRT-II complex	EhVps22	EHL_131120	EAP30	Q96H20	9e-22	47	30	Vps22p	Q12483	3.4e-15	48	25						
	EhVps25	EHL_137860	EAP20	Q9BRG1	9e-08	52	28	Vps25p	P47142	0.00038	46	19						
	EhVps36	EHL_045320	EAP45	Q86VN1	1.8e-15	49	25	Vps36p	Q06696	1.1e-09	48	26						
ESCRT-III complex	EhVps2	EHL_194400	CHMP2A	O43633	8.9e-24	55	29	Vps2p	P36108	2e-05	50	29						
	EhVps20	EHL_066730	CHMP2B	Q9UQN3	7.9e-16	48	25	—	—	—	—	—						
	EhVps24	EHL_048690	CHMP6	Q96FZ7	—	—	—	Vps20p	Q04272	0.00015	54	26						
	EhVps32	EHL_169820	CHMP3	Q9Y3E7	—	—	—	Vps24p	P36095	2.2e-05	48	22						
		—	—	CHMP4A	Q9BY43	0.0012	48	24	Vps32p	P39929	2.5e-12	48	25					
Vps4	EhVps4	EHL_118900	CHMP4B	Q9H444	9.6e-07	43	20	nd	—	—	—	—						
	—	—	CHMP4C	Q96CF2	1.4e-06	43	20	nd	—	—	—	—						
Other MVB proteins	EhVta1	EHL_010040	hVps4A	Q9UN37	1.1e-114	69	52	Vps4p	P52917	3e-114	78	60						
	EhVps46	EHL_093850	hVps4B	O75351	3e-114	78	60	—	—	—	—	—						
	EhDoa4	EHL_012290	hVta1	Q9NP79	2.8e-05	44	23	Vta1p	Q06263	—	—	—						
Ubiquitination components	EhADH112	EHL_181220	CHMP1A	Q9HD42	—	—	—	Vps46p	P69771	0.00032	48	18						
	nd	—	CHMP1B	Q7LBR1	—	—	—	nd	—	—	—	—						
	EhRsp5	EHL_011530	CHMP5	Q9NZZ3	5.6e-15	45	28	Vps60p	Q03390	9e-06	46	19						
	—	—	UBP4	Q13107	1e-77	58	36	Doa4p	P32571	2e-27	50	31						
Ubiquitination components	nd	—	ALIX	Q9UKL5	1e-21	40	20	Rim20p	Q12033	2e-12	38	21						
	EhRsp5	EHL_011530	NEED4	P46934	1.6e-50	53	33	BRO1p	P48582	0.003	50	32						
Ubiquitination components	nd	—	nd	—	—	—	—	Bullp	P48524	—	—	—						
	EhRsp5	EHL_011530	NEED4	P46934	1.6e-50	53	33	Rsp5p	P39940	7.8e-57	57	36						

^aPathema Entamoeba Bioinformatics Resource Center. ^bSwiss-Prot/TrEMBL databases. nd: not determined. —: Denote nonsignificant similarity/identity and e-values. S: Similarity. I: Identity.

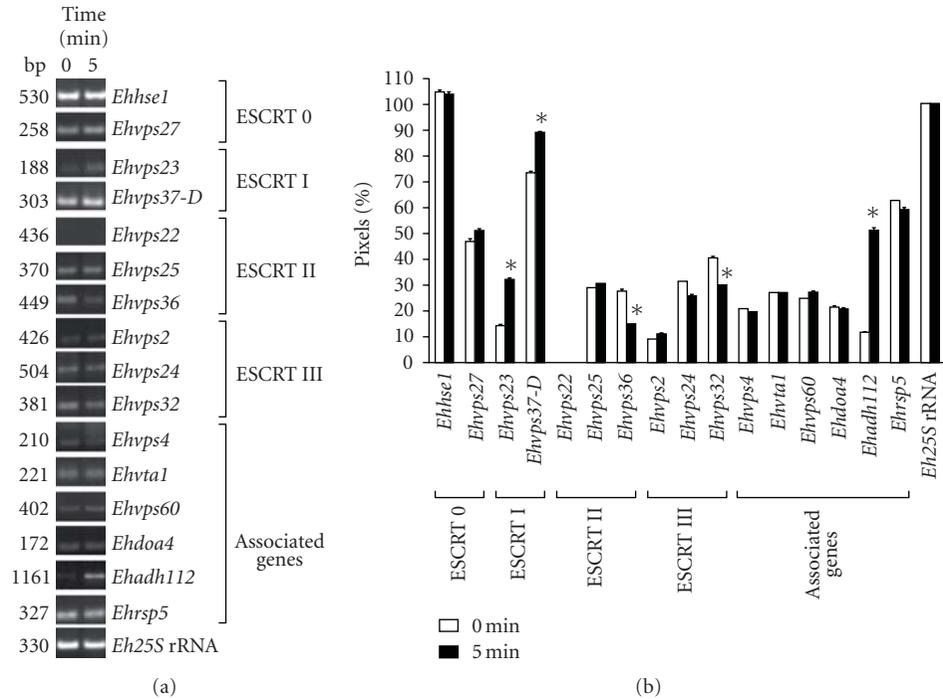


FIGURE 1: mRNA expression profile of *E. histolytica* putative ESCRT machinery genes. (a) RT-PCR products obtained from 1 μ g of total RNA from trophozoites growing in TYI-S-33 medium (0 minute) or after 5 minutes of erythrophagocytosis. (b) Densitometric analysis of RT-PCR products in (a). Pixels corresponding to *Eh25S rRNA* amplified product were taken as 100% in each lane. Data represent the mean of three independent experiments performed by duplicate for each gene. Asterisk, genes whose transcription is significantly changed according to *T* Student test.

untagged protein (47 kDa) (see Figure 3(c), lanes 2 and 3), which was also purified (see Figure 3(c), lane 4) and used to generate rabbit polyclonal antibodies. Anti-rEhVps4 serum recognized the untagged rEhVps4 protein, confirming the antibodies specificity (see Figure 3(d)). In addition, the same antibodies detected a single 47 kDa band in *E. histolytica* protein extracts (see Figure 3(e), lane 1), which corresponds to the expected molecular weight for the predicted EhVps4 polypeptide amino acid sequence. Antibodies did not detect any other AAA ATPases predicted in the *E. histolytica* genome [41]. Pre-immune serum, used as a control, did not recognize any band in trophozoite extracts (see Figure 3(e), lane 2). Through immunofluorescence and laser confocal microscopy assays, the specific rabbit polyclonal antibodies revealed EhVps4 as abundant small dots dispersed in the cytosol (see Figure 3(f)).

3.4. ATPase Activity Assays. To investigate whether the recombinant protein exhibited ATPase activity we used the rEhVps4 purified protein and a mutant version (EhVps4-E211Q) in which we changed the E211 residue of the AAA motif, essential for enzyme activity, by a Q residue (see Figure 4(a)) [18, 42]. Reactions were carried out using the PiPer Phosphate Assay Kit (Invitrogen) in the presence of 500 μ M ATP. Inorganic phosphate (Pi) release was measured spectrophotometrically at A_{562} . At zero time, absorbance values of samples containing rEhVps4-GST, rEhVps4-E211Q-GST and control rGST purified proteins (see Figure 4(b)) were

similar and they were taken as background. At 30 minutes, A_{562} values increased to 0.115 ± 0.008 and 0.162 ± 0.022 , using 3.5 μ g and 5 μ g of rEhVps4-GST protein, respectively. No significant A_{562} increase was detected when we used rEhVps4-E211Q-GST or rGST proteins at 30 minutes (see Figure 4(c)). These results showed that rEhVps4-GST has ATPase activity and that the AAA motif is essential for ATP hydrolysis.

3.5. Generation of Trophozoites Overexpressing the EhVps4-FLAG and Mutant EhVps4-E211Q-FLAG Proteins. To investigate the role of EhVps4 in *E. histolytica*, we generated transfectant trophozoites that overexpress the EhVps4 and the mutant EhVps4-E211Q FLAG-tagged proteins (see Figure 5(a)). Trophozoites transfected with pNEO vector were used as control in these experiments. Viability of transfected trophozoites was up to 90% as determined by trypan blue exclusion assays.

RT-PCR assays, using specific primers for *Ehvps4* gene, showed that trophozoites transfected with pEhVps4 or pEhVps4-E211Q plasmids expressed a higher amount of the *Ehvps4* transcript in comparison to pNEO transfected cells (see Figure 5(b), upper panel, lanes 1, 2, and 3). As control, we amplified the *Eh25S rRNA* transcript, which showed minimal changes among the different transfectants studied (see Figure 5(b), lower panel).

By Western blot assays, using the anti-FLAG antibodies, we detected the EhVps4-FLAG and EhVps4-E211Q-FLAG

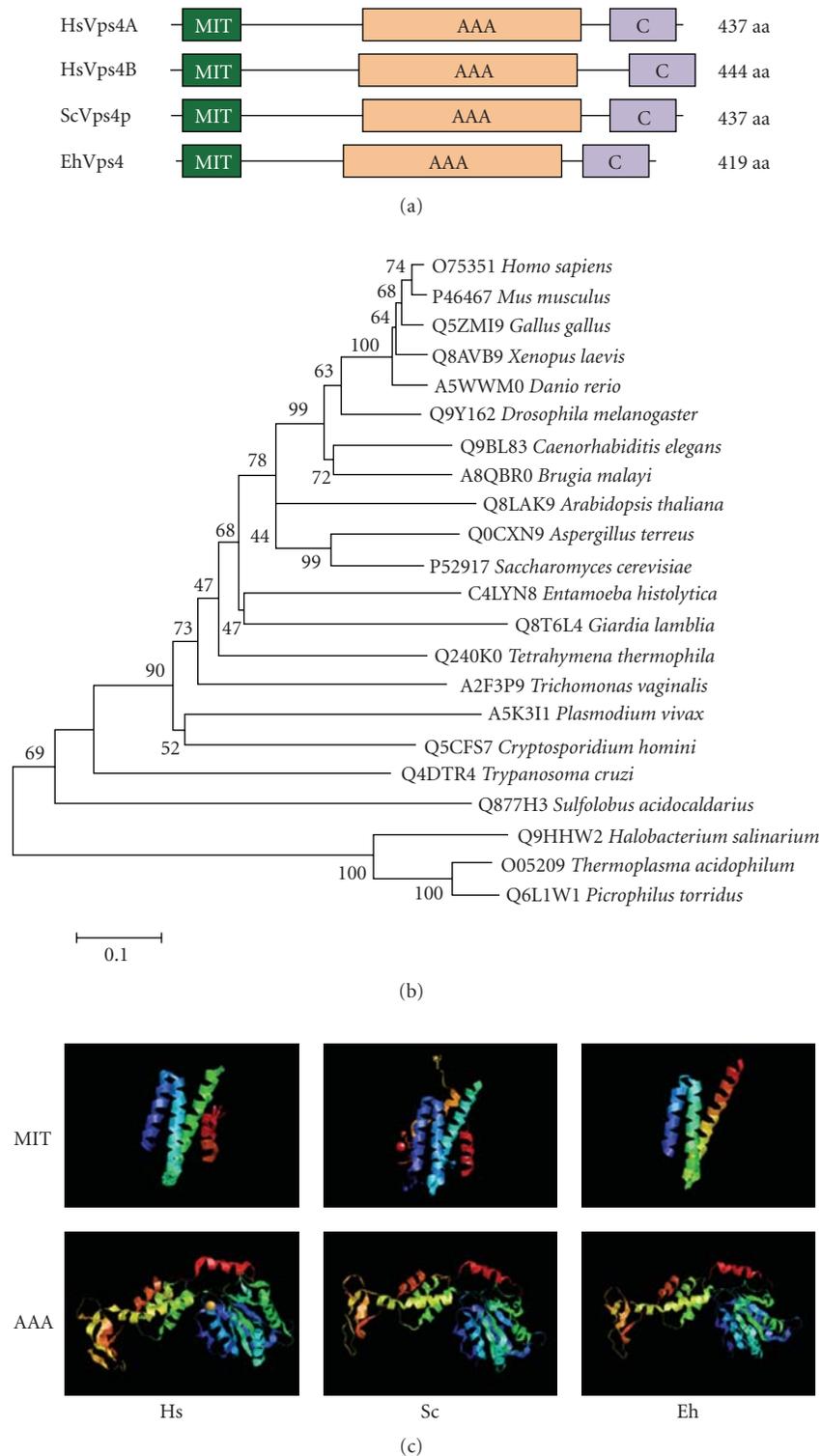


FIGURE 2: Comparison of EhVps4 with other Vps4 proteins. (a) Schematic representation of Vps4 proteins from human, yeast and *E. histolytica*. Conserved MIT, AAA, and Vps4 C-terminal (C) domains were predicted using PROSITE and Pfam programs. (b) Phylogenetic relationships between EhVps4 and homologous proteins from other organisms. The phylogenetic tree of Vps4 homologues was created with the MEGA 3.1 program using the Neighbor Joining algorithm based on ClustalW alignments of complete amino acids sequences. Numbers on tree nodes represent the bootstrap proportions (%) of 1000 replications. The UniProt KnowledgeBase database accession number for each protein is indicated before the organism. (c) Comparison of 3D structures of MIT and AAA domains from human (*Hs*), yeast (*Sc*) and *E. histolytica* (*Eh*) Vps4 homologues. 3D modeling of EhVps4 MIT and AAA domains was obtained using crystal data from yeast Vps4p MIT (2v6xA) and AAA (2qpAB) domains as template, respectively.

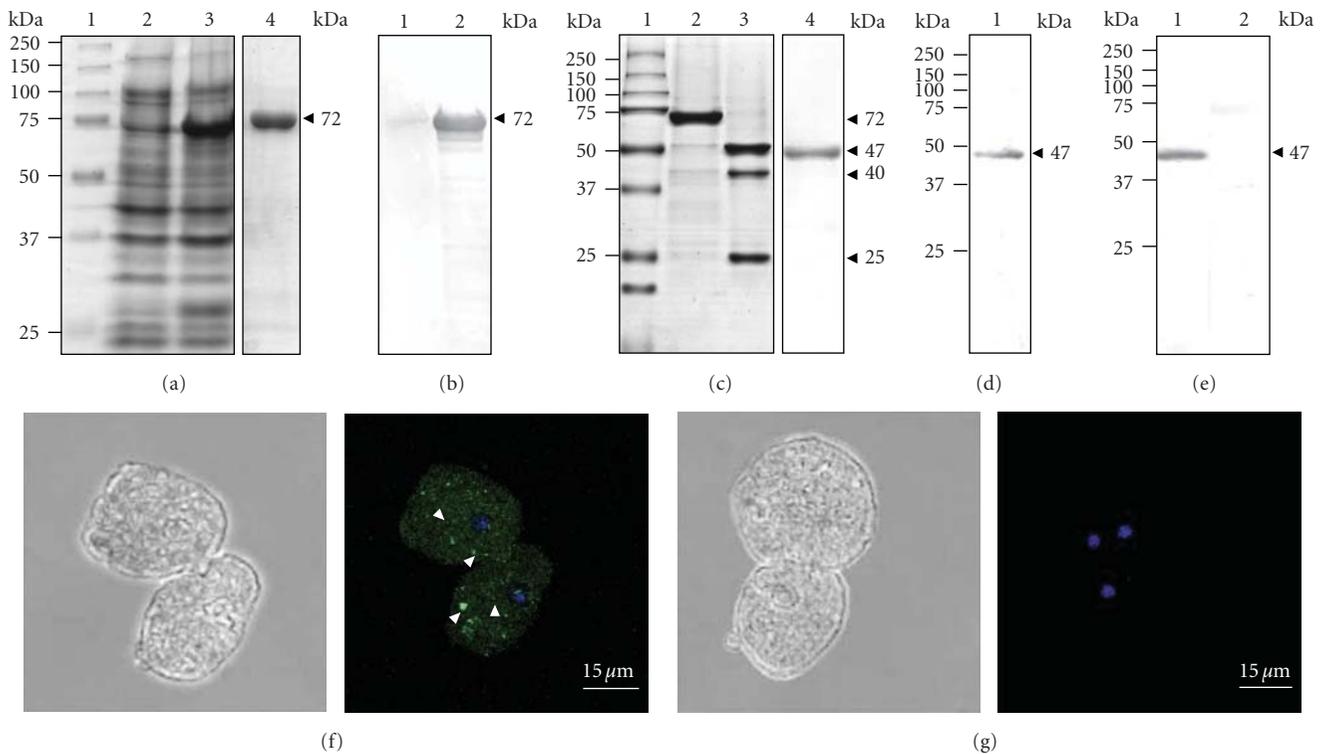


FIGURE 3: Expression and immunodetection of EhVps4. (a) Expression and purification of recombinant EhVps4-GST polypeptide. Proteins were separated through 10% SDS-PAGE and gels were stained with Coomassie blue. Lane 1, molecular weight markers; lane 2, noninduced bacteria extract; lane 3, IPTG-induced bacteria extract; lane 4, affinity purified rEhVps4-GST from IPTG-induced bacteria extract. (b) Immunodetection of rEhVps4-GST. Western blot assays were performed using noninduced bacteria extract (lane 1) and IPTG-induced bacteria extract (lane 2), with anti-GST antibodies. (c) PreScission Protease digestion of rEhVps4-GST revealed by Coomassie blue stained gels. Lane 1, molecular weight markers; lane 2, purified rEhVps4-GST protein; lane 3, rEhVps4-GST digested with PreScission Protease; lane 4, purified rEhVps4 protein. (d) Immunodetection of purified rEhVps4 protein by Western blot assays using specific anti-rEhVps4 antibodies (lane 1). (e) Immunodetection of EhVps4 in total extracts of trophozoites using specific anti-rEhVps4 antibodies (lane 1) and preimmune serum (lane 2). Proteins are indicated by arrowheads. (f) and (g) Cellular localization of EhVps4 in trophozoites. Trophozoites of clone A were incubated with rabbit anti-rEhVps4 (f) or preimmune (g) serum, treated with FITC-labeled secondary antibodies, counterstained with DAPI and analyzed through confocal laser microscopy. Left, cells observed in phase contrast; right, merge (trophozoites observed in the green (FITC) and blue (DAPI) channels). Arrowheads, EhVps4 signal in small cytoplasmic dots.

proteins in p*EhVps4* and p*EhVps4*-E211Q transfectant cells, whereas no signal was detected in p*NEO* transfectants, as expected (see Figure 5(c), upper panel). However, specific anti-rEhVps4 antibodies recognized endogenous EhVps4 protein in p*NEO* transfectants (see Figure 5(c), middle panel, lane 1), meanwhile signal increased 2.8- and 9.2-fold in p*EhVps4* and p*EhVps4*-E211Q transfectant cells, respectively (see Figure 5(c), middle panel, lanes 2, and 3). Anti-actin antibodies detected similar amounts of protein in the different transfectants (see Figure 5(c), lower panel). For densitometric analysis, actin band was taken as 100% in each lane and used to normalize EhVps4, EhVps4-FLAG, and EhVps4-E211Q-FLAG data (see Figure 5(d)).

3.6. Cellular Localization of EhVps4-FLAG and EhVps4-E211Q-FLAG Proteins in Transfected Trophozoites. To investigate the cellular localization of exogenous wild type EhVps4 and mutant EhVps4-E211Q FLAG-tagged proteins in transfected trophozoites, we performed immunofluorescence assays using anti-FLAG antibodies (see Figure 6).

In p*EhVps4* transfected trophozoites, anti-FLAG antibodies detected the overexpressed exogenous protein diffuse in the cytoplasm and at the plasma membrane (see Figure 6(d)). In contrast, in mutant p*EhVps4*-E211Q transfected cells, signal was not observed at the plasma membrane, but it was present as cytoplasmic structures of varying sizes (see Figure 6(f)). As expected, no signal was detected by anti-FLAG antibodies in p*NEO* transfected cells (see Figure 6(b)). Control assays without anti-FLAG primary antibodies did not show any signal in transfected trophozoites (see Figure 6(h)).

3.7. Cytopathic Activity, Erythrophagocytosis and Liver Abscesses Formation Are Impaired in Trophozoites Overexpressing the Mutant EhVps4-E211Q-FLAG Protein. To initiate the phenotypical characterization of trophozoites transfected with p*EhVps4* or p*EhVps4*-E211Q constructs, we first evaluated their cytopathic activity and rate of erythrophagocytosis. Nontransfected clone A and p*NEO* transfected cells were used as controls in these experiments. After 1 hour interaction of MDCK cells with trophozoites

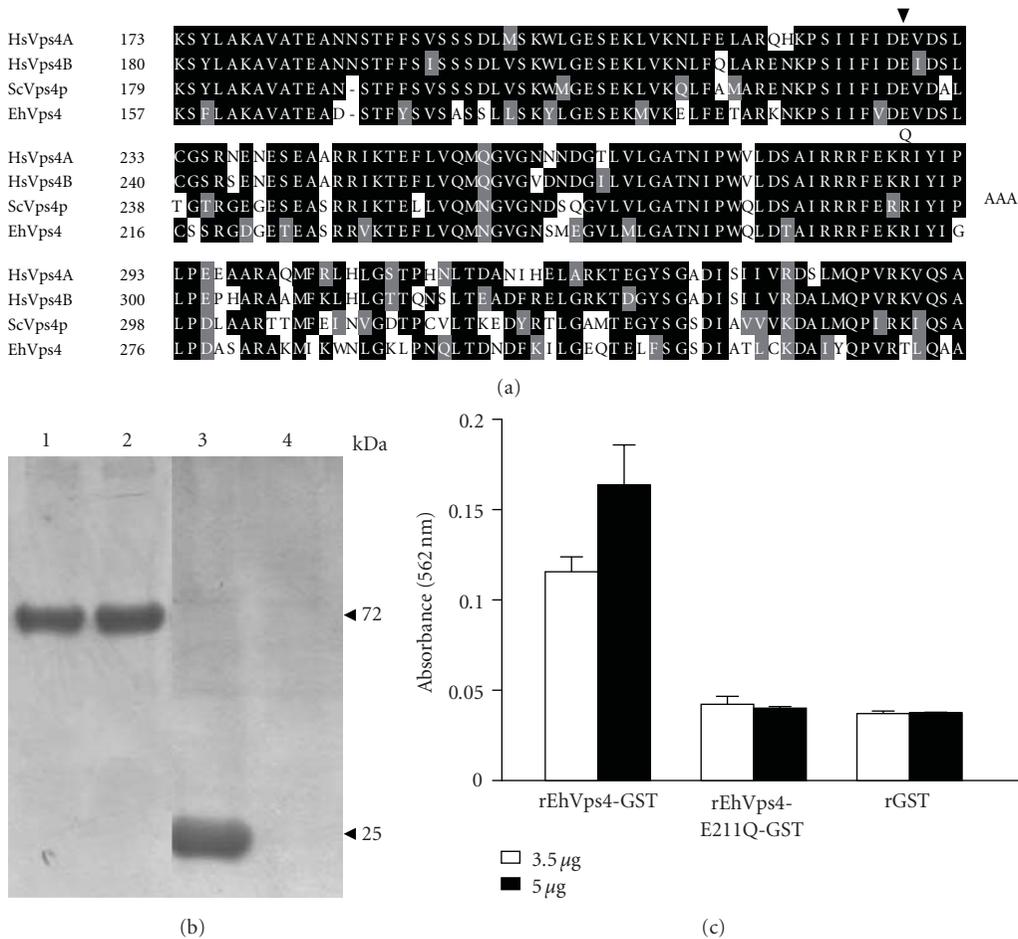


FIGURE 4: Measurement of ATPase activity of recombinant EhVps4. (a) Multiple sequence alignment of Vps4 AAA domain from *Homo sapiens* (HsVps4A and HsVps4B), *S. cerevisiae* (ScVps4p) and *E. histolytica* (EhVps4). Black boxes, identical amino acid (aa); grey boxes, conserved substitutions; open boxes, different aa. Numbers at the left are relative to the position of the initial methionine in each protein. Arrowhead indicates the position of the conserved E residue. (b) Purification of recombinant proteins. Proteins were expressed in *E. coli*, purified through affinity chromatography, separated through 10% SDS-PAGE and stained with Coomassie blue. Lane 1, rEhVps4-GST; lane 2, rEhVps4-E211Q-GST; lane 3, rGST; lane 4, purified fraction from noninduced bacteria extract. (c) ATPase assay. ATPase activity of rEhVps4-GST and rEhVps4-E211Q-GST was monitored as described in Section 2.7. rGST was included as control. Data are the mean of three independent assays.

of clone A, pNEO or pEhVps4 transfected trophozoites, monolayers presented in mean $86.8 \pm 13.2\%$ destruction. Interestingly, pEhVps4-E211Q transfected cells were less efficient to destroy MDCK cell monolayers, since they only produced $46.4 \pm 6.4\%$ cell destruction (see Figure 7(a)).

The rates of erythrophagocytosis of non-transfected clone A, pNEO and pEhVps4 transfected cells were similar, at each time tested. In contrast, pEhVps4-E211Q transfected trophozoites showed 60, 55%, and 57% decrease in erythrophagocytosis rate at 5, 10, and 15 minutes, respectively, when compared with the mean rate from the other trophozoites (see Figure 7(b)).

Then, we investigated the capacity of pEhVps4 and pEhVps4-E211Q transfected trophozoites to induce hepatic abscesses formation in hamsters. None of the six hamsters that were infected with pEhVps4-E211Q transfected trophozoites presented hepatic damage (see Figures 7(c)), whereas

the six animals infected with pNEO and pEhVps4 transfected trophozoites developed extensive abscesses.

3.8. EhVps4 Was Detected around Ingested RBC. To determine the localization of EhVps4 in pEhVps4 transfected trophozoites during erythrophagocytosis, we performed immunofluorescence assays with anti-rEhVps4 serum. Antibodies detected EhVps4 as a signal surrounding ingested erythrocytes, suggesting that this protein may be involved in phagocytosis (see Figure 8).

4. Discussion

In *E. histolytica* trophozoites, phagocytosis, and vesicular trafficking are important events for parasite nutrition and destruction of target cells. As in other eukaryotes, the endocytosis and phagocytosis pathways involve the formation of

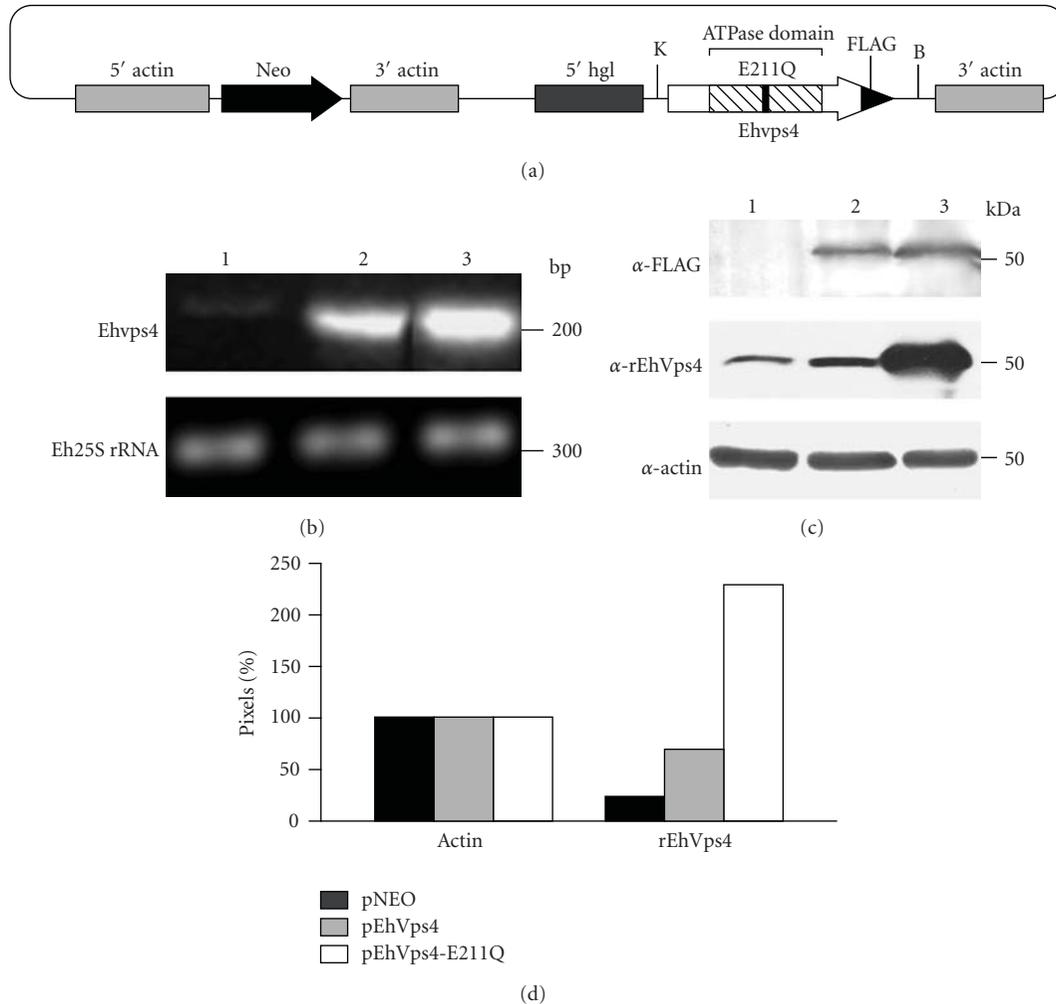


FIGURE 5: Expression of exogenous EhVps4 in transfected trophozoites. (a) Schematic representation of *pEhVps4-E211Q* construct. Mutant *EhVps4* gene was cloned into the *KpnI* and *BamHI* sites of *pNEO* vector, upstream the sequence coding for FLAG epitope. B, *BamHI* site; K, *KpnI* site. (b) 2% agarose gel with RT-PCR assays products. An internal fragment of the *EhVps4* gene (upper panel) or the *Eh25S rRNA* gene (bottom panel) was RT-PCR amplified using 1 µg of total RNA from trophozoites of clone A transfected with *pNEO* (lane 1), *pEhVps4* (lane 2) or *pEhVps4-E211Q* (lane 3) plasmid. (c) Western blot assays. Anti-FLAG (upper panel), anti-rEhVps4 (middle panel) and anti-actin (lower panel) antibodies were used to analyze protein lysates (30 µg) from *pNEO* (lane 1), *pEhVps4* (lane 2) and *pEhVps4-E211Q* (lane 3) transfected cells. (d) Densitometric analysis of bands corresponding to actin and EhVps4 in (c). Pixels corresponding to actin control were taken as 100% in each lane and used to normalize EhVps4 data.

endosomes, MVB and lysosomes [5, 43]. Indeed, MVBl-like structures have been detected in trophozoites [26, 27] and within isolated phagosomes [24], but proteins participating in their formation have not been identified.

Here, by mining the parasite genome sequence, we demonstrated the presence of 20 genes that encode putative ESCRT machinery components, which could participate in endosomal transport and MVB formation in *E. histolytica*. Most predicted proteins have the conserved functional domains and share high similarity and phylogenetic relationship with homologous proteins from other eukaryotes, strongly suggesting that they have similar functions. RT-PCR assays evidenced that 15 out of 16 ESCRT components tested are transcribed, which is consistent with the activity of endosome formation and vesicle trafficking exhibited

by *E. histolytica* trophozoites (reviewed in [5, 43]). Except for the *Ehadh112* and *EhVps23* genes, transcript amount was not significantly modified after 5 minutes of RBC interaction. Analysis of published *E. histolytica* microarrays data confirmed that most ESCRT genes are transcribed without any significant change under distinct experimental conditions [44, 45]. However, these experiments were not focused on phagocytosis. Additionally, in *Dictyostelium discoideum*, microarrays assays evidenced that expression of most genes involved in intracellular vesicle traffic is not significantly changed during phagocytosis [46]. In mouse macrophages, phagocytosis causes cellular redistribution of Hrs protein (the homologue of yeast Vps27p), but the protein amount remains unchanged [47]. It is possible that trophozoites could have enough ESCRT and associated

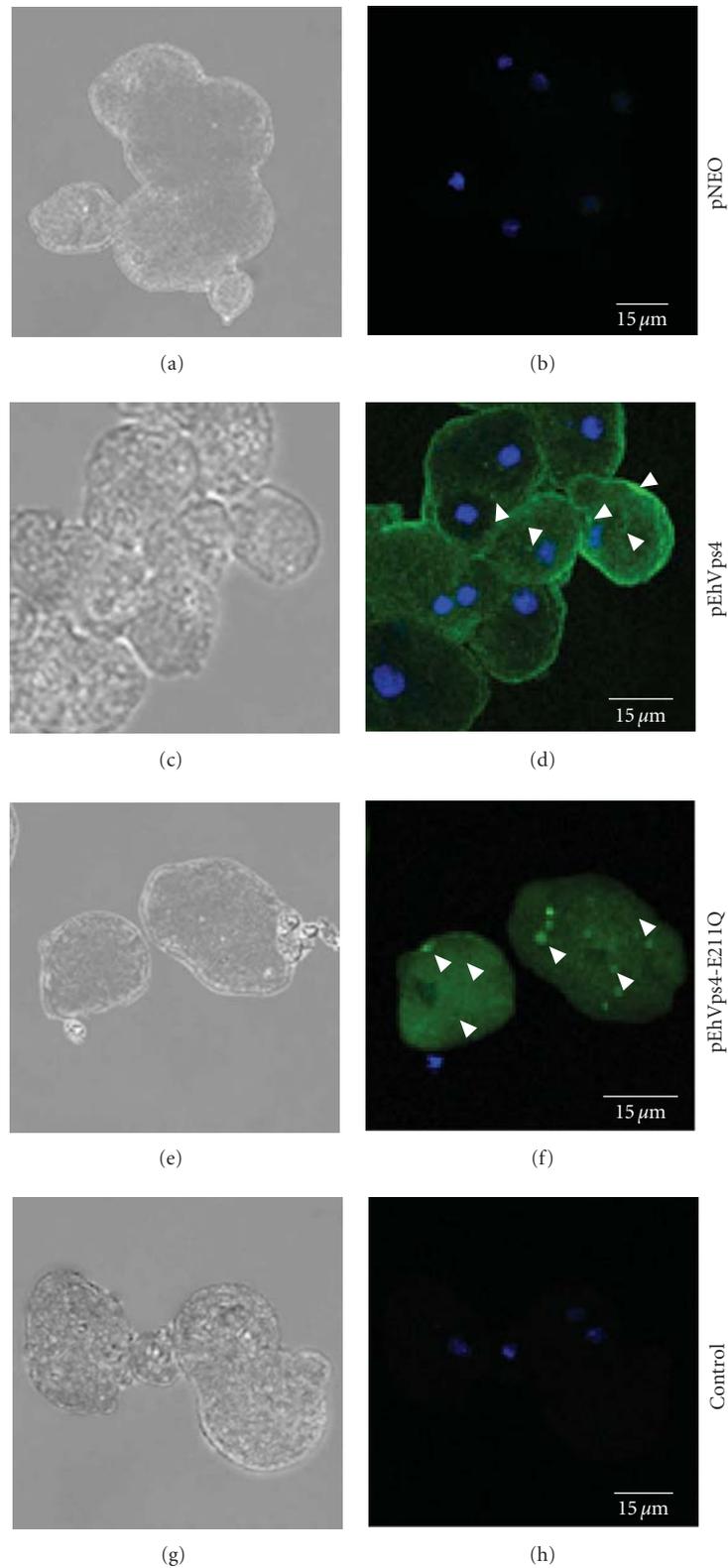


FIGURE 6: Cellular localization of exogenous EhVps4 in transfected trophozoites. Trophozoites transfected with *pNEO* ((a) and (b)), *pEhVps4* ((c) and (d)) and *pEhVps4-E211Q* ((e) and (f)) were incubated with mouse anti-FLAG antibodies, treated with FITC-labeled secondary antibodies, counterstained with DAPI and analyzed through confocal laser microscopy. *pEhVps4-E211Q* transfected trophozoites incubated with FITC-labeled secondary antibodies were used as control ((g) and (h)). (a), (c), (e) and (g) Cells observed in phase contrast; (b), (d), (f) and (h) Merge, trophozoites observed in the green (FITC) and blue (DAPI) channels. Arrowheads, EhVps4 signal in plasma membrane and cytoplasmic dots.

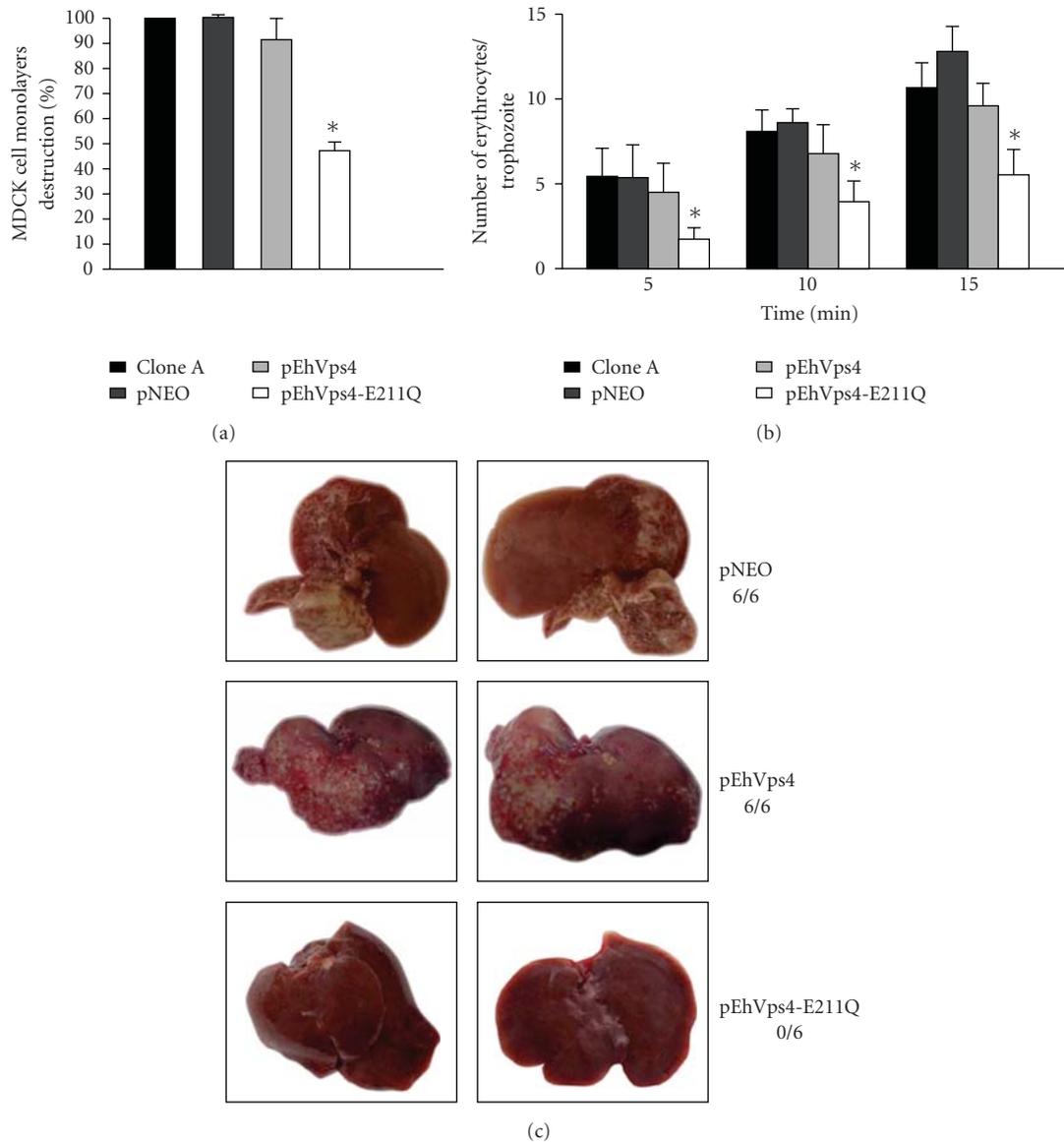


FIGURE 7: Virulence assays of transfected trophozoites. (a) Cytopathic activity. The destruction of MDCK cells monolayers by clone A trophozoites, pNEO, pEhVps4, or pEhVps4-E211Q transfected cells was determined as described [32, 33]. (b) Erythrophagocytosis. Rate of phagocytosis of clone A trophozoites, pEhVps4, pEhVps4-E211Q or pNEO transfected cells was evaluated at 5, 10, and 15 minutes [34]. Histograms show the mean count \pm SD of three independent experiments by duplicate. (c) Hepatic damage in hamsters infected with transfected trophozoites. Three groups of six hamsters were infected with pNEO, pEhVps4, or pEhVps4-E211Q transfected trophozoites. After 7 days, animals were sacrificed and liver damage was recorded. Pictures were taken from the liver side where the abscesses appeared to be larger and are representative for each group. Upper panels, livers from hamsters infected with pNEO transfected trophozoites (control); middle panels, livers from hamsters infected with pEhVps4 transfected trophozoites; lower panels, livers from hamsters infected with pEhVps4-E211Q transfected trophozoites. 6/6, 6/6 and 0/6 denote number of infected animals/number of inoculated animals.

proteins to perform erythrophagocytosis, if, as we think, these proteins are involved in phagocytosis in *E. histolytica*. However, changes in the gene expression of *E. histolytica* ESCRT members at shorter or larger times cannot be discarded.

In yeast and mammal cells, Vps4 is a key component for disassembly of ESCRT complexes from the endosomal membrane at the MVB invagination pathway [42, 48, 49]. We focused on EhVps4 protein to investigate its role in

endocytosis, specifically in one of the “professional function” of *E. histolytica* trophozoites: phagocytosis. As in yeast, *E. histolytica* has only one *vps4* gene, while higher eukaryotes have two *vps4* genes [21, 50, 51]. By confocal microscopy, EhVps4 was immunodetected as abundant small dots dispersed in the cytosol. In yeast, mammals, plants, and the protozoan *Leishmania major*, similar structures have been identified as MVB formed during endocytosis and vesicular trafficking [22, 42, 51, 52].

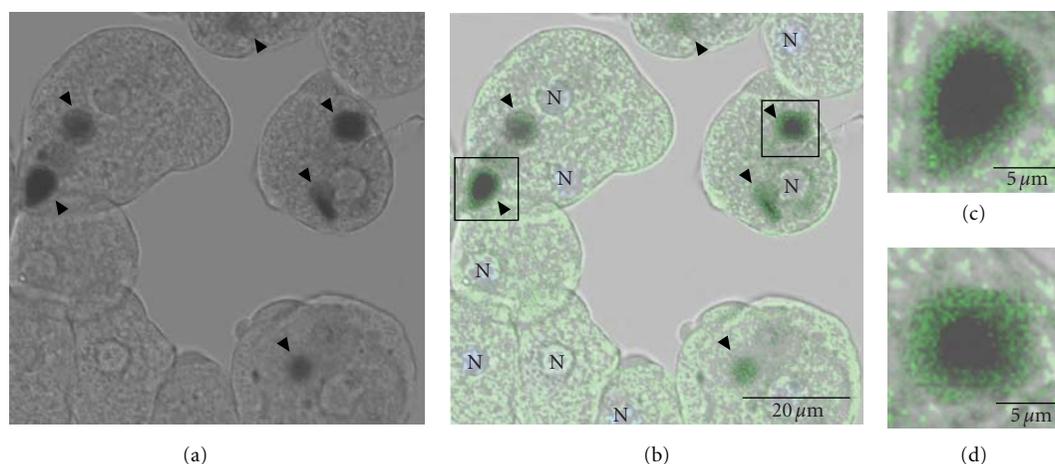


FIGURE 8: Cellular localization of EhVps4 protein during erythrophagocytosis. Trophozoites transfected with p*EhVps4* were incubated with RBC, treated with diaminobenzidine, anti-rEhVps4 and FITC-labeled secondary antibodies, and analyzed through confocal laser microscopy. (a) Cells observed in phase contrast. (b) Trophozoites observed in the green (FITC) channel and phase contrast. (c) Magnification of diaminobenzidine stained RBC squared in (b). Arrowhead, EhVps4 signal around RBC. N, nuclei.

Yeast Vps4p protein exhibits ATPase activity, which depends on the E233 residue present within the AAA domain [18, 42]. In this work, we showed that EhVps4 is an ATPase, which conserves the characteristic domains and folding of Vps4 homologues. This activity depends on the AAA domain since the mutant rEhVps4-E211Q-GST, in which E211 amino acid residue was substituted by Q residue, did not exhibit detectable ATP hydrolysis, as reported in yeast [18, 42].

We further investigated the biological relevance of EhVps4 by generating trophozoites that overexpress wild type EhVps4-FLAG and mutant EhVps4-E211Q-FLAG protein. As the endogenous protein, exogenous EhVps4-FLAG was located in abundant punctuate structures in the cytosol as reported in other organisms [42, 50, 52, 53]. However, overexpressed EhVps4-FLAG was also detected at plasma membrane. In transfected mammal cells that overexpress Sendai virus protein C and wild type Vps4, ALIX/AIP1 proteins are recruited at the plasma membrane to facilitate the budding of virus-like particles [54]. EhADH112, the *E. histolytica* homologue of ALIX, has been detected at the plasma membrane [55]. Experiments currently in progress will help us to define the role of Vps4 at the membrane of trophozoites.

Erythrophagocytosis and cytopathic activities were not improved in trophozoites overexpressing wild type EhVps4-FLAG, probably because other ESCRT proteins are limiting factors for cell destruction and RBC intake [42, 52, 56]. However, the dominant negative effect of mutant EhVps4-E211Q-FLAG protein in MDCK cells destruction, the rate of RBC intake, and liver abscesses formation in hamsters, suggest a role for EhVps4 in *E. histolytica* virulence properties. The localization of EhVps4-FLAG protein around ingested RBC strengthens this hypothesis.

In conclusion, we showed that *E. histolytica* has an ESCRT machinery, which is transcribed in trophozoites. Particularly, we presented evidence that the conserved EhVps4

is an ATPase that could participate in cytopathic activity and erythrophagocytosis, as well as in hepatic damage in hamster. Work currently in progress is focusing on the identification of cytoplasmic structures containing EhVps4, as well as its interaction with other components of the *E. histolytica* ESCRT machinery.

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Review Article

Toll-Like Receptor Initiated Host Defense against *Toxoplasma gondii*

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Toxoplasma gondii is an intracellular pathogen notable for its ability to establish a stable host-parasite relationship amongst a wide range of host species and in a large percentage of the human population. Toll-like receptor signaling through MyD88 is a critical pathway in initiating defense against this opportunistic protozoan and may also be a mediator of pathology during immune dysfunction. Other MyD88 independent signaling pathways are also involved in the host-parasite interaction. These responses can be triggered by the parasite itself, but interactions with the intestinal microbiota add additional complexity during enteric infection.

1. Introduction

Host defense against infection relies upon effective mechanisms of pathogen detection coupled with the ability to discriminate between infectious and noninfectious material. For the case of *Toxoplasma gondii*, an intracellular protozoan with worldwide distribution in humans and animals [1], recognition of infection elicits a rapid and strong Th1-polarized immune response that is necessary for host survival and long-term parasite persistence [2]. In the context of chronic infection, characterized by presence of parasite cysts in tissues of the central nervous system and skeletal muscle, IFN- γ -producing CD4⁺ and CD8⁺ T lymphocytes are required to maintain quiescent infection [3, 4]. The most dramatic evidence underlining this concept is found during AIDS progression, where cyst reactivation may occur as T cell numbers decline, often with devastating consequences [5].

Studies spanning the last two decades have established the importance of IL-12 as an early cytokine steering immunity to a polarized Th1 response that allows control of *Toxoplasma* [6–8]. In the absence of IL-12, mice rapidly succumb to infection due to overwhelming parasite replication and tissue destruction. The need for IL-12 to allow host survival extends into chronic infection [9]. During *T. gondii* infection, this cytokine is produced most prominently by dendritic cells, but also macrophages and

neutrophils [7, 10–12]. Each of these cell types is targeted for invasion during in vivo infection, but whether infected cells themselves are triggered to produce IL-12 or whether the cytokine is made by noninfected cells responding to parasite products is less clear. Regardless, it is also apparent that production of IL-12 must be tightly controlled to prevent proinflammatory pathology. The cytokine IL-10 is a key player in downregulating the response to *Toxoplasma*. Thus, IL-10 knockout mice succumb to infection with kinetics remarkably similar to IL-12 and IFN- γ gene deleted animals [13]. However, death in animals lacking IL-10 is associated with low parasite numbers and overwhelmingly high levels of IL-12, TNF- α , and IFN- γ [13, 14]. While it was originally thought that IL-10 was produced mainly by cells of the macrophage lineage, more recent data indicate that Th1 cells themselves are an important source of this cytokine during *Toxoplasma* infection [15].

How these complex and essential responses are initiated during infection with *T. gondii* and other microbes has been of long-standing interest to investigators in the field. The concept that cells of innate immunity use pattern recognition receptors (PRRs) to respond to pathogen-associated molecular patterns (PAMPs) was introduced by Janeway in the late 1980's [26]. Ten years later, Janeway and Medzhitov discovered Toll-like receptors (TLR), a major family of PRR [27]. Even though TLRs are structurally similar, they are

TABLE 1: Summary of findings in TLR/MyD88 knockout mice.

Mouse strain	Susceptibility	Phenotype	References
<i>MyD88</i> ^{-/-}	Highly susceptible	Uncontrolled parasite growth; defective IL-12 production; delayed emergence of Th1 response	[16, 17]
<i>ICE</i> ^{-/-}	Normal resistance	Normal	[18]
<i>TLR11</i> ^{-/-}	Intermediate resistance	Higher cyst burden; mice are resistant to ileitis during enteric infection	[19, 20]
<i>TLR2</i> ^{-/-}	Intermediate resistance	Mice are susceptible under high infectious dose conditions	[21]
<i>TLR4</i> ^{-/-}	Unclear	Absence of TLR4 confers resistance to gut pathology; absence of TLR4 results in increased susceptibility to peroral infection	[22, 23]
<i>TLR2x4</i> ^{-/-}	Intermediate resistance	Higher cyst burden in the absence of TLR4 and TLR2; decreased intestinal pathology during enteric infection	[20, 24]
<i>TLR9</i> ^{-/-}	Intermediate resistance	Deceased gut pathology; increased parasite burden	[25]

remarkable in that they recognize an extremely diverse array of microbial molecules, including proteins, lipids, DNA, and RNA [28]. Triggering of TLR by microbial ligands plays an important role in initiating immunity to many pathogens, including *T. gondii*.

2. Importance of MyD88-Dependent Toll-Like Receptor Signaling in Defense against *T. gondii*

Initial studies on TLR and their ligands focused on recognition of bacterial and viral molecules such as lipopolysaccharide and nucleic acids, but it soon became clear that TLRs were also central players in recognition of protozoan products and in triggering the immune response to this group of pathogens [29]. Studies in MyD88 gene knockout mice first suggested the importance of TLR in resistance to *T. gondii* [16]. The MyD88 molecule is referred to as the common adaptor of TLR signaling. This is because almost all TLRs use this molecule to relay signals in the host cell [30]. Accordingly, genetic inactivation of MyD88 results in loss of almost all TLR signaling. The exceptions are TLR3 and TLR4, which use another adaptor molecule called Toll-IL-1 receptor domain containing adaptor-inducing interferon (TRIF). In an intraperitoneal mouse injection model absence of MyD88 led to rapid death during infection with the normally low virulence ME49 parasite strain [16]. Mortality was associated with high parasite numbers and low levels of IL-12 and IFN- γ . In addition, neutrophil recruitment was found to be defective in the absence of MyD88, an effect that has been reported in other infectious disease models using this mouse strain [31, 32].

More recently, increased susceptibility in the absence of MyD88 was confirmed in animals undergoing *Toxoplasma* infection initiated through the oral route [17]. MyD88 is most often associated with signaling in innate immune cells, but there is evidence that cells of adaptive immunity also use this common adaptor molecule. Thus, studies in bone marrow chimera mice showed that T cell expression of MyD88 is necessary to prevent emergence of toxoplasmic encephalitis during chronic infection [33]. Although the susceptibility of MyD88 knockout mice to *T. gondii* implicates TLR in recognition of the parasite, this adaptor molecule is also

involved in signaling through receptors for IL-1 and IL-18. However, mice lacking IL-1 β -converting enzyme (ICE), a molecule that is required to produce bioactive IL-1 and IL-18, display normal resistance to infection [18]. This is an important result because it provides strong evidence that TLR signaling per se is required for resistance to *T. gondii* infection.

While results from studies using *MyD88*^{-/-} mice argue for their importance, there is as yet no evidence for a master TLR that controls the immune response to *Toxoplasma*. As described further in what follows, a parasite ligand for mouse TLR11 has been identified. Yet, mice lacking this TLR are only modestly increased in susceptibility relative to MyD88 knockout animals [19]. Along similar lines, *TLR2*^{-/-} mice have been reported to display increased susceptibility to *T. gondii*, but this effect is only observed using high inocula [21]. In a study using *TLR2x4*^{-/-} mice, the animals survived infection but were reported to harbor an increase in cyst number in the brain [24]. Another study suggested that lack of TLR4 increased susceptibility to enteric infection [22], although differing results were obtained by others [23]. Given the molecular complexity of this eukaryotic pathogen, it is reasonable to propose that *Toxoplasma* possesses multiple TLR ligands, such that knockout of any single TLR has minor effects on host resistance (Table 1). Similar conclusions have been reached in resistance to *Trypanosoma cruzi* and *Mycobacterium tuberculosis*, where TLR2 and TLR9 cooperate to provide optimal immunity to infection [34, 35].

3. Parasite TLR Ligands

Two *Toxoplasma* molecular structures serving as TLR ligands have been identified to date. The tachyzoite surface is uniformly covered with glycosylphosphatidyl inositol-(GPI)-anchored proteins [36]. Purification of parasite GPI anchors as well as core glycans and lipid moieties followed by stimulation of cells cotransfected with plasmids encoding TLR and NF κ B reporter genes revealed that GPI moieties triggered TLR2 and TLR4 [24, 37]. In macrophages, this leads to production of TNF- α and upregulation of major histocompatibility complex class II molecules [38]. Interestingly, fatty acids isolated from the parasite block TNF- α inducing properties of GPI moieties [39]. This could possibly be related to the observation that live parasites not

only do not induce TNF- α but actively suppress its synthesis during lipopolysaccharide-TLR4 triggering [40].

To date, GPI moieties come closest to serve as bona fide protozoan PAMP molecules [29]. Thus, in addition to those from *Toxoplasma*, GPI anchors isolated from *Trypanosoma cruzi*, *T. brucei*, *Leishmania major*, *L. donovani*, and *Plasmodium falciparum* all possess TLR2- or TLR4-activating properties [41–44]. In some cases, this promotes protective responses (*L. major*, *T. cruzi*), but in others this may be an underlying cause of pathology (*Plasmodium*) [45]. Why parasite GPI would activate TLR whereas (as far as is known) mammalian GPI do not trigger autoimmune reactions may relate to the fact that GPI molecules are expressed in great abundance on the surface of these protozoan parasites as well as the fact that the fine specificity of these structures varies amongst species [46].

A soluble parasite lysate (STAg) prepared by sonication of tachyzoites followed by high speed centrifugation is well known for its ability to stimulate dendritic cell IL-12 production in a manner dependent upon MyD88 [16]. Straightforward biochemical fractionation of the lysate resulted in identification of a parasite profilin as the MyD88-dependent IL-12-inducing molecule [19]. Interestingly, a profilin molecule with IL-12-inducing properties was simultaneously identified from the related apicomplexan protozoan *Eimeria* [47]. Nevertheless, not all apicomplexan profilins possess the capacity to trigger IL-12 production, because *P. falciparum* profilin has at most only weak IL-12-inducing activity [48]. For *Toxoplasma*, TLR11 was identified as the host cell receptor for profilin [19]. The TLR11 molecule, expressed in mice but not humans, was originally implicated in responses to uropathogenic bacteria [49], but to date *T. gondii* profilin is the only molecule identified as a ligand for this receptor. In addition to triggering host cell IL-12 synthesis, profilin is required for invasion, revealing a dual role in the host-parasite interaction [48, 50]. Profilin is not a secreted molecule but rather is expressed in the tachyzoite cytoplasm. This suggests that triggering of IL-12 synthesis may be mediated by cells responding to parasite degradation products rather than infection itself.

The TLR/MyD88 pathway is believed to play a role in controlling T cell immunodominance [51]. This is because professional antigen presenting cells such as dendritic cells can simultaneously process and present antigen as well as upregulate T cell costimulatory molecules in an MyD88-dependent manner. In support of this model, it was shown that intraperitoneal STAg injection triggered an immunodominant CD4⁺ T cell response to profilin that was dependent upon TLR11 expression and major histocompatibility class II recognition within the same dendritic cell population [52].

4. Role of TLR/MyD88 in the Gut—Who Is Doing the Driving?

It is well established that during high dose *Toxoplasma* infection of certain inbred mice, such as the C57BL/6 strain, animals develop severe pathology in the small intestine

mediated by proinflammatory cytokines [53]. A key role for IL-10 in preventing this pathology under low dose infection conditions has been shown using *IL-10*^{-/-} mice [14]. Similar intestinal pathology has been reported in other species infected with *T. gondii*, although whether this occurs in humans is not clear [54]. In several respects, pathology in the C57BL/6 gut resembles Crohn's disease in humans, which is now believed to involve dysregulated Th1 and Th17 responses in the intestinal mucosa [55–57]. As in inflammatory bowel disease in humans, pathology is associated with changes in the microbiota in the gut. Whether these changes are the result or the trigger of pathology is a current area of active interest.

During high dose *Toxoplasma* infection, gram-negative bacteria accumulate at sites of intestinal damage, and bacteria translocate into subepithelial regions of the intestine [58]. Interestingly, both TLR4 and TLR9 knockout mice are resistant to parasite-induced damage to the intestinal mucosa, and this is associated with decreased levels of proinflammatory cytokines [23, 25]. In addition, mice depleted of intestinal flora by treatment with broad-spectrum antibiotics are resistant to ileal damage triggered by *T. gondii* [58]. These combined results suggest that TLR-based recognition of bacteria, rather than sensing of the parasite itself, causes proinflammatory pathology in the intestinal mucosa.

Recent evidence also suggests that under low dose *Toxoplasma* infection conditions, the endogenous gut microbiota have an adjuvant effect on immunity to the parasite [20]. Thus, TLR11 knockout mice develop a robust Th1 response during enteric infection, despite being unable to respond to the IL-12-inducing TGPRF molecule. However, emergence of Th1 cells associated with protection is defective in *TLR2x4*^{-/-} and *TLR9*^{-/-} mice. Reinforcing these results, *Toxoplasma* infection of germ-free mice resulted in defective IL-12 responses in the intestinal mucosa but this response was restored by feeding mice with the TLR4 ligand lipopolysaccharide. Thus, it is possible that during orally initiated *Toxoplasma* infection, TLR ligands expressed by the parasite are less important than TLR ligands expressed by the intestinal microbiota [20, 59]. In this view, *T. gondii* infection would cause localized damage to the intestine, possibly as a result of the invasive and cell-lytic nature of the parasite. This would allow translocation of bacteria which would, under low dose conditions, promote differentiation of protective Th1 cells but that, under high dose conditions, and would lead to fulminant pathology. Nevertheless, it is interesting to note that *TLR11*^{-/-} mice were reported to be resistant to parasite-induced intestinal pathology, a result suggesting a role for *T. gondii* TGPRF in addition to the contribution of TLR4 and TLR9 to intestinal inflammation [20, 23, 25] (Table 1).

5. Immune Recognition and Protective Immunity without MyD88

While TLR and MyD88-dependent signaling is important in the host response to *T. gondii*, it is not the only pathway for recognition of the parasite. During in vitro infection

of bone marrow-derived macrophages, cells produce IL-12 independently of MyD88 signaling [60]. Interestingly, there is a parasite strain specificity to this effect because high virulence Type I parasites trigger low level IL-12 production that does not depend upon MyD88, whereas low virulence Type II strains induce higher amounts of this cytokine in partial dependence upon MyD88 [60, 61]. Control of IL-12 production has been linked to ROP16, a polymorphic rho-trypan kinase that mediates its effects through activation of signal transducer and activator of transcription (STAT)-3 [62, 63].

During enteric infection, we found that *MyD88*^{-/-} animals display uniform mortality that is associated with uncontrolled parasite replication and dissemination [17]. Nevertheless, while there is a delay in emergence of Th1 cells, IFN- γ -producing Th1 cell responses reach normal levels by one week after infection. We hypothesize that although MyD88 is required for controlling the parasite, it is not essential to trigger an adaptive response to the parasite in this situation. It is possible that in the absence of MyD88, immunity is triggered by bacterial recognition systems such as NOD2 that do not involve TLR/MyD88 signaling, or that non-TLR-based recognition of the parasite itself is sufficient to induce a delayed response.

In an effort to get at these issues and to test whether MyD88 knockout mice could generate a functionally protective response, we immunized mice with an avirulent uracil auxotrophic parasite strain called *cps1-1* and challenged with the virulent RH strain [17, 64]. In this case, immunization triggered emergence of parasite-specific Th1 cells and as predicted the animals were resistant to lethal challenge. Thus, this appears to be a case where MyD88-independent recognition of *T. gondii* results in fully functional adaptive immunity.

The molecular basis for recognition of *Toxoplasma* that does not rely on TLR/MyD88 is currently not clear. However, the parasite triggers G_i-protein coupled receptors (G_iPCR) leading to release of several chemokines including CCL17 and CCL22 [65, 66]. One particular chemokine receptor, CCR5, has been linked to IL-12 production [67]. As described above, the parasite activates STAT3 as well as STAT6 during invasion. Precisely how this occurs is not clear, but phosphorylation of these transcription factors does not require MyD88 (Butcher and Denkers, unpublished observation).

6. Importance of Human TLR in Resistance to *Toxoplasma*

Extensive studies in mice have shown the importance of the TLR/MyD88 pathway in resistance to a broad range of viral, bacterial, fungal, and protozoan infections. Nevertheless, the relevance of this pathway in human host defense is less clear. A cohort of 9 pediatric patients with inherited MyD88 deficiency was found to be susceptible to pyogenic bacterial infections but was unexpectedly resistant to other infections, including *Toxoplasma* [68]. Thus, TLR/MyD88 may play a more narrow role in human host defense relative to the

major effect of this signaling pathway mouse resistance to infection. However, another interpretation is that MyD88 deficiency results in early lethal susceptibility to infection, so that patients are therefore simply not available for study. For the specific case of *Toxoplasma*, lack of TLR11 expression argues that parasite profilin is not a major IL-12-inducing molecule in humans. Nevertheless, after transfection with human TLR2, CHO cells respond to derivatives of tachyzoite GPI molecules, arguing for a possible role of TLR2 in human infection [24]. Thus, the importance of TLR/MyD88 in humans for resistance to *Toxoplasma* and other infections is still an open question.

7. Future Directions

The mouse model has been extremely valuable in determining how the innate and adaptive immune system is coordinated to provide protection against *T. gondii*, and how dysfunction in the immune system may lead to immunopathology and death during infection with this opportunistic protozoan. The TLR/MyD88 pathway is clearly important in early recognition of infection. Yet, molecular details of how this occurs, and how, in some circumstances, the parasite evades detection, are still lacking. By applying genetic tools that are available to study this parasite, we can expect significant progress in this area in the future. It will also be important in the future to clarify the basis of *Toxoplasma* immune recognition in humans. Working towards these goals will permit a better understanding of this important human pathogen allowing us to develop more effective strategies to prevent and treat disease.

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Research Article

Reduction of Foxp3⁺ Cells by Depletion with the PC61 mAb Induces Mortality in Resistant BALB/c Mice Infected with *Toxoplasma gondii*

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Regulatory T cells (Tregs) are CD4⁺Foxp3⁺ cells that modulate autoimmune responses. Tregs have been shown to be also involved during the immune response against infectious agents. The aim of this work is to study the role of Tregs during the infection with the intracellular protozoan *Toxoplasma gondii*. Resistant BALB/c mice were injected with 200 µg of anti-CD25 mAb (clone PC61) and 2 days later they were infected with 20 cysts of the ME49 strain of *T. gondii*. We observed that depleted mice showed 50–60% mortality during the acute infection. When FACS analysis was carried out, we observed that although injection of PC61 mAb eliminated 50% of Tregs, infected-depleted mice showed a similar percentage of CD25⁺Foxp3⁻ (activated T cells, Tact) to those observed in infected nondepleted animals, demonstrating that in our depletion/infection system, injection of PC61 mAb did not hamper T cell activation while percentage of Tregs was reduced by 75% 10 days post infection. We concluded that Tregs are essential during protection in the acute phase of *T. gondii* infection.

1. Introduction

Toxoplasma gondii (*T. gondii*) is an intracellular parasite and the etiological agent of toxoplasmosis. Although the infection is asymptomatic in most immunocompetent individuals, toxoplasmosis may cause severe complications in immunocompromised individuals [1, 2]. If *T. gondii* infection occurs during pregnancy, transplacental transmission can occur, leading to abortion or congenital malformations [2–5].

The immune response against *T. gondii* has been largely characterized and it has been demonstrated that cell mediated immunity is essential to control infection [2, 6, 7], involving synergy between CD4⁺ and CD8⁺ T cells [8, 9]. *T. gondii* triggers the production of IL-12, mainly by dendritic cells [10–12], which stimulate NK cells and T lymphocytes to secrete large amounts of IFN-γ, a key cytokine for protection against this parasite [10, 13, 14]. Thus, protection against *T.*

gondii is dependent on a TH1 response [15]. However IL-10 is also required for prevention of development of IFN-γ mediated pathology [16].

Regulatory T cells (Tregs) are a subset of CD4⁺ T cells that control the immune response by suppressing many lymphocyte effector functions [17–19]. Natural Tregs constitutively express CD25 (the α chain of the IL-2 receptor) [20], CTLA-4 [21], and the forkhead family transcription factor Foxp3 [22, 23], which is required for their development and function [22]. Although initially described for preventing autoimmune responses [20, 24, 25], it has also been demonstrated that they can regulate the immune response against infectious agents [26–29]. For example, in vivo depletion of CD25⁺ cells leads to an increase in the production of IFN-γ in animals infected with *Plasmodium chabaudi adami* [30] and *Trypanosoma congolense* [31], or in animals infected with *Schistosoma mansoni* to an increased production of IFN-γ, IL-4, IL-5, and IL-13 [32], indicating that Tregs can

control both TH1 and TH2 responses. During infection with *Plasmodium berghei* [33] or *Litomosoides sigmodontis* [34], depletion of Tregs leads to control or elimination of the parasites, respectively.

The aim of this paper is to study the role of Tregs during the acute infection of *T. gondii* in the resistant BALB/c strain of mice. We carried out depletion experiments by injection of the PC61 mAb in mice followed by infection with the type II strain ME49, and analyzed mortality. Since PC61 mAb injection could also eliminate other cell subtypes expressing CD25, mainly activated T cells (Tact), we also studied the CD4⁺ T cell subsets affected by injection of the PC61 mAb.

2. Materials and Methods

2.1. Mice. Six—eight-week-old female BALB/cAnN mice, weighing 18–20 g, and Swiss mice, were bred in our animal house and maintained in pathogen-free conditions. All protocols depicted in this paper were approved by the local Bioethics Committee for Animal Research.

2.2. Parasites. The ME49 strain of *T. gondii* was maintained in Swiss mice as previously described [35]. Briefly, brains from infected mice were removed and homogenized in Dulbecco's Phosphate Buffered Saline (DPBS); the number of cysts was enumerated and mice were infected intraperitoneally with 10 cysts; this procedure was carried out every 2–4 months. For peroral infection, mice anesthetized with Sevorane (ABBOTT, Mexico City, Mexico) were infected with 20 cysts by gavage in 0.1 mL DPBS.

2.3. Hybridomas and mAbs. The PC61 hybridoma secreting rat IgG1 against murine CD25 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The F41D1 hybridoma, secreting an unrelated rat IgG1 mAb (isotype control), was a kind gift of Dr. Olivier Denis (Institut Scientifique de Santé Publique, Brussels, Belgium). Hybridomas were grown on CD Hybridoma Medium (GIBCO, Grand Island, NY, USA) and mAbs were obtained after ammonium sulfate (45% w/v) precipitation. After extensive dialysis against PBS, antibody concentration was determined by spectrophotometry at 280 nm. Antibodies were resuspended in PBS at 1–2 mg/mL and stored at –20°C until used.

2.4. CD25⁺ T Cells Depletion In Vivo and Infection. Unless otherwise stated, mice were injected intraperitoneally (ip) with 200 µg of purified PC61 mAb or control isotype at day –2. Two days later (day 0), mice were infected perorally as described above. Depletion was confirmed by analyzing CD4⁺CD25⁺ or CD4⁺CD25⁺Foxp3⁺ cells, in peripheral blood samples when mice were infected. Weight and cumulative mortality were recorded daily; survival curves were compared by the logrank test using the Prism software (GraphPad, San Diego, CA).

2.5. Immunofluorescence. Spleen cells (1×10^6) were incubated (30 minutes, 4°C) with anti-CD4-FITC or -TC (clone RM4-5, Caltag, Burlingame, CA), anti-CD4-PerCP (Biolegend, San Diego CA), anti-CD25-PE or –APC (clone PC61

5.3, Caltag), or anti-CD25-PE (Clone 7D4, Miltenyi Biotec, Auburn, CA) in 100 µL of washing buffer (DPBS + 1% FCS + 0.1% NaN₃). After washing 3 times, cells were suspended in DPBS and analyzed immediately. For Foxp3 detection, we used the Foxp3 Staining Buffer Set (eBioscience, San Diego, CA) with anti-Foxp3-Alexa Fluor 488 (clone FJK-16s, eBioscience) following the indications provided by the manufacturer.

2.6. Flow Cytometry. Flow cytometry analysis was performed on a FACScalibur or a FACScan cytometer (Becton Dickinson, San Jose, CA), running the Cell Quest program (Becton Dickinson). Lymphocytes were identified by forward scatter (FSC) and side scatter (SSC) characteristics, gated and further analyzed. Detailed analysis of each experiment is indicated in each figure legend. Samples were analyzed using the FlowJo software V. 5.7.2 (Tree Star, Ashland OR).

3. Results and Discussion

We carried out depletion studies by injection of the PC61 mAb to analyze the role of Tregs in the resistant BALB/c strain of mice during the infection with the type II strain ME49 of *T. gondii*. In the literature we found many protocols for Tregs depletion using the PC61 mAb in different models, using a wide range of mAb concentrations, from 100 µg to 1 mg [32, 33, 36–41], or even several injections of the PC61 mAb before and during infection [30, 42]. To reduce the possibility of Tact elimination due to high antibody concentrations, we tested the lowest doses reported of PC61 mAb (100 µg and 200 µg) by ip injection. Two days later we analyzed CD4⁺CD25⁺ cells to determine if depletion was achieved, since mice would be infected at this time point. We used the PC61 mAb in the FACS analysis because McNeill et al. [37], using Foxp3-GFP transgenic mice, demonstrated that detection of CD25⁺ cells in PC61-depleted mice using either the 7D4 or the PC61 mAbs showed similar results [37]. As can be seen in Figure 1, injection of either 100 (Figure 1(b)) or 200 µg (Figure 1(c)) of the PC61 mAb leads to similar levels of depletion of CD4⁺CD25⁺ cells in the spleen. Even though depletion efficiency was the same by the day we intended to infect the animals, we were concerned about how long would the depletion lasted. During the first week after injection of the PC61 mAb (Figure 2), depletion efficiency was very similar using both doses, but at 10 days post-depletion, a higher number of CD4⁺CD25⁺ cells were observed in animals treated with 100 µg when compared to cells from animals treated with 200 µg. At 14 days post depletion, about 83% of cells were already detected in both groups of animals. These results show that injection with 200 µg leads to a depletion of CD25⁺ cells for at least 10 days. Recovery of CD4⁺CD25⁺ is slightly faster in animals treated with 100 µg mAb; at 14 days, CD4⁺CD25⁺ cells from both groups reached almost normal levels. We thus chose 200 µg of PC61 mAb for the subsequent experiments.

In order to study the role of Tregs during infection with *T. gondii*, resistant BALB/c mice were depleted of CD25⁺ cells, and 2 days later they were perorally infected with 20 cysts of the ME49 strain. Since CD25 is a molecule expressed

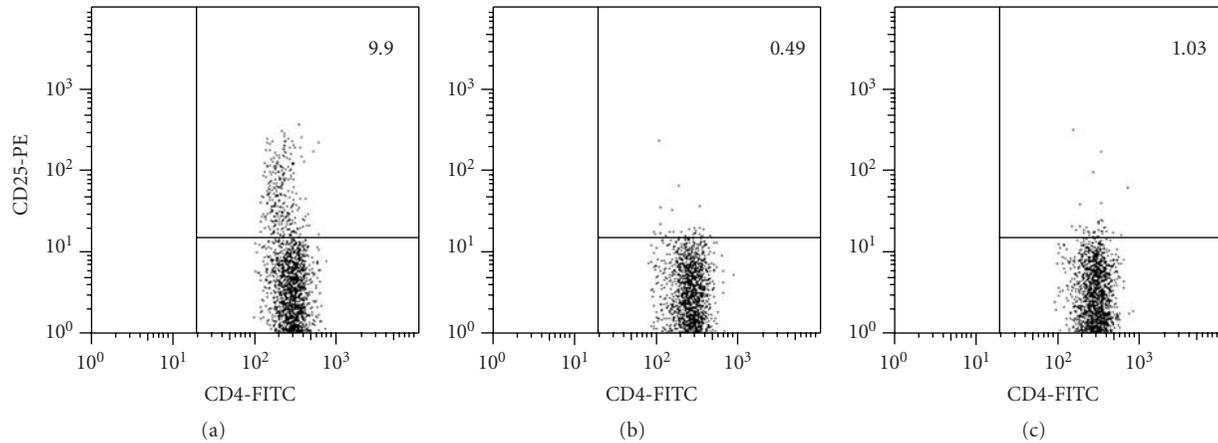


FIGURE 1: Injection of PC61 mAb depletes CD4⁺CD25⁺ cells. BALB/c mice (2 animals per group) were injected ip with DPBS (a), 100 μ g (b), or 200 μ g (c) of purified anti-CD25 mAb (PC61). Two days later, spleen cells were obtained; immunofluorescence was carried out using anti-CD4-FITC and anti-CD25-PE mAbs, and cells were analyzed by FACS. The lymphocyte region was first identified by FSC and SSC characteristics, gated, and 10,000 gated events were captured. Numbers indicate % of CD4⁺CD25⁺ cells within the CD4⁺ population.

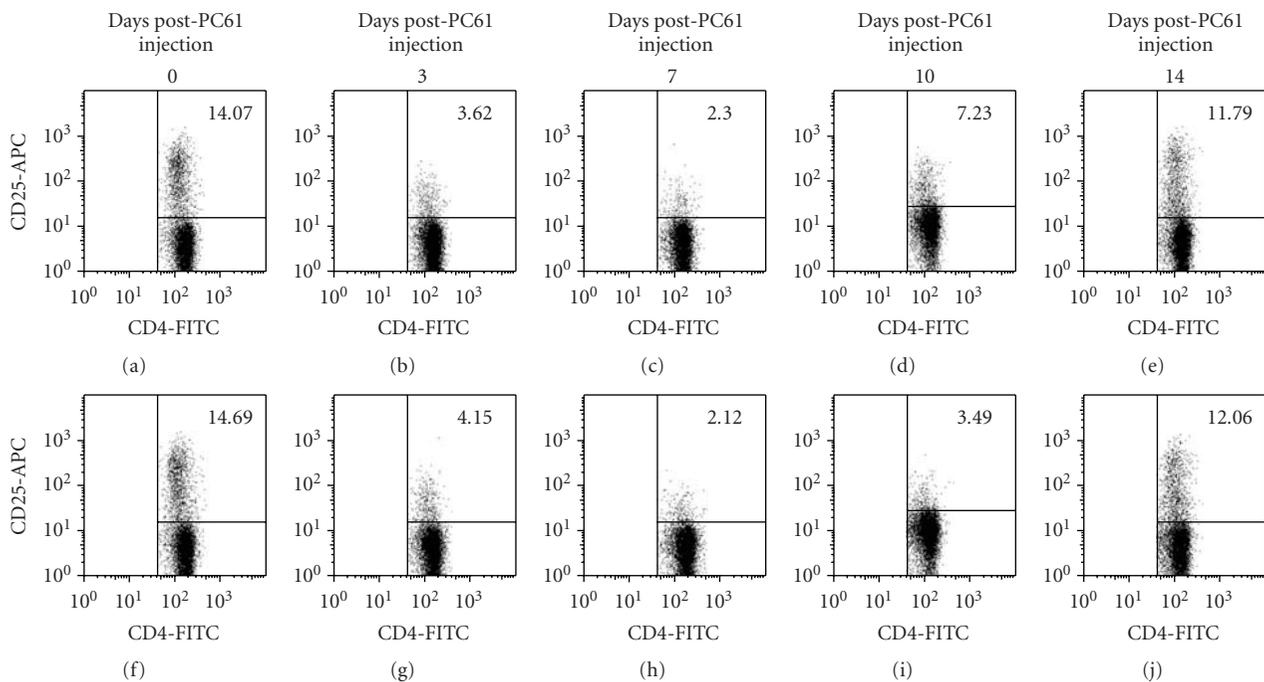


FIGURE 2: Kinetics analysis of the depletion of CD4⁺CD25⁺ cells. BALB/c mice were injected ip with 100 μ g ((a)–(e)) or 200 μ g ((f)–(j)) of PC61 mAb. Spleen cells were obtained at different time points and immunofluorescence was carried out as described in Figure 1 using anti-CD4-FITC and anti-CD25-APC mAbs. The lymphocyte region was first identified and gated by FSC and SSC characteristics, the CD4⁺ region was subgated and 10,000 CD4⁺ events were captured. Numbers indicate % of CD4⁺CD25⁺ cells within the CD4⁺ population.

by other cell types, including activated CD4⁺ T cells, and Foxp3 is exclusively expressed by Tregs and is required for their development and function [22], we thus analyzed the depletion of Foxp3⁺ cells (Tregs) the day of infection (2 days after depletion). We found that only 53% of Foxp3⁺ cells were eliminated by injection of the PC61 mAb (Figure 3), confirming results previously reported [37].

Weight changes and mortality were analyzed for 25 days after depletion and infection (Figure 4). All mice lost up to 25% weight due to the parasite; it has to be noted that depleted mice lost 5% less weight compared to isotype treated mice. After 15 days the latter started to gain weight (Figures 4(a) and 4(b)), but did not reach their initial weight.

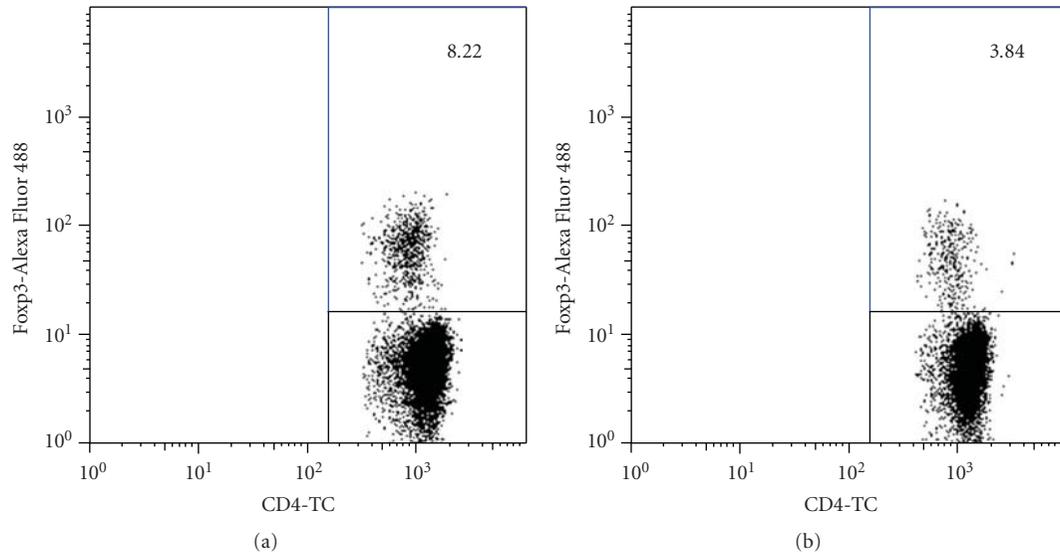


FIGURE 3: Depletion with PC61 mAb partially eliminates Foxp3⁺ cells. BALB/c mice were injected with 200 μ g of isotype (a) or PC61 (b) mAb ip. Two days later, before infection, animals were bled and immunofluorescence for detection of Foxp3 was carried out. The lymphocyte region was first identified and gated by FSC and SSC characteristics, the CD4⁺ region was subgated, and 10,000 CD4⁺ events were captured. Numbers indicate % of Foxp3⁺ cells within the CD4⁺ population. A representative analysis from one mouse per group is shown.

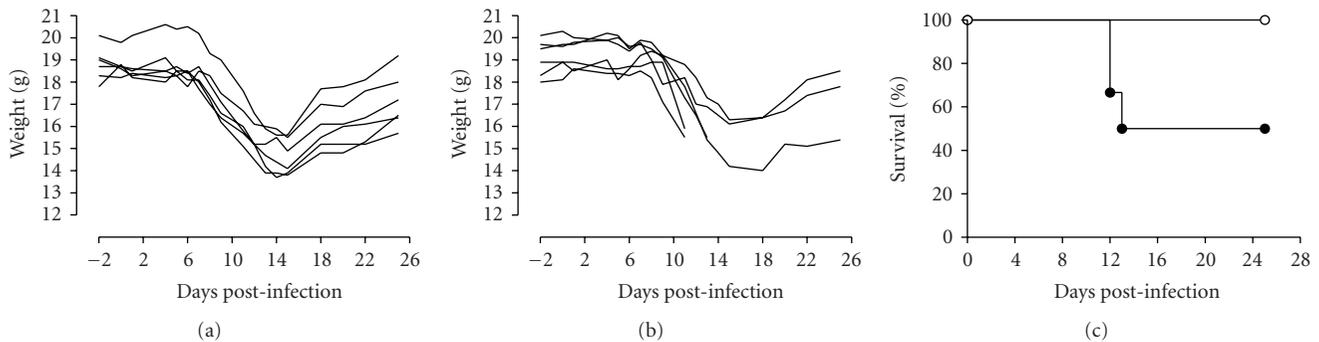


FIGURE 4: Depletion of CD25⁺ cells induces mortality in BALB/c mice infected with *T. gondii*. Animals ($n = 6$) were injected ip with 200 μ g of either isotype mAb (a) (\circ in (c)) or PC61 mAb (b) (\bullet in (c)) and 2 days later they were infected perorally with 20 cysts of the ME49 strain of *T. gondii*. Weight ((a),(b)) and cumulative mortality (c) were recorded daily for 25 days. In (a) and (b), each line corresponds to one mouse.

When mortality was analyzed, isotype-treated mice survived during the 25 day experiment, but 50% of PC61-treated mice died <13 days post infection (dpi) (Figure 4(c)), although this result was not statistically significant ($P = .0549$). A similar result was observed when mice were infected with 50 cysts, although we observed a higher mortality rate (data not shown).

Analysis of blood samples of the same animals from this experiment showed that although CD25⁺ cells were eliminated, including Foxp3⁺ and Foxp3⁻ cells, at later time points, a marked increase in CD25⁺Foxp3⁻ cells (Tact) was observed in depleted animals, while the percentage of Foxp3⁺ cells (Tregs) was still decreased (data not shown). Therefore, we carried out an experiment to confirm these observations. We depleted and infected mice as described above, they were killed 10 days pi, when animals showed symptoms of

toxoplasmosis (2–4 days before death) and an exhaustive analysis of spleen cells was performed.

Analysis of Tregs and Tact cells from infected animals at this time point (Figure 5) showed that infection induced an expansion of Tact cells (3.76 versus 24.79). Depleted infected animals, however, showed a dramatic expansion of Tact when compared to depleted/noninfected animals (0.94 versus 25.79), but no difference was detected between Tact from infected nondepleted or depleted mice (24.79 versus 25.79), demonstrating that depletion did not prevent activation of T cells. On the other hand, a nearly 50% reduction in percentage of Tregs was observed in infected-nondepleted animals, when compared to control mice (8.32 versus 16.35); depleted noninfected animals still showed a 50% decrease in Tregs at this time point (8.34 versus 16.35), while depleted infected animals had 4.85% of Tregs, which

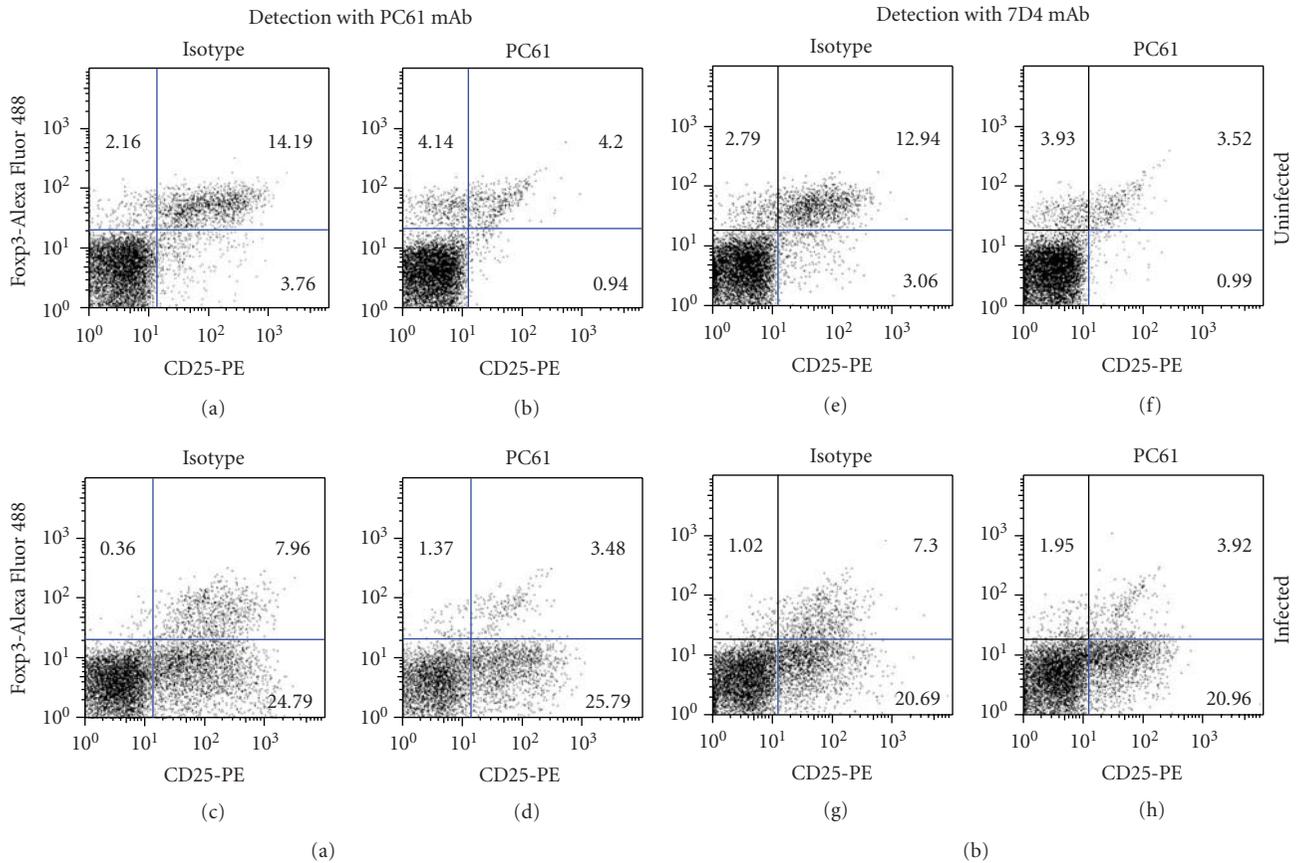


FIGURE 5: Analysis of Tregs and Tact cells after PC61 injection and infection with *T. gondii*. Animals were depleted and infected as described in Figure 4. At 10 dpi spleens were obtained and immunofluorescence was carried out using anti-CD4-PerCP, anti-Foxp3-Alexa Fluor 488 and anti-CD25-PE mAb, either PC61 (a)–(d) or 7D4 clone (e)–(h). The lymphocyte region was first identified and gated by FSC and SSC characteristics, the CD4⁺ region was subgated, and 10,000 CD4⁺ events were captured. Numbers indicate % of cells from the CD4⁺ gating. A representative analysis from one mouse per group is shown.

represents a 50% reduction when compared to noninfected-depleted mice (4.85 versus 8.34). All these results were similar when mAb 7D4 was used to detect CD25 cells (Figure 5), demonstrating no interference in the detection of CD25 with PC61 mAb.

A summary of the results obtained from all mice studied is depicted in Figure 6. Analysis shows that at 10 dpi (12 days post depletion) 50% of Tregs are still lacking in depleted noninfected animals. Interestingly, infection induces a 50% reduction of Tregs, but depleted infected animals have 50% less Tregs than nondepleted infected mice, and only 25% of Tregs when compared to control noninfected mice. Analysis of Tact cells confirmed that infection leads to a dramatic expansion of this subset, which is not altered by depletion, since mean percentage of Tact cells was unaffected, although a high dispersion in data was observed (Figure 6).

When the ratio of Tact/Tregs cells was calculated (Figure 7), we found that in noninfected animals, whether depleted or nondepleted, ratios remained nearly unchanged (≤ 0.28). In infected mice, a higher ratio was observed (1.2–4.0), indicating a expansion of Tact cells due to the parasite. In depleted/infected mice, however, the ratio was 0.5–12.7

(Figure 7(a)): a group of 4 mice showed a very high ratio (>5.0), while another group presented a low ratio (0.5–1.15). The day we carried out this experiment, one animal was moribund, and its spleen cells showed the highest proportion of Tact/Tregs (ratio = 12.7, Figure 7(a)). It has to be noted that in this experiment, an additional group of mice was used to evaluate mortality (Figure 7(b)), and we found that 60% of animals died 15 days pi, which correlates to the percentage of mice with the higher proportion of Tact/Tregs (Figure 7(a)). All these experiments show that although the generation of Tact cells due to infection is not altered by depletion, the percentage of Tregs remained very low, turning susceptible a resistant strain of mice.

In the present work we analyzed the role of Tregs during infection with *T. gondii* in the resistant BALB/c strain of mice. We observed that infected animals showed a decreased number of Treg cells, a result which agrees with that reported by Ge et al. [43], who showed that in pregnant and nonpregnant mice, infection with *T. gondii* induced a reduction in Foxp3 mRNA expression levels in spleen and a reduction in both percentage and absolute number of Tregs. Thus, this parasite induces a decline in Tregs percentage [43]. These

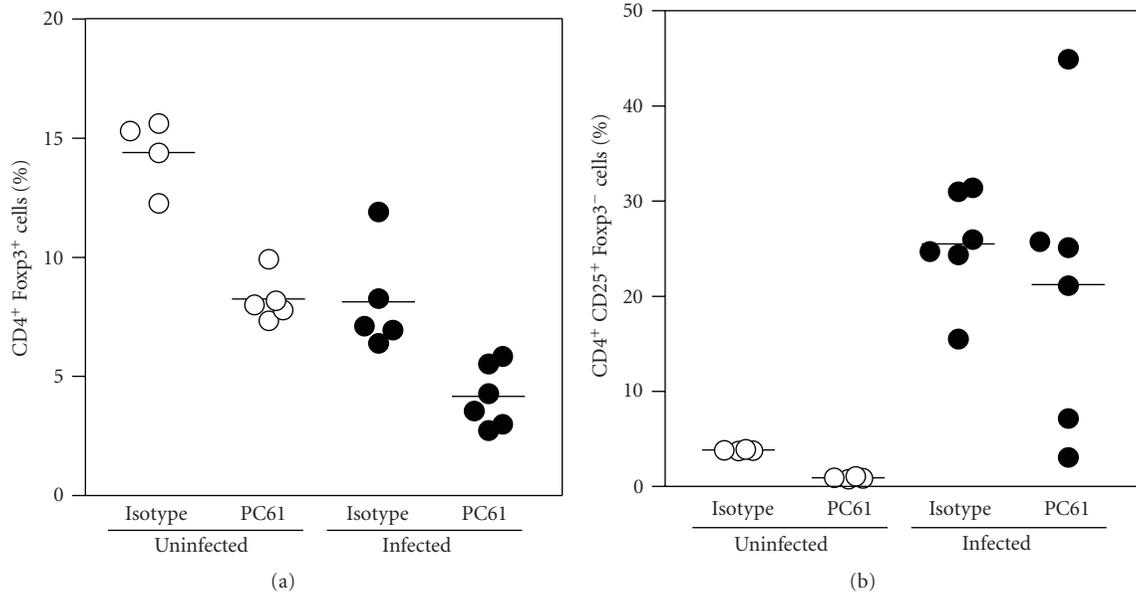


FIGURE 6: Summary of the Treg and Tact cell detection in mice after PC61 injection and infection with *T. gondii*. Percentage of Tregs (CD4⁺ Foxp3⁺) and Tact cells (CD4⁺ CD25⁺ Foxp3⁻) within the CD4⁺ gate obtained from all the animals described in Figure 5 is shown.

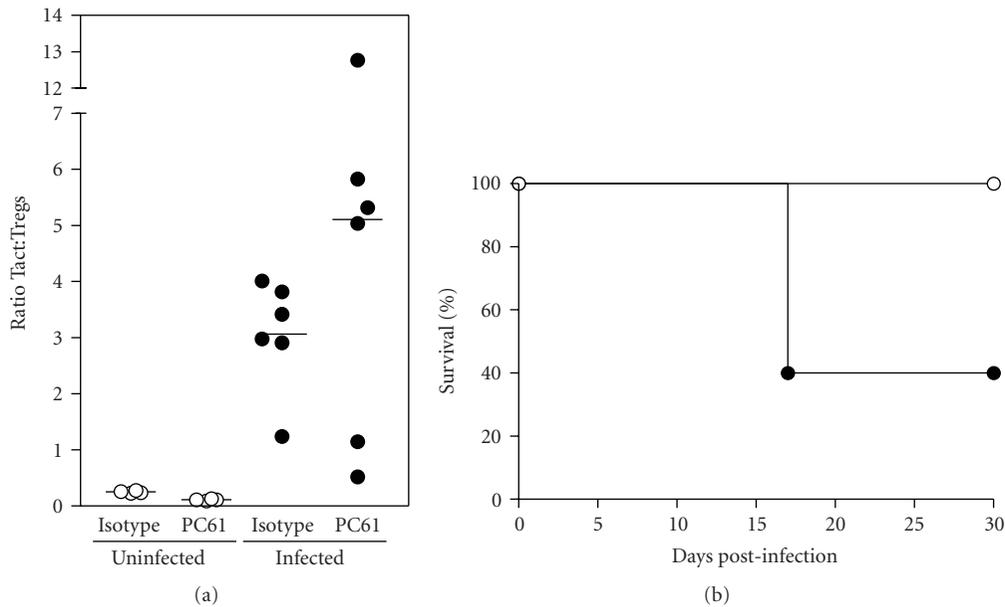


FIGURE 7: Infection with *T. gondii* induces an imbalance in Tact:Tregs cell ratio and is exacerbated by depletion. (a) Ratio of Tact:Tregs (CD4⁺ CD25⁺ Foxp3⁻ cells : CD4⁺ Foxp3⁺ cells) was calculated from data obtained from Figure 6. (b) An additional group of mice ($n = 5$) treated with isotype (○) or PC61 (●) was used to evaluate cumulative mortality for 30 days.

results differ from those observed during the acute phase of other infections (*P. berghei*, *S. mansoni*, *Heligmosomoides polygyrus*, *Mycobacterium tuberculosis*, *Plasmodium yoelii*, *Brugia malayi*, and *L. sigmodontis*), in which an increase in Foxp3⁺ cell number is observed [32, 39, 44–48]. In *T. gondii* infection, it is possible that Tregs die or migrate to other sites in order to control the immune response locally, as it has been shown during *P. berghei* infection [39]. It has to be

noted that *T. gondii* can infect all cell types and disseminates to most organs [49, 50]. The fate of Tregs during *T. gondii* infection, however, remains currently unknown.

Injection of PC61 to deplete CD25⁺ cells has been widely used to study the role of Tregs in different models [32, 36, 37, 39, 42, 51, 52]. In our depletion experiments, we observed that 50–60% of BALB/c mice died during the acute phase of infection, an observation that suggests that Tregs play an

important role in toxoplasmosis. However, since CD25 is also expressed by other cell types, injection of the mAb leads also to the depletion of Tact cells and interpretation of results should be taken carefully.

We used a low single dose (200 μ g) of PC61 mAb, which was enough to eliminate 50% of Tregs. These observations agree with those reported previously [37], in which a partial depletion of Tregs cells is observed after injection of PC61 mAb.

Injection of PC61 mAb initially eliminates 50% of Tregs, but in infected mice this reduction is exacerbated due to the infection, reaching 75% Tregs reduction in these animals at 10 days pi, when compared to nondepleted-noninfected mice. However, depletion did not hinder the activation of T cells after *T. gondii* infection. In fact, levels of CD69 expression and percentage of CD69⁺ cells were similar in Foxp3⁻ cells from nondepleted infected and depleted infected mice (data not shown), demonstrating that both groups generate the same proportion of Tact cells.

While this work was carried out, Couper et al. [41] reported that depletion using 1 mg of the PC61 mAb leads to an increased susceptibility of male C57BL/6J mice to the ME49 strain of *T. gondii*. Although the results in mortality obtained in our work are similar, we used the highly resistant BALB/c strain of mice, in contrast to the highly susceptible C57BL/6J strain. Moreover, we depleted animals with a lower concentration of PC61 mAb to lessen undesirable effects in other cell populations; a single dose of mAb (200 μ g), 2 days before infection was enough to achieve a 50% elimination of Tregs. We avoided the use of higher concentrations of PC61 mAb because we think that this would induce a broad and long lasting depletion of CD25⁺ cells (both Tact and Tregs) making more difficult the interpretation of the results.

The use of a suitable concentration of the PC61 mAb allowed us to detect a difference in the proportions of Tact and Treg cells 10 days pi. Thus, the amount of PC61 antibody did not prevent the generation of Tact cells due to the infection. The levels of Treg, however, remained low during infection, leading to the loss of resistance of the BALB/c strain. These results show that the absence of Tregs alters the protective immune response against *T. gondii* and thus mice become susceptible.

T. gondii induces the activation of CD4⁺ and CD8⁺ T cells that are crucial to control infection [53]. These cells could in turn be controlled by Tregs, as it has been demonstrated in the *T. congolense* infection [31]. Our results show that Tregs play an important role in the modulation of the protective immune response against *T. gondii*. Since infection with this parasite drives a powerful TH1 immune response [53], it is tempting to speculate that the absence of Tregs induces an uncontrolled inflammatory response by Tact cells. The specific molecules and cells directly responsible for the death of animals, however, remain to be established.

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Research Article

The Unexpected Role for the Aryl Hydrocarbon Receptor on Susceptibility to Experimental Toxoplasmosis

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The aryl hydrocarbon receptor (AhR) is part of a signaling system that is mainly triggered by xenobiotic agents. Increasing evidence suggests that AhR may regulate immunity to infections. To determine the role of AhR in the outcome of toxoplasmosis, we used AhR^{-/-} and wild-type (WT) mice. Following an intraperitoneal infection with *Toxoplasma gondii* (*T. gondii*), AhR^{-/-} mice succumbed significantly faster than WT mice and displayed greater liver damage as well as higher serum levels of tumor necrosis factor (TNF)- α , nitric oxide (NO), and IgE but lower IL-10 secretion. Interestingly, lower numbers of cysts were found in their brains. Increased mortality was associated with reduced expression of GATA-3, IL-10, and 5-LOX mRNA in spleen cells but higher expression of IFN- γ mRNA. Additionally, peritoneal exudate cells from AhR^{-/-} mice produced higher levels of IL-12 and IFN- γ but lower TLR2 expression than WT mice. These findings suggest a role for AhR in limiting the inflammatory response during toxoplasmosis.

1. Introduction

Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor [1]. Together with its transcriptional regulators, basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) nuclear partner, and aryl hydrocarbon receptor nuclear transporter (ARNT), it provides a powerful signaling system during a critical response to several environmental pollutants such as polyhalogenated aromatic and polyaromatic hydrocarbons [2]. The AhR function is particularly well-characterized in response to the exogenous compound 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [3]. Moreover, several lines of evidence suggest that a battery of proinflammatory cytokine genes can be upregulated upon interaction of TCDD with AhR. TCDD treatment causes increased expression of tumor necrosis factor (TNF)- α , interleukin

(IL)-1, IL-2, interferon (IFN)- γ , IL-18, IL-6, Chemokine (C-C motif) ligand 1 (CCL1), and plasminogen activator inhibitor-2 (PAI-2) [4–9]. Additionally, Negishi et al. demonstrated that a synthetic antiallergic agent M50354, which is another AhR agonist, increased the levels of IFN- γ associated with reduced expression of GATA-3, a key factor for Th2 differentiation [10].

In addition to its role in metabolizing exogenous compounds as part of an adaptive chemical response, there is growing evidence suggesting that AhR has normal physiological functions and that it likely has endogenous ligands. For example, there is evidence for the importance of AhR in normal development, liver functions, circadian rhythm, response to hypoxia, hormone signaling, and vascular regulation [11–14]. We previously reported that spleen cells from AhR-null mice overproduce IFN- γ and

IL-12 when challenged with concanavalin-A (ConA) or restimulated with ovalbumin in vitro [15]. This observation agrees with previous findings indicating that AhR plays an important role in normal development and function of the immune system [16]. Moreover, recently are emerging new evidences that AhR also plays a role in normal physiology, including certain immune responses [17]. In particular, Th17 cells and dendritic cells (DCs) express high levels of AhR [18, 19].

AhR has been implicated in the response to different infectious agents. For example, in influenza virus infection TCDD-induced AhR-activation diminishes the memory response but does not impair host resistance [20]. In lethal *Streptococcus pneumoniae* infection model, the survival rate is slightly enhanced in mice lacking AhR [21]. AhR^{-/-} mice infected with *Listeria monocytogenes*, an intracellular bacteria, are more susceptible to infection but develop enhanced resistance to reinfection [22], even though their serum levels of inflammatory cytokines such as IL-6, IFN- γ , and TNF- α are comparable to WT mice. Additionally, macrophages from AhR^{-/-} mice retain their ability to ingest *Listeria* and inhibit parasite growth [22]. These data suggest that AhR contributes to an optimal immune response, but its function appears to be distinct depending on the pathogen. Thus, establishment of the role of AhR in some parasitic infections may extend our understanding of the biological functions of AhR.

Toxoplasma gondii is an opportunistic protozoan parasite that causes toxoplasmosis, which is clinically asymptomatic in most individuals but can be fatal in immunocompromised hosts. Immunity to *T. gondii* is highly dependent on cell-mediated effector responses, that consist of high levels of type 1 cytokine production [23–26]. The IL-12/IFN- γ immune response axis plays a crucial role in determining resistance to *T. gondii* infection. Deficiencies in IFN- γ production, IFN- γ -receptor-mediated signaling pathway, cells that produce IFN- γ such as natural killer (NK) cells [27], CD4⁺ and CD8⁺ T cells [28], macrophage migration inhibitory factor (MIF) [29], or some other effector molecules such as nitric oxide (NO) [30], result in increased susceptibility to *T. gondii*. Furthermore, deficiencies in IL-12 [31], its receptor, or its intracellular signaling pathway (STAT-4) [32] render mice extremely susceptible to acute toxoplasmosis with survival rates similar to those observed in IFN- γ -deficient animals. On the other hand, exacerbated proinflammatory response can lead to immunopathology and death [33]. For this reason the immune system has evolved an elaborated series of pathways to downregulate proinflammatory responses. Since previous reports suggest that AhR participates in modulating Th1/Th2 balance and proinflammatory responses [10, 15], we analyzed the role of AhR in host control of experimental toxoplasmosis. We showed that AhR^{-/-} mice infected with ME49 strain of *T. gondii* develop fewer cysts in the brain but, paradoxically, succumb significantly faster than WT mice. The increased mortality rate of AhR^{-/-} mice upon *T. gondii* infection was associated with higher levels of TNF- α and IFN- γ and lower levels of IL-10 and GATA-3. These findings indicate that AhR plays an important role in downregulating inflammatory responses during *Toxoplasma gondii* infection.

2. Material and Methods

2.1. Mice. AhR-deficient (AhR^{-/-}) and WT (AhR^{+/+}) mice were generated as previously described [15, 16]. These mice lack a functional AhR, as the exon1 is replaced from the translational start site onwards with a neomycin gene. AhR^{-/-} mice were backcrossed with the C57BL/6 strain for at least 10 generations. AhR^{-/-} mice were maintained as heterozygotes (AhR^{+/-}) in our laboratory. AhR^{+/-} males were mated with AhR^{+/-} females to generate WT, AhR^{+/-}, and AhR^{-/-} mice. In the following experiments, we used eight- to 10-week-old male WT and homozygous mutant littermate mice (AhR^{-/-}). All of the mice were maintained in a pathogen-free environment at Centro de Investigación y Estudios Avanzados del Instituto Politécnico Nacional animal facility in accordance with institutional and national guidelines for animal research.

2.2. Parasite and Experimental Infections. Cysts from the avirulent ME49 strain were harvested from the brains of C57BL/6 mice that had been inoculated intraperitoneally (i.p.) with 20 cysts, 1 to 2 months before harvest. For experimental infections, brain suspensions were adjusted to 40 cysts per 200 μ L in PBS. WT and AhR^{-/-} mice received either 40 ME49 cysts or PBS via i.p. Control inoculations with uninfected brain suspensions failed to elicit detectable inflammatory responses or significant increase in cytokine levels. Soluble *Toxoplasma* antigen (STAg) from tachyzoites of *T. gondii* was prepared as described previously [34].

2.3. Quantification of Cysts in the Brain. To assess the disease progression, brains from *T. gondii*-infected WT and AhR^{-/-} animals were removed aseptically and homogenized in 2 mL of PBS at days 10, 15, 25, 57, and 60 postinfection. The total number of cysts was determined by examination under the microscope. The cysts were counted in a 10 μ L brain-suspension at least three times, and the averages were multiplied by 200. Parallel semiquantification of parasite-specific DNA sequences was performed on the same brain samples in order to confirm the microscopic findings, as described previously [15]. In brief, brains from *T. gondii*-infected animals were collected at day 40 post infection. DNA was extracted from tissues using the Qiamp tissue kit (Qiagen, Chatsworth, CA, USA), and 50 and 25 ng of each sample was analyzed by polymerase chain reaction (PCR). PCR amplification was performed on parasite DNA to amplify a 200- to 300-fold repeated fragment of 529 bp (primers TOX4, 5'-CGCTGCAGGGAGGAAGACGAAAGT-TG-3' and TOX, 5'-CGCTGCAGACACAGTGCATCTGGA-TT-3'). The 529 bp fragment was found in all 60 strains of *T. gondii* tested. This fragment is unique to *T. gondii* DNA and is distinct from that of other parasites [35]. The mouse GAPDH gene (primers, Table 1) was amplified in parallel as a control to monitor PCR inhibition and to control for DNA integrity.

2.4. Cell Preparations and Culture Conditions. Twenty-five days after *T. gondii* infection Peritoneal Exudate Cells (PECs)

TABLE 1: Oligonucleotide primers used for gene expression analysis by RT-PCR.

Target	Primer sequence (5'-3' direction) ^a	Product size (bp)	Cycles	Alignment (°C)	Reference
IFN- γ	F-AGCGGCTGACTGAACTCAGATTGTAG	243	30	57	[36]
	R-GTCACAGTTTTTCGCTGTATAGGG				
IL-10	F-ACCTGGTAGAAGTGATGCCCCAGGCA	237	30	56	[37]
	R-CTATGCAGTTGATGAAGATGTCAAA				
GATA-3	F-GAAGGCATCCAGACCCGAAAC	255	30	56	[38]
	R-ACCCATGGCGGTGACCATGC				
5-LOX	F-ATTGTTCCATTGCCATCCAGCTCA	529	30	56	[39]
	R-TCGTTCTCATAGTAGATGCTCACCA				
GAPDH	F-CTCATGACACAGTCCATGC	201	30	54	[40]
	R-CACATTGGGGGTAGGAACAC				

^a F: forward primer; R: reverse primer

and spleen cells were obtained from WT and AhR-/- *T. gondii* infected mice, in sterile conditions, and cultured as previously described [41]. In brief, spleen tissues were minced and filtered to obtain spleen cells, which were then washed and resuspended in DMEM culture medium supplemented with 10% Fetal Calf Serum (FCS), 2mM L-glutamine, 0.25 U/mL penicillin, and 100 mg/mL streptomycin (all from GIBCO, BRL Grand Island, NY, USA). Splenocytes were resuspended at 5×10^6 cells/mL in the same medium. One hundred μ L of the cell suspensions were transferred to 96-well flat bottom culture plates (Costar, Cambridge, MA, USA) and stimulated with either 100 μ L of Con-A mitogen solution (2 μ g/mL; Sigma, St. Louis, MO, USA) or with soluble *Toxoplasma* antigen (STAg) (2.5 μ g/mL). Plates were then incubated at 37°C, 5% CO₂ for 72 hours or 6 days with Con-A or STAg, respectively. Fifty-four hours after seeding the plates stimulated with STAg, 0.5 μ Ci of methyl-³H-TdR (specific activity 925 GBq/mmol. Amersham, UK) was added to each well. Cells were harvested, pipetted onto a glass fiber filter paper (Wallac), and analyzed by a liquid scintillation counter (Betaplate, Walac).

PECs were prepared as previously described [41]. In brief, 1×10^6 PECs were plated in 24-well plates. Two hours later nonadherent cells were washed-off twice with complete DMEM, and the remaining adherent macrophages (*M ϕ*) were replenished with complete medium. STAg was added at a final concentration of 2.5 μ g/mL for 24 hours. In both spleen and PEC cultures, supernatants were collected, centrifuged, aliquoted, and frozen at -20°C until use.

2.5. Cytokine Measurement. After *T. gondii* infection, WT and AhR-/- mice were bled from tail snips at various time points. Sera from blood samples and supernatants from cell cultures described above were analyzed to measure production of IL-2, IL-4, IL-10, IL-12p70, IFN- γ , and TNF- α by ELISA (Peprotech, Mexico) using paired monoclonal antibodies and murine recombinant cytokines to make standard curves, as previously described [42]. Optical density (OD) was measured after 5 minutes using an ELISA microplate reader (SpectraMax 250, Molecular Devices, USA) at 405 nm.

2.6. Nitric Oxide Quantification. Nitric oxide (NO) levels in the serum were determined indirectly by measuring the total serum nitrite (NO₂-) after reduction of nitrate (NO₃-) to nitrite (NO₂-) with nitrate reductase following the protocol described by Granger et al. [43] and adapting it to microwell plates (Costar). Briefly, 50 μ L of serum was incubated at room temperature with 50 μ L of substrate buffer (imidazole 0.1 mol/L, NADPH 210 μ mol/L, flavine adenine dinucleotide 3.8 μ mol/L: pH 7.6, all from Sigma-Aldrich) containing nitrate reductase (*Aspergillus niger*, Sigma) for 45 minutes to convert NO₃- to NO₂-. Total nitrite was then mixed with an equal volume of Griess reagent (1.5% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2.5% phosphoric acid; all from Sigma) [44], incubated for 10 minutes at room temperature in the dark, and the absorbance was measured at 570 nm in an automatic microplate reader (Organon Technika Microwell System). Values were quantified using serial dilutions of sodium nitrite.

2.7. Total IgE Determination. Peripheral blood was collected at various time points from tail snips of all experimental mice infected with *T. gondii*. Blood was centrifuged and stored at -20°C until use. Total IgE production was measured by ELISA, using a commercial kit (Opt-EIA ELISA-set, BD-Pharmingen).

2.8. Transaminase Enzyme Determination. The presence of the liver transaminase enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was evaluated in sera from WT and AhR-/- mice at 0, 3, 6, 14, and 25 days after *T. gondii* infection using ALT and AST kits (Spinreact, S. A. Ctra. Santa Coloma, Spain).

2.9. RT-PCR Assay to Evaluate IFN- γ , IL-10, and GATA-3 Gene Expression in Spleen Cells and Brains. At 25 days after *T. gondii* infection (when 40% the AhR-/- mice die), the brains were removed and total RNA was extracted using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). cDNA synthesis was performed with SuperScript One-Step reverse transcription-PCR (RT-PCR, Invitrogen). In brief, 3 μ g of total RNA was mixed

with 0.5 μg oligo (dT) 12 to -18 primers, 10 mM of each dNTP and 1X reaction buffer in a final volume of 20 μL . cDNA reactions were incubated at 65°C for 10 minutes to denature the RNA template and quench-cooled for 1 minute. 0.5 μL of SSII-RT reverse transcriptase was added, incubated at 42°C for 50 minutes and 70°C for 15 minutes. 0.5 μL of RNaseH was added and further incubated for 15 minutes at 36°C. cDNA samples were amplified for 30 cycles using the Red *Taq* polymerase (Invitrogen) and specific primers (Table 1). After amplification, PCR products were separated by gel electrophoresis on 1.5% agarose gels containing SYBR green I, a nucleic acid gel stain used at 1,000X (Amresco), and visualized with the FLA-5000 chemiluminescence detection system (Fujifilm). The data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and analyzed using the Multi-Gauge Image program. All reactions were repeated three times to ensure reproducibility.

2.10. Histopathology. Livers were removed and fixed in a solution that contained 10% formalin, 70% ethanol, and 5% acetic acid, embedded in paraffin blocks (all from Sigma-Aldrich). Sagittal sections of livers (5 μm thick) were obtained and mounted on slides and subsequently stained with hematoxylin and eosin (Sigma-Aldrich). Photomicrographs of representative sections were taken with an Axion-Star microscope equipped with a built-in digital camera (ZEISS).

2.11. Flow Cytometry Analysis. Fluorescence-activated cells sorting (FACS) analysis on CD4+ T cells, CD8+ T cells, and Treg cells in the spleen and TLR-2+ and CCR5+ macrophages (*M ϕ*) from WT and AhR-/- *T. gondii*-infected mice was performed. Briefly, at 25 days after *T. gondii* infection, spleen cells or peritoneal adherent *M ϕ* were stimulated in vitro for 6 days or 24 hours with 2.5 $\mu\text{g}/\text{mL}$ of STAg. The spleen cells were stained with fluorescein isothiocyanated (FITC) anti-CD8 antibody and phycoerythrin (PE) anti-CD4 antibody, and Treg cells were stained using a staining kit (Mouse Treg Flow kit) containing FITC anti-CD4 antibody, PE anti-CD25 antibody, and ALEXA anti-Foxp3 antibody, according to the manufacturer's instructions. The *M ϕ* were stained with FITC anti-F4/80 antibody and PE anti-TLR2 or PE anti-CCR5 antibody. The cells were fixed, and the proportion of cells staining positive for the appropriate markers was evaluated (10,000 events/sample) using a flow cytometer (FACS, Becton Dickinson, USA). Nonspecific binding was blocked with FcBlock, and the isotype controls were stained with rat antimouse IgG conjugated with -FITC, -PE, or -ALEXA (all from Biolegend, San Diego, CA).

2.12. Statistical Analysis. All statistical analyses were performed using Prism 4 (GraphPad Software, San Diego, CA). Comparisons between WT and AhR-/- animal groups were made using a nonparametric Mann-Whitney's *U*-test and Student's *t*-test as appropriate. For survival assays, log-rank test was used. Differences were considered statistically significant when *P* value was less than .05.

3. Results

3.1. Wild Type and AhR-/- Mice Exhibit Differential Resistance to Toxoplasmosis. To analyze the importance of AhR during acute toxoplasmosis, we first determined whether WT and knockout (AhR-/-) mice differed in their resistance to ME-49 *T. gondii* infection. WT and AhR-/- littermates were challenged i.p. with 40 cysts of *T. gondii* parasites, and we examined the course of the infection for 60 days. As shown in Figure 1(a), AhR-/- mice rapidly showed clinical signs of the disease that sustained for 5 days, while WT mice showed few symptoms. By day 7 after infection, AhR-/- mice started to lose weight (Figure 1(b)) and showed piloerection and prostrated behavior. *T. gondii*-infected AhR-/- mice succumbed as early as day 11 after infection and reached 89% mortality rate by day 60 after infection. In contrast, the mortality rate in WT mice was significantly lower, 6.7% (Figure 1(c)). Interestingly, despite the evident signs of sickness and death in AhR-/- mice, they developed fewer brain cysts compared to WT mice, even at day 60 postinfection (Figure 2(a), *P* < .05).

To further confirm our microscopic findings, we quantified the level of *T. gondii*-specific DNA in the brains from both groups of mice at day 25 post infection using semiquantitative PCR based on the 529 bp repeat element (REP; 200 to 300 copies/genome), as reported elsewhere [35]. The level of parasite DNA in the brains of infected WT and AhR-/- mice correlated with the number of cysts, confirming fewer parasite burdens in the brains of AhR-/- mice (Figures 2(b) and 2(c)). Taken together, these data suggest that AhR is critical in the host defense against *T. gondii* infection, and that the increased mortality rate in AhR-/- mice is not due to an inability to restrict parasite replication.

3.2. AhR-/- Mice Develop Significant Reduction of IL-10 and Increase of TNF- α in Sera. Next, we compared the levels of cytokines (IL-12, IFN- γ , TNF- α , and IL-10), nitric oxide (NO), and IgE, in sera from WT and AhR-/- mice after *T. gondii* infection. No significant differences between AhR-/- and WT mice in serum IL-12 and IFN- γ levels were detected at any of the time-points examined (Figures 3(a) and 3(b), resp.). However, significantly higher level of the inflammatory cytokine TNF- α was detected after 30 days of infection in sera from AhR-/- mice compared to WT mice (Figure 3(c), *P* < .05). This observation was in accordance with a low level of IL-10 observed after 15 days of infection in AhR-/- mice (Figure 3(d), *P* < .05). A higher level of total IgE was also observed after 6 days of infection in AhR-/- mice (Figure 3(f), *P* < .05). Interestingly, the serum nitric oxide level was higher in AhR-/- mice than WT mice on day 25 post infection alone.

3.3. Defective Proliferation and IL-2 Production by AhR-/- Spleen Cells. We next determined the functional capacity of spleen cells from both AhR-/- and WT mice to respond to *T. gondii*-specific stimulation. At day 25 post infection, proliferation of spleen cells from infected AhR-/- or WT mice in the presence of STAg or medium for 5 days was assayed

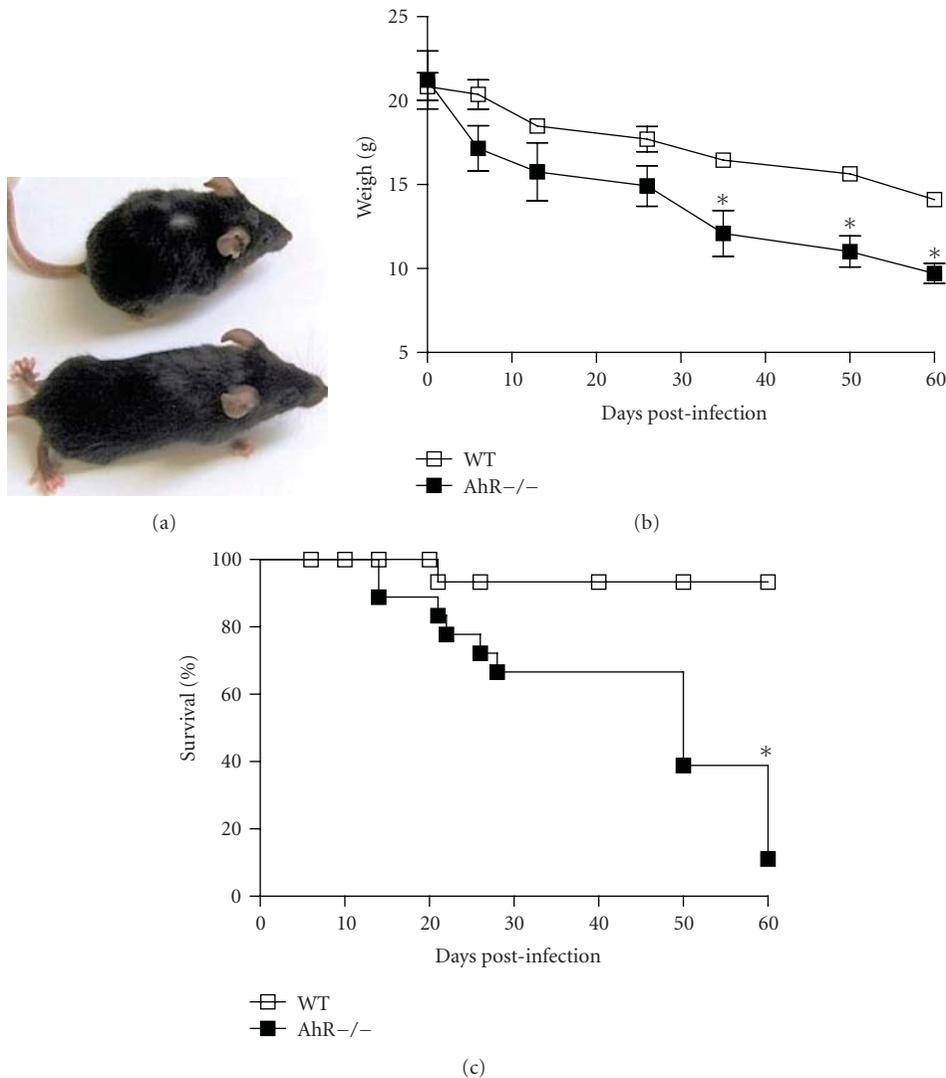


FIGURE 1: AhR^{-/-} mice infected with *T. gondii* display profound weight loss and accelerated mortality compared to infected-wild type mice. Physical appearance (a), body weight (b), and survival rate (c) of AhR^{-/-} and WT mice infected with 40 cysts of *T. gondii* were monitored during the times indicated. The values presented are the mean \pm SD of at least 6 animals per time point per group. The experiment shown is representative of at least four performed that gave similar results. * $P < .05$ for body weight by student's *t*-test; * $P < .0001$ for survival rate by log-rank test between the means of the values obtained with AhR^{-/-} versus wild-type control mice.

by [3H]thymidine incorporation. As shown in Figure 4(a), only primed-WT spleen cells were able to respond to STAg stimulation. Supernatants from the cell cultures were used to determine IL-2, IL-12, IFN- γ , and IL-4 production. The unresponsiveness of spleen cells in AhR^{-/-} mice was in accordance with low levels of IL-2 observed in the supernatants (Figure 4(b), $P < .05$). Interestingly, higher levels of IFN- γ were detected in supernatants of STAg-stimulated spleen cells from AhR^{-/-} compared to WT mice (Figure 4(d)). In contrast, no differences in IL-12 (Figure 4(c)) and IL-4 levels (data not shown) were observed in the same cultures.

3.4. Overproduction of IL-12 and IFN- γ in Peritoneal Exudate Cells from AhR^{-/-} Mice. It is well known that mononuclear phagocytes are important in controlling the early stage of *T.*

gondii infection by early and continuous production of IL-12, which is a key lymphokine that mediates host resistance to *T. gondii* infection [45, 46]. Therefore, we asked whether IL-12 production by mononuclear cells was altered in *T. gondii*-infected AhR^{-/-} mice. To assess this, we compared the ability of AhR^{-/-} and WT peritoneal exudate cells (PECs) to produce IL-12 and IFN- γ in response to STAg or medium alone for 48 hours. As seen in Figure 5, PECs from mice lacking AhR produced greater amounts of IL-12 and IFN- γ than WT mice (Figures 5(a)–5(b), resp.).

3.5. AhR^{-/-} Mice Exhibit Greater Liver Damage than WT Mice. From the above observations, we hypothesized that the immunopathology and death observed in infected AhR^{-/-} mice were due to overexpression of proinflammatory

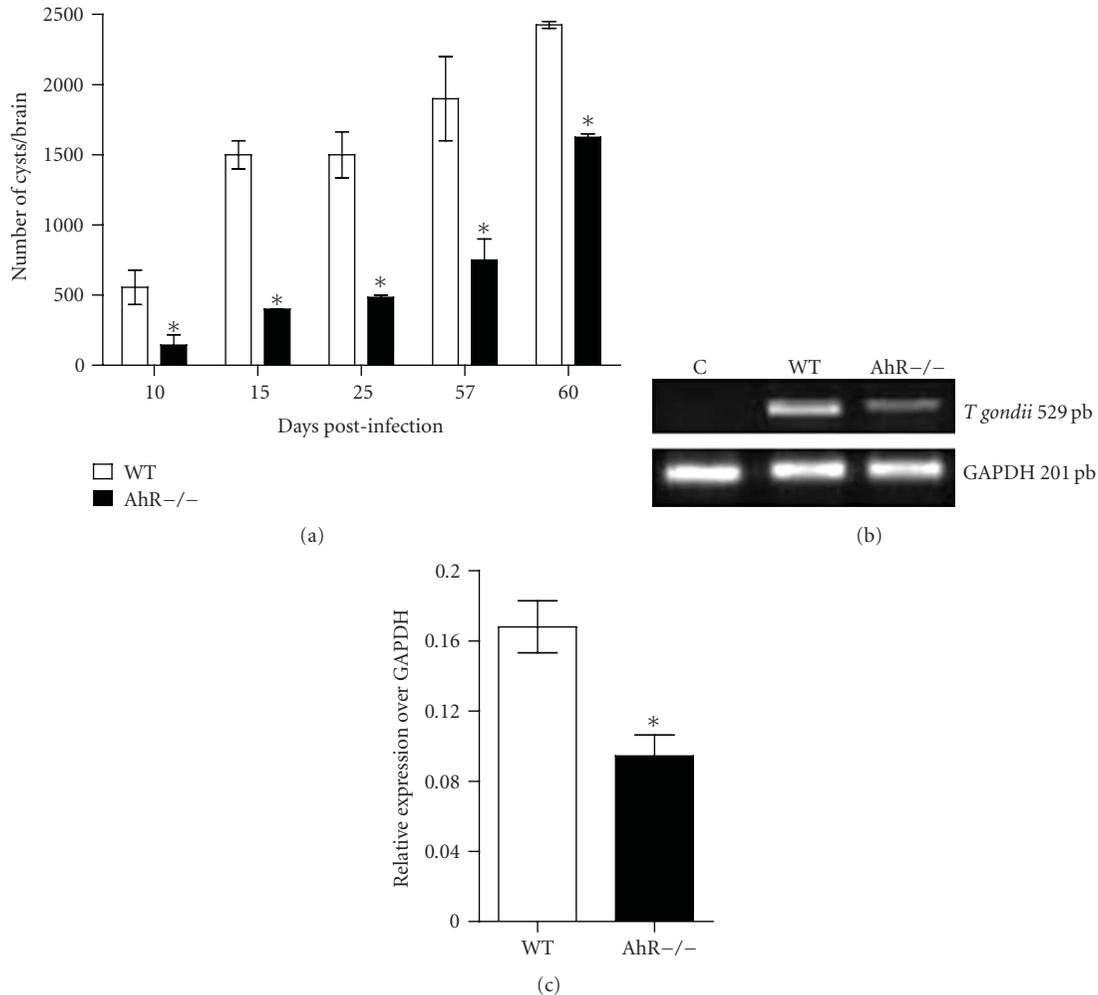


FIGURE 2: Parasite burden in *T. gondii*-infected AhR^{-/-} and WT mice after i.p. injection with ME49 strain of *T. gondii*. (a) Number of cysts per brain obtained after a kinetics of days p.i. as counted under the microscope (40X magnification). Data are representatives of three independent experiments where $n =$ five mice per group ($*P < .01$ with respect to WT, student's t -test). (b) *T. gondii* gene expression by semiquantitative PCR was performed in brains of noninfected mice (letter C) and WT and AhR^{-/-} mice at 25 days post *T. gondii* infection using primers specific for the sequence of the *T. gondii* gene: A representative gel electrophoresis from three independent experiments. (c) PCR analysis for detection of *T. gondii* in brains of WT and AhR^{-/-} mice at 25 days post *T. gondii* infection (Student's t -test).

cytokines. To confirm this hypothesis, portions of the livers from infected WT and AhR^{-/-} mice were subjected to histopathology. At day 10 post infection livers of infected WT mice presented a small number of mononuclear inflammatory foci; however, more number of small granulomas and inflammatory infiltrates were observed in livers from AhR^{-/-} mice at 10 days post infection (Figure 6(a)). At day 25 post infection, inflammatory infiltrate was present, but granulomas were rarely observed, and when present, were smaller in livers from infected WT mice (Figure 6(b)). In contrast, livers from *T. gondii*-infected AhR^{-/-} mice exhibited a large area of granulomas and had more mononuclear inflammatory infiltrates scattered by parenchyma and portal areas than *T. gondii*-infected WT mice. Additionally, detection of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) confirmed the extent of damages induced by the inflammatory response in livers, as liver

samples from infected AhR^{-/-} mice produced higher levels of AST and ALT at 25 days after infection, when inflammatory infiltrates and granulomas were detected in livers of *T. gondii*-infected AhR^{-/-} mice (Figures 6(c) and 6(d), resp.).

3.6. Quantification of IFN- γ , IL-10, and GATA-3 in Spleen Cells and Brains by RT-PCR. Given that GATA-3 is a key factor for Th2 differentiation, we investigated whether AhR deficiency had an effect on GATA-3 mRNA expression in spleen cells and brains from *T. gondii*-infected AhR^{-/-} and WT mice at 25 days post infection. Comparable levels of IFN- γ mRNA were detected in spleen cells and brains from AhR^{-/-} and WT mice. However, spleen cells and brains of AhR^{-/-} mice expressed lower transcript levels of both GATA-3 and IL-10 compared to WT mice (Figures 7(a) and 7(b), resp.).

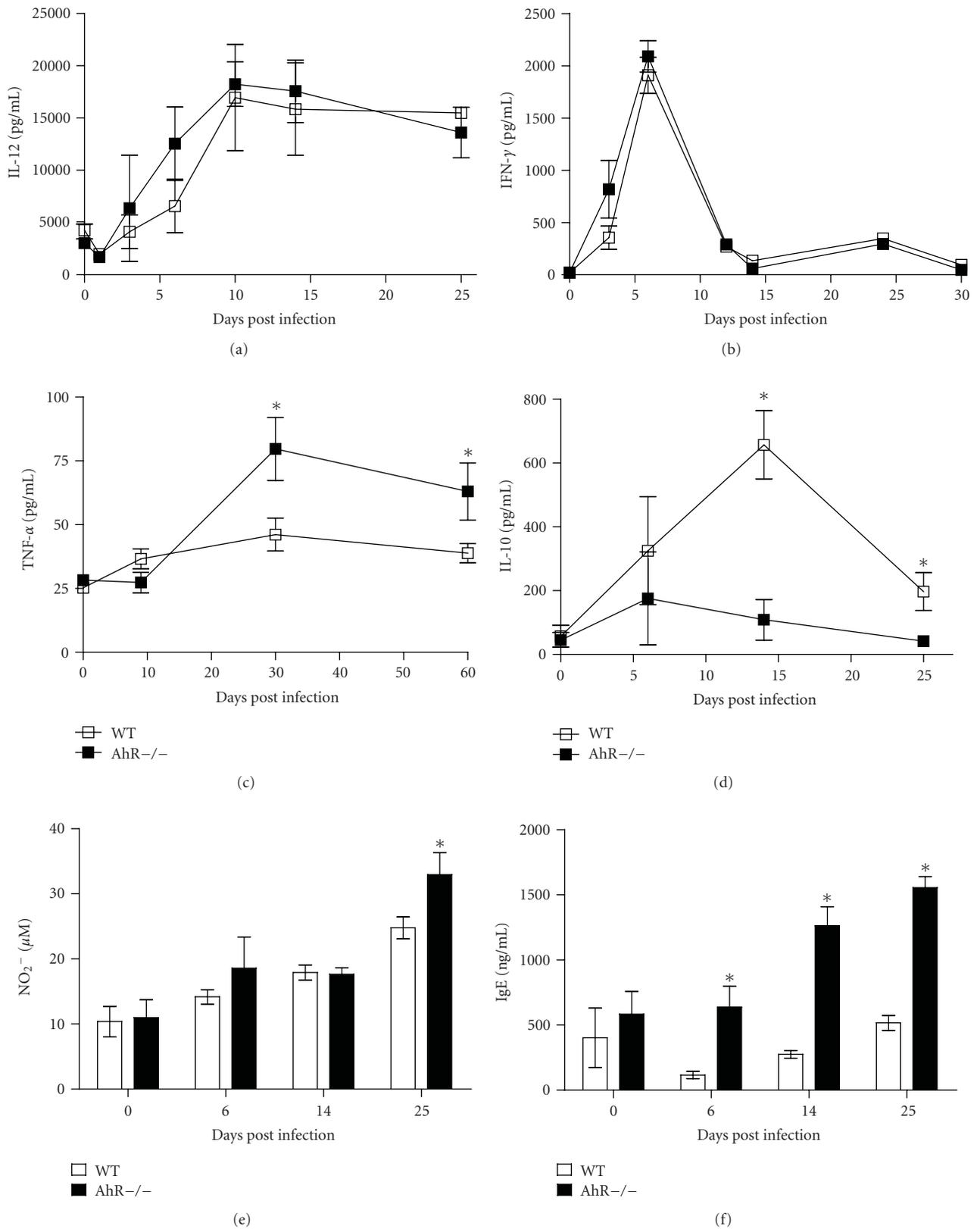


FIGURE 3: Levels of IL-12p70 (a), IFN- γ (b), TNF- α (c), IL-10 (d) nitric oxide (e), and total IgE (f) in sera from AhR-/- and WT mice infected with 40 cysts of *T. gondii*. For systemic cytokine, nitric oxide, and IgE production, mice were bled at the indicated time points and the levels of cytokines and total IgE (f) were measured in serum by ELISA and nitric oxide (e) was measured in serum by Griess assay as described above. The values presented are the mean \pm SD of triplicate samples of 6 animals per time point per group. *P < .05 with respect to WT, Student's *t*-test.

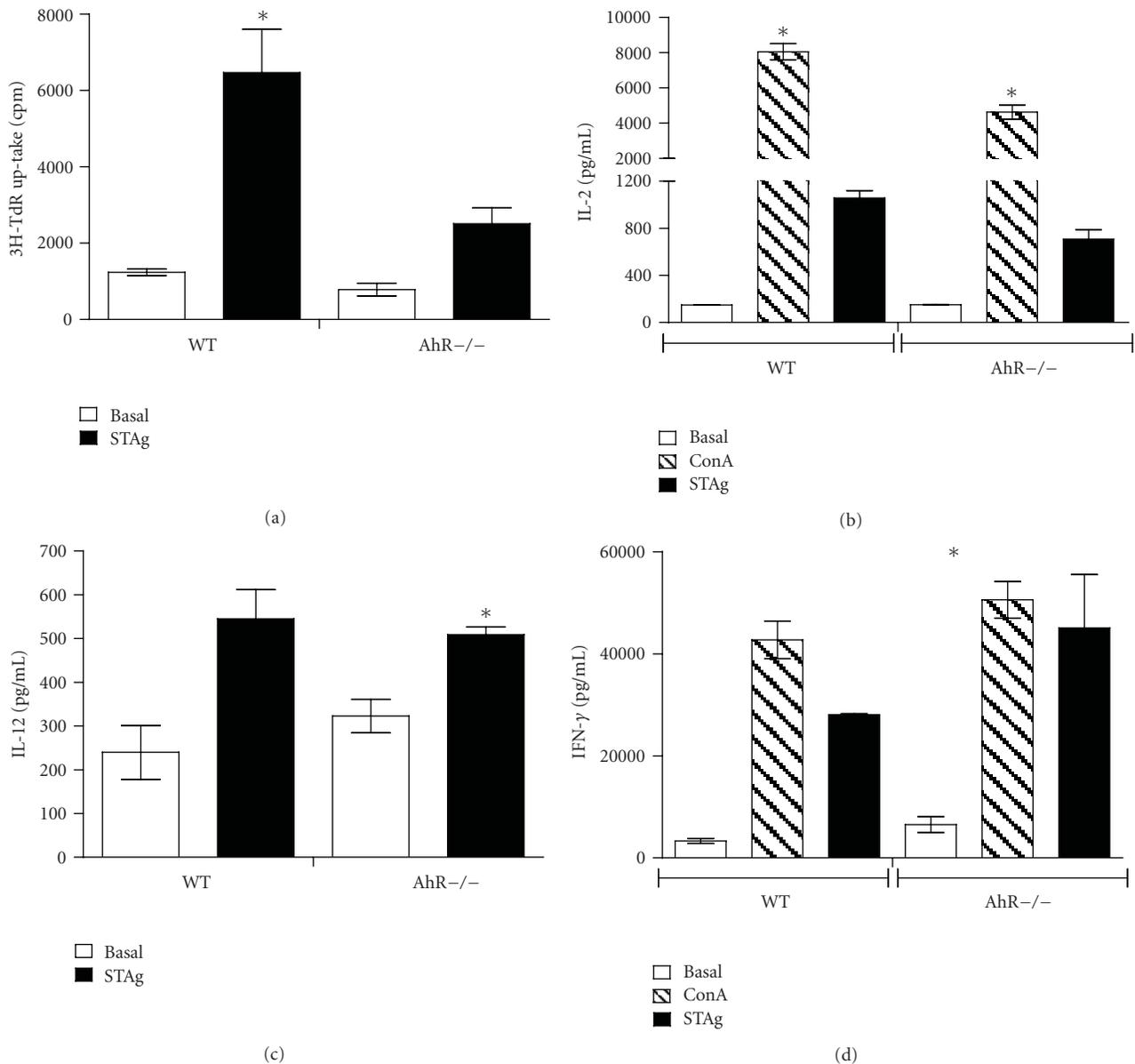


FIGURE 4: STAg-induced proliferative responses of spleen cells. Isolated spleen cells from *T. gondii*-infected (25 days p.i.) WT or AhR^{-/-} mice were stimulated with 2.5 μg/mL of STAg for 5 days in vitro. ³[H]TdrU was added (0.5 μCi/well) 20 hours before harvesting, and counts per minute (cpm) were calculated using a liquid scintillation counter (a). Secretion of IL-2 (b), IL-12 (c), and IFN-γ (d) in supernatants recovered from cell cultures were evaluated by an ELISA-sandwich. Means ± SE, n = 5. *P < .05, Student's *t*-test.

3.7. FACS Analysis. Upon establishing that deaths of AhR^{-/-} mice infected with *T. gondii* is likely due to a high proinflammatory response that may control parasite replication but, at the same time, cause severe systemic damage to the host, we asked whether AhR deficiency had a role on the immunophenotyping of T cell subpopulations. To test this, spleen cells were obtained at 25 days post infection and incubated with 2.5 μg/mL of STAg ex vivo for 5 days, and CD4⁺, CD8⁺, and CD4⁺/CD25⁺/Foxp3⁺ (T regulatory lymphocytes-T_{reg}) lymphocyte subpopulations were quantified. As shown in Table 2, the proportion of STAg-specific CD4⁺ or CD8⁺ T cells (Table 2) were comparable between AhR^{-/-} and WT

mice. Interestingly, a slight, but not significant, decrease in STAg-specific T_{reg} cells was observed in AhR^{-/-} mice compared to WT mice (Table 2). Taken as a whole, these data show that AhR^{-/-} mice are capable of developing an adaptive immune response.

Macrophages are very important innate immune cells that respond promptly to *T. gondii* infection as well as to its soluble antigen. Moreover, it is well known that CCR5 is one of the main receptors for STAg that is involved in triggering the early production of IL-12 and TNF-α [47]. Hence, to determine whether AhR deficiency phenotypically and functionally alters these cell populations, we analyzed the

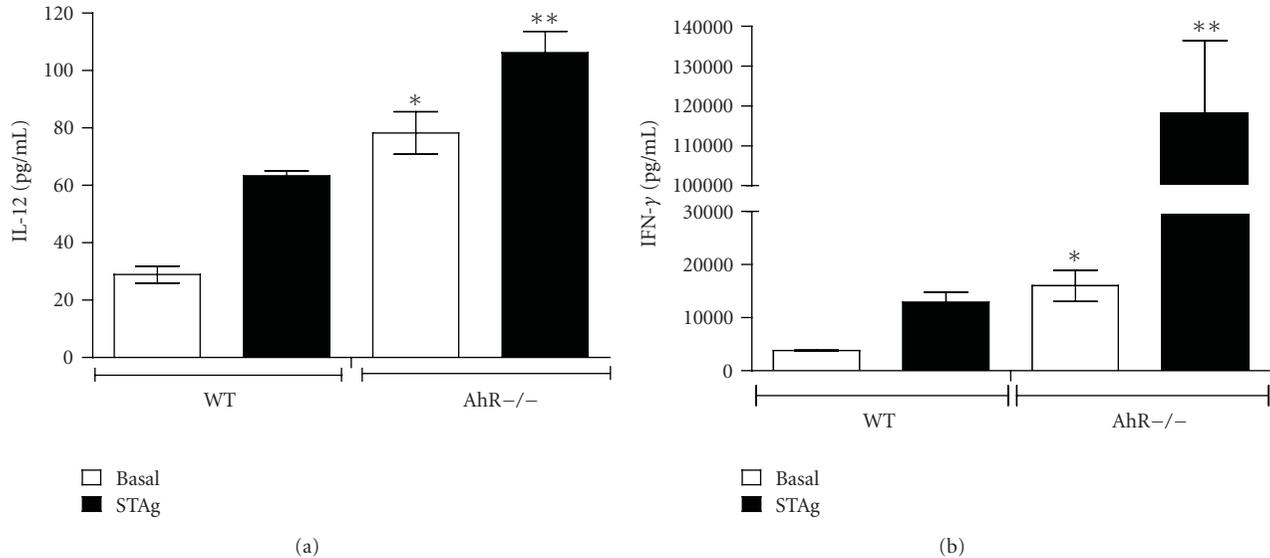


FIGURE 5: IL-12 and IFN- γ levels of PECs from *T. gondii*-infected AhR^{-/-} and WT mice. After 25 days of *T. gondii* infection, PECs from AhR^{-/-} or WT mice were recovered and restimulated in vitro with 2.5 μ g/mL of STAg for 24 hours. The IL-12 and IFN- γ levels were determined by ELISA-sandwich on supernatants recovered from cultures. Means \pm SE, $n = 5$. * $P < .05$ with respect to basal bars values. ** $P < .05$ with respect to STAg stimulus, Student's t -test.

TABLE 2: Flow cytometric profiles of spleen cells stained for CD4+, CD8+, and CD4+/CD25+/Foxp3+ (Treg). Data are shown as % of *T. gondii*-specific CD4+, CD8+, and Treg cells from 4 to 5 mice per group.

	WT (%)	AhR ^{-/-} (%)
CD4+	26.0 \pm 2.3	25.4 \pm 2.7
CD8+	12.7 \pm 1.5	13.2 \pm 0.9
CD4+/CD25+/Foxp3+	9.5 \pm 2.37	7.4 \pm 2.8

expression of CCR5 and TLR2, another molecule that may be involved in *T. gondii* recognition, on peritoneal adherent macrophages isolated from AhR^{-/-} and WT mice 25 days after infection. AhR^{-/-} mice displayed at least 50% lower expression of TLR2 mainly on F4/80+ cells (Figure 8(a)). In contrast, expression of CCR5 was unaltered in the same population (Figure 8(b)).

3.8. AhR^{-/-} Mice Exhibit Decreased 5-LOX mRNA Expression. It has been demonstrated that the eicosanoid called lipoxinA₄ (LXA₄) plays a role in the anti-inflammatory response against *T. gondii* [33]. Interestingly, LXA₄ is an endogenous ligand for AhR, and the binding of AhR with LXA₄ controls the expression of proinflammatory cytokines such as IL-12 and IFN- γ [48]. Given that the expression of LXA₄ is dependent on the expression of 5-lipoxygenase (5-LOX) [33, 48], we asked whether expression of 5-LOX may be affected in *T. gondii*-infected AhR^{-/-} mice. As seen in Figure 8(c), the transcript levels of 5-LOX in spleen cells were significantly lower in AhR^{-/-} mice compared to WT-infected mice (Figure 8(c)).

4. Discussion

AhR has been suggested to play an important role in the immune response to virus and bacterial infections, where a functional innate immune response is pivotal for complete resistance to the pathogens [20, 22, 49]. However, in those studies the molecular mechanism associated with AhR was not too clear. Thus, establishment of the role of AhR in response to parasitic infections may help in understanding the endogenous immune function of AhR.

Here, we showed that AhR deficient mice (AhR^{-/-}) are more susceptible to *T. gondii* infection than WT mice. After peritoneal infection with 40 cysts AhR^{-/-} mice succumbed to *T. gondii* infection faster than WT mice; however, AhR^{-/-} mice developed fewer brain cysts, despite higher serum levels of TNF- α , nitric oxide (NO) and IgE and lower serum levels of IL-10 compared to infected WT mice. The high mortality rates in AhR^{-/-} mice suggest that AhR is critical in the host defense against toxoplasmosis; however, the lower number of cysts in the brain in conjunction with high levels of TNF- α , and NO in AhR^{-/-} mice suggests that the higher mortality rate is not caused by the inability to restrict parasite replication.

Resistance to experimental toxoplasmosis has been shown to be dependent on production of several proinflammatory cytokines (MIF, IL-1 β , IL-12, TNF- α , and IFN- γ) and NO [25, 29, 30, 50]. Furthermore, the powerful proinflammatory immune response together with IgE, an antibody that has been correlated with early acute inflammation in toxoplasmosis [51, 52], and NO [53] restricts dissemination of the parasite and prevents death by parasitic infection. After parasite dissemination has been contained by IFN- γ -dependent responses, the onset of the

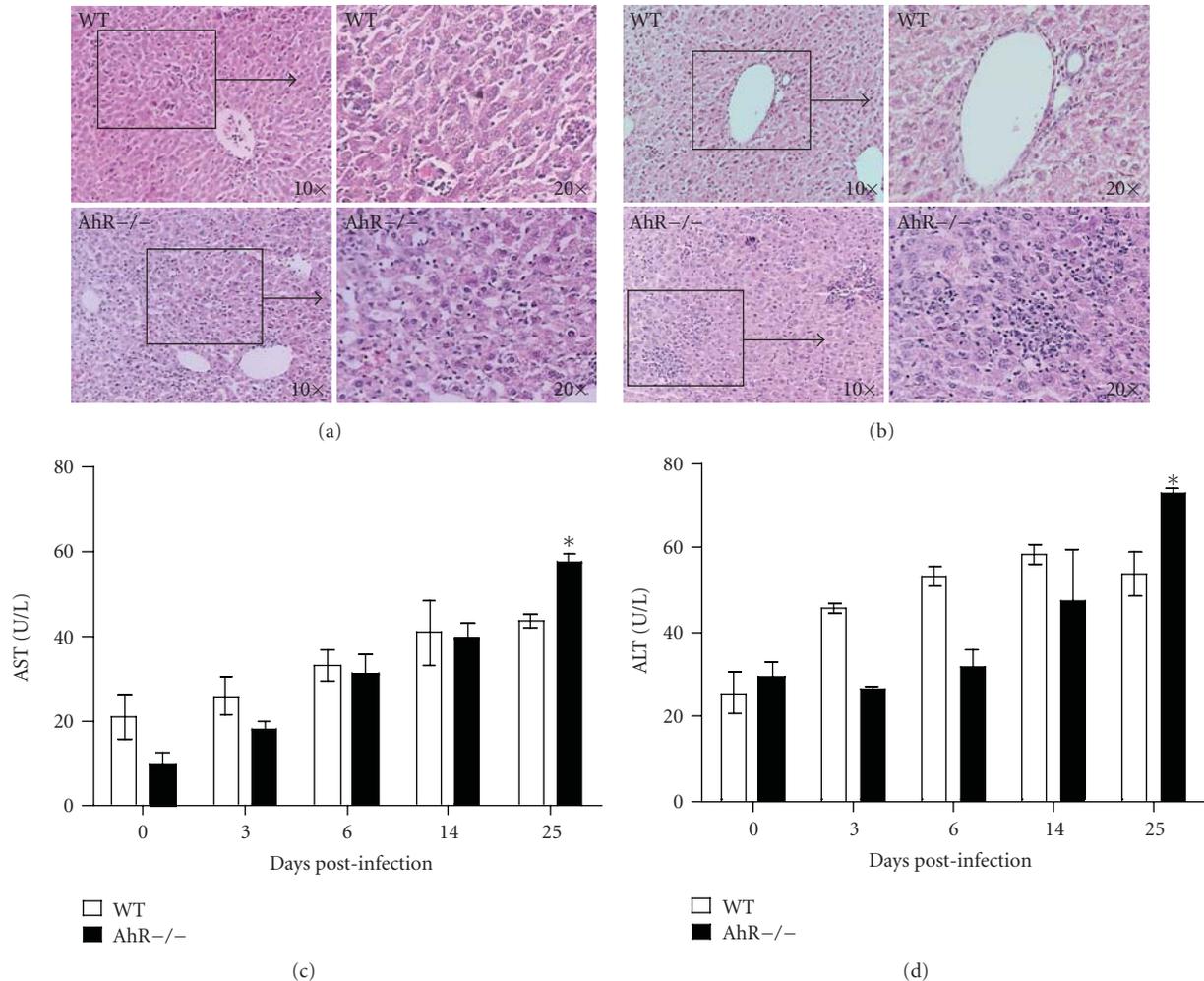


FIGURE 6: Representative histopathological liver changes after 10 days (a) or 25 days (b) of infection with 40 cysts of *T. gondii*. Note the mass of necrosis observed by H & E staining (arrow). (c) Serum transaminase levels on AhR^{-/-} and WT infected mice. * $P < .05$, Student's *t*-test.

chronic phase of infection is characterized by continuous cell-mediated immunity. Such potent responses are kept under tight control by anti-inflammatory cytokines such as IL-10, which is required for preventing necrosis in the small intestine and death in both genetically resistant BALB/c and susceptible C57BL/6 mice following infection with *T. gondii* [54]. Thus, these first results suggest that high production of inflammatory cytokines in conjunction with low levels of IL-10 could reduce dissemination of brain cysts without preventing mortality, possibly because the inflammatory response causes systemic damage.

To test the above hypothesis, we analyzed the T cell response by comparing the proliferative capacity and cytokine production in spleen cells from AhR^{-/-} and WT infected mice in the presence of STAg. The proliferation and IL-2 production in AhR^{-/-} infected mice were diminished. These observations were in line with previous report that show that embryonic fibroblasts from AhR^{-/-} mice exhibit a lower proliferation rate and impaired IL-2 production

[55] associated with the fact that the IL-2 promoter region contains distal regulatory elements that can be addressed by the AhR to induce IL-2 and cooperate with the proximal promoter in this [8]. Moreover, it is well known that TNF- α and IFN- γ have antiproliferative properties [52]. We detected high levels of TNF- α in serum, which could have contributed to low levels of proliferation in *T. gondii*-infected AhR^{-/-} mice.

In accordance with our initial hypothesis, we found high levels of IFN- γ in the supernatant of spleen cells from infected AhR^{-/-} mice. Somewhat to our surprise, no difference in IL-12 levels was detected in supernatants of spleen cell cultures between AhR^{-/-} and WT mice. However, this inconsistency was not observed in supernatants from restimulated STAg PECs. The levels of IL-12 and IFN- γ in supernatants of AhR^{-/-} PECs were significantly higher than those in WT-PECs. These observations were consistent with the extensive histopathological damage in livers and high levels of ALT and AST detected in sera. Together, the high production of inflammatory cytokines in conjunction with

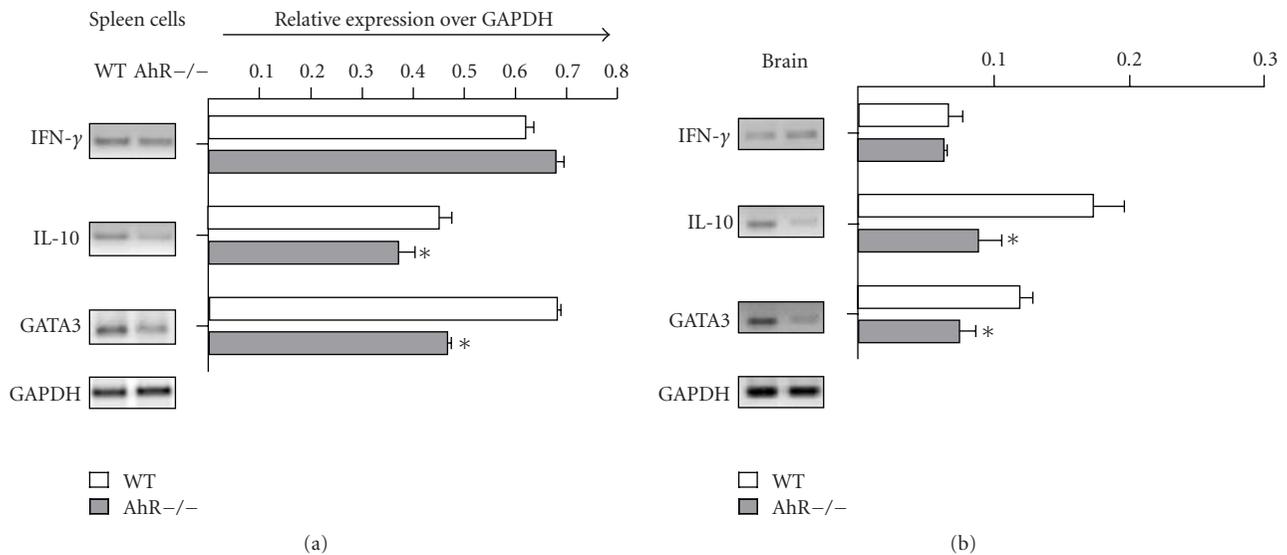


FIGURE 7: Gel electrophoresis on IFN- γ , IL-10, GATA-3, and GAPDH-amplified products. Total RNA was isolated from Splenocytes (a) or brain tissue (b) from WT and AhR^{-/-} mice at 25 days after *T. gondii* infection as described in *Materials and Methods*. The resulting fragments migrated along with the 200–500 bp fragment of the 100 kb marker used. The exact fragment sizes are listed in Table 1.

low levels of IL-10 may mediate the damage in AhR^{-/-} mice and may contribute to the high mortality rate observed in *T. gondii*-infected AhR^{-/-} mice.

Previous studies have suggested that AhR has a role in modulating the balance between Th1 and Th2 response [10, 15]. Therefore, we asked whether high levels of proinflammatory cytokines favor Th1 polarization in AhR^{-/-} mice. The mRNA expressions of IFN- γ , IL-10, and GATA-3 were determined by RT-PCR in spleen cells and brains from infected AhR^{-/-} and WT mice. Contrary to what we expected, we did not observe any significant difference in IFN- γ mRNA levels in either spleen cells or the brains between AhR^{-/-} and WT mice. In contrast, reduced levels of IL-10 and GATA-3 were observed in AhR^{-/-} mice compared to WT mice. We suggest that the reduction of GATA-3 and IL-10 observed in *T. gondii*-infected AhR^{-/-} mice is responsible for potentiating the biological activity of IFN- γ , rather than favoring polarization of the immune response toward a Th1 response.

Recent results suggest that AhR can regulate the generation of regulatory T cells (T_{regs}), a main source of IL-10, since AhR activation by TCDD induces differentiation of T-cell progenitor cells into T_{regs} [56]. Given that infected AhR^{-/-} mice display low levels of IL-10, one possibility is that T_{regs} subpopulation is reduced in the absence of AhR. However, this idea was disproved by our observation that no significant differences were observed in the proportion of T_{regs} cell subpopulations between AhR^{-/-} and WT mice. Furthermore, no significant differences were noted in CD4⁺ and CD8⁺ T cell subpopulations between *T. gondii*-infected AhR^{-/-} and WT mice. These results suggest that AhR is not required for adaptive T cell response during toxoplasmosis, at least in those subpopulations analyzed. The production of cytokine IL-12 is critical for the development of IFN- γ dependent

resistance to *T. gondii* infection (Aliberti J Alan Sher JEM 196 No.9 2002). The signaling pathway through CC chemokine receptor 5 (CCR5) plays a critical role in triggering IL-12 production, mainly by CD8 α ⁺ subset of dendritic cells (DC) upon stimulation with STAg [33, 47]. Since macrophages also display CCR5 expression, we considered the possibility that overexpression of CCR5 on macrophages might be responsible for the high levels of IL-12 secreted by PECs from *T. gondii*-infected AhR^{-/-} mice. However, in the present study, there was no significant difference in CCR5 expression on F4/80⁺ cells between infected AhR^{-/-} and WT mice. This observation shows that, at least in macrophages, CCR5 expression is not affected by the lack of AhR.

Toll-like receptors (TLRs), which are innate immune receptors, are also involved in the recognition of *T. gondii* profilin. TLR11 is the main receptor that plays a major role in IL-12-dependent control of *T. gondii*, although other TLR family members also contribute to host resistance to this protozoan pathogen [57]. Thus, while TLR2 deficient mice display a normal IL-12 production and resist *T. gondii* infection at conventional doses, they are susceptible when challenged with higher infective doses, arguing for a cooperative role of TLR2 in controlling the parasite [58]. Moreover, recently it has been described that both human and murine bone marrow-derived DC expressing high levels of TLR2 favor an anti-inflammatory response characterized by enhanced IL-10 production [59–61]. Therefore TLR2 triggering mediates IL-10 upregulation. Here we showed that macrophages from *T. gondii*-infected AhR^{-/-} mice expressed significantly less TLR2 as compared to WT mice. This observation supports the notion that lower expression of TLR2 on macrophages contributes to reduce IL-10, favoring the robust proinflammatory response observed in *T. gondii*-infected AhR^{-/-} mice.

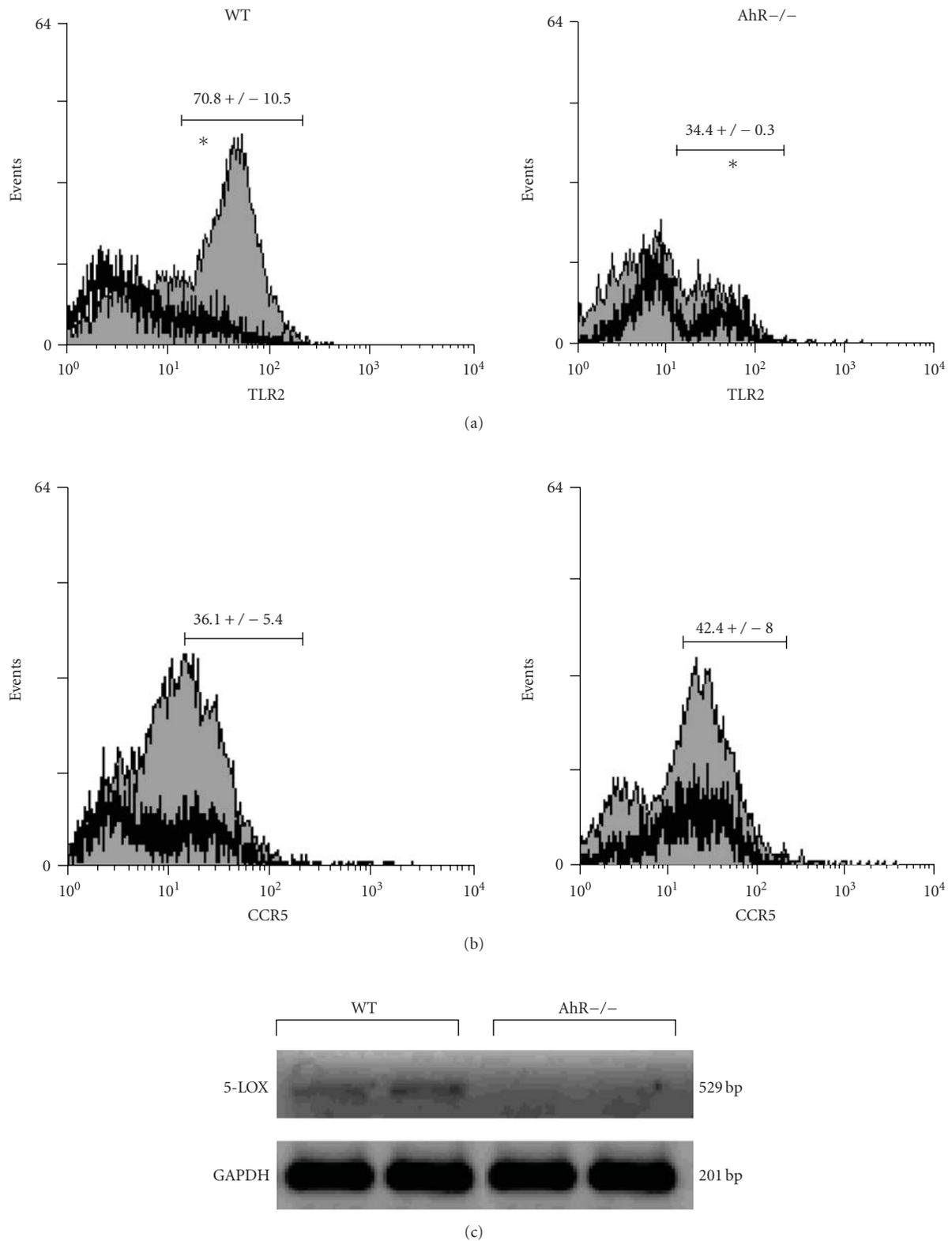


FIGURE 8: Flow cytometric profiles of adherent exudate macrophages stained for TLR2 and CCR5. The histograms represent TLR2 (a) or CCR5 (b) versus F4/80 expression on adherent macrophages from AhR^{-/-} or WT mice challenged with STAg for 24 hours after 25 days of *T. gondii* infection: Nonstimulus (dark line) and STAg stimulus (gray curve). Shown are representative data from three to four independent experiments. Significances were calculated by Student's t test. * $P < .05$ WT versus AhR^{-/-}. Gel electrophoresis of 5-LOX and GAPDH-amplified products (c). Total RNA was isolated from splenocytes from WT and AhR^{-/-} mice at 25 days after *T. gondii* infection as described in *Materials and Methods*.

Modulation of CCR5 on DCs involves ligation of G protein-coupled receptor formyl peptide receptor-like 1 (FPRL-1). LXA₄, an arachidonate-derived inhibitor of acute inflammation, has been suggested to bind to two receptors: FPRL-1 (therefore could induce CCR5 downmodulation) and AhR [62].

In vivo injection of STAg triggers production of endogenous LXA₄ in a 5-lipoxygenase-(5-LOX-) dependent manner causing suppression of IL-12 production by DCs [33]. Furthermore, upon infection with *T. gondii*, serum levels of LXA₄ increase steadily in WT mice over the course of the acute phase and remain high during the chronic phase [33]. Moreover, induction of SOCS-2, an intracellular mediator of anti-inflammatory response, upon STAg injection requires 5-LOX and AhR [48]. Interestingly, 5-LOX-deficient mice succumb to *T. gondii* infection at the early onset of chronic disease with excessive production of proinflammatory cytokines and substantially fewer brain cysts, suggesting that the excessive proinflammatory response in brains of 5-LOX-deficient hosts is responsible for the mortality [63]. In line with this, we found that 5-LOX expression in spleen cells was significantly decreased in *T. gondii*-infected AhR^{-/-} mice. Thus, one possible mechanism by which the absence of AhR may cause these enhanced inflammatory responses can be the lack of interaction between AhR and LXA₄, through the absence of expression of 5-LOX, which leads to a failure to control the magnitude of the inflammatory response induced by *T. gondii* infection. Further research will be necessary to prove this hypothesis.

In summary, we presented here experimental evidence for a regulatory role of AhR during experimental toxoplasmosis using AhR^{-/-} mice. Our data suggest that AhR is not required for adaptive T cell response to *T. gondii* infection but may play a constitutive role in the innate immune response to toxoplasmosis by dampening the inflammatory responses. These studies represent the first demonstration that AhR is critically involved during a protozoan infection.

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Review Article

The Prominent Role of Neutrophils during the Initial Phase of Infection by *Leishmania* Parasites

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Neutrophils are rapidly and massively recruited to the site of *Leishmania* inoculation, where they phagocytose the parasites, some of which are able to survive within these first host cells. Neutrophils can thus provide a transient safe shelter for the parasites, prior to their entry into macrophages where they will replicate. In addition, neutrophils release and synthesize rapidly several factors including cytokines and chemokines. The mechanism involved in their rapid recruitment to the site of parasite inoculation, as well as the putative consequences of their massive presence on the microenvironment of the focus of infection will be discussed in the context of the development of the *Leishmania*-specific immune response.

1. Introduction

Obligate intracellular protozoa of the genus *Leishmania* are sand fly-transmitted parasites capable of infecting various mammalian hosts including rodents, dogs, and humans. In man, infection with different species of *Leishmania* leads to a large spectrum of clinical manifestations including cutaneous, mucocutaneous, and visceral forms. Spontaneous cure of skin ulcers and life-long immunity to reinfection are the most common outcome in cutaneous leishmaniasis (CL), but diffuse or mucocutaneous forms of the disease may also develop. Mucocutaneous leishmaniasis (MCL) is mostly related to *Leishmania* species of the New World and can lead to partial or total destruction of the mucosal epithelia of the mouth, nose, throat, and associated tissues. Visceral leishmaniasis (VL) may display several clinical manifestations ranging from subclinical infection to potentially lethal forms if not treated, with heavy parasite burdens in the spleen, liver, and bone marrow, associated with anemia, splenomegaly, hepatomegaly, fever, and loss of weight. Differences in clinical outcome and manifestations of leishmaniasis result from several parameters including the nature of the infecting *Leishmania* species and host genetic factors. There are currently no available vaccines.

In order to decipher the mechanisms of the immune response involved in susceptibility or resistance to infection, the most widely used experimental model of cutaneous leishmaniasis relies on the infection of mice with *L. major*. Subcutaneous infection with *L. major* promastigotes leads in most strains of mice (the so-called “resistant” strains, e.g., C57BL/6, C3H, CBA) to the development of a small, self-healing lesion, to the control of parasite replication, and to immunity against reinfection. In contrast, in a few (susceptible) strains of mice such as BALB/c, sustained inflammatory lesions develop and parasite replication is not controlled, with spreading of parasites to non-draining lymph nodes and spleen. Susceptibility or resistance to infection was shown to result from the development of a subset of T cells distinguished by the cytokines they secrete. Emergence of *Leishmania*-specific Th2 cells, characterized by the secretion of IL-4 and IL-13, was shown to correlate with susceptibility to infection. In contrast, resistance to infection was associated with the IL-12 driven secretion of IFN γ by Th1 cells. IFN γ activates the microbicidal properties of phagocytes leading to parasite clearing and healing of the lesions [1, 2].

The driving events leading to the development of either a protective response or nonhealing lesions were reported to

occur within the first hours to 3 days after infection with *L. major*, at a time during which neutrophils are massively recruited as a result of infection. Indeed neutrophils are the first cells to be mobilized and arrive within hours to the site of tissue damage and parasite entry. Together with macrophages they phagocytose *Leishmania*, but only macrophages function as definitive host cells for *Leishmania*. The rapid recruitment of neutrophils to the site of infection was first described following needle inoculation of a large number of parasites into the skin [3, 4] and elegantly confirmed following infection with the natural vector (infected *Phlebotomus dubosqui* sand flies) using two photon intravital microscopy [5]. Importantly, in resistant strains of mice, neutrophils are recruited within hours of parasite inoculation but their level decreases to 1%–2% of the cellular infiltrate 3 days after infection. In contrast, in susceptible BALB/c mice neutrophils are still recruited and detected in large numbers at the site of infection more than 10 days after parasite inoculation. The importance of the newly migrating polymorphonuclear leukocytes (PMN) in the subsequent development of *Leishmania*-protective immune response will be the subject of this review.

2. Interactions between Neutrophils and *Leishmania* during the First Days of Parasite Inoculation

One of the classical functions attributed to neutrophils is their capacity to phagocytose and kill microorganisms. However, some pathogens including *Leishmania* can survive transiently within neutrophils. To this end, the parasite has developed several protective mechanisms including the prevention of the activation of an oxidative burst, thus avoiding the generation of highly toxic reactive oxygen species [6] and the ability to be targeted to nonlytic compartments of neutrophils, as recently reported for *L. donovani* [7].

Neutrophils have a short lifespan and become rapidly apoptotic, leading to their phagocytosis by macrophages. However, following infection, their lifespan can be increased to several days. Indeed, infection of human neutrophils in vitro with *L. major* increased their lifespan to two days, inhibiting the processing of procaspases in the infected cells [8, 9]. In order to test if infection of mouse neutrophils with *L. major* also delays apoptosis, highly purified inflammatory neutrophils were isolated from the peritoneal cavity of mice four hours after injection of *L. major* i.p. and cultured for 24 hours alone or in the presence of *L. major* promastigotes. Neutrophil apoptosis was measured by FACS. Early apoptosis, characterized by the presence of phosphatidyl serine on the cell surface, was detected by Annexin-V staining, while staining with both Annexin-V and 7AAD was indicative of late apoptosis/necrosis. Exposure to *L. major* decreased markedly the percentage of both early and late apoptosis (Figure 1(a)). Coculture of neutrophils with an excess of macrophages (2:1 m ϕ :PMN ratio) was previously reported to increase significantly neutrophil apoptosis [10]. To investigate if macrophage-induced neutrophil apoptosis was modulated by *L. major*,

neutrophils and macrophages were cocultured with or without metacyclic *L. major* promastigotes. No decrease in neutrophil apoptosis was measured in *L. major*-infected relative to noninfected cultures; indeed an increase in early apoptosis (Annexin-V⁺, 7AAD⁻ neutrophils) was noted in neutrophils incubated with both macrophages and *L. major* (Figure 1(b)) as already shown [10]. The acceleration of neutrophil apoptosis by macrophages was reported to be mediated by the transmembrane form of Tumor necrosis factor (mTNF) on macrophages. Using mice that express a functional mTNF but do not release soluble TNF, it was shown that the sole presence of transmembrane TNF allowed the control (decrease) of neutrophil number at the site of parasite inoculation seven days postinfection, resulting in the resolution of the inflammatory lesion [10, 11].

Another study has revealed a further effect of the neutrophil-macrophage interaction on the fate of the intracellular parasite. Indeed in susceptible BALB/c mice, interaction between macrophages and dead neutrophils was reported to exacerbate parasite growth through the production of PGE₂ and TGF β by macrophages. In contrast, using cells from resistant C57BL/6 mice, interaction of dead neutrophils with macrophages promoted parasite killing, through secretion of TNF by macrophages [12].

Thus, it appears that at the onset of infection, neutrophils provide transiently a shelter to *L. major*, which in turn delays apoptosis of the cells as long as the increasing number of monocytes/macrophages at the site of parasite inoculation does not reverse this trend. When the ratio between leukocytes of the monocyte/macrophage and neutrophil increases two or three days following needle inoculation with a high dose of *L. major* in C57BL/6 mice, or following infection through the bite of *L. major*-infected sand flies, macrophages become the dominant cell population in the cellular infiltrate [3–6], favouring neutrophil apoptosis. When neutrophils become apoptotic, the parasites are transferred to macrophages where they will replicate.

The exact way live parasites present within neutrophils are transferred to macrophages is currently not clear and may include several modes of entry. Data obtained in vitro showed that macrophages can phagocytose apoptotic neutrophils containing intact *Leishmania*, providing the parasite with a silent “Trojan horse” mode of infection [9, 13].

Recently, Peters et al. purified *Leishmania*-infected neutrophils and injected them into the skin of mice that had been exposed to the bites of uninfected sand flies (to condition the site of inoculation of the infected cells to resemble as closely as possible that of a natural infection). In vivo visualization of the interaction between neutrophils and macrophages revealed that neutrophil-containing *Leishmania* were not directly phagocytosed by macrophages, but rather, neutrophils released the parasites that subsequently entered macrophages [5]. More studies will be needed to evaluate if there exists distinct modes of transfer from neutrophils to macrophages between different *Leishmania* species or between strains of differing virulence within a given species.

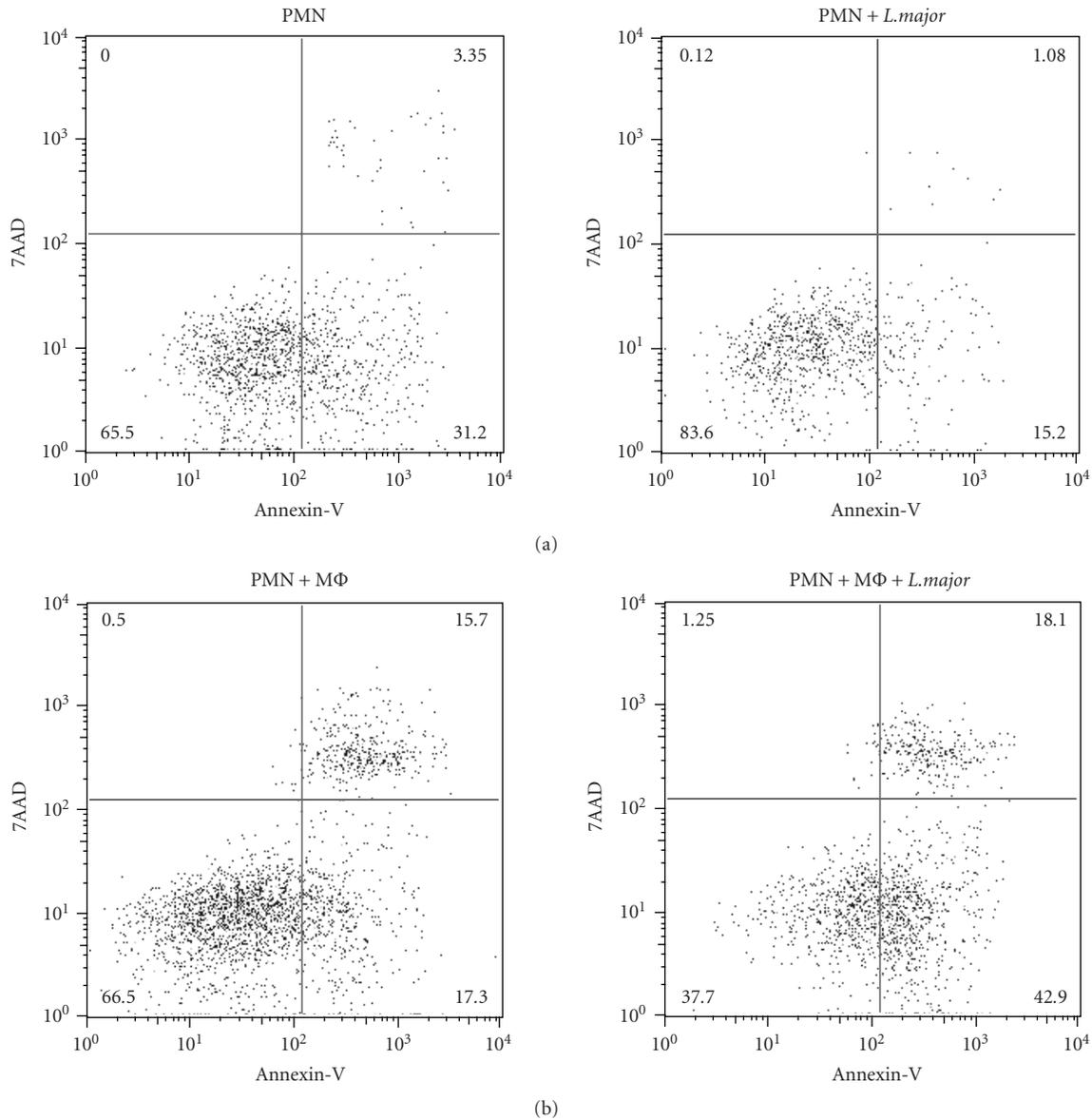


FIGURE 1: Exposure of neutrophils to *L. major* decreases spontaneous but not macrophage-induced apoptosis. *L. major*-recruited C57BL/6 neutrophils (PMNs) and macrophages (M ϕ) were isolated from the peritoneal cavity 4 hours or 24 hours after *L. major* injection i.p., respectively. (a) MACS-purified PMNs were cultured for 24 hours in presence or in absence of metacyclic *L. major* (*L. major* : PMN ratio 1 : 5). Cells were collected, labeled with Annexin-V, 7AAD, and the 1A8 mAb (Ly6G) and PMN apoptosis analyzed by FACS, gating on the 1A8⁺ PMN population. Early apoptotic cells are Annexin-V⁺ 7AAD⁻, and late apoptotic/necrotic cells are Annexin-V⁺ 7AAD⁺. (b) PMNs were cultured in presence of fixed M ϕ (PMN : M ϕ 1 : 2) with or without parasites, and 24 hours later, neutrophils were analyzed as in A. Data are representative of three independent experiments.

That neutrophils play a decisive role at the onset of infection by *Leishmania* parasites is also suggested by studies on experimental visceral leishmaniasis. Indeed, neutrophils were shown to have both a direct impact on parasite killing and on the protective immune response developing following infections with *L. donovani* or *L. infantum* [14–16]. A small percentage of *L. donovani* parasites was reported to escape direct killing in neutrophils, being directed to nonlytic compartments of the cells. Macrophages appeared to be able to phagocytose the parasitized neutrophils in vitro [7],

but whether this or additional mechanism(s) of *L. donovani* entry exist during infection remains to be determined.

3. Neutrophils as Orchestrators of the Immune Response

In addition to their phagocytic function, neutrophils contribute to the initiation of inflammation, a process which is

recognized as essential in launching immunity. The importance of neutrophils as decision shapers in the development of an immune response is only emerging as they have long been considered by immunologists as short lived, nondividing cells of poor interest [17]. This view is now changing, and neutrophils currently appear not only as key components of the inflammatory response but also as cells that display important immunoregulatory roles in different microbial infections [4, 18–21]. In this part of the review, we will focus (1) on the parameters controlling neutrophil early recruitment following *L. major* infection and (2) on the release by neutrophils of cytokines and chemokines that may influence the development of a protective immune response against the parasite.

3.1. Mediators of Early Neutrophil Recruitment. During homeostasis, neutrophils are circulating in the blood. Upon deposition of *L. major* in the skin, the sand fly (or the needle) causes tissue injury, favouring within hours massive neutrophil influx from the bloodstream to the site of *Leishmania* inoculation. The factors involved in their rapid recruitment are still not well defined and may involve chemokines, cytokines and other molecules secreted by the host and/or the parasite, as discussed below.

Neutrophils are predominantly responsive to members of the CXC chemokine family such as IL-8, a chemokine not only primarily secreted by epithelial cells, keratinocytes, fibroblasts, and endothelial cells but also by neutrophils. Indeed, in response to *L. major*, human neutrophils were shown to release IL-8 *in vitro* [22], a process that should favour their own recruitment. In the mouse IL-8 has two functional homologues, MIP-2 (CXCL2, Gro β) and KC (CXCL1, Gro α). Upon *L. major* infection, KC mRNA has been reported to be rapidly and transiently induced in *L. major* infected skin, suggesting a possible association between KC transcription and granulocyte recruitment [23]. Thus IL-8 in humans and KC in mice may contribute to the early neutrophil recruitment at the site of parasite inoculation but their direct involvement during infection remains to be demonstrated *in vivo*.

Neutrophil recruitment can also be induced by cytokines such as IL-17 and Tumor necrosis factor (TNF). Among the family of IL-17 cytokines, IL-17A and IL-17F are able to promote the recruitment of monocytes and neutrophils via the induction of other cytokines and chemokines such as G-CSF and IL-8 by various cell types (reviewed in [24]). The role of IL-17 on neutrophil recruitment to the site of *L. major* inoculation has been investigated in BALB/c mice genetically deficient in this cytokine (IL-17^{-/-} mice). During the first three weeks following *L. major* inoculation, neutrophil recruitment at the site of infection did not differ between IL-17^{-/-} and control mice. However, from four weeks after infection neutrophil numbers in the infected skin were significantly lower in absence of IL-17 [25]. Thus IL-17 does not seem to be a major contributor of the early neutrophil recruitment occurring during the first days of infection but appears to be involved in promoting neutrophil influx into the infection site at later time points.

Following infection with *L. major*, mice deficient in TNF or in one of its receptor (TNFR1 or p55) develop nonhealing ulcers, revealing an essential role for TNF in the control of inflammatory lesions [26–31]. Furthermore, using mTNF-knock-in (mTNF Δ/Δ) mice, which express functional mTNF but do not release soluble TNF, the transmembrane form of TNF was shown to be crucial in resolution of the inflammation associated with the leishmanial ulcer. Indeed following *L. major* infection, (mTNF Δ/Δ) mice, unlike TNF^{-/-} mice, were able to heal their lesion, a process associated with clearance of neutrophils from the infection site [11]. In addition, we further showed that transmembrane TNF was essential in the control of neutrophil presence one week after infection. Neutrophil numbers were still elevated at the site of infection 7 days after parasite inoculation in TNF^{-/-} mice while *L. major* infected mTNF Δ/Δ mice controlled significantly better the number of neutrophils present at the infection site, which declined to levels comparable with those observed in C57BL/6 mice. Thus TNF appears to be an essential player in the control of neutrophils in the lesion however, its contribution in the rapid recruitment of neutrophils within hours of *L. major* inoculation still remains to be investigated.

Mast cells are thought to play a role of sentinels in the skin and are known to be activated by different factors, including live *L. major* [32]. When mice deficient in mast cells were inoculated with *L. major*, they exhibited decreased neutrophil recruitment to the infection site three weeks after infection. However no apparent difference in neutrophil numbers was detected during the first week of infection [33], suggesting that, as seen for IL-17, mast cells and the products they secrete are not major players in the rapid neutrophil influx occurring within hours of *L. major* inoculation but may contribute to neutrophil recruitment in a later phase of infection.

A role for the complement component C3 in neutrophil recruitment following *L. major* infection was investigated using BALB/c mice that express cobra venom factor (CVF) under the control of the α 1-antitrypsin promoter, leading to continuous activation and consumption of C3 in the serum. Upon infection, these mice did not develop any inflammatory lesions and had lower neutrophil infiltration at the site of infection [34]. It remains difficult to assess if such decreased neutrophil recruitment is the cause or the result of the observed inhibition of lesion development.

Finally, neutrophil recruitment can presumably also be actively induced by the parasite. Indeed, *Leishmania* parasites, including *L. major*, have been shown to secrete *in vitro* a factor that is chemotactic for granulocytes (*Leishmania* chemotactic factor, LCF) and capable of attracting human neutrophils [22]. However, if factors secreted by *Leishmania* species may play a role in the process of neutrophil recruitment, skin damage caused by uninfected sand fly bites or by a sterile needle is sufficient to induce a rapid neutrophil influx to the lesion site, suggesting that factors released by the parasites contribute only in part to the initial neutrophil ingress observed following *L. major* inoculation [5].

Altogether, despite the identification of many putative candidate molecules that could participate in the initial

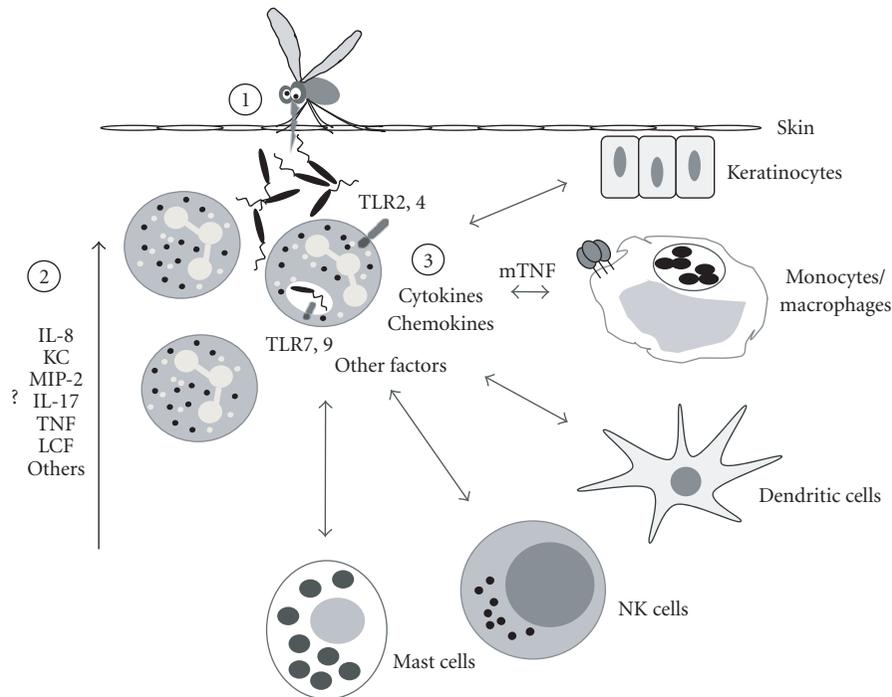


FIGURE 2: Early neutrophil recruitment to the site of *L. major* infection and its potential influence on the development *L. major* specific-immune response. (1) *L. major* parasites are transmitted by the bite of the sand fly. (2) This induces rapid and massive recruitment of neutrophils to the site of parasite inoculation, a process not defined, that may involve different host and/or parasite-derived factors such as IL-8, KC(Gro- α), MIP-2 (Gro- β), IL-17, TNF, and LCF. (3) *L. major* parasites induce chemokine and cytokine secretion by neutrophils that attract and/or activate inflammatory cells at the site of infection. Crosstalk between neutrophils and the different cell types present or recruited to the site of infection, as well as interaction between these cells, will contribute to determine the type and magnitude of the *L. major* specific immune response that will develop.

neutrophil recruitment occurring within hours of parasite inoculation, the exact contribution of individual factors and/or their combination in this process still needs to be clarified.

3.2. Influence of Neutrophils on the Local Environment during the First Days after Leishmania Inoculation. As discussed above, neutrophils are the first cells to arrive at the site of entry of infectious agents, where they are stimulated to phagocytose the foreign bodies and also to secrete factors involved in the recruitment and/or activation of other inflammatory cells. It has been shown that neutrophils can express a large number of factors including chemokines and cytokines [35] that could influence the microenvironment at the site of infection and thus the subsequent immune response. One of the strategies designed to explore the role of neutrophils during *Leishmania* infection has been to deplete these cells prior to infection by the injection of specific mAbs. To this end, essentially two mAbs have been used, that is, RB6-8C5 [36] and NIMP-R14 [37]. RB6-8C5 is an antibody that reacts mainly with the granulocyte differentiation Ag (Gr-1), although it also recognizes the Ly-6C- and Ly-6B.2 antigens. Thus injection of this mAb will deplete not only neutrophils but also other Gr-1-expressing cells such as monocyte-derived macrophages, eosinophils,

dendritic cells, and subpopulations of lymphocytes and monocytes. The NIMP-R14 mAb [37] recognizes a 25- to 30-kDa protein present on the neutrophil membrane and depletes neutrophils more selectively, as it does not recognize Ly6C (Charmoy M. and Tacchini-Cottier F. unpublished results). Another mAb (1A8) [38] recognizes the LY6G and not the LY6C molecules, and thus it does not affect Gr-1⁺ blood monocytes. It has recently been used to deplete neutrophils in vivo [39]. Thus, depending on the mAb used in vivo and on the regimen of administration, one has to take into account that cells other than neutrophils may be depleted that could contribute to the phenotype observed.

The role of neutrophils in infection of mice with *L. major* has been investigated by several groups including ours. Depletion of neutrophils in *L. major* susceptible BALB/c mice prior to inoculation of the parasite dramatically changed the course of infection, leading to a significantly lower lesion size than in control mice and to modifications of the developing immune response. Indeed, more IFN γ and less IL-4 were found to be secreted by lymph nodes cells in neutrophil-depleted mice compared to their similarly infected controls [4, 12]. Neutrophil depletion in *L. major*-resistant C57BL/6 mice prior to parasite inoculation leads to an increase parasite burden after 35 days of infection using the RB6-8C5 or the NIMP-R14 mAb. However, this is a transient effect,

as mice are finally healing their lesions with no change in the Th1-associated immune response [4, 40, 41]. In another experimental model [16], neutrophil depletion using the NIMP-R14 mAb during the first days of infection with *L. donovani* was shown to have an impact both on the control of parasite replication and on the development of a parasite-specific immune response. Neutrophil-depleted mice had increased parasite load in the spleen, bone marrow, and then to a lesser extent in the liver, and in these mice, development of the *L. donovani*-induced immune response was altered, with a significant increase in interleukin 4 (IL-4) and IL-10 levels and reduced IFN γ secretion by CD4⁺ and CD8⁺ T cells as compared to similarly infected mice injected with a control mAb.

As indicated above, the outcome of NIMP-R14 mAb injection prior to inoculation of *L. major* differed in BALB/c and C57BL/6 mice. In order to understand the contribution of neutrophils in the distinct phenotypes observed, BALB/c and C57BL/6 neutrophils, respectively, from *L. major*-susceptible or resistant mice, were exposed to *L. major* in vitro. Different responses were observed: *L. major* induced selectively in C57BL/6 neutrophils TLR2, 7 and 9 mRNA, and the autocrine secretion of IL-12p70 and IL-10. In contrast exposure of BALB/c neutrophils to *L. major* did not increase these TLRs mRNAs, nor the secretion of IL-12p70. However, these BALB/c neutrophils secreted high levels of IL-12p40, forming the IL-12 inhibitory IL-12p80 complex [42]. TLR4 was induced similarly in neutrophils from both C57BL/6 and BALB/c mice [42]. Following infection with *L. major*, C57BL/6 and BALB/c neutrophils were also shown to differ in their secretion of neutrophil elastase (a molecule that contributes to parasite killing within macrophages through activation of TLR4 [43]) as indeed only C57BL/6 neutrophils secreted this molecule [12]. In most of the studies investigating neutrophil biology, the careful identification of this cell population is of utmost importance for the conclusions reached. Indeed, several cell surface neutrophil proteins are shared with other cells of the myeloid lineage. Thus, the proper identification of neutrophils requires the combination of a mAb directed against Ly-6G with several other mAbs directed against cell surface proteins also present (Ly-6C, CD11b) or absent (F4/80, MHCII, CD11c) on these cells.

The secretion of cytokines and chemokines by neutrophils is not as high as that of other cell types such as dendritic cells. However, considering the massive number of neutrophils present at the site of parasite inoculation during the first days of infection, these cells and the products they release are likely shaping the microenvironment in a way that can markedly impact on the developing immune response.

4. Conclusions

We have reviewed evidences in literature demonstrating that neutrophils play an essential role during *Leishmania* infection, providing a transient safe shelter for the parasite during the first day of infection, until the macrophages become the dominant population in the cellular infiltrate.

In addition, factors released by these massively and rapidly recruited neutrophils likely contribute to determining the type and magnitude of the *Leishmania*-specific immune response. Further studies will be required to decipher the cross-talks between neutrophils and other cells, that leads to the eventual resolution of the lesion, or on the contrary to unimpaired progression of the infection (Figure 2). In this line, factors contributed by the sand fly vector also need to be considered. Indeed, recent experiments by Peters et al. revealed that sand fly inoculation maintains a localized, prolonged neutrophilic response which is different from that observed after needle injection of the parasite. This neutrophilic response impaired protection afforded in mice vaccinated using a killed *Leishmania* vaccine and challenged by the bite of infected sand flies, while such mice exhibited resistance against challenge infection induced through needle inoculation of live parasites [44]. Removal of the neutrophils promoted resistance against sand fly-induced infection, further pointing to the importance of neutrophils as a decisive parameter in shaping the outcome of infection by *Leishmania* parasites.

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Review Article

Cytokines and Their STATs in Cutaneous and Visceral Leishmaniasis

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Cytokines play a critical role in shaping the host immune response to *Leishmania* infection and directing the development of protective and non-protective immunities during infection. Cytokines exert their biological activities through the activation and translocation of transcription factors into the nucleus whether they drive the expression of specific cytokine-responsive genes. Signal transducer and activator of transcription (STATs) are transcription factors which play a critical role in mediating signaling downstream of cytokine receptors and are important for shaping the host immune response during *Leishmania* infection. Here we discuss the signature cytokines and their associated STATs involved in the host immune response during cutaneous and visceral leishmaniasis.

1. Introduction

Leishmaniasis is a zoonotic disease resulting from infection with protozoan parasites of the species *Leishmania*, of which approximately 30 different species have been described [1]. The clinical manifestations of disease differ depending on causative species and occur in three major forms: cutaneous, mucosal, and visceral leishmaniasis [1–3].

Leishmania infection is transmitted to susceptible mammalian hosts by sand flies of the genus *Phlebotomus* and *Lutzomyia* in the Old and New World, respectively [3]. *Leishmania* parasites exist as an extracellular promastigote within the gut of the sand fly, transforming into an intracellular amastigote within macrophages of mammalian hosts. As obligate intracellular pathogens in mammals, parasite survival and replication requires that parasites are rapidly internalized by host cells. While macrophages are considered to be critical effector cells in eliminating parasites and resolving disease, these cells play an important role during infection as the definitive host cell in which *Leishmania* species survive and replicate [4].

The genetic background of the host plays an important role in determining host resistance or susceptibility and dis-

ease outcome [5]. T lymphocytes play critical role in shaping the host immune response by secreting cytokines, which may act both synergistically and antagonistically through complex signaling pathways to direct both protective and non-protective immunities against intracellular parasites. Early studies using mouse models of experimental CL have revealed a clear dichotomy between Th1-associated cytokines mediating protection and Th2-associated cytokines mediating susceptibility [5, 6]. On the other hand during VL, Th2 response and cytokines such as IL-4 and IL-13 seem to be necessary for immunity and efficient response to antileishmanial chemotherapy [7, 8].

The ability of the host to control infection and resolve disease requires the generation of cell-mediated immune responses capable of activating host macrophages to eliminate intracellular parasites. In both human and experimental models of CL, control of infection is mediated by T lymphocytes and is critically dependent upon the early induction of an IL-12-driven Th1-type immune response and the production of IFN- γ by CD4+ T cells [5, 9]. IFN- γ plays a critical role in the activation of macrophages to kill intracellular parasites by inducing production of nitric oxide which is critical for elimination of parasites [6].

Previous studies have shown that susceptibility to infection and disease progression in CL is driven largely by the induction of a non-protective, IL-4-driven Th2-type immune response and the production of Th2-associated cytokines IL-4, IL-10, IL-13, and TGF- β [9–12]. In experimental models of murine CL, IL-4 was shown to play a critical role in mediating susceptibility to *Leishmania* infection by down-regulating the expression of protective Th1-associated cytokines IL-12 and IFN- γ and by inhibiting NO production and parasite killing by macrophages [6]. Nonetheless, some studies using IL-4-deficient BALB/c mice have shown that IL-4-mediated exacerbation of CL is dependent upon the particular strain of *Leishmania* [13]. IL-10 and TGF- β have been shown both in human and experimental models of disease to suppress healing responses and prevent parasite clearance by the host [12, 14–16].

The clear Th1/Th2 dichotomy observed in murine cutaneous leishmaniasis has not been demonstrated in humans, as the factors governing the nature of the host immune response and disease outcome during leishmaniasis are far more complex in men than in mice [14]. It is clear however, in both human and experimental models of disease, that cytokines play a critical role in shaping the nature of the host immune response in response to *Leishmania* infection.

Cytokines signal through a cascade of intracytoplasmic proteins known as STATs (*signal transducer and activator of transcription*) [17]. These transcription factors are indispensable for mediating biological activities of cytokines and for induction of cytokine-responsive genes. STATs are activated by tyrosine phosphorylation by cytokine receptor-associated Jak kinases. Upon activation, the STATs are phosphorylated and dimerize through their SH2 domain to form homo- or heterodimers. These dimers translocate to the nucleus where they bind to the promoter region of genes via specific DNA binding domains and thus bring about the transcription of their respective genes [17–20].

Here, we discuss the signature cytokines and complex STAT-mediated signaling networks involved in regulating the host response and determining disease outcome during cutaneous and visceral leishmaniasis.

2. Interferon- γ (IFN- γ) and STAT1

The development of cell-mediated immune responses capable of controlling *Leishmania* infection and resolving disease is critically dependent upon signaling by interferon gamma (IFN- γ), a type II interferon secreted primarily by activated T cells and NK cells in response to IL-12 signaling [21]. The major biological function of IFN- γ is to activate macrophages and enhance the microbicidal activity of these cells to kill intracellular pathogens [22]. IFN- γ induces iNOS (inducible nitric oxide synthase) expression and NO production by phagocytes harboring intracellular parasites and is required for activating macrophages to eliminate parasites and resolve *Leishmania* infection [9, 10].

The biological effects of IFN- γ are largely dependent upon the activation of STAT1 transcription factors. Upon IFN- γ /IFN γ R ligation, STAT1 is phosphorylated and STAT1

homodimers associate and translocate into the nucleus to initiate gene transcription [21]. IFN- γ -dependent immune responses against microbial pathogens are severely impaired in mice genetically deficient in STAT1 [23]. In experimental models of cutaneous *L. major* infection, STAT1^{-/-} mice developed extensive tissue destruction and severe inflammatory infiltrates composed heavily of neutrophils and parasitized macrophages [24]. STAT1/IFN- γ signaling pathway also induces the expression of Th1-associated transcription factor, T-bet. Both STAT1 and T-bet are required to mount an efficient Th1 response and as such, are indispensable for host defense against *Leishmania* infection in mice [19, 25]. In contrast, STAT1^{-/-} mice are resistant to visceral infection with *L. donovani* even in the absence of a strong Th1 response and develop minimal liver pathology. Studies in these mice have opened the possibility that resistance in STAT1^{-/-} mice is due to a defect in the trafficking of monocytes and an inability of parasites to establish a successful infection [19]. In fact, a recent study by our group has shown that STAT1 in CD4+ T cells plays a critical role in immunity to *L. major* by mediating recruitment of T cells to the site of infection [26]. This is perhaps not surprising since STAT1 is required for efficient induction of C-X-C chemokine receptor 3 (CXCR3) on CD4+ T cells [27] which is critical for efficient recruitment to the site of infection [28]. Interestingly, STAT1 in T cells is not necessary for Th1 development and IFN- γ production during *L. major* infection [26, 29].

3. Interferon- α/β (IFN- α/β) and STAT2

Type I interferons α and β (IFN- α/β) are proinflammatory cytokines that signal through the activation and phosphorylation of STAT1 and STAT2. Studies using mice deficient of STAT1 and STAT2 have shown that both these transcription factors are required for mediating most of the proinflammatory effects of these cytokines [30]. In cutaneous infection with *L. major*, IFN- α/β have been shown to act as early regulators of the innate response to infection and are required for initiating the expression of nitric oxide synthase type 2 (NOS2) and the production of NO [30]. The expression of NOS2 by macrophages has been shown to play an important role in protection against intracellular parasites such as cryptococci, *Toxoplasma*, mycobacteria, and *Leishmania*. A study by Bogdan et al. found that type I IFN- α/β are required for initial NOS2 expression [30]. Via NOS2, IFN α/β play a critical role in the innate response to CL infection by mediating events involved in parasite containment, IFN- γ expression, and the activity of cytotoxic NK cells [30].

Mice deficient of STAT2 are known to demonstrate an increased susceptibility to viral infections and impaired responsiveness to type I interferons [31]. In CL caused by *L. amazonensis*, infection with intracellular amastigotes was found to reduce STAT2 phosphorylation and enhance STAT2 degradation through the activity of parasite-derived proteases in DC from infected C57BL/6 and BALB/c mice. The role of STAT2 in VL is largely unknown [31].

4. Interleukin-12 (IL-12) and STAT4

The proinflammatory cytokine, IL-12, is a heterodimer composed of two subunits, p35 and p40 and is produced primarily by macrophages and dendritic cells (DCs) in response to microbial pathogens [32]. IL-12 functions as the main physiological inducer of gamma interferon (IFN- γ) by activated T cells and promotes Th1-type CD4+ T cell differentiation, and therefore plays an important role in inducing cell-mediated protection in response to *Leishmania* infection [33].

The importance of IL-12 in immunity to CL has been clearly demonstrated in experimental models of *L. major* infection: animals genetically deficient in the IL-12 gene or genetically resistant mice treated with anti-IL-12 antibodies fail to control parasite replication and are unable to resolve infection, and treatment of genetically susceptible BALB/c mice with recombinant IL-12 is sufficient to confer resistance in these animals [34]. Similarly, a comparison between *L. donovani*-infected IL-12^{+/+} and IL-12^{-/-} C57BL/6 mice showed a higher parasitic burden in the livers of infected IL-12^{-/-} mice [35].

The specific cellular effects of IL-12 are due to the activation of Janus kinase (JAK)-STAT pathways, primarily to the activation of the specific transcription factor, STAT4 [32]. STAT4 is one of seven members of the STAT family of transcription factors and is the major STAT activated by IL-12 [33]. In activated T cells and NK cells, STAT4 functions to induce IFN- γ production in response to IL-12 signaling. IL-12 signaling leads to activation of STAT4 by Jak kinase Jak2 and Tyk2. STAT4 dimers translocate into the nucleus and bind DNA sequences at IFN- γ activation site (GAS) [17, 18]. IL-12-induced IFN- γ mediates most of the proinflammatory activities of IL-12 and is critical for Th1 differentiation and directing the cell-mediated immune response required for protection against *Leishmania* infection.

STAT4-deficient mice are viable but have impaired Th1 differentiation, IFN- γ production, and cell-mediated immunity, phenotypes shared by mice lacking IL-12 or IL-12R subunits [33]. In experimental models of cutaneous leishmaniasis, STAT4-dependent IL-12 signaling was found to be crucial for the development of protective immunity as evidenced by the increased susceptibility observed in STAT4-deficient mice to *L. major* [32], and STAT4/STAT6^{-/-} mice to *L. mexicana* [36].

5. IL-4, IL-13 and STAT6

IL-4 and IL-13 are the signature cytokines associated with Th2-type immune responses and are associated with nonhealing forms of cutaneous disease in mice [9]. IL-4 stimulates the differentiation of naïve CD4+ T cells into Th2 cells capable of producing Th2-associated cytokines IL-5, IL-10 and IL-13, and promotes antibody production and IgE class switching by B cells. IL-4 also functions as a powerful inhibitor of IFN- γ -producing CD4+ T cells and suppressor of protective Th1 immune responses [37]. IL-4, along with related cytokine IL-13, trigger macrophages to

undergo alternative activation and is associated with parasite survival and persistence of infection [9, 10, 24].

The IL-4R and IL-13R share a common γ c receptor chain involved in signal transduction. Both IL-4 and IL-13 are the principal activators of transcription factor STAT6 which mediates most biological activities of these cytokines [38]. STAT6^{-/-} mice show impaired Th2 differentiation and lose responsiveness to IL-4 and IL-13 while maintaining normal responses to other cytokine signals [23].

The importance of IL-4, IL-13, and STAT6 in mediating susceptibility during cutaneous infection was clearly demonstrated in experimental models of *L. mexicana* infection using genetically susceptible mice deficient in the genes for IL-4, IL-13, and STAT6. IL-4^{-/-}, IL-13^{-/-}, IL-4^{-/-}/IL-13^{-/-}, and STAT6^{-/-} mice all mount a robust Th1 response and effectively control parasite growth and replication, indicating that IL-4, IL-13, and STAT6 mediate susceptibility by preventing the development of protective Th1 responses [5, 39].

L. donovani-infected mice have been demonstrated to produce IL-4 in some models and not in others [40, 41]. However, IL-4 does not seem to play a part in promoting susceptibility to *L. donovani* as susceptible phenotypes have been shown even in the absence of IL-4 [41]. In experimental models of visceral infection in mice, IL-4 has been found to play no antagonistic role in eliminating infection [40]. Some studies have actually shown that IL-4^{-/-} mice are more susceptible to infection with *L. donovani* than wild type mice and suggest a protective role for IL-4 in VL [8, 39]. Since IL-4 does not seem to exacerbate *L. donovani* infection, it appears that Th2 responses are not important in suppressing the protective Th1 immune response. Furthermore, the assumption that treatment of VL should be enhanced in absence of IL-4 was shown not to be true. In fact, *L. donovani*-infected IL-4^{-/-} mice were seen to be as susceptible to infection as wild type mice. Rather, resolution of infection upon drug treatment was more effective in wild type mice as compared to IL-4^{-/-} mice [8]. The findings suggest a role for IL-4 in enhancing the protective role of IFN- γ . Experimental studies in BALB/c mice genetically deficient in IL-4 were found to respond poorly to chemotherapy with sodium stibogluconate and these mice were also found to produce little IFN- γ . A recent study has shown that IL-4 plays a critical role in vaccine induced protection against VL by enhancing IFN- γ production by CD8+ T cells [42].

6. IL-10 and STAT2

The anti-immune and anti-inflammatory cytokine, IL-10 is produced by a variety of cells including T cells, monocytes, macrophages, DCs, and B cells [15]. While many cells can produce IL-10, the main biological functions of IL-10 appear to be on macrophages and DCs. IL-10 functions to inhibit the production of proinflammatory cytokines IL-1, IL-6, IL-12, and tumor necrosis factor (TNF) by macrophages and DCs [23] and thus prevents the expansion of Th1-type cells required for protective immunity during *Leishmania* infection [14–16].

IL-10 also promotes activation, survival, and antibody production by B cells and the development of humoral immune responses which play a detrimental role in host defense against *Leishmania* infection by facilitating parasite entry into host cells [43]. In both human and experimental models of CL, high levels of IL-10 production are strongly associated with nonhealing forms of disease [9, 15].

In the absence of IL-10 (IL-10^{-/-}), genetically susceptible C57BL/6 mice mount an immune response capable of controlling parasite replication and resolving cutaneous infection with *L. mexicana*. IL-10^{-/-} mice were found to express higher levels of IFN- γ and produced more NO than C57BL/6 IL-10^{+/+} mice. IL-10 also mediates susceptibility and promotes parasite persistence in cutaneous infections with *L. major*, however its role in *L. amazonensis* and *L. pifanoi* infection remains unclear [15].

Increased IL-10 production has been reported in patients suffering from VL as well as mice infected with *L. donovani* [44]. IL-10 levels decline upon resolution of VL following chemotherapy indicating that IL-10 is a susceptibility factor in VL [45, 46]. In fact, IL-10^{-/-} mice are highly resistant to *L. donovani* [45, 47]. Furthermore, a correlation was found between decreasing levels of IL-10 and resolution of VL in these models. Since IL-10 can act as an inhibitor of IFN- γ induced NO synthesis, it is likely that antagonistic effect of IL-10 is mediated by its ability to suppress production of NO, which is critical for elimination of parasites [45].

Various STAT transcription factors appear to be involved in IL-10 production by T cells, B cells, NK cells, monocytes, and macrophages; however, STAT3 plays a conserved role in IL-10 signaling in all cell types [48]. STAT3 is a common transcription factor in signaling by a variety of cytokines, including members of the IL-6 family of cytokines, granulocyte CSF, EGF, IFN- γ , and IL-2 [49].

STAT3-deficient mice are not viable and experience early fetal death. However, conditional cell-specific knockouts of the STAT3 gene using the Cre-loxP system have demonstrated an important role for STAT3 signaling in IL-6- and IL-2-induced proliferation by T cells [23, 49]. In similar studies, IL-10 was demonstrated to play an essential role in the deactivation of macrophages and neutrophils. IL-10-deficient mice exhibited increased production of proinflammatory cytokines such as TNF α , IL-1, IFN- γ , and IL-6 and a polarized immune response towards a Th1-type response [23, 50]. The specific role of STAT3 signaling in leishmaniasis has not been demonstrated and requires further investigation.

7. Conclusion

The pathology and resolution of leishmaniasis is dependent to a large extent on the infecting species, and the model used. Cytokines do have a role in all cases either by initiating the development of a Th1 response as in VL or development of Th2 response in case of CL. The major cytokines in both cases are IL-12, IFN- γ , IL-10, IL-4, the effects of which are mediated by specific STATs.

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Review Article

***Leishmania* Interferes with Host Cell Signaling to Devise a Survival Strategy**

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The protozoan parasite *Leishmania spp.* exists as extracellular promastigotes in its vector whereas it resides and replicates as amastigotes within the macrophages of its mammalian host. As a survival strategy, *Leishmania* modulates macrophage functions directly or indirectly. The direct interference includes prevention of oxidative burst and the effector functions that lead to its elimination. The indirect effects include the antigen presentation and modulation of T cell functions in such a way that the effector T cells help the parasite survive by macrophage deactivation. Most of these direct and indirect effects are regulated by host cell receptor signaling that occurs through cycles of phosphorylation and dephosphorylation in cascades of kinases and phosphatases. This review highlights how *Leishmania* selectively manipulates the different signaling pathways to ensure its survival.

1. Introduction

Leishmaniasis, caused by the protozoan parasite of the genus *Leishmania*, is an infection that occurs primarily in the tropical and subtropical regions of the world. *Leishmania* is a dimorphic protozoan parasite that resides as an extracellular flagellate-promastigotes—in its sand fly vector and as an intracellular aflagellate-amastigotes—in macrophages of its mammalian host [1]. Leishmaniasis is characterized by the parasite-induced immunosuppression executed not only by active subversion but also by immune deviation such that the resulting immune responses suppress the antileishmanial immune response further. Because macrophages are not only the host cells for the parasite but also sentinels of the immune system, these cells are targeted by the parasite for immune modulation to ensure their survival. The parasite interferes with the signaling system of the cell such that the effector functions triggered by various cell surface receptors are either actively suppressed or are altered to result in the immune responses that promote parasite survival. A variety of mechanisms potentially contributing to mononuclear phagocyte deactivation during intracellular infection have been identified [2]. Of considerable interest is the evidence that intracellular pathogens are able to impair cell signaling

pathways required for host cell activation that may eventuate in their elimination [2, 3]. Cell signaling is regulated by two principal classes of enzymes, protein kinases and phosphoprotein phosphatases [4, 5] (Figure 1).

As the signals are transduced in cascades of kinases and phosphatases through cycles of phosphorylation and dephosphorylation, the parasitic interference often targets these signaling intermediates [6]. Herein, we will analyze the alterations in the signaling of some receptors in *Leishmania*-infected macrophages and will associate those alterations with the altered responsiveness of the macrophages to the ligands of those receptors.

2. *Leishmania* Modulates the Receptor Responsiveness in Macrophages

2.1. Regulation of CD40 Responsiveness and Mitogen Activated Protein Kinase Family. The interaction between CD40, a costimulatory molecule expressed on macrophages, B cells, and dendritic cells [7], and its ligand CD40 ligand (CD154) on T cells [8] results in Th subset skewing to Th1 type. Consistent with the proposition that Th1 cells are responsible for protection against *Leishmania major* infection, the CD40-deficient

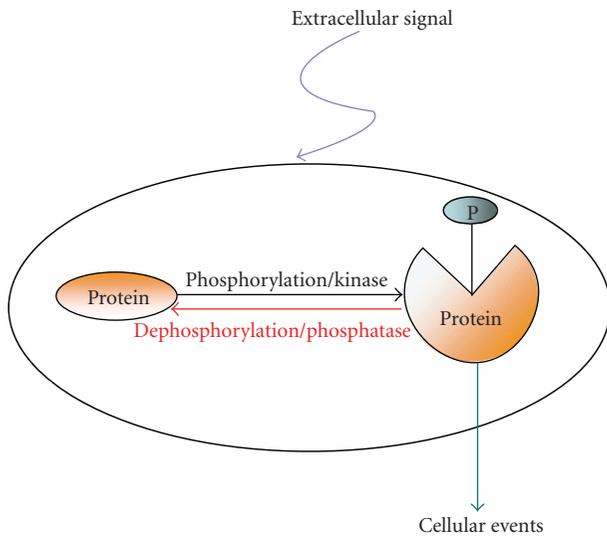


FIGURE 1: General principles of signal regulation by kinases and Phosphatases: the phosphorylation and dephosphorylation of the protein, the former being catalyzed by a kinase, and the latter by a phosphatase. Phosphorylation usually causes a conformational change in the protein.

mice fail to develop a Th1 response and are susceptible to *Leishmania* infection [9]. The susceptibility to *Leishmania* infection can be prevented by IL-12 administration in these mice suggesting that CD40-CD154 interaction is required for the production of IL-12, which polarizes the Th cells to Th1 type [9–11]. Thus, the host-protective function of CD40 was attributed to setting a Th1 bias [9, 10, 12]. Beside their role in Th1 immune response, CD40-CD40L interactions were also shown to stimulate macrophages to produce a number of cytokines and inflammatory mediators including nitric oxide (NO), which plays a key role in parasite killing [13]. As CD40-L binds to CD40, it triggers the signal through several signaling intermediates [14] to result in mitogen-activated protein kinase (MAPK) phosphorylation [15, 16]. The MAP kinases play an important role as signal kinases and their activity is elicited upon phosphorylation of threonine and tyrosine residues in a Thr-X-Tyr motif in their regulatory domain and thereby controls the activation status of transcription factors [17]. There are three major groups of MAP kinases in mammalian cells—the extracellular signal-regulated protein kinases (ERK) [18], the p38MAP kinases [19], and the c-Jun NH2-terminal kinases (JNK) [20]. MAPKs phosphorylate selected intracellular proteins, including transcription factors, which subsequently regulate gene expression by transcriptional and posttranscriptional mechanisms [21]. Each of these kinases is regulated by other upstream kinases [22]. These three families of MAPKs form three parallel signaling cascades activated by distinct or sometimes overlapping sets of stimuli. Activated by mitogens and growth factors, the ERKs mediate signals promoting cell proliferation, differentiation, and survival. JNK and p38 MAPKs are predominantly activated not only by stress such as osmotic changes and heat shock but also by inflammatory

cytokines TNF- α and IL-1 β and bacterial lipopolysaccharide (LPS) [23–25].

Several studies show that MAPKs are actively repressed and cannot be activated when *Leishmania*-infected macrophages are stimulated with a variety of agonists. Inhibition of MAPK phosphorylation resulted in less expression of IL-12 and iNOS2 (inducible nitric oxide synthetase type 2), the enzyme that catalyzes the production of NO [26, 27] which has been shown to play crucial role in the development of immunity to *Leishmania* [28]. In naive macrophages, *Leishmania donovani* promastigotes failed to activate the phosphorylation of p38 MAPK, ERK1/2, and JNK, as well as the degradation of $\text{I}\kappa\text{B-}\alpha$ [29] affecting the activation of proinflammatory cytokines. The parasite surface molecule LPG has been implicated in the inactivation of MAPKs, since phagocytosis of LPG-deficient *L. donovani* promastigotes caused MAPK activation, without the requirement for subsequent macrophage stimulation [29].

One of the studies showed that ERK and p38 MAPKs play differential roles in the regulation of LPS-stimulated inducible NO synthase and IL-12 gene expression [30]. LPS stimulated ERK, JNK, and p38 MAP kinases in J774 macrophages but with different activation kinetics. It was also demonstrated that p38 plays an essential role in the induction of inducible NO synthase, and ERK MAP kinases play only a minor role in promoting NO generation by using inhibitors selective for ERK (PD98059) and p38 (SB203580). It was also demonstrated that synthetic *Leishmania* lipophoglycans act by stimulating ERK MAP kinase to inhibit macrophage IL-12 production thus promoting parasite survival and thus underlining the physiological relevance of these regulatory signals [30].

In addition, the CD40-induced p38MAPK phosphorylation, iNOS2 expression, and antileishmanial function were impaired in *Leishmania*-infected macrophages but were restored by anisomycin, a p38MAPK activator, suggesting a crucial role of p38MAPK in CD40 signaling. Anisomycin's effects were reversed by SB203580, a p38MAPK-specific inhibitor, emphasizing the role of p38MAPK in CD40-induced iNOS2-dependent leishmanicidal function. Thus anisomycin's ability to restore CD40 signaling and eliminate amastigotes not only highlighted the susceptibility of amastigotes to killing after p38MAPK activation but also suggested a potential use of anisomycin as an antileishmanial drug [31].

While interference with CD40-induced p38MAPK is consistent with the general suppressive scheme of parasitism, the observation does not explain the CD40-induced IL-10 production from macrophages [32] and increased IL-10 production from *Leishmania*-infected macrophages [33]. Since IL-10 is a suppressive cytokine, these observations support the proparasitic role of IL-10 but contradict our results. This is because *Leishmania* interference with the CD40 signaling through MAPK, if it were associated with IL-10 production as well, would inhibit IL-10 production and clearly, that was not the case. So, it is possible that there are other signaling pathways or MAPKs carrying the CD40 signal and associate with IL-10 production. Indeed, it was observed

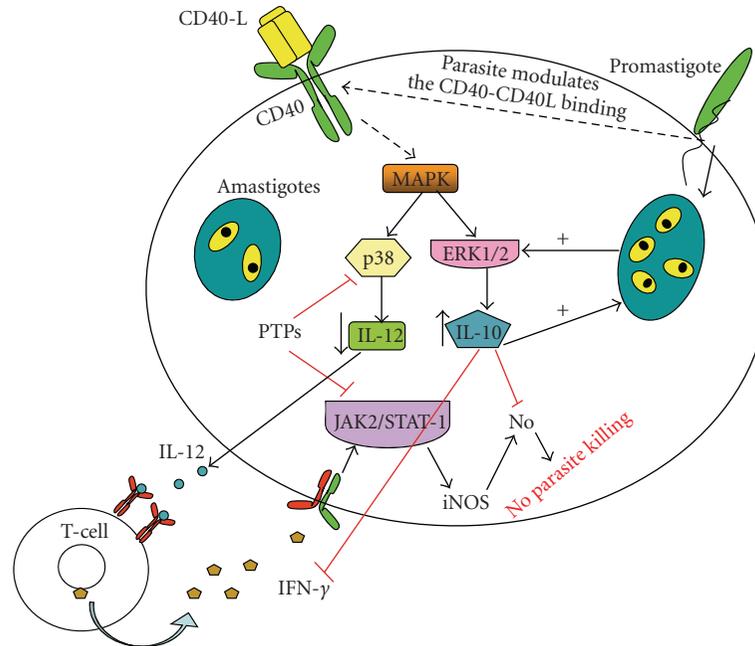


FIGURE 2: Modulation of CD40 responsiveness in *Leishmania*-infected macrophages: crosslinking of anti-CD40 antibody activates p38 MAPK-induced leishmanicidal function via iNOS2 induction. *Leishmania* infection downregulates CD40-induced p38 MAPK phosphorylation and uses the capability of this receptor to signal along an ERK1/2-dependent pathway to produce the proparasitic Th2 cytokine IL-10 from macrophages. iNOS2: inducible nitric oxide synthase 2; NO: nitric oxide; PTP: Protein tyrosine phosphatases.

that CD40 induced ERK-1/2 activation, inhibition of which resulted in decreased CD40-induced IL-10 production [21]. In *Leishmania*-infected macrophages, CD40-induced ERK-1/2 activation was increased suggesting a reciprocal interaction between the p38MAPK and ERK-1/2 activation [34]. Indeed, inhibition of one MAPK activated the other MAPK. In macrophages, higher strengths of stimulation induced p38MAPK phosphorylation but weaker strengths resulted in ERK-1/2 phosphorylation [34]. During *Leishmania* infection, the level of CD40-induced ERK1/2 phosphorylation and IL-10 production increases, whereas p38MAPK activation and IL-12 production decrease, demonstrating a reciprocal modulation of the CD40 signaling pathway by the parasite [34] (Figure 2). IL-10 produced during the infection inhibits CD40-induced IL-12 production by impairing p38MAPK activation [34]. Neutralization of CD40-induced IL-10 enhances the antileishmanial functions of CD40. Thus, the anti-leishmanial function of CD40 is self-limited by induction of IL-10. The work of Yang et al. also demonstrated a critical role for ERK activation in the induction of IL-10 production by *Leishmania* and showed that parasite immune complexes bind to macrophage FcγR and induce this activation via the macrophage FcγR [35].

Although first observed in *Leishmania* infection in macrophages, the same principle of differential CD40 signaling holds true in dendritic cells and in tumor models [36–38]. In one study it was shown that infection with *L. amazonensis* amastigotes inhibited the ability of DCs to undergo proper maturation in vivo characterized by significantly low CD40 surface expression and significantly

decreased IL-12p40 production through activation of the MAP kinase ERK1/2 [39].

While the differential CD40 signaling and its selective manipulation by *Leishmania* solved the apparent paradox of inducing counteractive cytokines by CD40 stimulation, the question remained to be solved is how a single receptor induces reciprocal signaling pathways and counteractive effector functions. We have shown that such differential signaling depends on the composition of the signalosomes assembled on the membrane. When CD40 binds TRAF-2,3,5, it signals primarily through p38MAP kinase whereas binding to TRAF-6 signals primarily through ERK-1/2. We have shown that cholesterol influences the assembly of distinct CD40 signalosomes. Depletion of membrane cholesterol inhibited the assembly of the p38MAP kinase inducing CD40 signalosome but enhances the ERK-1/2 activation [40]. Consistent with these observations, *Leishmania* is found to deplete membrane cholesterol and enhance CD40 binding to TRAF-6 [40]. However, how *Leishmania* interferes with macrophage cholesterol metabolism remains to be elucidated.

It was demonstrated that the *Leishmania* surface molecule, lipophosphoglycan, stimulates the activation of ERKs, JNK, and the p38 MAP kinase simultaneously but with differential kinetics in J774A.1 macrophage cell line. It was shown that both p38 and ERK MAP kinase activation appears to be necessary for AP-1 activation by LPG and it also induced IL-12 production and generation of nitric oxide demonstrating that *L. donovani* LPG activates pro-inflammatory, endotoxin-like response pathway in J774A.1

macrophages [41]. A recent report [42] pointed to the importance of the metalloprotease GP63 in regulating several important signaling proteins, contributing to downstream changes in global protein tyrosine phosphorylation levels as well as a specific effect on p38 MAPK activation. p38 was inactivated upon infection in a GP63- and protein degradation-dependent manner, which likely involves cleavage of the upstream adaptor TAB1 [42].

2.2. TLR Responsiveness. Of the growing number of receptors involved in the recognition of pathogen-associated molecular patterns (PAMPs) [43], TLRs are considered key players of the innate immune response [44, 45]. This family of receptors is comprised of thirteen members that recognize most of the molecular patterns on pathogens. The recognition of the ligands results in the secretion of inflammatory mediators such as TNF- α and IL-12 as well as the induction of iNOS2 expression [45–47], leading to host protection.

Following the recognition of a PAMP, the adaptor myeloid differentiation factor 88 (MyD88) is recruited to the TIR (toll-interleukin 1 receptor) domain of the TLR [48]. Next, IL-1 receptor-associated-kinase-1 (IRAK-1) is recruited to the complex and is phosphorylated by IRAK-4 and by autophosphorylation. IRAK-1 dissociates from MyD88 to interact with TRAF6 and activates various cascades, ultimately leading to the activation of MAP kinase pathways, the translocation of NF- κ B to the nucleus as well as the secretion of proinflammatory cytokines [49, 50]. Another pathway, termed “MyD88-independent”, is implicated in signaling following engagement of TLR3 and TLR4. This cascade uses TRIF as an adaptor protein and allows the translocation of NF- κ B to the nucleus and the activation of MAP kinase pathways with a slower kinetics as well as the activation of IP-10 and IFN- α/β via the activation of IRF3 [51, 52].

Substantial studies demonstrated that different receptors mediate the uptake and phagocytosis of *Leishmania* spp. by macrophages, although the initial signaling events are unknown [53]. As LPG of *Leishmania* promastigotes interacts with NK cell-expressed TLR2 [54], it is possible that the leishmanial LPG may interact with the macrophage expressed TLR2 and modulates cellular functions to ensure its survival within the host cell. For example, *L. major*-induced IL-1 α expression was substantially decreased in MyD88-deficient mice [55]. Similarly, the genetically resistant C57BL/6 mice became susceptible to *Leishmania* parasite in absence of MyD88 due to increased level of IL-4 and decreased level of IFN- γ and IL-12p40 [56]. Furthermore, silencing of TLR2, TLR3, IRAK-1, and MyD88 expression by RNA interference also revealed the involvement of both TLR2 and TLR3 in the production of NO and TNF- α by macrophages in response to *L. donovani* promastigotes [57]. TLR2-mediated responses are dependent on Gal β 1, 4Man α -PO $_4$ containing phosphoglycans, whereas TLR3-mediated responses are independent of these glycoconjugates. TLR3 also plays a role in the leishmanicidal activity of the IFN- γ -primed macrophages [57]. It is quite possible that *Leishmania* may modulate MyD88 expression and recruitment to

TLRs resulting in altered TLR responsiveness of the infected macrophages.

An impaired resistance to *L. major* was also reported in TLR4-deficient mice. Compared to wild type controls, the growth of parasites in the cutaneous lesions was drastically increased in mice from a resistant background carrying a homozygous mutation of the *tlr 4* gene (TLR4 *e/e*) as early as one day after inoculation of *L. major*. Later in the infection, an enhanced arginase activity leads to the production of compounds essential for parasite proliferation in macrophages and its increase in mutant mice indicating that TLR4 signaling could enhance the microbicidal activity of macrophages harboring parasites [58]. Results from studies comparing TLR4 deficient mice with TLR4 and IL-12 β 2 double deficient mice suggested an IL-12 independent role of TLR4 in anti-*Leishmania* immunity [59]. The IL-12 dependent NK cell IFN- γ response was severely compromised in TLR9-deficient mice as well. In studies with *L. infantum* infection, in mature dendritic cells- (mDCs-) depleted mice, the IFN- γ response was abolished due to low IL-12 production that could be rescued by CpG and IL-12 [60]. *L. major* is also shown to modulate TLR9 signaling for activating NK cells [61]. Likewise, *L. donovani* infection caused suppression of TLR2- and TLR4-stimulated IL-12p40, with an increase in IL-10 production in cells of monocyte/macrophage lineage by suppressing p38MAPK phosphorylation and activating ERK-1/2 phosphorylation through a contact-dependent mechanism [62]. These studies imply how *Leishmania* modulates the TLR responsiveness that might help their survival in macrophages.

2.3. IFN- γ Receptor Responsiveness. It is widely accepted that IFN- γ plays a critical role in controlling *Leishmania* infection by inducing macrophage leishmanicidal activity as well as by favoring Th1 development [63, 64]. The biological functions of IFN- γ are mediated via IFN- γ R- (IFN- γ receptor-) mediated pathway involving receptor-associated kinases JAK1/JAK2 and STAT-1 [65, 66]. IFN- γ binding to the receptor activates JAK1/JAK2 kinases and phosphorylates STAT-1, which translocates to the nucleus and enhances transcription of IFN- γ -induced genes to increase macrophage microbicidal activity (Figure 3). IFN- γ R is comprised of IFN- γ R α and IFN- γ R β chains. While IFN- γ R α chain plays a critical role in ligand binding, IFN- γ R β is required for IFN- γ signal transduction [67]. The critical role of IFN- γ R in development of IFN- γ -mediated host immunity is evident in studies showing that IFN- γ R $^{-/-}$ mice are highly susceptible to pathogens such as *Mycobacterium avium* [68], *Listeria monocytogenes* [69], *Candida albicans* [70], and *Plasmodium berghei* [71]. It has been shown that IFN- γ R $^{-/-}$ mice are highly susceptible to high as well as low dose *L. major* infection indicating that IFN- γ R is essential for control of cutaneous leishmaniasis [72]. *L. donovani* has also been shown to attenuate IFN- γ R expression in human monocytes [73]. Some of the important macrophage functions suppressed by *Leishmania* which are IFN- γ inducible are NO production, MHC class II expression. One of the major results of *Leishmania* infection

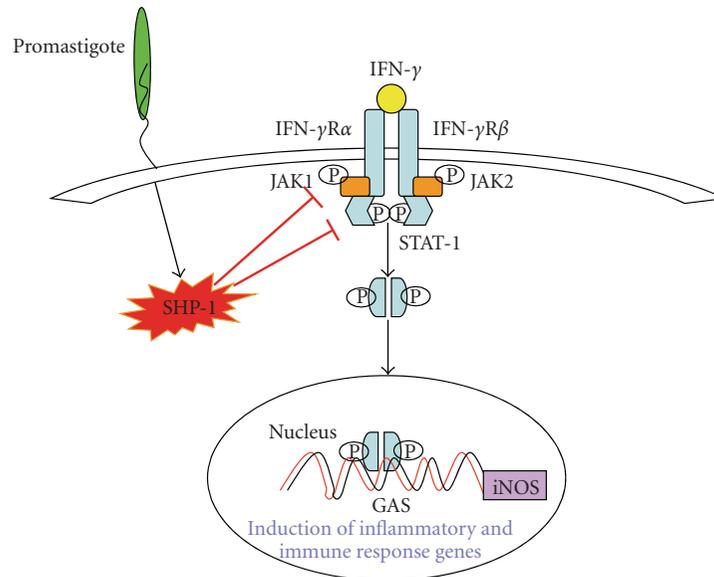


FIGURE 3: STAT1 phosphorylation regulation through IFN γ receptor: infected macrophages display reduced levels of total and phosphorylated JAK1 and JAK2 and attenuate IFN- γ induced STAT-1 phosphorylation in macrophages, aiding the parasites in escaping host immunity. GAS: Interferon gamma-activated site.

is the inhibition of the JAK2/STAT1 signaling cascade. Infected macrophages show defective phosphorylation of JAK1, JAK2, and STAT1 on IFN- γ stimulation [73, 74]. This inactivation depends on the activation of phosphotyrosine phosphatases (PTPs), in particular the PTP, SHP-1. One study has shown that inactivation of JAK2/STAT1 is caused by the negative regulation of the IFN- γ receptor in infected cells [75]. However, this was not observed following *L. amazonensis* amastigote infection, where IFN- γ -dependent regulation of MHC class I was not affected by infection, indicating that the primary signaling lesion lays downstream of the IFN- γ receptor [76]. Certain bacterial and viral pathogens have been shown to evade host immunity by downregulating IFN- γ R α expression on effector cells [77–79]. Similarly, both *L. major* and *L. mexicana* suppressed IFN- γ R α and IFN- γ R β expression, reduced levels of total Jak1 and Jak2, and downregulated IFN- γ -induced Jak1, Jak2, and STAT1 activation, with the effects more profound with *L. mexicana* infection than *L. major*. In addition *L. mexicana* preferentially enhanced tyrosine phosphorylation of dominant negative STAT1 β , which may be one of the several survival mechanisms used by this parasite to evade the host defense mechanisms [80]. Recently it was shown that infection of DCs with *L. amazonensis* parasites resulted in multiple alterations in innate signaling pathways, including a protease- and proteasome-dependent decreased phosphorylation of STAT1, 2, 3 and ERK1/2, and markedly reduced expression of interferon regulatory factor-1 (IRF-1) and IRF-8. Furthermore, it was shown that alterations in intracellular signaling and suppression of IL-12 production were caused by direct effects of amastigotes rather than by the induction of endogenous IL-10 [81].

2.4. IL-10 Receptor Responsiveness. IL-10 is a homodimer with 160aa and belongs to class II α -helical cytokine. IL-10 is produced by many cell types including T cells, monocytes, and macrophages. IL-10 interacts with its tetrameric receptor complex consisting of two IL-10R1 and two IL-10R2 polypeptide chains [82]. IL-10 is a potent immunosuppressant of macrophage functions, suppresses the production of proinflammatory cytokines by activated monocytes/macrophages, and enhances B lymphocyte proliferation and antibody secretion. IL-10 decreases expression of MHC classes I and II affecting antigen presentation [83] and reduces the transcription and translation of proinflammatory cytokines TNF α , IL-12, and IL-18 from macrophages [84]. IL-10 also suppresses the induction of iNOS2 that catalyzes the production of NO, the leishmanicidal free radical [85–87]. IL-10 affects T cells mostly in an indirect manner, by its effects on antigen presenting cells [88].

IL-10 is a critical cytokine in determining the host susceptibility to *Leishmania* infection. In murine models of cutaneous [89] and visceral [90] leishmaniasis, IL-10 contributes to disease progression. IL-10-deficient or anti-IL-10 receptor antibody-treated mice are relatively resistant to *Leishmania* infection [91], while the administration of exogenous IL-10 [92] or the induction of endogenous IL-10 exacerbates the disease [93]. It has been reported that the susceptibility of BALB/c mice to *L. major* infection is dependent on IL-10 as IL-4R $\alpha^{-/-}$ BALB/c mice, despite the absence of IL-4/IL-13 signaling remains highly susceptible to *L. major* infection [94]. Similarly, the IL-10 levels in patients with *L. donovani* infection directly correlate with the disease severity [95].

It was demonstrated that macrophage IL-10 is turned-on by *Leishmania* amastigotes itself and the virulence factor responsible for this induction was found to be host IgG [33]. The host IgG present on the surface of *Leishmania* amastigotes interacts with FcγR on the surface of macrophage to trigger signaling events that leads to the induction of IL-10; however, unopsonised amastigotes fail to do so. IL-10 produced by infected macrophages prevents macrophage activation and thus inhibits production of proinflammatory cytokine IL-12 and TNFα. FcγR KO (mice lacking all FcγRs) mice similar to IL-10 KO mice are resistant to *L. mexicana* infection and also control parasites as a result of strong IFNγ response [96]. Ligation of FcγR on macrophages with IgG on the parasite surface induces IL-10, which in turn suppresses IFNγ response and NO production in *L. mexicana* infection. Further it was also shown that FcγR III is crucial for macrophage to produce IL-10 and disease progression in *L. mexicana* infection [97].

MAPK ERK1/2 has been shown to be involved in induction of IL-10 from macrophages [35]. IgG opsonised amastigotes interact with FcγR on macrophages to induce ERK1/2 activation. The hyperactivation of ERK1/2 results in histone H3 phosphorylation of IL-10 promoter making the promoter accessible to transcription factor, and the result is secretion of high levels of inhibitory cytokine IL-10. However lesion-derived amastigotes alone are not sufficient to induce IL-10; despite their activity to rapidly activate ERK1/2, some inflammatory signal is required for induction of IL-10. This inflammatory stimulus can be small fragments of hyaluronan called as LMW-HA which is a major component of extracellular matrix. Also leishmanial lesions are generally superinfected with bacteria which can provide inflammatory signal through TLR2 or 4. Role of ERK1/2 MAPK in inducing IL-10 has also been demonstrated in CD40-CD40L interaction; lower dose of anti-CD40 stimulation induces more ERK1/2 MAPK phosphorylation and IL-10 secretion in *L. major* infection while inhibition of ERK1/2 reduces CD40 induced IL-10 secretion and disease progression [34].

The IL-10/IL-10R interaction engages the phosphorylation and activation of receptor-associated janus tyrosine kinases, JAK1 and Tyk2, which in turn phosphorylate transcription factor STAT3. It then homodimerizes and translocates to the nucleus where it binds with high affinity to STAT-binding elements (SBEs) in the promoters of various IL-10-responsive genes [98]. STAT3 plays a dominant mediator of majority of IL-10 functions [99]. Overexpression of dominant negative STAT3 suppresses the IL-10 promoter activity while wild type STAT3 leads to enhancement of this activity [100]. The anti-inflammatory functions of IL-10 are STAT3 dependent as in STAT3 deficient murine macrophages IL-10 is unable to suppress LPS-induced TNF-α and IL-6 production [101, 102]. In human macrophages, IL-10 rapidly induces SOCS3 protein expression and this expression requires STAT3 as STAT3 dominant negative human macrophages failed to induce IL-10-mediated SOCS3 expression [103].

3. Alterations of Host Cell Kinases and Phosphatases by *Leishmania*

3.1. Protein Kinase C. PKC, serine-threonine kinases with several isoforms are involved in a wide variety of immune cell functions and are classified as classical, novel, and atypical PKC depending on their structure and cofactor requirement [104]. A number of studies have implicated PKC in the control of host defense against intracellular infections. Indeed, *Leishmania* infection inhibits PKC activation and subsequent intracellular signaling. PKC-dependent oxidative burst activity and protein phosphorylation were found to be markedly attenuated in *Leishmania*-infected human monocytes [105]. Promastigote LPG has been shown to inhibit PKC activation and PKC-dependent phosphorylation of both the PKC-specific VRKRTRLLR substrate peptide and MARCKS (Myristoylated alanine-rich C kinase substrate) [106]. Another PKC substrate protein, MRP (MARCKS-related protein), levels were also found to be decreased in infection with all species or strains of *Leishmania* parasite, including lipophosphoglycan-deficient *L. major* L119 [107]. LPG-mediated inhibition of PKC activation may be due to the ability of LPG to interfere with binding of regulators, including Ca²⁺ and diacyl glycerol; in addition LPG can also block PKC membrane insertion [108]. LPG also inhibits phagosomal maturation, by inhibiting PKC-α dependent depolymerization of periphagosomal F-actin [109, 110]. Further infection with *L. major* inhibited PKC-dependent c-fos and TNFα gene expression [111]. *L. donovani* infection selectively inhibited Ca²⁺-dependent PKC activity but Ca²⁺-independent PKC activity was enhanced. *Leishmania* infection reduced the Ca²⁺-dependent PKC isoform-PKCβ-expression whereas expression of PKC zeta, a Ca²⁺-independent PKC isoform, was enhanced [112]. This decrease in Ca²⁺-dependent PKC activity can be due to IL-10 produced by *L. donovani* infection as pretreatment with anti-IL-10 neutralizing antibody significantly restored Ca²⁺-dependent PKC activity [113]. Infection of macrophages with *L. donovani* enhanced the level of intracellular ceramide largely due to its de novo synthesis and the enhanced ceramide was found to be responsible for the downregulation of classical PKC activity, upregulation of Ca²⁺-independent atypical PKC-zeta expression, and activity of calcium independent PKC [114]. Also C-C chemokines particularly macrophage inflammatory protein- (MIP-) 1 alpha and macrophage chemoattractant protein- (MCP-) 1 were found to restrict the parasitic burden via the regulation of impaired PKC signaling and induction of free-radical generation in murine leishmaniasis. These chemokines restored Ca²⁺-dependent PKC activity and inhibited Ca²⁺-independent atypical PKC activity in *L. donovani*-infected macrophages under both in vivo and in vitro conditions [115]. de Almeida-Amaral et al. reported the presence of protein kinase C-like (PKC-like) protein in *L. amazonensis* and found that this PKC-like protein is activated by phorbol ester (PMA) and has both calcium dependent and independent PKC-like activity. Further they studied the role of this PKC-like protein in modulation of promastigotes (Na⁺ K⁺)ATPase activity and found that activation of Ca²⁺-dependent PKC-like protein

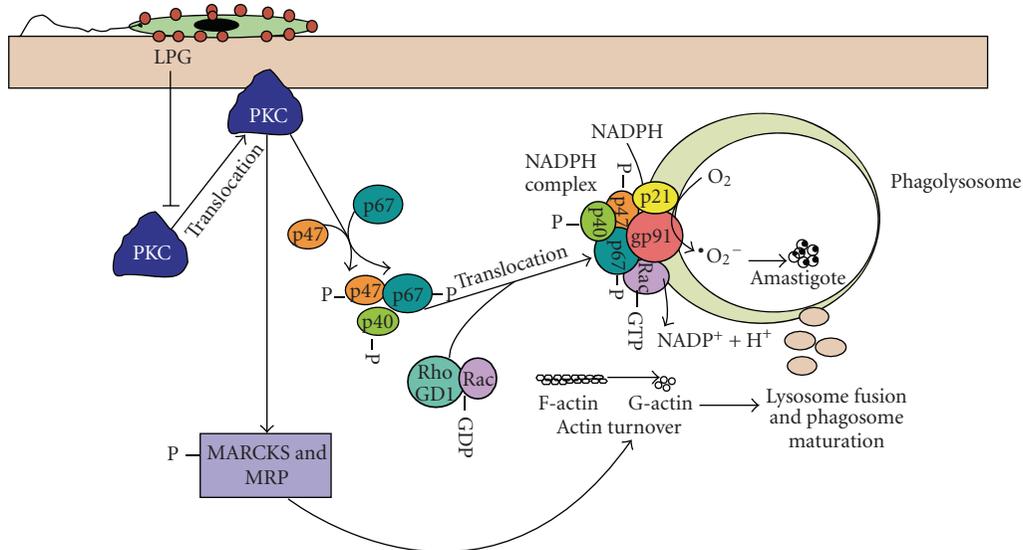


FIGURE 4: PKC regulation in *Leishmania* infected macrophages: LPG of *Leishmania* inhibits PKC activation and translocation to the membrane. PKC is responsible for phosphorylation of p47phox and p67phox components of NADPH oxidase which are subsequently translocated to phagosomal membrane to form NADPH oxidase complex, which is responsible for superoxide anion generation and hence parasite killing. Further PKC phosphorylates myristoylated alanine-rich C kinase substrate (MARCKS) and MARCKS-related protein (MRP) which are involved in actin turnover and finally in phagosomal maturation and lysosomal fusion resulting in parasite killing. As PKC activation is inhibited by *Leishmania*, this results in subsequent inhibition of all the above mentioned processes thereby favoring parasite survival.

increased ($\text{Na}^{++} \text{K}^{+}$)ATPase activity while activation of Ca^{2+} -independent PKC-like protein has inhibitory effect [116]. Recently a study by the same group reported presence of ecto-PKC in different *Leishmania* species. They found higher PKC activity in infective stationary stage of *Leishmania* promastigotes and that this PKC-like plays a critical role in attachment and internalization steps involved in the parasite invasion process [117]. Some of the *Leishmania*-PKC interactions are summarized in Figure 4.

3.2. Other Kinases. PI3K signaling activated by *Leishmania* infection is a negative signaling pathway which helps in progression of disease. It has been shown that PI3K signaling negatively regulates IL-12 production and inhibition of PI3K signaling by specific inhibitor or its downstream kinase Akt reverses the IL-12 blockade in macrophages [118]. PI3K^{-/-} DCs show enhanced IL-12 production and PI3K^{-/-} mice elicit an enhanced Th1 response upon *L. major* infection [119].

4. Modulation of Phosphatases

Leishmania can also activate various molecules that inhibit intracellular signaling cascades (Figure 5) thereby evading host immune machinery to inhibit immune responses.

4.1. SHP-1 Protein Tyrosine Phosphatase. An important negative regulatory molecule of numerous signaling pathways, such as those related to the actions of interferons [120, 121], erythropoietin [122, 123], and many others, is SHP-1 (Src

homology 2 domain containing tyrosine phosphatase) which is expressed principally in haematopoietic cells but also in smooth muscle [124] and epithelial cells [125]. Many of the interactions of SHP-1 with its substrates involve the binding of either one or both of its tandem SH2 domains to tyrosine phosphorylated, immunoreceptor tyrosine-based inhibitory motifs (ITIMs). These specialized motifs are known to be present in many signaling molecules [126, 127]. Multiple types of ITIMs exist and display-specific abilities to recruit and activate SH2 containing PTPs.

SHP-1 is responsible for the negative regulation of many signaling pathways in all hematopoietic cell types, by acting in a variety of fashions. For instance, SHP-1 can bind to receptors and dephosphorylate them directly; it can also associate with a receptor and dephosphorylate other members of the receptor binding complex. The PTP can also interact with other cytosolic proteins and tyrosine dephosphorylates them or their associated proteins [126]. Macrophages infected with *Leishmania* in vitro have elevated SHP-1 activity as well as total PTP activity, resulting in widespread dephosphorylation of high-molecular-weight proteins [74]. Furthermore, infection causes colocalization of SHP-1 and JAK2 and prevents tyrosine phosphorylation of JAK2 in response to IFN- γ [74]. Dephosphorylation of JAK1/2, TYK2, and STAT1 α , -2, -3, -5 α/β , and -6 has already been documented [121–123, 128, 129]. Forget et al. showed that activation of the host PTP SHP-1 is responsible for the dephosphorylation and inactivation of ERK1/2, as SHP-1-deficient macrophages showed normal JAK2 and ERK1/2 activity following infection with *L. donovani*, and responded to IFN γ by increased NO production [130].

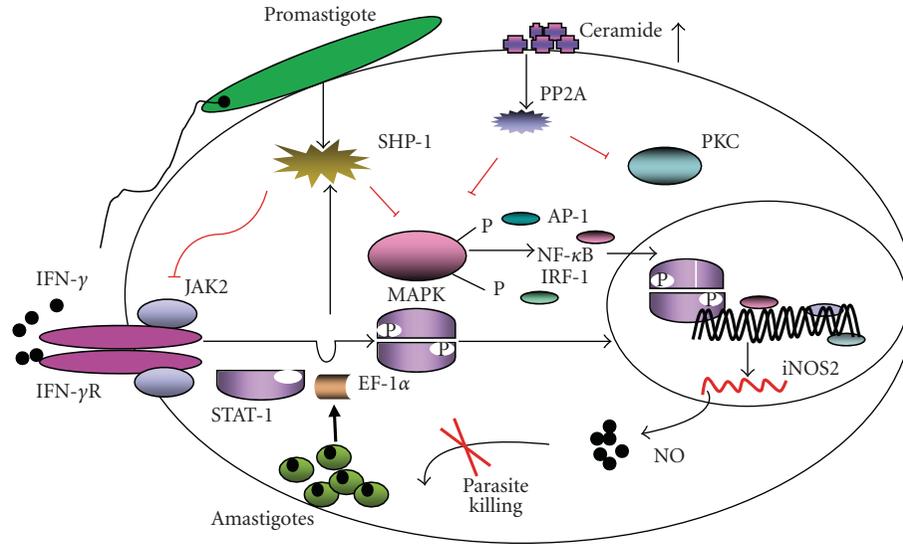


FIGURE 5: MAPK phosphorylation in *Leishmania*-infected macrophages: *Leishmania* infection of macrophages represses the most important MAPK family members: ERK1/2, p38, and JNK. MAPK inactivation is accompanied by inhibition of transcription factors Elk-1, c-fos, IRF-1, AP-1, and NF- κ B and IFN- γ -dependent NO generation.

In viable motheaten mice, whose SHP-1 phosphatase activity is deficient, increased nuclear translocation of the transcription factor NF- κ B has been reported [131, 132], which seems to provoke an exacerbated inflammatory response. Macrophages derived from SHP-1^{-/-} mice show elevated iNOS induction and NO generation and are more efficient at killing *Leishmania* [133]. This is reflected in vivo by increased NO generation and reduced parasite load in both SHP-1-deficient mice and mice treated with chemical PTP inhibitors like peroxovanadium [133–135]. Some studies have demonstrated that the inhibition of IFN- γ -dependent phosphorylation cascades following infection is due to activation of host cell tyrosine phosphatases [74, 134]. This was associated with a phenotype of cell deactivation in which MAP kinase signaling, c-FOS, and iNOS expression were each defective. Importantly, inhibition of phosphotyrosine phosphatase activity with sodium orthovanadate before infection prevented development of the deactivated phenotype [136]. Studies aimed at understanding the mechanism responsible for the change in activation state of SHP-1 led to the identification of *Leishmania* EF-1 α as a modulator of host SHP-1 and also suggesting it to be a novel virulence factor contributing to macrophage deactivation [137].

Recently one study revealed that upon *Leishmania* infection, SHP-1 is able to rapidly bind to and inactivate a critical kinase (IRAK-1) in TLR signaling pathway. This regulatory binding was shown to be mediated by an evolutionarily conserved motif identified in the kinase. This motif was also present in other kinases involved in Toll signaling and therefore could represent a regulatory mechanism of relevance to many kinases. This work reports a unique mechanism by which *Leishmania* can avoid harmful TLR signaling [138].

4.2. Other Phosphatases. Ceramide is also capable of activating protein phosphatases such as protein phosphatase 1 (PP1) and PP2A [139–141]. It is through these protein phosphatases that ceramide inhibits kinases such as the classical as well as novel PKC isoforms and Akt [138–142]. It was observed that endogenous ceramide generated during leishmanial infection led to the dephosphorylation of protein kinase B (Akt) in infected cells. Ceramide induced the PKC ζ -Akt interaction along with the serine/threonine phosphatase PP2A [143].

However a phosphotyrosine phosphatase PTEN (phosphatase and tensin homologue deleted on chromosome ten) is reported to play a protective role against *L. major* infection as the mice lacking PTEN are more susceptible to the infection than the WT mice. PTEN deficient macrophages have reduced ability to kill parasites in response to IFN- γ treatment, showing decreased TNF- α production, iNOS expression, and NO secretion but more IL-10 secretion than WT cells. Thus the study shows that phosphatase PTEN is required for efficient clearance of intracellular parasite in macrophages [144].

5. Conclusion

There are multiple ways by which intracellular pathogens like *Leishmania* make use of host cell's machinery in order to survive and replicate. One such mechanism is the distortion of host macrophage's own signaling pathways to selectively repress or enhance the expression of various cytokines and microbicidal molecules and antigen presentation. The interplay between various signaling molecules is complex. As signaling pathways can be pharmacologically manipulated, a better knowledge of their role and the mechanisms whereby

they regulate host immune cell functions and pathogen growth should permit the development of new therapies to control infectious agents.

Acknowledgment

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Research Article

BALB/c Mice Vaccinated with *Leishmania major* Ribosomal Proteins Extracts Combined with CpG Oligodeoxynucleotides Become Resistant to Disease Caused by a Secondary Parasite Challenge

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Leishmaniasis is an increasing public health problem and effective vaccines are not currently available. We have previously demonstrated that vaccination with ribosomal proteins extracts administered in combination of CpG oligodeoxynucleotides protects susceptible BALB/c mice against primary *Leishmania major* infection. Here, we evaluate the long-term immunity to secondary infection conferred by this vaccine. We show that vaccinated and infected BALB/c mice were able to control a secondary *Leishmania major* challenge, since no inflammation and very low number of parasites were observed in the site of reinfection. In addition, although an increment in the parasite burden was observed in the draining lymph nodes of the primary site of infection we did not detect inflammatory lesions at that site. Resistance against reinfection correlated to a predominant Th1 response against parasite antigens. Thus, cell cultures established from spleens and the draining lymph node of the secondary site of infection produced high levels of parasite specific IFN- γ in the absence of IL-4 and IL-10 cytokine production. In addition, reinfected mice showed a high IgG2a/IgG1 ratio for anti-*Leishmania* antibodies. Our results suggest that ribosomal vaccine, which prevents pathology in a primary challenge, in combination with parasite persistence might be effective for long term maintenance of immunity.

1. Introduction

Protozoa of the genus *Leishmania* are obligate intracellular parasites of the mononuclear phagocytic lineage. *Leishmania* infection causes a group of diseases ranging from self-healing cutaneous ulcers to potentially lethal fatal visceral infection, globally known as leishmaniasis [1]. *L. major* is the main causative agent of cutaneous leishmaniasis (CL) in the Old World. In humans, CL due to *L. major* infection is self-limiting and healing is associated with resistance to reinfection. This acquired immunity to reinfection in natural

Leishmania hosts suggests that a vaccine is feasible. However, there are no available vaccines against human leishmaniasis [2].

Effective primary immunity against *L. major* in mouse requires an IL-12 dependent production of IFN- γ from CD4⁺T cells (Th1 response) and CD8⁺ T cells that mediates a nitric oxide-dependent killing by infected macrophages [3, 4]. In contrast, susceptibility correlates with the dominance of an IL-4 driven Th2 response, as it has been observed in certain mice strains like BALB/c [3, 4]. Subcutaneous (s.c.) experimental infection of BALB/c mice with a high

dose inoculum of stationary phase promastigotes induces rapidly evolving lesions that correlated with the generation of strong Th2 responses [5]. This model of experimental CL has been extensively used to explore the protective role of several parasite antigens combined with different adjuvants [2, 6, 7]. The immunization with certain parasite proteins, irrespective of their cellular location (surface or intracellular parasite antigens), inoculated with Th1 modulating adjuvants can induce immune responses that resulted in protection [8, 9]. The production of parasite specific IFN- γ combined with the control of the production of the disease associated IL-4 and IL-10 cytokines has been correlated to protection against the development of CL in vaccinated BALB/c mice [10]. Protective cell mediated immunity can also be induced in BALB/c mice after s.c. infection using a nonpathogenic challenge of *L. major* promastigotes (leishmanization) [11–14]. Leishmanized mice developed very low or no pathology after primary infection and acquired resistance against a pathogenic rechallenge [11, 13, 14]. Leishmanization induced parasite specific Th1 responses that were able to control the secondary challenge made in a distant site [13, 14].

In a previous work, we have shown that during *L. major* infection, susceptible BALB/c mice develop a Th2 response against parasite ribosomal crude extracts purified from promastigotes [15]. Vaccination with the parasite ribosomal proteins (LRP) combined with CpG oligodeoxynucleotides (CpG ODN) as adjuvant induced a specific Th1 response, since vaccinated mice developed anti-LRP antibodies of the IgG2a isotype and their splenocytes produced high amounts of IFN- γ , but not IL-4, after in vitro stimulation with LRP [15]. The immune state induced by vaccination conferred protection against a primary challenge with *L. major* parasites in the footpad. After infection, a *Leishmania* specific IL-12 dependent production of IFN- γ and a reduced production of IL-4 and IL-10 were associated to protection [15].

In this work, we have analyzed whether or not vaccinated and protected mice were able to control the development of CL after a secondary challenge. To this end, mice vaccinated with LRP + CpG ODN were infected in the footpad with a pathogenic challenge of *L. major* parasites. The development of footpad swelling was analyzed over a period of 18 weeks as a stringent test of vaccine induced protection. Since no CL pathology was found during the follow up, mice were reinfected into the ear dermis with a low dose pathogenic challenge of *L. major* metacyclic promastigotes. Our results showed that vaccinated and infected mice developed a resistant phenotype to parasite associated disease at a secondary site of infection.

2. Materials and Methods

2.1. Animals and Parasites. Female BALB/c mice (4–6 week-old) were purchased from Harlan Interfauna Ibérica S.A. (Barcelona, Spain). *L. major* parasites (WHOM/IR/-/173) and clone V1 (MHOM/IL/80(Friedlin)) were kept in a virulent state by passage in BALB/c mice. *L. major* amastigotes

were obtained and transformed to promastigote by culturing at 26°C in Schneider's medium (Gibco, BRL, Grand Island, NY, USA) supplemented with 20% foetal calf serum. Metacyclic promastigotes of *L. major* (clone V1) were isolated from stationary cultures by negative selection as described in [16] using peanut agglutinin (Vector Laboratories, Burlingame, CA, USA).

2.2. Parasite Antigens, Adjuvant and Immunizations . Soluble *Leishmania major* antigen (SLA) was prepared as described [17]. Briefly, *L. major* promastigotes were harvested from culture and washed four times in phosphate-buffered saline (PBS). The parasites were suspended in PBS and subjected to three freezing and thawing cycles and sonicated with five cycles of 30 seconds at 38 MHz. After cell lysis, soluble antigens were separated from the insoluble fraction by centrifugation for 15 minutes at 12,000 \times g using a microcentrifuge. *L. major* ribosomal proteins (LRP) were prepared as described [15]. Phosphorothioate-modified CpG ODN (5'-TCAACGTTGA-3' and 5'-GCTAGCGTTAGCGT-3') were synthesized by Isogen (The Netherlands).

Six mice were s.c. immunized in the right footpad with 12 μ g of *L. major* LRP combined with 25 μ g of each CpG ODN in a volume of 30 μ l. Control groups ($n = 6$) received either CpG ODN or phosphate saline buffer PBS. Mice were immunized three times at two-week intervals.

2.3. Parasite Challenge. The primary parasite challenge was done by s.c. inoculation in the left footpad with 5×10^4 stationary-phase promastigotes of *L. major* (WHOM/IR/-/173) in a volume of 30 μ l, four weeks after the last vaccine inoculation. The secondary infection was done at week 18 after primary infection with 1000 metacyclic promastigote of *L. major* (clone V1) isolated from stationary cultures by negative selection using peanut agglutinin (Vector Laboratories, Burlingame, CA). Metacyclic forms were injected into the dermis (i.d.) of both ears of each mouse in a volume of 10 μ l.

Footpad swelling was measured with a metric calliper and calculated as thickness of the left footpad minus thickness of the right footpad. Evolution of the ear lesion was monitored by measuring the diameter of the indurations with a metric calliper.

2.4. Estimation of Parasitic Load. The number of parasites was determined by limiting dilution assay [18]. Briefly, ears were recovered from infected mice and the ventral and dorsal sheets were separated. Ear sheets were deposited in RPMI medium containing Liberase CI enzyme blend (50 μ g ml⁻¹) (Roche, Mannheim, Germany). After an incubation period of 2 hours at 37°C, the tissues were cut into small pieces, homogenized and filtered using a cell strainer (70 μ m-pore size). The homogenized tissue was serially diluted in a 96-well flat-bottomed microtiter plate containing Schneider's medium plus 20% FCS. The number of viable parasites was determined from the highest dilution at which promastigotes could be grown up to 7-day incubation at 26°C. The number of parasites was also determined in the local draining

lymph nodes (DLN) of infected ears (retromaxillar) and footpad (popliteal) and in the spleen. Organs were recovered, mechanically dissociated, homogenized and filtered and then serially diluted as above. Parasite load is expressed as the number of parasites in the whole organ.

2.5. Measurement of Cytokines in Supernatants. Splenocytes and DLN cells suspensions were seeded in complete RPMI medium (RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, and 10 mM 2-mercaptoethanol). 3×10^6 cells were seeded in 48-well plates during 48-hour at 37°C in the presence of LRP ($12 \mu\text{g ml}^{-1}$) or SLA ($12 \mu\text{g ml}^{-1}$). The release of IFN- γ , IL-10, and IL-4 was measured in the supernatants of splenocytes and DLN cell cultures using commercial ELISA kits (Diaclone, Besançon, France).

2.6. Analysis of the Humoral Responses. Reciprocal end-point titre (defined as the inverse of the highest serum dilution factor giving an absorbance >0.2) against LRP and SLA was determined by serial dilution of the sera assayed by ELISA using anti-IgG1 (1/1000) and anti-IgG2a (1/500) horseradish peroxidase-conjugated anti-mouse immunoglobulins as secondary antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands). Plates were coated with $100 \mu\text{l}$ of LRP ($5 \mu\text{g ml}^{-1}$ in PBS) or SLA ($2 \mu\text{g ml}^{-1}$ in PBS).

2.7. Statistical Analysis. Statistical analysis was performed by a Student's *t*-test. Differences were considered significant when $P < .05$.

3. Results and Discussion

3.1. Protective Immunity Generated by s.c. Vaccination with LRP + CpG ODN in the Footpad. In a previous work we showed that BALB/c mice vaccinated with LRP combined with CpG ODN were protected against the development of cutaneous lesions in the footpad 8 weeks after parasite challenge [15]. The absence of footpad swelling was correlated with a 3-log reduction in parasite burden in the ipsilateral popliteal DLN when compared with mice immunized with the adjuvant or the excipient (control groups) [15]. In addition, no parasites were found in the spleen of the LRP + CpG ODN vaccinated animals whereas control groups contained approximately 10^4 parasites. It was concluded that the Th1 immune response induced in BALB/c mice by the vaccination of the LRP combined with CpG ODN resulted in a solid immunity that efficiently controlled parasite induced cutaneous disease maintaining a chronic infection in the local DLN [15]. In this work, we decided to analyze the footpad swelling of LRP + CpG ODN vaccinated mice after a longer period of time. After parasite challenge, vaccinated mice did not develop lesion for up to eighteen weeks (Figure 1(a)). Since control groups were sacrificed at week seven after challenge (because they began to develop severe necrotic lesions) a comparative analysis between controls and LRP + CpG ODN vaccinated mice was not possible. However, the parasite burden in the spleen and in the popliteal DLN of the LRP + CpG ODN vaccinated mice was

analyzed at week 18 after parasite challenge in the footpad. As it is shown in Figure 1(b), no parasites could be detected in the spleen of the vaccinated mice. The number of parasites located at the popliteal DLN at week 18 after challenge (5.41 ± 0.99 ; \log_{10} scale) represents a 1.12-log increment ($P = .23$) when compared with the number of parasites detected in the same organ in LRP + CpG ODN vaccinated mice 8 weeks after challenge (4.84 ± 0.26 ; \log_{10} scale) [15]. Although a slightly increment in the number of parasites was detected, the presence of high levels of IFN- γ measured in the supernatants of DLN cells cultures after stimulation with SLA and LRP in the absence of detectable levels of IL-4 and IL-10 (Figure 1(c)), should be taken as an indication that the parasite-specific Th1 response observed at week 8 [15] was maintained at week 18 after challenge. Thus, the Th1 response elicited by LRP + CpG ODN vaccination was able to induce an immunological status that protects mice against the development of cutaneous lesions during the 18 weeks of follow up. In addition, a chronic infection was patent in the vaccinated mice, being the parasites maintained located in the local DLN without dissemination to the internal organs.

This study reinforces that CpG ODN provides protection when used in combination with LRP extracts. Previous studies using this adjuvant in combination with parasite lysates showed a different degree of protection against *L. major* infection in the susceptible BALB/c [19–21] and in the resistant C57BL/6 [19] mice strains. The identification of a protein fraction composed by ribosomal proteins that provide protection against the development of cutaneous leishmaniasis lesions represents a substantial step in defining the protective immunogens within SLA, helping to identify new protective parasite antigens for the development of molecularly defined vaccines against leishmaniasis.

3.2. Protected Mice Became Resistant to Disease Caused by a *L. major* Rechallenge in the Ear Dermis. Next, we analyzed if protected mice were able to control a second parasite challenge. For that purpose, vaccinated and protected mice ($n = 6$) were rechallenged in the ear dermis with 1000 *L. major* metacyclic promastigotes, parasite infective forms that seem to be similar to the promastigotes that are inoculated during the insect vector blood feeding [22]. Since vaccines were inoculated in the contralateral footpad of the primary infection site, rechallenge was made by i.d. infection in the ears. BALB/c mice infected with a low dose of *L. major* metacyclic promastigotes develop progressive inflammatory lesion in the spot of infection that increased in size, accompanied by ulceration and tissue necrosis [23–26] as occurred in mice challenged in the footpad with a high dose inoculum of stationary promastigotes [3, 4]. As control six naïve BALB/c mice were also infected in the ear dermis with the same dose of parasites.

Very low dermal lesion development was observed in reinfected mice (Figure 2(a)). In some cases (in four of six mice) a complete absence of inflammation in the ears was observed for up to seven weeks. Two mice developed low dermal lesions (<1 mm) that reached a peak at week 5 and were almost completely healed at week seven. On

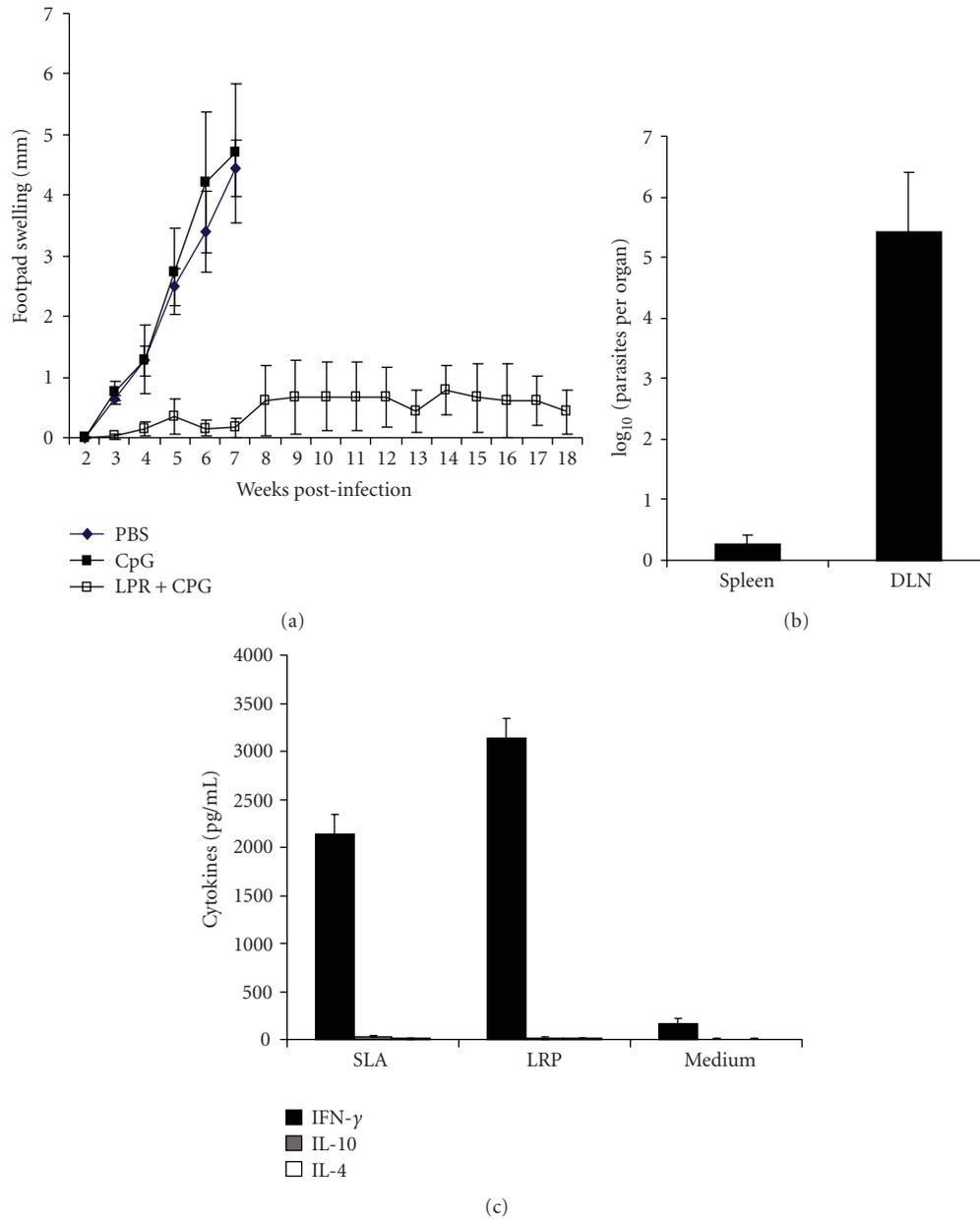


FIGURE 1: Course of *L. major* infection in BALB/c vaccinated mice. Mice (six per group) were s.c. immunized in the right footpad with three doses of the LRP adjuvanted with CpG ODN (LRP + CpG), with the CpG ODN adjuvant (CpG) or with PBS. One month after the last immunization, the animals were infected in the left hind footpad with 5×10^4 *L. major* stationary phase promastigotes. (a) Footpad swelling is given as the difference of thickness between the infected and the uninfected contralateral footpad. (b) The number of viable parasites in the spleen and the popliteal DLN of the LRP + CpG ODN vaccinated were individually determined by limiting dilution at week eighteen post challenge. Results are expressed as the mean \pm SD of six spleens and popliteal DLN. (c) At week eighteen after footpad infection the level of IFN- γ , IL-10 and IL-4 was measure by ELISA in the supernatants of popliteal lymph node cells cultures from LRP + CpG ODN vaccinated mice. Cells were in vitro stimulated for 48 hours with 12 μ g/ml of SLA or LRP and medium alone. Results are expressed as the mean \pm SD.

the other hand, infection in all control naïve mice leads to the development of progressive inflammatory lesions in the ears (Figure 2(a)). The parasite load in the ear dermis and retromaxillar DLN was analyzed at week seven after challenge. Vaccinated and reinfected mice showed very low parasite loads in the ears and in the retromaxillar DLN, correlating to the absence of parasite in the spleens

(Figure 2(b)). These data contrast with the parasite burdens found in the ear dermis and in the retromaxillar DLN in the control group mice. Also, as an indication of parasite dissemination, parasites were detected in the spleen of the control mice (Figure 2(b)). These data indicate that the immune state generated after the first infection in the LRP + CpG ODN vaccinated mice is extremely potent, leading

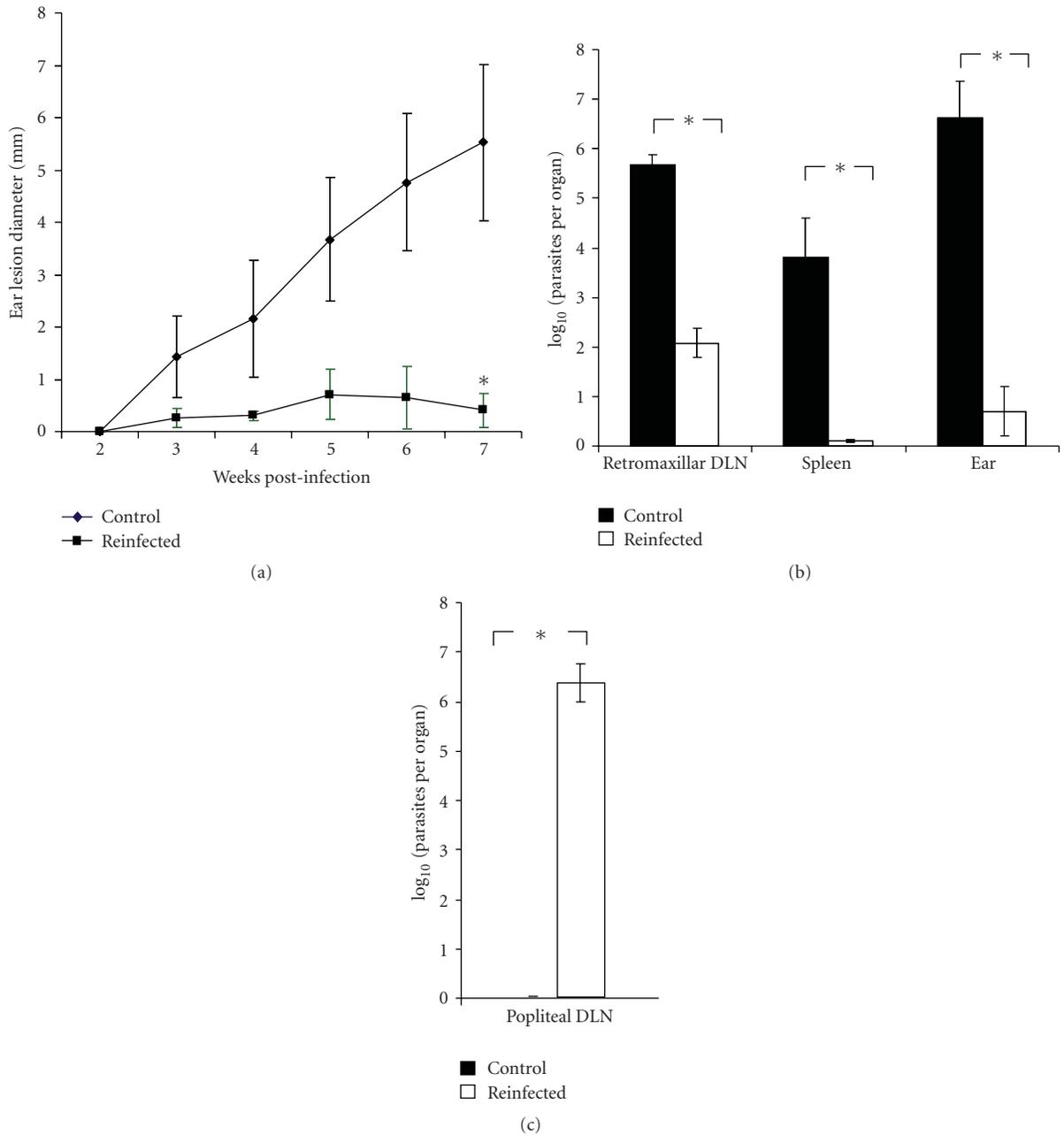


FIGURE 2: (a) Course of *L. major* infection in protected and reinfected BALB/c mice. Values represent the mean lesion diameter ± standard deviation (SD). *P < .001: significant differences in inflammation for protected versus control mice at week seven postchallenge. (b) Seven weeks after reinfection, mice were euthanized and parasite burden in the ear dermis, spleen and in the retromaxillar DLN was individually quantitated. Results are expressed as the mean ± SD of twelve ears and six spleens and DLN. *P < .001: significant decrease for reinfected versus control mice. (c) The parasite burden at week seven after rechallenge was individually quantitated in the popliteal lymph nodes of control and reinfected mice. Results are expressed as the mean ± SD of six DLN. *P < .001: significant decrease for reinfected versus control mice.

to a rapid and efficient control of parasite growth in the site of reinfection, that resulted in the generation of a moderate dermal pathology.

In the vaccinated reinfected mice the primary challenge site was also analyzed, since in immune genetically resistant mice an *L. major* secondary challenge can cause disease reactivation in the primary site despite efficient parasite

clearance in the site of reinfection [27, 28]. The parasite load found in the popliteal DLN of the reinfected mice (6.68 ± 0.63 ; log₁₀ scale) (Figure 2(c)) represents an increment of 1.23-log ($P = .028$) when compared with the number of parasites detected at the moment of the secondary challenge. Since no parasites were found in the popliteal DLN of control mice (Figure 2(c)), parasite dissemination from the ear to

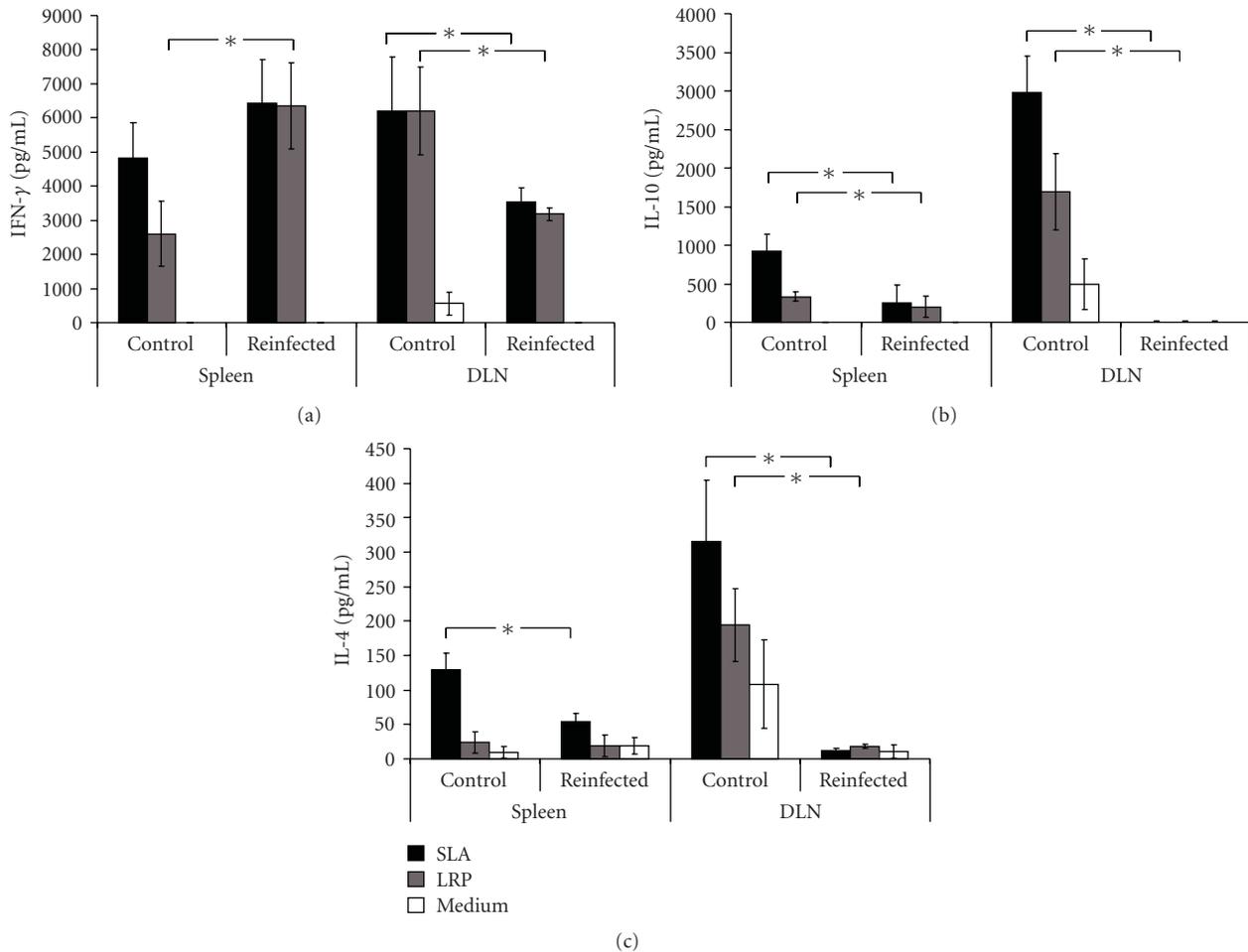


FIGURE 3: Analysis of the cellular responses. At week seven after ear infection the level of IFN- γ (a), IL-10 (b), and IL-4 (c) was measured by ELISA in the supernatants of spleen and retromaxillar lymph node cells cultures from both mice groups. Cells were in vitro stimulated for 48 hours with 12 μ g/ml of SLA or LRP and medium alone. Results are expressed as the mean \pm SD of twelve ears and DLN. (* $P < .001$).

these lymph nodes seems to be unlikely and the increase in the number of parasites found in the popliteal DLN may be indicating that the secondary challenge induced some parasite replication. However, we did not detect an increment in the footpad swelling in the vaccinated reinfected BALB/c mice for up to seven weeks after secondary challenge (not shown). Thus, we conclude that secondary challenge in the ear dermis did not produce a disease reactivation in these vaccinated mice.

3.3. Analysis of the Cellular Immune Response. To determine which immunological parameters are related to resistance after the secondary challenge, the SLA and the LRP driven production of IL-4, IL-10, and IFN- γ was assayed at week seven after ear infection. Spleen cell cultures from control and reinfected mice were established to analyze the systemic response and DLN cells (retromaxillar) were cultured to analyze the local response induced by the ear infection. Spleen cells from reinfected mice produced higher amounts of IFN- γ after SLA or LRP stimulation than control mice, but only the level of LRP specific IFN- γ was significantly

different between the two groups. We observed that the level of SLA- and LRP-specific IFN- γ detected in the DLN cell cultures was higher in control than in the reinfected mice (Figure 3(a)). Most likely, the high level of IFN- γ detected in retromaxillar DLN could be related to the high number of parasites found in control animals (Figure 2(b)) that may be stimulating the production of IFN- γ by Th1 cells, since in this model of infection the presence of parasites is correlated with IFN- γ production [26]. The IL-10 and IL-4 production after stimulation with SLA or LRP was barely detected in the spleen and DLN cells from reinfected mice whereas in the spleen cell cultures and especially in the DLN cell culture supernatants from control mice high levels of these cytokines were measured (Figures 3(b) and 3(c)).

Those data are compatible with the fact that Th1/Th2 mixed responses were elicited after infection in control mice, characterized by the production of parasite specific IFN- γ and IL-4 cytokines. In addition, the presence of high levels of parasite specific IL-10 may be also implicated in the progression of the disease, since the inactivating effect of this cytokine in infected macrophages has been related

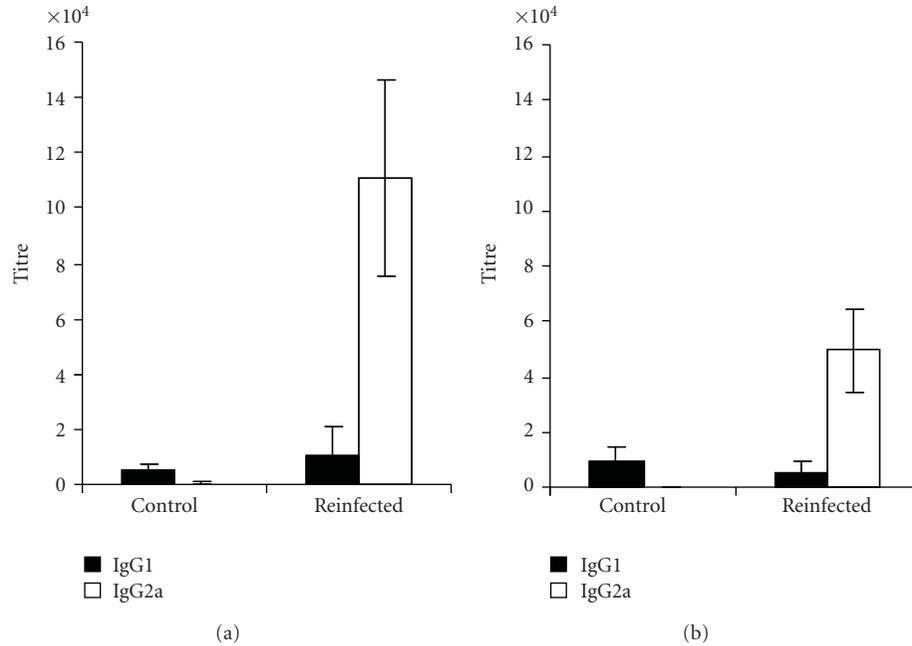


FIGURE 4: Analysis of the humoral responses. Serum samples from control and vaccinated reinfected mice were obtained seven weeks after challenge in the ear dermis. The titre for IgG1 and IgG2a antibodies against LRP (a) and SLA (b) was determined individually by ELISA. Results are expressed as the mean \pm SD.

TABLE 1: Cytokine production by popliteal DLN cells from vaccinated reinfected mice at week seven after secondary challenge.

	SLA	LRP	Medium
IFN- γ	5837.16 \pm 834.82	5773.52 \pm 1411, 14	1635, 11 \pm 607, 89
IL-10	526.31 \pm 214.98	408, 92 \pm 233.36	104, 39 \pm 57, 13
IL-4	229.54 \pm 58.78	44.36 \pm 31.22	54.38 \pm 37.89

The level of cytokines was determined by ELISA in the supernatant of popliteal DLN cells obtained from reinfected mice at week seven post rechallenge, after *in vitro* stimulation with 12 μ g/ml of SLA and LRP. Mean \pm SD of samples from six mice is shown (pg/ml).

with BALB/c mice susceptibility against *L. major* infection [29–32]. The pattern of cytokine production observed in infected control mice, with detectable level of parasite specific production of IFN- γ , IL-10 and IL-4 was previously observed after infection with a pathogenic challenge of *L. major* in BALB/c ears [25, 26, 33]. On the contrary, a Th1-mediated IFN- γ production was elicited in the reinfected mice group in the absence of Th2 responses and IL-10 mediated regulatory responses.

The SLA and the LRP driven production of IL-4, IL-10, and IFN- γ was also assayed in the popliteal DLN of the reinfected mice. Although detectable levels of the three cytokines were observed, the level of IFN- γ was higher than the levels of IL-10 and IL-4 (Table 1). A high ratio of IFN- γ /IL-10 and IFN- γ /IL-4 for both parasite proteins preparations (11.1 and 25.5 for SLA; 14.1 and 130.15 for LRP, respectively) was obtained, indicating that a parasite-specific IFN- γ response was still maintained at week seven after secondary challenge in the popliteal DLN, yet in the presence of IL-4 and IL-10 cytokines. This Th1/Th2 mixed response may account for the increment observed in the number of parasites after secondary infection in the popliteal DLN.

3.4. Analysis of the Humoral Responses. The humoral response elicited in control mice and in the reinfected mice was analyzed at week seven after parasite challenge in the ear dermis. The titre of anti-LRP and anti-SLA specific IgG1 and IgG2a antibodies were determined, since the presence of IgG1 and IgG2a antibodies is considered a marker of Th2 and Th1 type responses, respectively [34]. In the sera from control mice the anti-*Leishmania* predominant antibodies were of the IgG1 isotype and very low but detectable levels of IgG2a were observed (Figures 4(a) and 4(b)). On the contrary, vaccinated reinfected mice showed high titres of IgG2a antibodies against LRP (Figure 4(a)) and SLA (Figure 4(b)). These humoral responses are in agreement with the nature of cellular responses observed after *in vitro* stimulation with both antigenic preparations. A strong Th1 response was elicited in vaccinated reinfected mice after parasite rechallenge having a resistant phenotype. On the contrary, antibodies found in the sera from mice of the control group were mainly of the IgG1 isotype as expected for their nonhealing phenotypes.

Altogether, our data showed that the immune response elicited in the LRP + CpG ODN vaccinated mice after

the primary infection was able to control a secondary challenge. Acquisition of the resistant phenotype was correlated to the capacity to induce a Th1 response (large amounts of parasite specific production of IFN- γ and a high anti-leishmanial proteins IgG2a/IgG1 ratio) in the absence of Th2 or IL-10 mediated responses. The immune responses associated with the resistance after secondary infection in the vaccinated-infected mice were similar to that obtained in BALB/c mice that controlled a secondary infection in the ear after a primary infection in the contralateral ear, showing a Th1 response after rechallenge [26]. It is important to note that protection in these mice only occurred when lesions were developed in the primary site of infection [26], whereas LRP + CpG ODN vaccinated mice became resistant after primary challenge without the development of dermal lesions. Also, protection to reinfection achieved by BALB/c mice infected with a low infection dose in the footpad was dependent on the induction of Th1 responses [11, 35]. Here we show that that the immune response elicited by the LRP + CpG ODN vaccine after primary challenge was able to control the development of lesions and generated a long-term immune state necessary for the maintenance of immunity to further infection. The presence of parasites in the popliteal DLN may be related with this Th1 response, since it has been demonstrated that the presence of parasite antigens is necessary for the maintenance of cell mediated immunity in BALB/c mice [14, 36].

4. Conclusion

The data reported here provided evidence that BALB/c mice protected against the development of dermal pathology due to *L. major* s.c challenge after LRP + CpG ODN vaccination have acquired an immunological status which conferred them the capacity to resist a further infection (an appealing feature for a vaccine that might be employed in endemic areas, where reexposure to the parasite would be very frequent). After a secondary challenge in the ear dermis, these mice showed a robust protection against *L. major* infection. Very low parasite burdens and development of dermal lesions in the site of reinfection were found. A specific Th1 protective response after the secondary challenge was correlated to resistance to reinfection. Thus, the immune state generated by the combination of vaccination with LRP + CpG ODN and the primary infection is extremely potent, leading to a rapid and efficient elimination of the parasite from the site of reinfection. Despite extensive research efforts, leishmanization with viable *Leishmania* parasites is the only vaccine with proven efficacy in humans [2, 37]. The induction of long-term immune responses by vaccines based on parasite extracts or recombinant parasite products that protected mice against the development of leishmaniasis after a primary challenge has been extensively reported [8, 38]. However, there is scarcity of studies analyzing the long-term maintenance of resistance to reinfection of vaccinated mice. Extrapolation of this approach to other animal or human models is hazardous but our findings may be relevant to develop effective tools against leishmaniasis based on defined *Leishmania* subunits.

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Review Article

Drug Resistance in Visceral Leishmaniasis

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Visceral leishmaniasis remains a public health problem worldwide. This illness was included by the World Health Organization in the list of neglected tropical diseases targeted for elimination by 2015. The widespread emergence of resistance to pentavalent antimonials in India where half cases occur globally and the unavailability of a vaccine in clinical use constitute major obstacles in achieving this goal. The last decade new antileishmanials became available, including the oral agent miltefosine. However, in poor endemic countries their wide use was curtailed because of the high costs, and also due to concerns of toxicity and emergence of resistance. Various mechanisms of antileishmanial resistance were identified recently in field isolates. Their elucidation will boost the design of new drugs and the molecular surveillance of resistance. Combination regimens should be evaluated in large trials. Overall, the development of antileishmanials has been generally slow; new drugs are needed. In order to control visceral leishmaniasis worldwide, treatment advances should become affordable in the poorest countries, where they are needed most.

1. Introduction

Visceral leishmaniasis (VL; also known as kala azar) is a protozoan systemic infection, which is almost always fatal if left untreated. This illness is endemic in several tropical and subtropical regions and in the Mediterranean basin. The estimated annual global burden of VL is 500 000 new cases and more than 50 000 deaths, of which 90% occur just in five countries—India, Bangladesh, Nepal, Sudan, and Brazil [1]. VL is transmitted through hematophagous sandflies and is caused by *Leishmania donovani* in the Indian subcontinent, Asia, and Africa, *L. infantum* in the Mediterranean basin, and *L. chagasi* in South America. After an incubation period of several months, typical VL manifests with intermittent fever, weight loss, massive hepatosplenomegaly, and progressive deterioration of the host; hemorrhages and edemas may develop late in the course [2–4]. Leishmaniasis was selected by the World Health Organization for elimination by 2015, along with other neglected tropical diseases [5]. Since there is no antileishmanial vaccine in clinical use, control of VL relies almost exclusively on chemotherapy.

For almost seven decades pentavalent antimonials constituted the standard antileishmanial treatment worldwide,

however the last 15 years their clinical value was jeopardized due to the widespread emergence of resistance to these agents in Bihar, India, where half of VL cases occur globally [6]. The last decade novel formulations of conventional antileishmanials as well as new drugs, including the oral agent miltefosine, became available or are under investigation. In practice, however, their wide use in poor countries is hampered mainly due to high costs and also due to concerns of toxicity and emergence of resistance [6]. In response to concerns about preserving the currently available antileishmanials, especially in regions with anthroponotic parasite transmission, there is growing interest on combination regimens. Control of VL in poor countries is further compromised by the emergence of human immunodeficiency virus (HIV)-VL-coinfection [7]. This article will review recent publications on antileishmanial drugs, with emphasis on resistance issues. Strategies to preserve the activity of currently available drugs will be addressed.

2. Pathogenesis and Immune Response

Leishmaniasis are obligatory intracellular protozoan parasites. The parasites remain within their vectors as extracellular promastigotes [8]. Following sandfly bite, neutrophils migrate

locally and capture the parasites, however the latter have the ability to escape and subsequently invade the macrophages of the skin, where they differentiate and replicate as amastigotes [9, 10]. From there, parasites disseminate and invade additional macrophages of the reticulo-endothelial system, and finally infiltrate the bone marrow, liver, and spleen [8]. VL should be regarded as a state of long-term parasitism, since leishmanias are not eradicated completely but rather remain in skin macrophages for lifetime, even after successful treatment in hosts with intact T-cell immune responses. In skin, leishmanias act as a reservoir for the potential relapse of symptomatic VL. The risk for relapse increases when T-cell immune responses are impaired and irrespectively of prior antileishmanial treatment, as noted in HIV-infected patients [7, 11, 12]. Relapses usually peak 6–12 months after treatment.

Following *Leishmania* infection, host immune responses are elicited. Immune responses are characterized by a mixed T-helper cell-type 1 (Th1) and Th2 response, the production of cytokines, and the activation of macrophages [13–15]. High levels of specific antibodies are also detected however their exact role remains unclear [8]. Recent evidence indicates that the first weeks following infection, neutrophils play a significant role in the killing of parasites and the development of a protective Th1 immune response [9, 10].

The immunologic mechanisms that underlie the resolution of infection or the progression and systemic dissemination of leishmanias have not been elucidated completely so far. Following infection, T-cell-dependent immune responses are elicited in an integrated fashion. Interleukin 12 (IL12) promotes cell-mediated immunity. Activated CD4 T cells are recruited to cutaneous or visceral sites of infection and direct the local inflammatory responses. CD4 T-cell responses are associated with the interferon (IFN)- γ -induced macrophage activation through participation of cytokines, mainly IL12 and also IL2 and tumor necrosis factor, and the intracellular parasite killing by activated macrophages [8]. IL4 also plays an important role in effective antileishmanial chemotherapy, which appears to be modulated by IFN- γ -production [16]. Deactivation of macrophages, suppression of Th1 responses, and dissemination of leishmanial infection are induced by IL10 [14]. Increased IL10 levels have been detected repeatedly in human VL and are considered crucial in uncontrolled leishmanial infection [13, 14]. Targeting IL10 has been associated with activation of Th1 responses and parasite killing, whereas IL10 suppression constituted a critical step in vaccine-mediated immunotherapy [17]. Most data on cellular immune responses and cytokines have been observed in murine models; similar results have been found in humans.

3. Antimonials

Although pentavalent antimonials (meglumine antimoniate and sodium stibogluconate) are in clinical use for several decades, there are aspects on their mechanism of action that remain unclear. It is generally accepted that pentavalent antimonials (SbV) are the prodrug, and that they

should convert to trivalent antimonials (SbIII) in order to demonstrate their antileishmanial activity [18–20]. Recent evidence indicates that antimonials kill leishmanias by a process of apoptosis [20]. Thiol metabolism is critical in their mechanism of action. Trypanothione is the major thiol in leishmanias. SbIII inhibits trypanothione reductase in vitro, inducing the loss of intracellular thiols and a lethal imbalance in thiol homeostasis, leading to accumulation of reactive oxygen species [20–22]. In order for antimonials to exert their action, an almost intact immune system of the host is required.

Initially antimonials were given at 10 mg/kg for 6–10 days with >90% cure rates, however after the first treatment failures occurred in India two decades ago, higher doses and prolonged schemes (up to 20 mg/kg for 30 days) were introduced gradually and in parallel with the increasing rates of antimony unresponsiveness [23]. However, the dose escalation policy did not prevent further emergence of resistance, but rather selected resistant parasites. During the last decade, antimonial resistance and therapeutic failures reached epidemic dimensions in Bihar, India; nowadays, up to 60% of newly diagnosed VL cases in this area do not respond to antimonials [23]. Inadequate treatment in terms of dosing and duration, and poor compliance promote the widespread antimonial resistance in India. In this country, the high incidence rate of unresponsiveness to antimonials is further sustained by the anthroponotic transmission of leishmanial infection, which increases the chances for the rapid spread of resistant parasites among humans once they emerge [24, 25]. Low rates of antimonial resistance have been reported in Sudan also [26]. Pentavalent antimonials were abandoned in India, however they remain the first treatment choice in most VL-endemic areas in the rest of the world, with efficacy rates exceeding 90%–95% and low case fatality and relapse rates [2–4, 27]. Low cost is their main advantage. Disadvantages include intramuscular administration, prolonged treatment, and transient, but occasionally life-threatening adverse effects, such as cardiac arrhythmias, increased hepatic transaminases, pancreatitis, and pneumonitis [2–4, 23].

While several experimental studies on antimonial resistance have been conducted with parasite mutants selected in vitro using step-wise increasing drug concentrations, resistance mechanisms in field parasites have not been elucidated in details. Mechanisms of in vitro antimonial resistance may differ from mechanisms in field isolates [28]. Similarly, in vitro unresponsiveness does not necessarily translate to clinical resistance [29]. Reduction of drug concentration within the parasite, either by decreasing drug uptake or by increasing efflux/sequestration of the drug, constitutes the primary mechanism of antimonial resistance; other potential resistance mechanisms include inhibition of drug activation, inactivation of active drug, and gene amplification [18, 20, 28, 30–32].

Thiol metabolism possesses a key role in both clinical and laboratory-generated resistance mechanisms. It has been found that elevated intracellular thiol levels and overexpression of trypanothione peroxidase are associated with high levels of SbIII resistance [22, 31, 33]. However, it appears that

more than one step in thiol metabolism should be impaired in order for resistance to emerge, indicating that antimonial resistance is multifactorial. In natural antimonial resistance, the impaired thiol metabolism results in inhibition of SbV activation and decreased uptake of the active form SbIII by amastigotes; these processes are accomplished by the lower expression of the genes γ -glutamylcysteine synthetase, ornithine decarboxylase, and aquaglyceroporin 1, which are involved in the metabolisms of glutathione and trypanthione, and uptake of SbIII, respectively [18, 19, 28]. It has been suggested that decreasing the intracellular thiol concentrations through thiol depletors may increase the leishmanicidal action of drugs and thus reverse parasite resistance [33].

Overexpression of the membrane-bound ATP-binding cassette (ABC) transporters on the surfaces of leishmanias is another mechanism of antimonial resistance. In addition to leishmanias, this transport system modulates the efflux and intracellular accumulation of various drugs and thus resistance in other parasites (e.g., *Plasmodium* spp.) and also in cancer cells. Overexpression of ABC transporters concerns laboratory-derived and in-field resistant parasites [31, 34]. It has been found that, in contrast to infection with Sb-sensitive *L. donovani* isolates, infection with Sb-resistant *L. donovani* isolates upregulates the multidrug resistance-associated protein 1 (MRP1) and the permeability glycoprotein (P-gp) in host cells, thus inhibiting intracellular drug accumulation by decreasing antimony influx [31, 34, 35]. In animal models, inhibition of the proteins MRP1 and P-gp by lovastatin reverses their action on drug accumulation, and allows them to escape a fatal outcome [35]. These results indicate that lovastatin, which can inhibit P-gp and MRP1, might be beneficial for reverting Sb resistance in VL [35]. Flavonoid dimers are also known to reverse antimonial resistance in leishmanias in vitro by inhibiting ABC transporters and increasing the intracellular accumulation of the drug [36]. These findings should be confirmed in animal models.

In conclusion, the overall phenomenon of antimonial resistance is multifactorial. Several mechanisms of resistance to antimonials have been detected among clinical leishmanial isolates. However, the modes of emergence and spread of antimonial resistance in field remain largely unknown. A monoclonal or oligoclonal distribution of resistant parasites would be expected, given the anthroponotic nature of leishmanial transmission in the Indian subcontinent. However, a study of 13 Sb-resistant and 11 Sb-sensitive *L. donovani* clinical isolates collected from Nepal using DNA fingerprinting methods in a population genetics approach revealed a polyclonal distribution of resistant isolates and three major clusters, each containing both sensitive and resistant isolates [37]. Analysis of isolates of paired samples collected from the same patients before treatment and after treatment failure showed primary as well as acquired resistance [37]. Based on these findings, the hypothesis of independent events of emergence of drug resistance appears likely, which suggests a pleiotropic answer of leishmanias to drug pressure, as indicated by the various existing mechanisms of antimonial resistance. High genomic variability among *L. donovani* clinical isolates from India was also found with the use of amplified fragment length polymorphism, suggesting that

various point genetic rearrangements provide the frame for the transition of a parasite from sensitive to resistant [38].

4. Amphotericin-B and Its Lipid Formulations

Conventional amphotericin B has been used as a second-line treatment for VL since the 1960s. This drug exhibits an excellent antileishmanial activity with >90%–95% cure rates in Indian VL cases. Unresponsiveness and relapses occur rarely, except among HIV-infected patients [3, 11, 12]. In this population, secondary episodes of VL are common and are attributed mainly to relapse but also to reinfection [11]. A recent study failed to disclose decreased susceptibility among leishmanias collected from HIV-infected patients during repeated VL episodes (mean follow-up period: 35.6 months; range: 3–137 months), despite repeated courses of amphotericin B; these data indicate that amphotericin B will remain a very useful drug for the treatment or secondary prophylaxis in this group of patients, even after repeated use [11].

The routine scheme of conventional amphotericin B is 1/mg/kg administered on alternate days for a total of 30 days, however, a recent study in India showed 96% cure rates with a dose of 0.75 mg/kg/day for 15 days [6]. Major disadvantages of conventional amphotericin B are its prolonged administration and the frequent adverse effects, such as infusion-related fever and chills, nephrotoxicity, and hypokalemia, which necessitate administration in hospital [6]. Conventional amphotericin B is used extensively in India for cases unresponsive to antimonials or even as a first line drug. However, outside India this drug does not offer any advantage over pentavalent antimonials.

Lipid formulations of amphotericin B improved highly the safety profile of this drug. Lipid formulations are taken selectively by the reticulo-endothelial system, and exhibit a highly localized enhanced antileishmanial action. There are three lipid formulations of amphotericin B: liposomal amphotericin B, amphotericin B lipid complex, and amphotericin B cholesterol dispersion. Currently, liposomal formulations of amphotericin B are the first treatment choice in southern Europe endemic countries as well as in other developed countries, because of their rapid and up to 100% cure rates with 3–5 days schemes, improved convenience for the patient, and reduction of health-care costs [27, 39, 40]. However, in poor countries even short courses of liposomal formulations are unaffordable, and the selection of antileishmanial treatment turns more to a question of cost than of efficacy or toxicity [6, 27]. The use of nanoparticles and microspheres for the delivery of conventional amphotericin B also increased its efficacy against experimental VL [41–43]. Similar results have been reported with the heat-induced reformulation of amphotericin B [44].

5. Miltefosine

Miltefosine (hexadecylphosphocholine) is the first orally administered drug for VL and the latest to enter the market. This agent is associated with high efficacy rates,

including cases unresponsive to antimonials [45, 46]. In a phase IV multicenter trial in India of 1132 adults and children with VL treated with miltefosine, cure rates were 82% per intention-to-treat analysis and 95% per protocol analysis [47]. In this study, 3% of patients developed adverse effects, mainly gastrointestinal toxicity, and elevated hepatic transaminases and creatinine [47]. So far, miltefosine is licenced in India, Germany, and Colombia. The scheme of miltefosine is 100 mg/kg/day for 28 days in adults weighing ≥ 50 kg, 50 mg/kg/day in adults < 50 kg, and 2.5 mg/kg/day in children (maximum dose: 100 mg/day). Major concerns for the wide use of miltefosine include its teratogenic potential and its long half-life (approximately 150 hours) which may facilitate the emergence of resistance. Miltefosine is strictly forbidden in women of child-bearing age who may become pregnant up to two months following drug discontinuation. In India miltefosine is available over the counter, a fact that may expose this drug to misuse and emergence of resistance. Once generated, resistant parasites could spread rapidly, endangering the life span of miltefosine in a country where it is needed most.

The exact antileishmanial mechanism of miltefosine remains largely unknown. The intracellular accumulation of the drug appears to be the critical step for its action. The intracellular accumulation of miltefosine includes the following steps: binding to plasma membrane, internalization in the parasite cell (two proteins, the miltefosine transporter LdMT and its beta subunit LdRos3, are the most significant), and intracellular targeting and metabolism [48]. It has been found that miltefosine induces an apoptosis-like cell death in *L. donovani*, by producing numerous defects [48]. Miltefosine also induces several immunologic and inflammatory effects on macrophages. In animal models, miltefosine does not require T-cell-dependent immune mechanisms in order to act, indicating that this agent can be used in T-cell-deficient patients [12, 48]. Recently, it was found that miltefosine enhanced IFN- γ receptors and thus IFN- γ responsiveness in *L. donovani*-infected macrophages; in the same model, miltefosine induced an IL-12-dependent Th1 response and reversed the Th2 response to Th1 response [49].

Resistance to miltefosine may emerge easily during treatment due to single point mutations [50, 51]. Decrease in drug accumulation is the common denominator in all miltefosine resistant *Leishmania* lines studied to date, and this could be achieved through decreased uptake, increased efflux, faster metabolism, or altered plasma membrane permeability; the first two mechanisms have been already described in models of experimental miltefosine resistance [48, 50]. Two proteins, miltefosine transporter LdMT and its specific beta subunit LdRos3, form part of the miltefosine translocation machinery at the parasite plasma membrane, and are required for miltefosine uptake [48]. Experimental mutations at LdMT or LdRos3 rendered the parasites remarkably less sensitive to miltefosine, and this resistance persisted in vivo; cross-resistance with other antileishmanials was not detected [48, 50]. The overexpression of ABC transporters is another mechanism for acquisition of miltefosine resistance, through reduction of the drug intracellular

accumulation [48, 52]. Recently, a novel flavonoid derivative was designed and it was shown that the use of suboptimal doses in order to overcome the overexpression of LtrMDR1 (a P-glucoprotein-like transporter belonging to the ATP-binding cassette superfamily) was associated with a four-fold increase of intracellular miltefosine accumulation in the resistant *Leishmania* lines [53]. Furthermore, modifications in lipid compositions of membranes and sterol biosynthesis have been detected in miltefosine-resistant *L. donovani* promastigotes [54]. Since membrane fluidity and permeability are influenced by lipid composition, their modification may affect drug-membrane interactions [54]. A case of a healthy patient with VL who relapsed 10 months after successful treatment with miltefosine for 28 days was reported recently [55].

6. Paromomycin

Paromomycin (aminosidine) is an aminoglycoside with antileishmanial activity. In a phase III study of VL in India, this drug was associated with 94.6% cure rates, similar to amphotericin B [56]. Adverse effects were more frequent in the paromomycin-treated group compared with the amphotericin B-treated group (6% versus 2%, resp.); paromomycin-related adverse effects included elevated hepatic transaminases, ototoxicity, and pain at injection-site [56]. Currently, paromomycin is under phase IV clinical trials. Paromomycin is inexpensive but requires daily intramuscular injections for 21 days [6].

Paromomycin inhibits protein synthesis and modifies membrane fluidity and permeability. An in vitro study showed that following a 72-hour exposure to *L. donovani* promastigotes and amastigotes to paromomycin, the mitochondrial potential was decreased, which indicates that mitochondria are the targets of the drug [57]. In laboratory-derived resistant parasites developed through serial-passage increasing-drug concentrations, paromomycin uptake was decreased compared to the wild-type parasite, in association with inhibition of protein synthesis; no cross-resistance with other antimonial agents was detected [57]. Since paromomycin is an aminoglycoside, it is possible that resistance will emerge rapidly if used as monotherapy.

7. Combination Regimens

The rationale for using combination regimens with different resistance mechanisms over monotherapy relies on the expected enhanced efficacy (through synergy or additive activity without drug interaction), shorter treatment duration, less toxicity, improved compliance, reduced likelihood of emergence of resistance, and reduced costs. A combination policy for VL is supported by the fact that antileishmanial drugs belong to different chemical classes. Recent studies have investigated this option. In a retrospective study conducted among Sudanese patients with VL, it was found that combination of sodium stibogluconate and paromomycin administered for 17 days was associated with higher cure and survival rates compared to sodium stibogluconate

monotherapy administered for 30 days (44%–86% lower odds of death in the combination group) [58]. Combinations of miltefosine with amphotericin B, paromomycin or pentavalent antimonials have been evaluated in an in vivo model and revealed that the combinations of miltefosine with amphotericin B or paromomycin were efficacious [59]. These preliminary data justified a recent study in Bihar, India, comparing 5 mg/kg of liposomal amphotericin B administered once (group A; 45 patients), 5 mg/kg of liposomal amphotericin B administered once plus miltefosine for either 10 days (group B; 46 patients) or 14 days (group C; 45 patients), 3.75 mg/kg of liposomal amphotericin B administered once plus miltefosine for 14 days (group D; 45 patients), and 5 mg/kg of liposomal amphotericin B administered once followed by miltefosine for 7 days (group E; 45 patients); in this study, similar final cure rates (91%–98%) were noted in all treatment groups [60]. These data indicate that a single dose of liposomal amphotericin B followed by 7–14 days of miltefosine is active against Indian VL [60]. In this study, all patients were treated in an outpatient setting. Large, randomized-controlled trials are required before adaptation of combination regimens.

Several combination regimens with investigational agents have been tested in vitro and in animal models [61]. The plant-derived immunostimulant agent picroliv has no antileishmanial activity, however when administered with half-dose miltefosine increases significantly the activity of the later [62]. The combination of verapamil (a calcium channel blocker) and diperoxovanadate (a potent antileishmanial agent) with sodium antimony gluconate reversed the in vitro antimonial resistance among clinical *L. donovani* isolates [63, 64]. Diperoxovanadate also demonstrated immunomodulating effects by increasing IFN and decreasing IL-10 [64]. These combinations deserve further testing in VL cases unresponsive to antimonials.

8. Strategies to Preserve the Efficacy of Currently Available Antileishmanials

In addition to intrinsic pharmacologic features, there is a number of human parameters that may favor the emergence and spread of leishmanial resistance. These include poor compliance, expensive treatment, availability of antileishmanial drugs over the counter, and limited access to health-care facilities for early diagnosis and treatment. Given the current situation of the widespread emergence of antimonial resistance in India, there is growing concern to preserve the efficacy of novel antileishmanials. Such a strategy should focus on the following axons.

- (1) Treatment of VL should be based on guidelines for prompt diagnosis, selection of first-line drugs, management of cases unresponsive to antimonials, and HIV-coinfected cases. A recent study of Indian VL cases revealed that a strategy of treatment with antimonials (first choice) or amphotericin B (second choice), based on culture and susceptibility results, compared with an empiric treatment strategy, was associated with higher cure rates (86.21% versus

35.71%), and reduced expenses, duration of hospitalization, and likely period of spread of parasites in the community [65].

- (2) In order to enhance compliance, directly observed therapy for antileishmanials should be implemented, like in tuberculosis control programs.
- (3) VL cases should be treated early in order to avoid further transmission of resistant parasites in the community.
- (4) Distribution and clinical response of antileishmanials should be monitored.
- (5) Antileishmanial treatment should be provided free-of-charge through the health-care system.
- (6) The emergence and spread of antileishmanial resistance should be monitored.
- (7) The efficacy and safety of combination regimens should be evaluated in large trials.

9. Conclusions

The control of VL globally is challenged by the widespread emergence of antimonial resistance in India. The last decade new formulations of conventional antileishmanial drugs as well as new agents became available. The wide use of the oral agent miltefosine was hampered by the potential for teratogenicity and emergence of resistance. Combination regimens should be evaluated in large trials. The last years several mechanisms of in field antileishmanial resistance were identified. Understanding their molecular and biochemical characteristics will lead the design of new drugs and also the molecular surveillance of resistance. In order not to jeopardize the life span of available antileishmanials, their delivery, clinical response, and resistance should be monitored. Overall the development of antileishmanials has been generally slow; new drugs are needed.

Author's Statement

The findings and opinions in this review are those of the author and do not necessarily represent those of the Hellenic Center for Disease Control and Prevention.

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Research Article

A Multifactorial Mechanism in the Superior Antimalarial Activity of α -C-GalCer

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We have previously shown that the C-glycoside analog of α -galactosylceramide (α -GalCer), α -C-GalCer, displays a superior inhibitory activity against the liver stages of the rodent malaria parasite *Plasmodium yoelii* than its parental glycolipid, α -GalCer. In this study, we demonstrate that NK cells, as well as IL-12, are a key contributor for the superior activity displayed by α -C-GalCer. Surprisingly, the diminished production of Th2 cytokines, including IL-4, by α -C-GalCer has no effect on its superior therapeutic activity relative to α -GalCer. Finally, we show that the in vivo administration of α -C-GalCer induces prolonged maturation of dendritic cells (DCs), as well as an enhanced proliferative response of mouse invariant V α 14 (V α 14i) NKT cells, both of which may also contribute to some degree to the superior activity of α -C-GalCer in vivo.

1. Introduction

Malaria remains one of the most serious and prevalent infectious diseases in the world. Approximately 500 million people acquire the disease annually, which leads to 2-3 million deaths per year. Malaria is caused by species of *Plasmodium*, an apicomplexan parasite transmitted in nature by mosquito vectors of the genus *Anopheles*. The life cycle of *Plasmodium* is the same no matter what the species, consisting of both sexual stages that occur in the mosquito vector and asexual stages that occur in a vertebrate host. The asexual stages can be divided into the pre-erythrocytic stages, which consist primarily of developmental events occurring within infected hepatocytes (the so-called liver stages), and the erythrocytic, or blood, stages, which occur in red blood cells, and which result in the signs and symptoms of malaria [1].

Numerous studies have shown that T cells, including CD8+T cells, CD4+T cells, $\gamma\delta$ -T cells, and natural killer (NKT) cells play a key role in the immune response to the pre-erythrocytic stages of *Plasmodium* [2]. Of recent

interest is the role of NKT cells, a unique population of lymphocytes that coexpress markers of NK cells along with a semi-invariant TCR. In mice, the TCR of most NKT cells consists of an invariant V α 14J α 18 α chain paired with a variable set of β chains consisting primarily of V β 8.2, V β 7, or V β 2. This invariant V α 14 TCR (V α 14i) enables NKT cells to recognize the MHC class I-like molecule CD1d, which is capable of presenting hydrophobic molecules such as lipids and hydrophobic peptides to V α 14i NKT cells [3, 4].

To date, only a few compounds have been shown to bind CD1d and stimulate V α 14i NKT cells. Of these, the prototype molecule is α -galactosylceramide (α -GalCer), a glycolipid originally derived from a marine sponge extract. Previously, using a murine malaria model, we showed that α -GalCer is able to directly mediate protection to the pre-erythrocytic stages of malaria in a manner dependent on iNKT cells, CD1d, and IFN- γ [5]. More recently, we demonstrated that a structurally similar C-glycoside analog of α -GalCer, α -C-galactosylceramide (α -C-GalCer), is also capable of mediating protection to the pre-erythrocytic stages of malaria in a manner dependent on V α 14i NKT

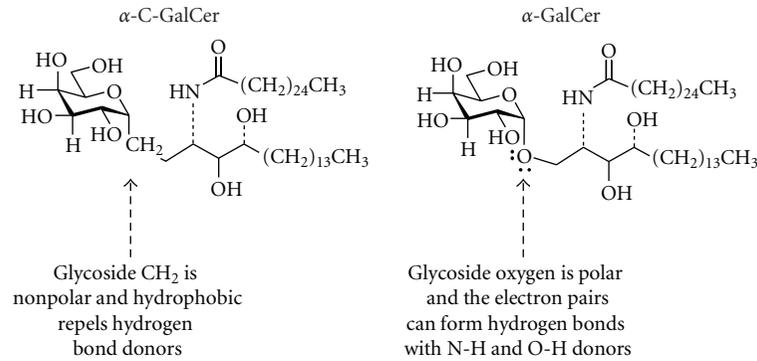


FIGURE 1: Structural comparison between α -C-GalCer and α -GalCer showing the main differences between the two molecules. Glycoside CH₂ is nonpolar and hydrophobic; it repels hydrogen bond donors. In contrast, glycoside oxygen is polar, and the electron pairs can form hydrogen bonds with N-H or O-H donors.

cells, CD1d, and IFN- γ [6]. Moreover, we showed that α -C-GalCer, which differs from α -GalCer only in the identity of the chemical group involved in the glycosidic linkage of the galactose and ceramide moieties of the molecule (Figure 1), exhibits a much more potent and longer lasting antimalarial effect than α -GalCer, an effect which appears to stem from prolonged downstream IFN- γ production by NK cells, and which requires the presence of IL-12 [6]. In a follow-up study, we showed that CD8 α ⁺ dendritic cells (DCs) are the cells responsible for producing this IL-12 in response to glycolipid injection, and that depletion of these cells results in attenuated downstream IFN- γ production by NK cells [7]. Our findings were corroborated by a more recent study by Fujii et al., which demonstrated that α -C-GalCer induces higher degree of DC activation than α -GalCer [8].

In the present study, we further examine the physiologic mechanism underlying the superior antimalarial activity of α -C-GalCer. We find that the mechanism involves IL-12, NK cells and DCs, but not the Th2 cytokines IL-4 and IL-10, nor the TCR β used by the V α 14i NKT cells.

2. Materials and Methods

2.1. Chemicals. α -galactosylceramide [(2'S,3'S,4'R)-1'-O-(α -D-galactopyranosyl)-2-(N-hexacosanoylamino)-1,3,4-octadecanetriol] was synthesized by Kirin Brewery (Gumma, Japan). The stock solution was dissolved in a 0.5% polysorbate-20 (Nikko Chemical, Tokyo), 0.9% NaCl solution at a concentration of 200 μ g/mL, and diluted in PBS to the desired concentration just prior to injection into mice.

α -C-galactosylceramide [(2'S,3'S,4'R)-1'-CH₂-(α -D-galactopyranosyl)-2-(N-hexacosanoylamino)-3,4-nonadecanediol] was synthesized as described previously [9]. The stock solution, originally dissolved to a concentration of 1 mg/mL in 100% DMSO, was diluted to a working concentration of 200 μ g/mL in a 0.5% polysorbate-20 (Nikko Chemical, Tokyo), 0.9% NaCl solution. Before injection into mice, the working solution was further diluted to the desired concentration in PBS.

2.2. Mice. Six- to eight-week-old female BALB/c and C57BL/6 mice were purchased from the National Cancer Institute

(Bethesda, MD). IL-12p40-deficient mice of BALB/c and C57BL/6 backgrounds, IL-4-deficient mice of BALB/c background, and IL-10-deficient mice of C57BL/6 background were all purchased from the Jackson Laboratory (Bar Harbor, ME).

2.3. Injections. For glycolipid treatments, mice were injected intraperitoneally with 1 μ g of either α -GalCer or α -C-GalCer. In some experiments, treated and untreated mice were challenged with live *P. yoelii* sporozoites 3 days later.

2.4. Parasites and Their Use for Challenge. *P. yoelii* (17XNL strain) was maintained by alternate cyclic passages in *Anopheles stephensi* mosquitoes and Swiss Webster mice. Sporozoites obtained from dissected salivary glands of infected mosquitoes 2 weeks after their infective blood meal were used for challenge of the mice. Challenge of mice to determine the development of liver-stage malaria infection was performed by an intravenous injection of 10,000 viable sporozoites into the tail vein. The outcome of the challenge was determined 40–42 hours later by measuring the parasite burden in the livers of the mice using a quantitative real-time RT-PCR method [10].

2.5. Culture Medium. DMEM media supplemented with 10% heat-inactivated fetal calf serum (FCS) (DMEM-10) and RPMI 1640 media supplemented with 10% heat-inactivated FCS (RPMI-10) were used in all experiments involving cell suspensions. In addition to FCS, the media were supplemented with 100 IU/mL of penicillin/streptomycin, 10 μ g/mL of gentamicin, 10 mM HEPES buffer, 50 μ M of β -mercaptoethanol, 2 mM of L-glutamine, 1mM of sodium pyruvate, and 100 μ M of nonessential amino acids. All reagents were obtained from GIBCO (Carlsbad, CA) except FCS, which was obtained from HyClone (Logan, UT).

2.6. Determination of Malaria Liver Stage Development. The degree of liver stage development in challenged mice was determined by quantifying the amount of *P. yoelii*-specific 18S ribosomal RNA (rRNA) molecules in the livers of the

mice by way of a recently developed real-time RT-PCR technique [10]. 40–42 hours after sporozoite challenge, livers from challenged mice were dissected and homogenized in 4 mL/liver of denaturing solution (4 M guanidium thiocyanate, 25 mM sodium citrate pH 7.0, 0.5% sarcosyl, 0.1 M β -mercaptoethanol) using a PowerGen 125 biohomogenizer (Fisher, Pittsburgh, PA). RNA was then purified from 600 μ L aliquots of the homogenates using the method developed by Chomczynski and Sacchi [11]. 2 μ g samples of the RNA were then reverse-transcribed, and aliquots of the resulting complementary DNA (cDNA) (133 ng) were used for real-time PCR amplification of *P. yoelii* 18S rRNA sequences. This amplification was performed in a GeneAmp 5700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). For this purpose, we used primers 5'-GGGGATTGGTTTTGACGTTTTTGCG-3' (54 nM) and 5'-AAGCATTAATAAAGCGAATACATCCTTAT-3' (60 nM) together with the double-stranded DNA (dsDNA)-specific dye SYBR Green I incorporated into the PCR reaction buffer (PE Biosystems, Foster City, CA) in order to detect the PCR product generated. The temperature profile of the reaction was 95°C for 10 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute.

2.7. Isolation of Splenocytes and Intrahepatic Lymphocytes from Mice. Mouse splenocytes were prepared by gently grinding spleens between the frosted ends of two microscope slides in a petri dish containing 10 mL of DMEM-10 or RPMI-10 medium. The resulting cell suspension was filtered through nylon mesh, pelleted, and incubated with 5 mL of ACK lysis buffer for 5 minutes at room temperature to remove red blood cells. The cells were then washed three times with DMEM-10 or RPMI-10 medium, filtered once more through nylon mesh, and counted using a hemocytometer and trypan blue (GIBCO, Carlsbad, CA).

Intrahepatic lymphocytes from mice were prepared by grinding livers in a steel mesh screen using a 3 mL syringe pestle, and filtering the resulting homogenate into a 50 mL tube using 45 mL of Hank's Balanced Salt Solution (HBSS) (GIBCO, Carlsbad, CA). After centrifugation of the homogenate, the pellet was resuspended in 45 mL of a 35% Percoll (Amersham Biosciences, Piscataway, NJ) solution (29.1 mL HBSS, 14.2 mL Percoll, 1.6 mL 10X PBS, 200 U heparin), and centrifuged at 500 g for 10 minutes at 20 degrees Celsius. The resulting high-density liver lymphocyte pellet was resuspended in 5 mL of ACK lysis buffer for 5 minutes at room temperature to remove red blood cells, and then washed three times with DMEM-10 or RPMI-10 medium. After the final wash, the cells were filtered through nylon mesh, and counted using a hemocytometer and trypan blue.

2.8. Flow Cytometry Experiments. In order to measure the degree of DC maturation induced by α -GalCer and α -C-GalCer, freshly isolated splenocytes from BALB/c mice were first incubated for 15 minutes at 4°C with unlabeled anti-mouse Fc γ III/II receptor mAb clone, 2.4G2 (PharMingen, San Diego, CA) in staining buffer (PBS containing 1%

FBS and 0.1%NaN₃) to block Fc receptors. Next, the cells were surface stained with PE-conjugated anti-CD11c mAb clone, HL3, and FITC-conjugated anti-CD86 mAb clone, GL1, FITC-conjugated anti-CD40 mAb clone, HM40-3, or syngeneic MHC-class II-specific FITC-conjugated anti-IA^d mAb clone, AMS-32.1 (all PharMingen, San Diego, CA) in staining buffer for 30 minutes at 4°C. After two washes in staining buffer, the cells were then analyzed using a FACSCalibur instrument (Becton Dickson, San Diego, CA) with CELLQuest software (Becton Dickson, San Diego, CA).

Next, in order to specifically detect V α 14i NKT cells, we first loaded mouse CD1d-IgG₁ dimer X molecules (PharMingen, Sand Diego, CA) with a 10–20-fold molar excess of α -GalCer overnight in 1X PBS at room temperature. After preparation of splenocytes and liver lymphocytes, we incubated the cells with the loaded dimers for 60 minutes in staining buffer at 4 degrees Celsius (after Fc receptor block), using 4 μ g of loaded dimer for every 1×10^6 cells stained. Next, after washing the cells once with staining buffer, we surface stained the cells with FITC-labeled anti-CD3 ϵ mAb clone, 145-2C11, and PE-labeled antimouse IgG₁ clone, A85-1 (both PharMingen, San Diego, CA) for 30 minutes in staining buffer at 4 degrees Celsius. Finally, after washing the stained cells twice with staining buffer, we analyzed them by FACS as detailed above.

Finally, in order to measure the TCR β usage of V α 14i NKT cells before and after in vivo α -GalCer or α -C-GalCer stimulation, we first incubated splenocytes and liver lymphocytes with unlabeled antimouse Fc γ III/II receptor mAb clone, 2.4G2 (PharMingen, San Diego, CA) and unlabeled streptavidin (Molecular Probes, Eugene, OR) for 15 minutes in staining buffer at 4 degrees Celsius in order to block Fc receptors and surface biotin molecules. After washing the cells twice with staining buffer, we incubated the cells with α -GalCer-loaded dimers in the manner indicated above. After dimer incubation, we surface stained the cells with FITC-labeled anti-CD3 ϵ mAb clone, 145-2C11, and either biotin-labeled anti-V β 8.1/8.2 clone, MR5-2, biotin-labeled anti-V β 7 clone, TR310, or biotin-labeled anti-V β 2, clone B20.6 (all PharMingen, San Diego, CA) for 30 minutes in staining buffer at 4 degrees Celsius, followed by two washes with staining buffer. We then stained the cells with PE-labeled antimouse IgG₁ clone A85-1 and APC-labeled streptavidin (both from PharMingen, San Diego, CA) for 30 minutes in staining buffer at 4 degrees Celsius. Finally, after washing the cells twice with staining buffer, we analyzed them by FACS as detailed above.

3. Results

3.1. IL-12 Is a Key Factor in the Antimalarial Activity of Both α -C-GalCer and α -GalCer. In a previous study, we showed that α -C-GalCer's ability to stimulate prolonged IFN- γ production is abrogated in the absence of IL-12 [6]. Since IFN- γ is required for α -C-GalCer's anti-malaria effect, we wanted to see if its superior therapeutic activity against malaria liver stages was abrogated in the absence of IL-12 as well. To address this issue, we first treated both WT and IL-12-deficient mice with equal doses of either glycolipid three

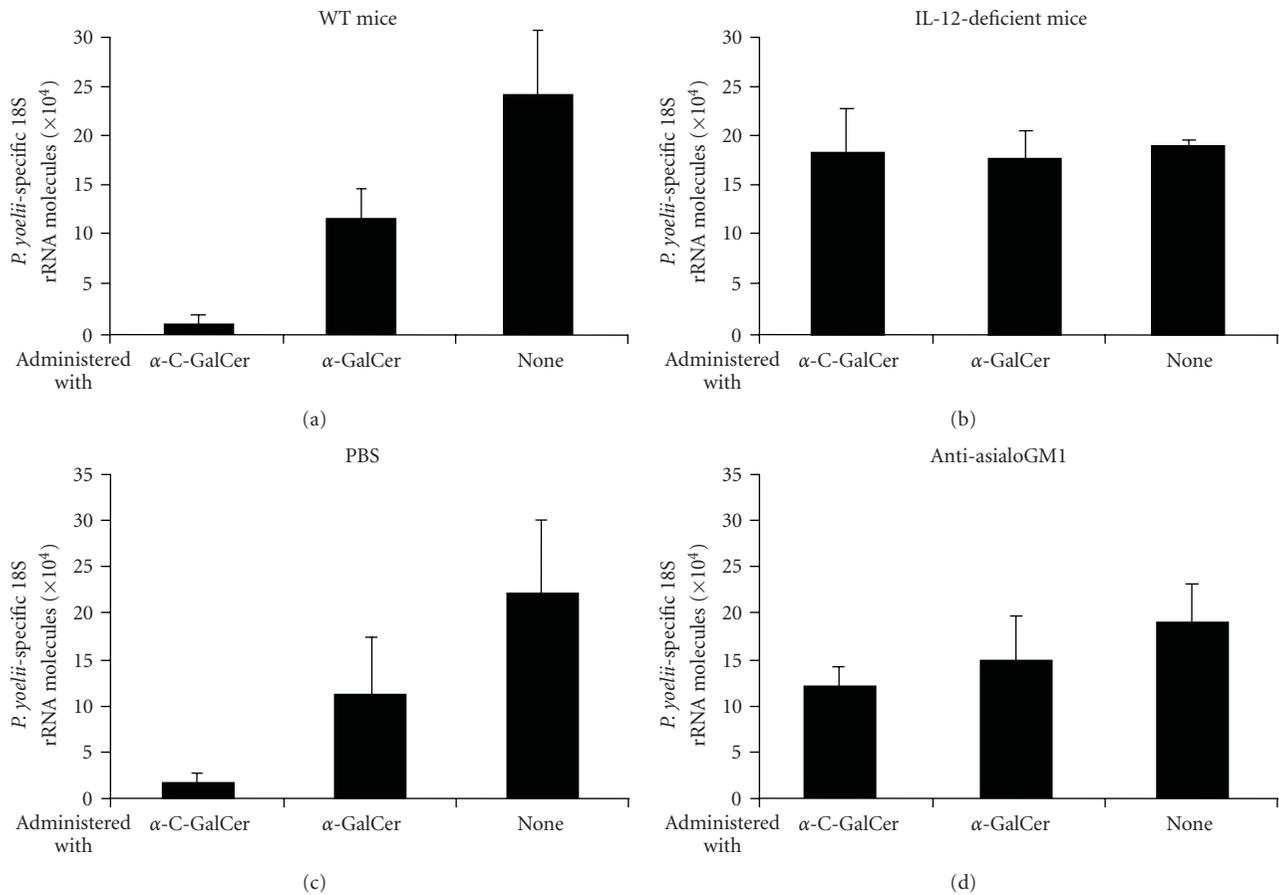


FIGURE 2: α -C-GalCer's superior therapeutic activity against malaria liver stages requires IL-12 and NK cells. (a) Groups of 5 WT or IL-12-deficient BALB/c mice were treated intraperitoneally (i.p.) with $1 \mu\text{g}$ of either α -C-GalCer or α -GalCer or with nothing 3 days before challenge intravenously with live *P. yoelii* sporozoites, and then checked for malaria liver stage development. The results are expressed as the average \pm SD of 5 mice. (b) Groups of 5 WT C57BL/6 mice were treated i.p. with PBS or anti-asialoGM1 antibody 1 day prior to i.p. injection with $1 \mu\text{g}$ of α -C-GalCer or α -GalCer, or with nothing. Three days later the mice were challenged with live *P. yoelii* sporozoites, and then checked for malaria liver stage development. The results are expressed as the average \pm SD of 5 mice. The data shown come from one of three experiments with similar results.

days before challenge with sporozoites, and then measured malaria liver stage development. As expected, in WT mice α -C-GalCer suppressed liver stage development to a much greater degree than α -GalCer; however, in IL-12-deficient mice the anti-malaria activity of both glycolipids was totally abolished (Figure 2(a)). Thus, IL-12 is a key factor not only driving α -C-GalCer's superior antimalarial effect, but also mediating the antiplasmodial effect of both glycolipids.

3.2. α -C-GalCer's Enhanced Antimalarial Response Requires the Presence of NK Cells. Our finding that α -C-GalCer's superior antimalarial activity requires IL-12 (Figure 2(a)), which is required for optimal IFN- γ production by NK cells following glycolipid administration [6], strongly suggests that NK cells are also required for α -C-GalCer's superior antimalarial activity. To assess the role of NK cells in α -GalCer- and α -C-GalCer-mediated protection against malaria, we pretreated mice with anti-asialoGM1 antibody, which is known to selectively deplete NK cells [12], or with PBS, and one day later treated the mice with equal

doses of α -GalCer or α -C-GalCer, or with nothing. Three days after glycolipid treatment, we challenged the mice with sporozoites and then measured liver stage development as before. We found that in nondepleted PBS-treated control mice, α -C-GalCer exhibited better antimalarial activity than did α -GalCer, as expected. In contrast, we observed an abrogation of α -C-GalCer's superior antimalarial activity in mice depleted of NK cells with anti-asialoGM1 antibody (Figure 2(b)). This result strongly suggests that α -C-GalCer's superior antimalarial effect stems from its ability to stimulate enhanced IL-12 production, which then triggers NK cells to produce more IFN- γ needed to suppress malarial liver stage development.

3.3. α -C-GalCer's Enhanced Antimalarial Response Does Not Involve the Th2 Cytokines IL-4 and IL-10. In an earlier study, we and others showed that mice injected with α -C-GalCer produce a far less IL-4 than did those injected with α -GalCer [6, 8]. Given that IL-4 is a Th2 cytokine with known inhibitory activity against Th1 cytokines [13], we were

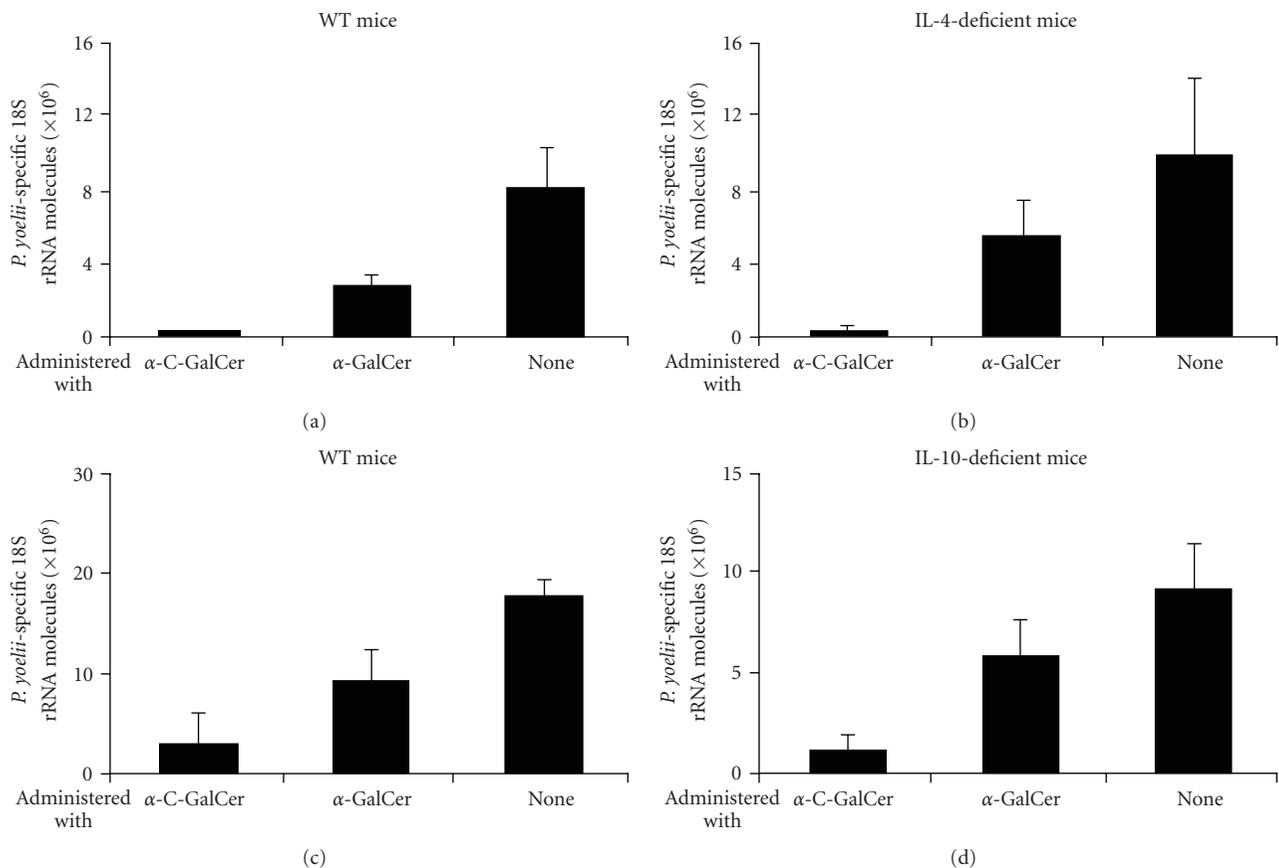


FIGURE 3: α -C-GalCer's superior antimalarial activity does not involve IL-4 or IL-10. (a) Groups of 5 WT or IL-4-deficient BALB/c mice, or (b) groups of 5 WT or IL-10-deficient C57BL/6 mice, were treated i.p. with $1 \mu\text{g}$ of either α -C-GalCer or α -GalCer or with nothing 3 days before challenge with live *P. yoelii* sporozoites, and then checked for malaria liver stage development. The results are expressed as the average \pm SD of 5 mice. The data shown come from one of three experiments with similar results.

curious to see if α -GalCer's enhanced therapeutic activity against diseases ameliorated by Th1 cytokines stems from its ability to stimulate lower amounts of IL-4. To address this issue, we injected both WT mice and mice deficient in IL-4 with equal doses of α -GalCer or α -C-GalCer, or with nothing, and 3 days later challenged them with live *P. yoelii* sporozoites. Forty-two hours after the challenge, we obtained livers from all the mice and determined the degree of malaria liver stages that developed by way of quantitative real-time RT-PCR. We found that the ability of α -C-GalCer to better inhibit liver stages was the same in both IL-4-deficient mice and WT mice (Figure 3(a)). This result indicates that α -C-GalCer's superior antimalarial activity does not involve its reduced IL-4 production.

Another Th2 cytokine known to be produced following α -GalCer injection in mice is IL-10 [14, 15]. One of the important effects of IL-10 is the direct inhibition of IL-12 production by APCs, and the consequent downregulation of Th1-type responses [13, 16, 17]. Given IL-10's production consequent to glycolipid injection in mice, and its inhibitory affect on Th1-type responses, we wanted to see if IL-10 plays a role in α -C-GalCer's enhanced therapeutic activity against

diseases ameliorated by Th1 cytokines. To address this issue, we injected IL-10-deficient mice and WT mice with equal doses of α -GalCer or α -C-GalCer, or with nothing, and 3 days later challenged them with live sporozoites for a liver stage protection experiment. As with IL-4 deficient mice, we found that IL-10-deficient mice injected with α -C-GalCer inhibited malarial liver stages better than those injected with α -GalCer—the same as WT mice (Figure 3(b)). Thus, it appears that IL-10 also plays no role in α -C-GalCer's enhanced antimalarial activity relative to α -GalCer.

3.4. α -C-GalCer Induces a Prolonged In Vivo Maturation of DCs Compared to α -GalCer. In a previous study, we showed that CD8 α ⁺ DCs are the cells responsible for producing IL-12 in response to α -GalCer and α -C-GalCer injection, and that depletion of these cells results in attenuated downstream IFN- γ production by NK cells [7]. Given the importance of DCs in the in vivo physiological response to α -GalCer and α -C-GalCer, we wanted to see how these two glycolipids affect these cells in vivo in WT mice. To address this issue we looked at the ability of α -GalCer and α -C-GalCer to induce maturation of DCs by following the expression patterns of

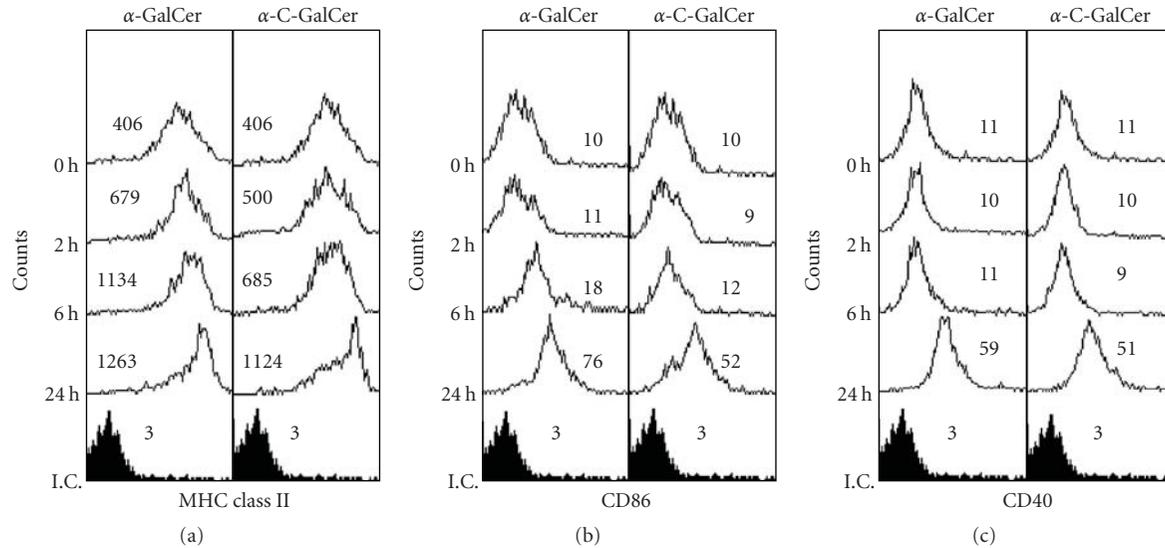


FIGURE 4: α -C-GalCer induces a prolonged in vivo maturation of DCs compared to α -GalCer. Groups of 3 WT mice were injected i.p. with 1 μ g α -GalCer or α -C-GalCer, or with nothing, and 2, 6, or 24 hours later splenocytes were collected and subjected to FACS analysis. CD11c+ cells were gated and analyzed for their levels of (a) MHC class II, (b) CD86, and (c) CD40 at the different time points after glycolipid administration. Also shown are isotype control stainings obtained from the maximally activated, 24-hour, α -GalCer-treated splenocyte populations, gated on CD11c+ cells. The numbers next to each histogram tracing represent the mean fluorescence intensity for that tracing. The data shown come from one of four experiments with similar results.

various surface markers known to be upregulated during DC maturation, namely, MHC class II, CD86, and CD40. More specifically, we injected WT mice with α -GalCer, α -C-GalCer, or nothing, and 2, 6, and 24 hours later obtained splenocytes for FACS analysis of cells coexpressing CD11c and MHC class II, CD86, or CD40.

We found that the first marker to show upregulation on CD11c+ DCs after injection of either glycolipid was MHC class II. α -GalCer-treated mice showed increased expression of this marker as soon as 2 hours after injection, while α -C-GalCer-treated mice showed increased expression 6 hours after injection (Figure 4(a)). By 24 hours posttreatment, we saw the highest MHC class II expression on CD11c+ DCs from mice injected with either α -GalCer or α -C-GalCer, with α -GalCer-treated mice expressing slightly more marker than α -C-GalCer-treated mice (Figure 4(a)).

The next marker to show upregulation after glycolipid injection was CD86. In α -GalCer-treated mice, CD86 expression first started showing an increase at 6 hours postinjection; whereas in α -C-GalCer-treated mice this marker did not show increased expression until 24 hours postinjection (Figure 4(b)). Again, as with MHC class II, we observed the highest expression of CD86 24 hours after injection of α -GalCer or α -C-GalCer, with α -GalCer-treated mice expressing more marker than α -C-GalCer-treated mice at this time point (Figure 4(b)).

Finally, the last DC marker to show upregulation following glycolipid injection was CD40. In both α -GalCer- and α -C-GalCer-injected mice, increased expression of this marker was only observed 24 hours after treatment. At 2 and 6 hours postglycolipid injection, CD40 expression on DCs was the same as untreated controls (Figure 4(c)). Interestingly,

at 24 hours postinjection the expression levels of CD40 on DCs from both α -GalCer- and α -C-GalCer-treated mice were more or less the same, in contrast to MHC class II and CD86 (Figure 4(c)). Overall, the upregulation data indicates that α -GalCer induces a faster maturation of CD11c+ DCs than does α -C-GalCer, which appears to induce a more prolonged maturation of this cell type.

3.5. α -C-GalCer Induces a Slower and Shorter In Vivo Downregulation of V α 14i TCRs and a Greater In Vivo Proliferation of V α 14i NKT Cells Compared to α -GalCer. A number of recent studies have shown that V α 14i NKT cells proliferate in vivo following injection of α -GalCer [18–21]. This proliferation is accompanied by early downregulation of V α 14i TCRs and NK1.1 on the surface of NKT cells, followed by reappearance of these markers two days later. A finding from earlier studies that α -C-GalCer stimulates less cytokine production by NKT cells than α -GalCer [6, 8] suggests that α -C-GalCer is a weaker NKT cell stimulus than α -GalCer. To see if α -C-GalCer also stimulates poorer proliferation of NKT cells when compared to α -GalCer, we injected WT mice with equal doses of either glycolipid, and 5, 24, 48, 72, 120, and 168 hours later obtained splenocytes and liver lymphocytes for FACS analysis of V α 14i NKT cells. To detect V α 14i NKT cells we made use of recently developed mouse CD1d-IgG1 dimers, which, when loaded with α -GalCer, stain V α 14i NKT cells [22].

We found that V α 14i NKT cells from α -GalCer-treated mice rapidly downregulated their TCRs, becoming undetectable in both spleen and liver by 5 hours, and remaining so at 24 hours (Figure 5(a)). Not until 48 hours did V α 14i NKT cells from α -GalCer-treated mice become detectable again in

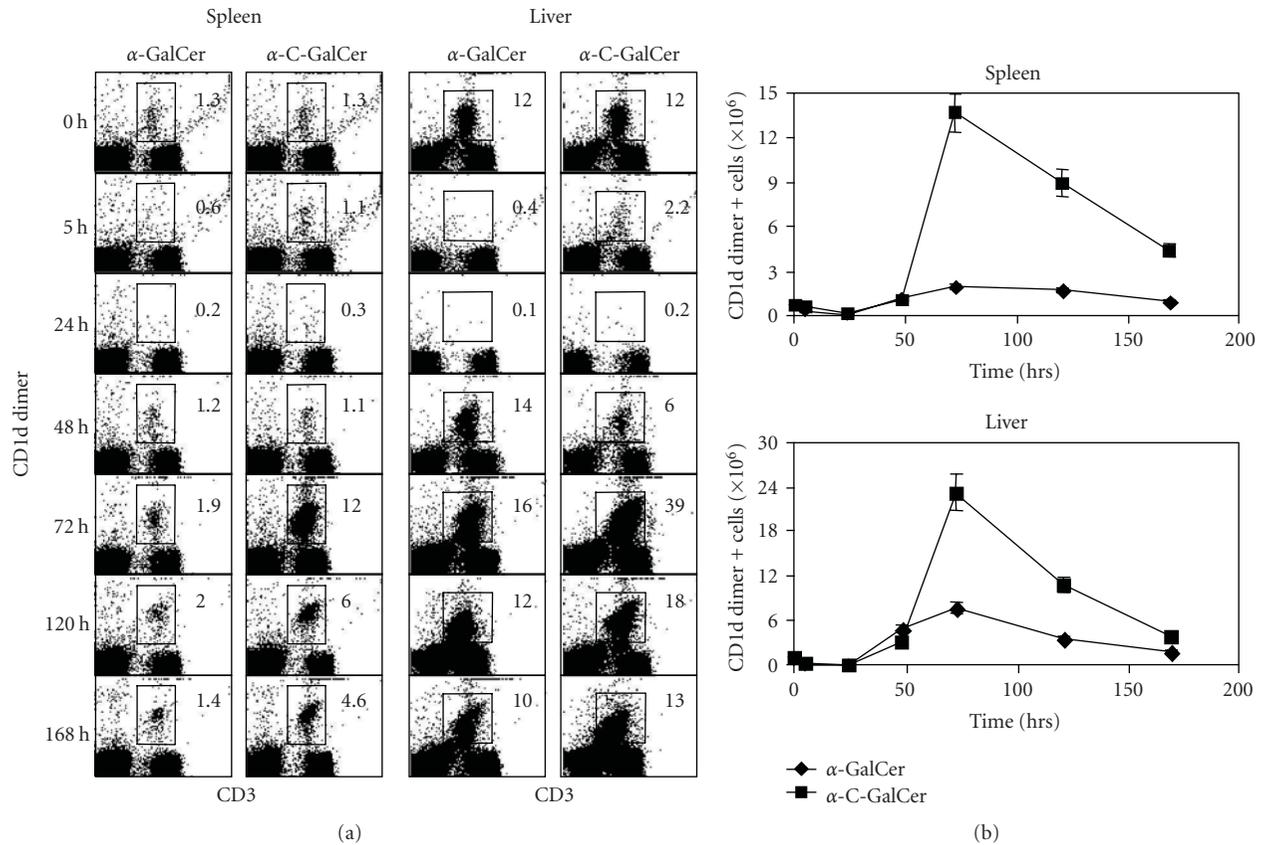


FIGURE 5: α -C-GalCer induces a slower and shorter in vivo downregulation of V α 14i TCRs and a greater in vivo proliferation of V α 14i NKT cells compared to α -GalCer. (a) Groups of 2 WT C57BL/6 mice were injected i.p. with 1 μ g of α -GalCer or α -C-GalCer, and 0, 5, 24, 48, 72, 120, and 168 hours later splenocytes and liver lymphocytes were isolated and stained with α -GalCer-loaded mouse CD1d-IgG₁ dimers to assess the level of V α 14i NKT cells present. The numbers shown in the individual panels represent the percentage of V α 14i NKT cells present in the gated lymphocyte population. The data shown are representative of three independent experiments. (b) The average absolute numbers of V α 14i NKT cells in the spleens and livers of α -GalCer- and α -C-GalCer-treated mice at the various time points were calculated according to the following equation: (percentage of V α 14i NKT cells in the gated lymphocyte population) * (percentage of total isolated cells represented by the gated lymphocyte population) * (total number of isolated cells). The data shown are the average values calculated from three independent experiments \pm SE.

both spleen and liver. At this time point the cells returned to levels comparable to that detected prior to injection, and remained so at 72, 120, and 168 hours postinjection (Figure 5(a)).

In contrast to α -GalCer, V α 14i NKT cells from mice treated with α -C-GalCer exhibited a slower and shorter TCR downregulation, with small percentages of cells still present at 5 hours in both spleen and liver (Figure 5(a)). At 24 hours the cells were almost completely undetectable, but started reappearing at 48 hours, although at lower levels than that detected prior to injection (Figure 5(a)). Strikingly, at 72 hours the levels of V α 14i NKT cells in the spleens and livers of α -C-GalCer-treated mice were greatly increased over that observed prior to injection. In the spleen, the percentage of cells was approximately 10 times higher than that observed at the start; similarly, in the liver the percentage was about 3 times higher (Figure 5(a)). At 120 hours, the levels of V α 14i NKT cells started to come down in both the spleen and liver, but still remained higher than that observed prior to

injection. By 168 hours, the percentages continued to come down, approaching baseline in the liver, but remaining high in the spleen (Figure 5(a)).

Using the percentages of V α 14i NKT cells we were able to follow the overall proliferative response of the cells stimulated by the glycolipids. We found that the overall pattern of V α 14i NKT cell expansion was the same for both glycolipids, with peak cell numbers occurring 72 hours postinjection followed by a return towards baseline (Figure 5(b)). Surprisingly, we found that α -C-GalCer stimulated a far greater expansion of V α 14i NKT cells in both spleen and liver than did α -GalCer. The difference was most striking at 72 hours when the numbers of V α 14i NKT cells in the spleens and livers of α -C-GalCer-treated mice were approximately 5 times higher than those in α -GalCer-treated mice (Figure 5(b)). Overall, this data indicates that despite its poor ability to stimulate cytokine production by NKT cells [6], α -C-GalCer is a better in vivo stimulus for V α 14i NKT cell proliferation than α -GalCer.

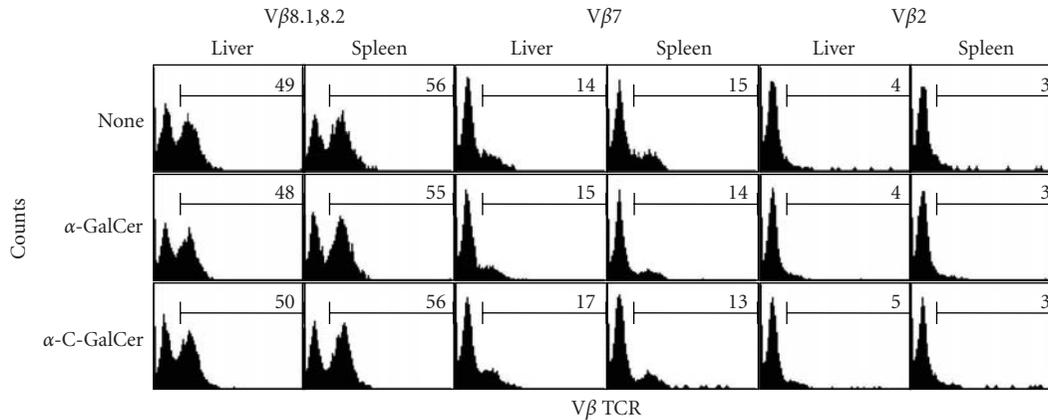


FIGURE 6: TCR β usage does not affect in vivo V α 14i NKT cell activation stimulated by α -GalCer or α -C-GalCer. Groups of 2 WT C57BL/6 were injected i.p. with 1 μ g of α -GalCer or α -C-GalCer, or with nothing, and 72 hours later splenocytes and hepatic lymphocytes were isolated and stained for V α 14i NKT cells using α -GalCer-loaded mouse CD1d-IgG1 dimers, as well as for V β 8.1/8.2, V β 7, and V β 2. V α 14i NKT cells were gated, and FACS analyzed for the three different V β gene segments. The numbers shown represent the percentage of cells expressing the indicated V β segment. The data shown comes from one of two independent experiments with similar results.

3.6. TCR β Usage Does Not Affect In Vivo V α 14i NKT Cell Activation Stimulated by α -GalCer or α -C-GalCer. Two recent studies show that TCR β usage by V α 14i NKT cells affects the avidity of V α 14i TCRs for CD1d-glycolipid complexes [22, 23]. In particular, they indicate that TCR β chains encoded by the V β 8.2 gene segment confer higher avidity to V α 14i TCRs than V β 7 or V β 2, and that glycolipid ligands preferentially stimulate these higher avidity cells. Despite these findings, our data showing a complete disappearance of V α 14i NKT cells by 24 hours post- α -GalCer or α -C-GalCer injection (Figure 5(a)) suggests that in vivo both glycolipids stimulate all V α 14i NKT cells regardless of their TCR β usage. To confirm this suspicion, we treated WT mice with α -GalCer, α -C-GalCer, or with nothing, and 72 hours later obtained splenocytes and liver lymphocytes for staining with CD1d-IgG1 dimer loaded with α -GalCer. In addition to dimer staining, we also stained the cells for V β 8.1/8.2, V β 7, and V β 2 to see if the distribution V α 14i NKT cells changes after glycolipid-mediated stimulation. More specifically, if α -GalCer or α -C-GalCer preferentially stimulates the higher avidity V β 8.2-containing NKT cells in vivo, then there should be an increase in the proportions of these cells in the expanded NKT cell population that results after glycolipid-induced proliferation. We found that there was no difference in the proportions of TCR β usage by V α 14i NKT cells before or after stimulation with α -GalCer or α -C-GalCer (Figure 6). Prior to glycolipid administration about 50% of V α 14i NKT cells in the livers and spleens of mice expressed V β 8.1/8.2, while about 15% expressed V β 7 and 3-4% expressed V β 2. These proportions did not change after α -GalCer- or α -C-GalCer-induced proliferation (Figure 6). In all, these results suggest that TCR β usage does not affect in vivo V α 14i NKT cell activation stimulated by α -GalCer or α -C-GalCer.

4. Discussion

The current study further defines the physiologic mechanism by which α -C-GalCer exhibits its superior antimalarial activity when compared to its structurally similar analog α -GalCer. As reported previously α -C-GalCer's enhanced antimalarial effect appears to stem from prolonged downstream IFN- γ production by NK cells requiring IL-12 [6]. This requirement for IL-12 is reconfirmed in the present study by our results showing the abrogation of α -C-GalCer's enhanced therapeutic effect against malaria liver stages in mice lacking IL-12 (Figure 2(a)). The current study also definitively defines the important role of NK cells in the mechanism of α -C-GalCer's enhanced antimalarial effect. Our finding that NK cell depletion abrogates α -C-GalCer's superior antimalarial activity when compared to α -GalCer attests to the key role these cells play (Figure 2(b)). In addition, despite a previously reported finding that α -C-GalCer stimulates less IL-4 than α -GalCer [6, 8], the ability of α -C-GalCer to stimulate diminished production of Th2 cytokines like IL-4 and IL-10 has no effect on its superior therapeutic activity relative to α -GalCer (Figure 3). Taken together, these data, along with our prior results showing that CD8 α ⁺ DCs are the cells responsible for producing IL-12 in response to glycolipid injection [7], indicate that α -C-GalCer's superior antimalarial activity stems from its ability to stimulate prolonged IL-12 production by DCs thereby resulting in enhanced downstream IFN- γ production by NK cells, and enhanced protection (Figure 7).

Our previous finding that α -C-GalCer stimulates prolonged IL-12 by DCs [7] correlates with our current finding that α -C-GalCer induces prolonged maturation of DCs after injection into mice (Figure 4). The fact that DCs mature at a slower rate after α -C-GalCer administration than after α -GalCer administration implies that the NKT cell inputs into

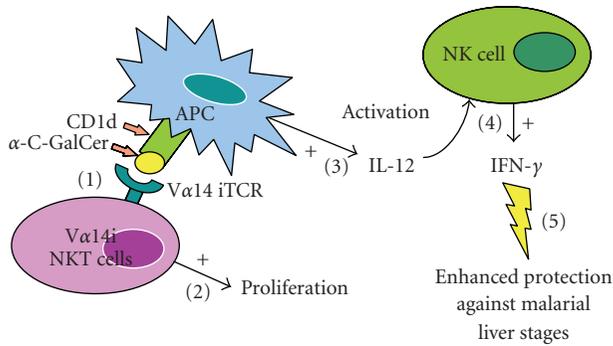


FIGURE 7: Mechanism of α -C-GalCer's enhanced therapeutic effect against malarial liver stages: (1) α -C-GalCer presented by CD1d molecules expressed on APCs stimulates, (2) enhanced (+) proliferation by V α 14i NKT cells relative to α -GalCer, and (3) enhanced (+) activation and IL-12 release by APCs compared to α -GalCer, which (4) induces augmented IFN- γ production by NK cells. This increased IFN- γ is responsible for (5) the better protection against malarial liver stages exhibited by α -C-GalCer.

α -C-GalCer-bearing DCs are weaker than those delivered to α -GalCer-bearing DCs. These weaker inputs might stem from a lower affinity interaction between the V α 14i TCR and the CD1d- α -C-GalCer complex. In light of studies demonstrating NKT cell TCR downregulation hours after encounter with α -GalCer-bearing APCs [19–21], as well as other studies showing that high affinity interactions result in faster and greater TCR downregulation [24, 25], the weaker V α 14i TCR:CD1d- α -C-GalCer interaction would be expected to result in slower and lesser downregulation of V α 14i TCRs on NKT cells than that seen with α -GalCer. Indeed, our experiment looking at the disappearance and reappearance of V α 14i NKT cells after α -GalCer or α -C-GalCer administration demonstrated precisely this phenomenon (Figure 5). As a consequence of shorter/lesser TCR downregulation, longer contact between NKT cells and α -C-GalCer-bearing DCs probably occurs, which would result in a temporal summation of NKT cell inputs by DCs that might exceed that achieved by α -GalCer-bearing DCs, which receive stronger- but shorter-lived inputs from NKT cells due to the higher affinity interaction between the V α 14i TCR and the CD1d- α -GalCer complex. Since glycolipid-induced IL-12 production by DCs requires contact with NKT cells [26–31], α -C-GalCer's ability to stimulate prolonged IL-12 might stem from longer contact time between NKT cells and α -C-GalCer-bearing DCs.

In addition to stimulating a slower and shorter downregulation of TCRs on V α 14i NKT cells, the interaction between CD1d- α -C-GalCer complexes and V α 14i TCRs also stimulates a greater *in vivo* expansion of V α 14i NKT cells compared to α -GalCer (Figure 5). This result is surprising because earlier data from us and others demonstrating that α -C-GalCer stimulates less cytokine production by NKT cells than α -GalCer [6, 8] implies that α -C-GalCer is a weaker agonist for NKT cells. It is likely that the striking differences we observed in the abilities of α -GalCer and α -C-GalCer to induce cytokine synthesis and cellular expansion by NKT

cells are the result of differential TCR signal transduction events, which probably stem from the difference in affinities between the V α 14i TCR:CD1d- α -GalCer interaction and the V α 14i TCR:CD1d- α -C-GalCer interaction.

This situation is analogous to a previous study showing that a partial T cell agonist incapable of stimulating early T cell activation events was capable of stimulating later events, such as proliferation, to the same degree or better as the full agonist [32]. These findings were explained by the so-called kinetic proofreading model for T cell activation, which proposes that while early T cell activation events depend on the affinity of TCR:MHC-peptide interactions, later T cell activation events depend more on the temporal summation of successive TCR signals delivered by MHC-peptide complexes serially engaging different TCRs over time [24, 32–36]. Based on this model, it is possible that α -C-GalCer is a partial NKT cell agonist, whose ability to stimulate enhanced V α 14i NKT cell expansion is due to enhanced temporal summation of APC inputs by NKT cells made possible by slower and shorter TCR downregulation and prolonged contact between NKT cells and α -C-GalCer-bearing APCs. While more research is needed to verify this mechanism, it is interesting to note that TCR β usage does not appear to effect α -C-GalCer's stimulation of NKT cells (Figure 6) despite two recent studies showing that TCR β chains encoded by the V β 8.2 gene segment confer higher avidity to V α 14i TCRs than V β 7 or V β 2, and that glycolipid ligands preferentially stimulate these higher avidity cells [22, 23].

5. Conclusions

We have previously shown that the C-glycoside analog of α -GalCer, α -C-GalCer, displays a superior inhibitory activity against the liver stages of the rodent malaria parasite *P. yoelii*. In the present study, we not only confirmed that IL-12 is a key factor that mediates the antiplasmodial activity of both glycolipids, but also defined the important role of NK cells as mediators of α -C-GalCer's superior effect. We found that the Th2 cytokines IL-4 and IL-10 are not involved. Moreover, we demonstrated a differential activity of α -C-GalCer in its stimulation of DCs as compared to α -GalCer, and correlated this with an enhanced proliferative response of V α 14i NKT cells to α -C-GalCer. Finally, we showed that V β usage does not influence the response of V α 14i NKT cells to either glycolipid.

Acknowledgments

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Review Article

The Coming-Out of Malaria Gametocytes

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The tropical disease malaria, which results in more than one million deaths annually, is caused by protozoan parasites of the genus *Plasmodium* and transmitted by blood-feeding Anopheline mosquitoes. Parasite transition from the human host to the mosquito vector is mediated by gametocytes, sexual stages that are formed in human erythrocytes, which therefore play a crucial part in the spread of the tropical disease. The uptake by the blood-feeding mosquito triggers important molecular and cellular changes in the gametocytes, thus mediating the rapid adjustment of the parasite from the warm-blooded host to the insect host and subsequently initiating reproduction. The contact with midgut factors triggers gametocyte activation and results in their egress from the enveloping erythrocyte, which then leads to gamete formation and fertilization. This review summarizes recent findings on the role of gametocytes during transmission to the mosquito and particularly focuses on the molecular mechanisms underlying gametocyte activation and emergence from the host erythrocyte during gametogenesis.

1. Introduction

With an annual death toll of more than one million people, the tropical disease malaria is considered one of the most significant infectious diseases worldwide. Malaria is caused by protozoan parasites of the genus *Plasmodium* and transmitted by blood-feeding Anopheline mosquitoes. During their life cycle, plasmodia alternate between the human host and the insect vector, and thus the transmission stages of the parasite had to develop mechanisms for rapid adaptation to the new environment in order to coexist with the respective host.

Like most apicomplexan parasites, plasmodia further switch between tissue-specific multiplication cycles and a phase of sexual reproduction, which mediates the transition from the human to the mosquito and thus plays a crucial part in the spread of the disease. The malaria sexual phase begins with the differentiation of gametocytes in human erythrocytes, followed by their uptake during the blood meal of the mosquito and the formation of gametes within the insect midgut. The transformation of the fertilized zygote into the infective ookinete subsequently marks the end of the malaria sexual phase (reviewed in [1]).

Historically, scant research has been devoted to the malaria sexual stages, namely, gametocytes, gametes, and zygotes, since they neither contribute to the clinical picture of patients nor do they play a role for vector control. However, within the last two decades the dramatic increase of drug resistance in malaria parasites has forced researchers to broaden their consideration of tactics to combat the disease, including transmission blocking strategies aimed at the sexual stages. Such transmission blocking strategies, on the level of either drugs or vaccines, are designed to disrupt parasite reproduction and further development in the mosquito midgut, thus breaking the life cycle of the parasite. Research on the malaria transmission stages, however, was formerly hampered by cost- and time-consuming cultivation, as well as by the technically challenging infections of mosquitoes with parasites. This was particularly true for work on *P. falciparum*, the causative agent of malaria tropica.

In recent years knowledge on the malaria sexual phase has benefited from a dramatic resurgence provided by proteomic, microarray, and annotation projects that arose out of the genome sequence projects for multiple malaria species (e.g., [2–7]). As a result, a number of new sexual stage antigens have been identified, and progress has been

made in the identification and functional characterization of enzymes and regulatory proteins that are involved in gametocyte differentiation and fertilization (reviewed in [1]).

Nowadays, three main questions regarding the malaria sexual phase are in the focus of interest. (1) Which are the mechanisms that cause a subset of erythrocytic parasites to enter the sexual stage pathway and to differentiate to gametocytes?. (2) How do gametocytes become activated within the mosquito midgut and how do they transform into gametes?. (3) In which way do the sexual stages interact with factors of the mosquito midgut? This review addresses the role of gametocytes during malaria transmission and particularly discusses the recent findings on gametocyte activation following entry of the mosquito midgut, as well as their egress from the host erythrocyte and transformation into gametes. Additional aspects of gametocytogenesis, sexual stage proteins, and malaria transmission can be found in other recent reviews [1, 8–11].

2. Gametocyte Differentiation in the Human

The gametocytes are the only stages within the life cycle of malaria parasites that are able to mediate the transition from the human host to the insect host. The development of asexual blood stage parasites to intraerythrocytic gametocytes, which is referred to as gametocytogenesis, starts approximately 7–15 days after the appearance of parasites in the human blood. It is not known to which degree gametocytes develop stochastically, with a small proportion of committed parasites leaving the asexual cycle and entering the sexual pathway, versus gametocytogenesis as a response to complex environmental signals during infection. Gametocytogenesis was previously shown to be influenced by different kinds of stress, including parasite density, anemia, host immune response or drug treatment (e.g., [12–20]; reviewed in [8, 10]). Up to date, little is known about parasite genes that regulate gametocytogenesis, but it was observed that reduced levels of gametocytes in parasite cultures are often associated with the loss of genetic information following subtelomeric deletion in the right arm of chromosome 9 [21].

While in most *Plasmodium* species the sexual stages mature within less than two days, a time period of about 10 days is required for gametocyte development in the human malaria pathogen *P. falciparum* [22]. Gametocyte maturation can be classified into five morphological stages (stages I–V) [23], and mature *P. falciparum* gametocytes show an eponymous falcipare form. In these stages, the host erythrocyte has conformed to the crescent shape of the parasite and is reduced to a small cytoplasmic hem. The intraerythrocytic gametocyte lies within the parasitophorous vacuole (PV) and is shielded from the erythrocyte cytoplasm by the PV membrane (PVM), which is located adjacent to the parasite plasma membrane (PPM) (Figure 1(a)). Underneath the PPM is a typical gametocyte feature, the pellicular complex, which consists of a subpellicular membrane (SPM) vacuole subtended by an array of longitudinally oriented microtubules [24]. These structures probably

give the gametocyte stability, and the electron-dense SPM disappears during gametogenesis (Figures 1(a), 1(b), 1(c), and 1(d)). Besides morphological changes, maturation of gametocytes also includes alterations on the molecular level in order to prepare the parasites for the rapid adaptation to the mosquito midgut. For instance, a large amount of mRNA is transcribed and stored in the cytoplasm of female gametocytes, as shown for the transcripts of the sexual stage surface proteins *Pbs25* and *Pbs28* in the rodent malaria model *P. berghei* [25], which will be transcribed only in the mosquito vector (see below).

The gender is predetermined in the developing gametocytes [26]. Gender specificity becomes established in the schizont committed to gametocytogenesis [27], and the gender ratio is typically female-biased with one male for about five female gametocytes, depending on the respective parasite clone [28]. This difference might be explained by the fact that one male gametocyte forms approximately eight microgametes, thus establishing a roughly 1 : 1 ratio of micro- and macrogametes in the mosquito midgut, thereby leading to most efficient fertilization in a monoclonal infection [8, 28]. Recently, Reece et al. demonstrated in the mouse model that parasites were able to adjust the sex ratio according to parasite density and the number of parasite clones coinfecting the mammalian host [29]. For instance, a less female-biased sex ratio would increase the probability of a successful fertilization of females of the respective clone, when competing with others [29–32]. In contrast, in vitro studies on *P. falciparum* did not show an adjustment of sex ratio to gametocyte density. However, an impact of the sex ratio on the infection rate, depending on gametocyte density, was observed [33].

The fact that single, haploid asexually replicating malaria parasites are able to develop into gametocytes of both sexes in the absence of sex chromosomes indicates that gametocyte gender determination is governed by differential gene expression [34]. Gametocyte stages I to IV were reported to sequester in the bone marrow and spleen, while terminally differentiated stage V gametocytes are then released in the peripheral blood system [35, 36] and only become infectious to mosquitoes after a further two or three days of circulation [37, 38]. Bloodstream gametocytes might not be distributed homogeneously, as evidenced by a significant aggregation pattern observed in midgut smears of *P. falciparum*-fed mosquitoes [39], and it is an intriguing hypothesis that parasites increase the likelihood of fertilization in the mosquito midgut by promoting uptake in preformed complexes of gametocytes.

3. Gametocyte Activation in the Mosquito Midgut

While feeding on an infected human, the female mosquito takes up malaria gametocytes together with the blood meal. By entering the midgut, the parasites receive environmental signals, which indicate the switch from warm-blooded host to insect vector and which initiate the development

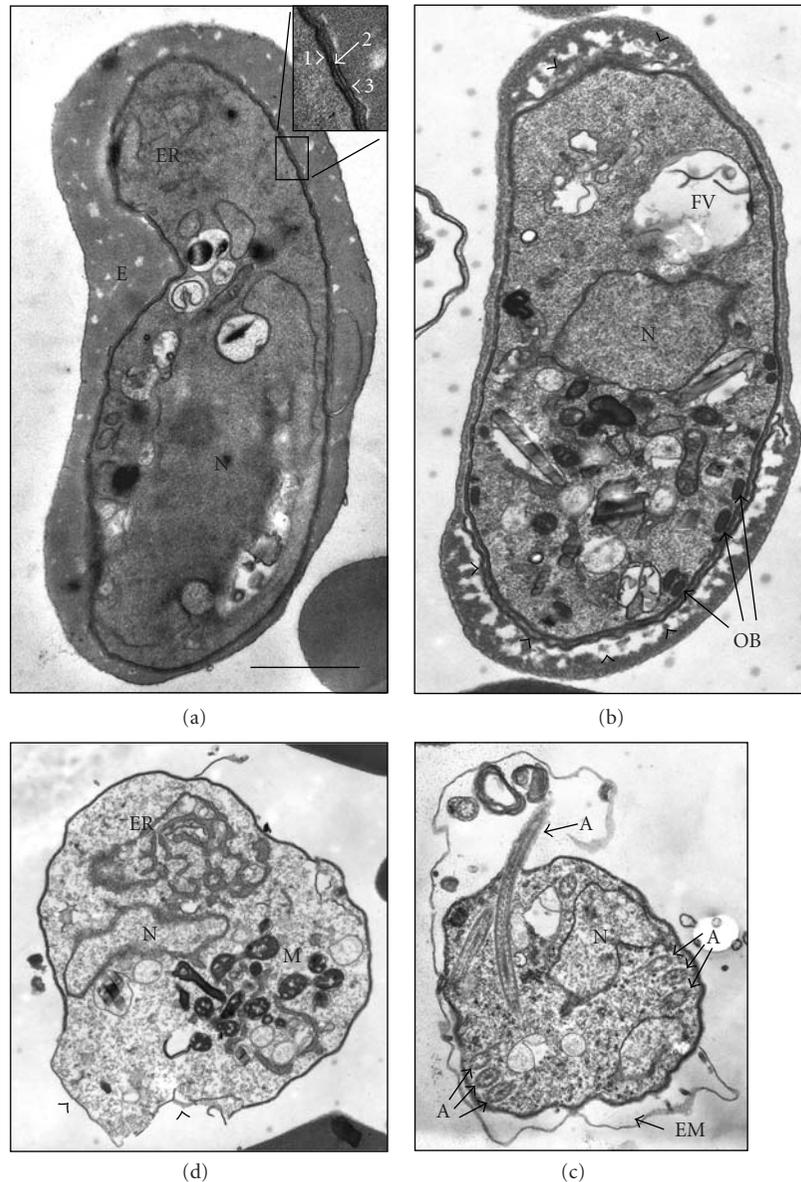


FIGURE 1: Ultrastructural changes in *P. falciparum* gametocytes during activation. (a) Transmission electron micrograph of a mature, non-activated gametocyte. The erythrocyte is reduced to an electron-light hem. The PVM is located adjacent to the PPM and the PV is therefore not discernable. No osmiophilic bodies are detectable, indicating that a male microgametocyte is pictured. Inset shows the pellicular membrane complex, depicting the SPM (1), the PPM (2) and the PVM (3). (b) A female macrogametocyte two-minute postactivation. The gametocyte is in the process of rounding up, thereby losing its crescent shape. The osmiophilic bodies become closely associated to the parasite surface. At the poles, the PVM separates from the PPM and the erythrocyte is in the process of degrading (arrowheads). (c) Ultrastructure of an exflagellating microgametocyte. The axoneme of a forming microgamete is visible as longitudinal section, and several other cross sections of axonemes are detectable inside the microgametocyte. The PVM has disappeared and the EM ruptured. (d) A female macrogamete after emergence. This stage is marked by pronounced ER and the highly branched single mitochondrion. The SPM is in the process of disintegrating (arrowheads). A, axoneme; EM, erythrocyte membrane; ER, endoplasmic reticulum; FV, food vacuole; M, mitochondrion; N, nucleus; OB, osmiophilic body; PPM, parasite plasma membrane; PV, parasitophorous vacuole; PVM, PV membrane; SPM, subpellicular membrane. Bar, 1 μm .

of gametes. Such signals include a drop of temperature by approximately 5°C [40], and the presence of the mosquito-derived molecule xanthurenic acid (XA), a byproduct of eye pigment synthesis [41, 42]. An additional signal reported to induce gametocyte activation is an increase of pH from 7.2 to

about 8 [40, 43], but such a pH shift was later discussed to be an artificial inductor of exflagellation [9]. While XA appears to initiate a number of signaling events in the parasite (see below), the quest for a receptor that binds XA was hitherto unsuccessful.

Gametocyte activation is routinely measured by the formation of exflagellation centers, although a time period of almost 15 minutes lies between these two events [44]. This is due to the fact that exflagellation can easily be observed under the light microscope and quantified by counting of exflagellation centers. Exflagellation is the process when the activated male microgametocyte forms motile flagellar microgametes, which detach from the residual body by binding to erythrocytes (see below). Two previous studies showed that induction of exflagellation involves a fast increase in intracellular calcium and cGMP [45, 46]. An initial benchmark in elucidating sexual stage signaling was the identification of two guanylyl cyclases ($GC\alpha$ and $GC\beta$) as integral membrane proteins in *P. falciparum* [47], which are activated by addition of XA (Figure 2) [48]. Noteworthy, the subsequent disruption of the $GC\beta$ ortholog in *P. berghei* resulted in normal exflagellation, but motility-impaired ookinetes, indicating that the role of $GC\beta$ is not essential for gametocyte activation [49]. The increase of cGMP triggers the activation of a cGMP-dependent protein kinase, PKG. Activation of PKG leads to rounding up of the gametocyte, a process that appears to be independent from calcium increase [50]. Gametocyte exflagellation further involves the presence of the second messengers diacylglycerol and inositol triphosphate (IP_3), hydrolysis products of phospholipase C activity [51]. The latter eventually mediates the release of intracellular calcium from the endoplasmic reticulum (ER) (Figure 2).

It is not yet known how the signaling pathway involving IP_3 and calcium release and the pathway involving cGMP and PKG activation are linked together, and whether PKG has an additional effect on calcium release from the ER (Figure 2). Current data suggest that at least three effector pathways exist (discussed in [50]): (1) a PKG-dependent, calcium-independent pathway that mediates rounding up of the activated gametocytes, (2) a calcium-dependent pathway that initiates microgamete formation, and (3) a calcium-dependent pathway that regulates emergence of activated gametocytes of both genders.

4. Gametocyte Egress from the Host Erythrocyte

Following uptake by the mosquito, both male and female gametocytes round up and then escape from the enveloping erythrocytes within about 10 minutes postactivation (Figures 1(b), 1(c), and 1(d)). In this period the microgametocyte replicates its genome three times in order to produce eight motile microgametes (reviewed in [1, 9]). Egress of the activated gametocyte from the host erythrocyte has been linked to the presence of osmiophilic bodies, gametocyte-specific secretory organelles that were first identified by electron microscopy due to their electron-dense features (Figure 1(b)) [24, 52]. They appear first in stage IV gametocytes and are particularly present in the female sexual stages. The osmiophilic bodies migrate to the PPM during activation and disappear within a few minutes post-activation, coevally with the rupture of the PVM (Figures 1(b), 1(c), and 1(d)) (G. Pradel, unpublished observations).

Osmiophilic bodies contain a gametocyte-specific and highly hydrophilic protein, *Pfg377*, which is considered a marker for these organelles [53, 54]. Only recently has the function of *Pfg377* been investigated by reverse genetic methods. Gene-disruption studies showed that female *P. falciparum* gametocytes lacking this protein reveal a reduced number of osmiophilic bodies and fail to egress from the host erythrocyte, pointing to a pivotal role of this protein in gametocyte emergence [55].

Another protein, which was only recently identified, MDV-1/Peg3, has also been implicated with gametocyte egress. In *P. falciparum*, expression was reported to be initiated in stage I gametocytes in association with all membranous structures of the PVM and to persist until gametocyte maturation [56–58]. First gene disruption studies on *P. falciparum* described a reduced formation of particularly male gametocytes [57]. Two subsequent studies, however, indicated a role of the protein post-activation of gametocytes. Lal et al. [59] reported the presence of MDV-1/Peg3 in *P. berghei* gametocytes of both sexes and a subsequent focal localization at the anterior pole of the developing ookinete. Studies on parasites in which the respective gene was knocked out resulted in reduced ookinete formation. A study by Ponzi et al., on the other hand, showed that *P. berghei* MDV-1/Peg3 was associated with the gametocyte osmiophilic bodies [60]. Gametocytes lacking this protein failed to egress from the host erythrocyte, thus resulting in reduced fertilization and ookinete formation. Ponzi et al. [60] therefore suggested that MDV-1/Peg3 plays a major role in disrupting the PVM and the erythrocyte membrane (EM).

Independent from the life-cycle stage, host cell egress of malaria parasites involves rupture of two membranes, PVM and EM. The time line of rupture, however, was recently object to several brisk discussions. Particularly two models are currently under investigation, the inside-out model, in which the PVM ruptures prior to the EM [61], and the outside-in model, in which the EM is degraded first [62, 63] (reviewed in [64]). The timeline of parasite egress was hitherto mainly investigated in the asexual blood and liver stages, and no data are available for the egress of gametocytes. In the above mentioned study, Ponzi et al. [60] suggested that MDV-1/Peg3 is involved in PVM destabilization and that EM rupture depends on the absence of the PVM. In accord with this hypothesis, new studies from our laboratory indicated that the PVM disappears within a few minutes after gametocyte activation, and that the rupture of the EM follows several minutes later (G. Pradel, unpublished observations), thus supporting the inside-out model of egress.

The coming-out of malaria parasites from the host cell requires protease activity. A number of new studies engaged with the identification of proteases that mediate emergence of asexual blood stage merozoites. Data point to the involvement of the cytoskeleton-degrading malaria proteases falcipain-2 and plasmepsin II [64]. Particularly SERA (serine-rich antigen) proteins, which were identified in the PV of blood stage schizonts (e.g., [65, 66]), are supposed to mediate PVM rupture. It was shown for

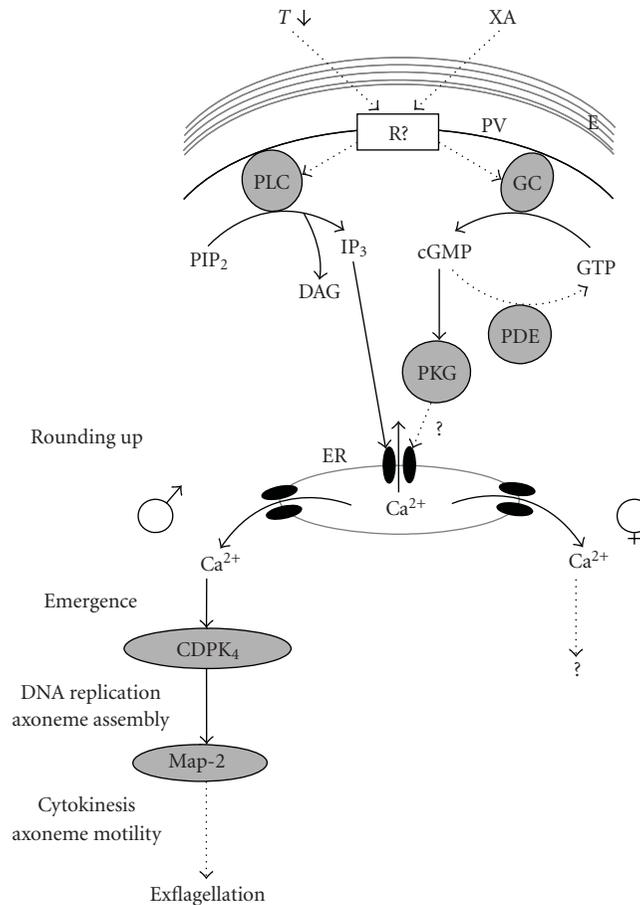


FIGURE 2: Schematic overview of signaling pathways identified to date that are involved in gametocyte activation (modified from [83]). Gametocyte activation is induced by a decrease in temperature and the presence of the mosquito-derived molecule XA. So far, a receptor involved in activation has not been identified. Activation effects PLC and GC, resulting in an increase of IP₃ and cGMP. The latter activates a PKG. IP₃ mediates release of intracellular calcium from the ER, which activates CDPK₄ and consequently Map-2 in the male microgametocyte, eventually leading to exflagellation. A possible link between PKG and calcium release has not yet been confirmed. Signaling pathways in the activated macrogametocyte that are downstream of calcium release were not yet identified. Black lines indicate direct interactions and dashed lines indicate indirect interactions. Ca²⁺, calcium ion; CDPK, calcium-dependent protein kinase; cGMP, cyclic guanosine monophosphate; DAG, diacylglycerol; DNA, deoxyribonucleic acid; E, erythrocyte; ER, endoplasmic reticulum; GC, guanylyl cyclase; GTP, guanosine triphosphate; IP₃, inositol triphosphate; Map-2, Mitogen-activated protein kinase 2; PDE, phosphodiesterase; PIP₂, phosphatidylinositol-4,5-bisphosphate; PKG, cGMP-dependent protein kinase; PLC, phospholipase C; PV, parasitophorous vacuole; R, receptor; T, temperature; XA, xanthurenic acid.

SERA-5 of *P. falciparum* that the protease is proteolytically activated by the serine-like subtilisin protease *PfSUB1* [67]. While no detailed studies were yet performed on malaria gametocytes, it is worth mentioning that transcripts of select representatives of the above mentioned protease families are expressed in these stages, including falcipain-1, plasmepsin VI, SERA-6, SERA-7, and *PfSUB3* [68, 69].

A popular strategy in investigating protease activity during rupture is the treatment of parasites with protease type-specific inhibitors. Again, these studies were mostly performed on blood stage parasites, particularly using cysteine protease inhibitors like E64. Treatment with this inhibitor, however, resulted to date in contradictory results, and it was reported that the inhibitor blocked

either degradation of the PVM [62, 63] or rupture of the EM [70]. A similar egress study using protease inhibitors was recently performed on activated *P. berghei* gametocytes and showed that exflagellation can be blocked by the cysteine/serine protease inhibitors TPCK and TLCK [71]. Our laboratory subsequently confirmed these results in *P. falciparum*. Treatment of activated gametocytes with TPCK, TLCK, PMSE, or two novel falcipain-targeting cysteine protease inhibitors during activation reduced the formation of microgametes [72]. Furthermore, the aspartic protease inhibitor EPNP appeared to interfere with rounding up of gametocytes. The exact modes of action for these proteases during gametocyte egress from its host cell remain to be investigated.

5. Exflagellation and Gamete Formation

is the process in which the newly formed microgametes adhere to neighboring erythrocytes, thus forming rosettes called exflagellation centers, and then detach from the residual body of the activated male microgametocyte. The exflagellating microgamete adheres to sialic acids and glycoporphin A of the erythrocyte surface [73] and this binding is probably mediated by *Pfs230*, an abundantly expressed adhesion protein that is associated with the gamete surface [74]. Interestingly, *Pfs230* is proteolytically processed during gametocyte activation, and this processing can be inhibited by the metalloprotease inhibitor 1,10-phenanthroline [75, 76]. The same inhibitor blocks exflagellation by leaving the microgamete amotile [72], and it is tempting to speculate that processing of *Pfs230* increases the adhesive properties of this protein, which are needed for the binding of the exflagellating microgamete to erythrocytes [74].

Astonishing advances have been made in unveiling the signaling cascades during gametogenesis of plasmodia. This might be explained by the fact that sexual stage proteins can easily be disrupted because of their nonessentiality for parasite proliferation, and thus a functional characterization can be obtained by phenotype analysis of parasites, in which the respective genes have been knocked out. Genome annotation has revealed an extensive catalog of parasite-encoded kinases, the malaria kinome, with at least 86 hypothetical kinases identified in *P. falciparum* [77, 78]. Several orthologs of these kinases were disrupted in *P. berghei*. An initial elegant study showed that the calcium-dependent protein kinase *PbCDPK4* is involved in sexual stage signaling and regulation [79] (Figure 2). The kinase becomes activated by calcium increase following XA activation, resulting in genome replication in microgametocytes [79]. In *P. falciparum*, *PfCDPK4* was reported to be gametocyte-specific and activated by phospholipase C [80]. In a subsequent step, the mitogen-activated protein kinase *Pbmap-2* controls formation of male gametes at the stage of cytokinesis [81–83]. Downstream of these events, the protein kinases *Pbnek-2* and *Pbnek-4* trigger genome replication to the tetraploid level in the zygote stage [81, 84, 85]. Furthermore, *PbCDPK3* is required for ookinete motility and engagement with the mosquito midgut epithelium [86, 87]. When highlighting these novel signaling pathways during gametogenesis, it has to be taken under consideration, however, that in some cases the results obtained for *P. berghei* and *P. falciparum* might differ. For example, a recent reverse genetics approach on the *P. falciparum* ortholog *Pfmap-2* pointed to an essential function of this kinase for the parasite asexual blood cycle [88], contradictory to the abovementioned results on *Pbmap-2*. This indicates that insights gained by studying the rodent malaria model *P. berghei* cannot be as easily applied to human malaria pathogens as has so far been assumed.

Ingestion by the blood-feeding mosquito triggers molecular changes in the sexual stages of *P. falciparum*, with approximately 20% of stage-specific genes being activated during sexual stage development and parasite transmission [3, 5, 6]. This molecular switchover adjusts the gametocytes to the invertebrate host and on one hand initiates

reproduction but on the other hand prepares the emerging gametes for the hostile environment of the mosquito midgut. Gametocyte development and gamete formation are particularly accompanied by the coordinated expression of numerous surface-associated proteins, including the EGF domain-containing proteins *Pfs25* and *Pfs28*, the cysteine motif-rich proteins *Pfs230* and *Pfs48/45*, as well as the multiadhesion domain *PfCCp* proteins. These proteins and their potential as transmission blocking targets were discussed previously [1] and will therefore not be focus of this review. Noteworthy is that the majority of these surface proteins have adhesive properties and can be divided in two classes. One class of sexual stage proteins, including *Pfs230*, *Pfs48/45*, and the six *PfCCp* proteins, is expressed within the PV of the developing gametocyte and subsequently present on the gamete surface, but expression of these proteins usually ceases during fertilization (Figure 3). The expression of the second class of surface proteins starts at the time point of fertilization, as was shown for *Pfs25* and *Pfs28*, and expression often persists until the ookinete has formed [1]. The reason for this sudden onset of protein expression during fertilization is the translational repression of messenger RNA encoding for these proteins. This was interalia shown for the repression of *Pbs25* and *Pbs28* by the *P. berghei* RNA helicase DOZI (development of zygote inhibited) as part of a ribonucleoprotein complex [25]. Furthermore, transcript of the transcription factor AP2-O is present in female gametocytes. The factor, however, is only translated in the ookinete stage, where it then activates a set of genes encoding for adhesion proteins important for midgut invasion [89].

The reason for such a high number of adhesive proteins in the malaria parasite sexual stages remains elusive, but a new study from our laboratory might provide a first step towards answering this question. We showed that the six *PfCCp* proteins, which are characterized by a high number of adhesion modules, assemble to form multiprotein complexes during their expression in the PV, and these complexes are subsequently present on the surface of the newly emerged macrogametes [90]. Preliminary data point to an additional involvement of other surface-associated adhesion proteins in these complexes, like the transmembrane protein *Pfs48/45*, which might link the complex to the gamete surface [91] (S. Scholz, A. Kuehn, N. Simon, and G. Pradel, unpublished observations). We hypothesize that these protein complexes cover the macrogamete in the form of a sticky coat and that they are involved in important adhesive processes during malaria transmission to the mosquito. The complexes might play a role in promoting contact between the emerging gametes within the blood meal or in protecting the gametes from the aggressive environment of the mosquito midgut. Noteworthy, the gametes and zygotes are the only stages within the parasite's life cycle that, for more than one day, have to persevere outside a host cell. Here they are exposed to factors of the blood meal, including midgut bacteria and digestive enzymes, as well as components of the human immune system. This exposure results in an approximate 300-fold loss of parasite abundance during transmission to the mosquito, and the malaria transmission stages are

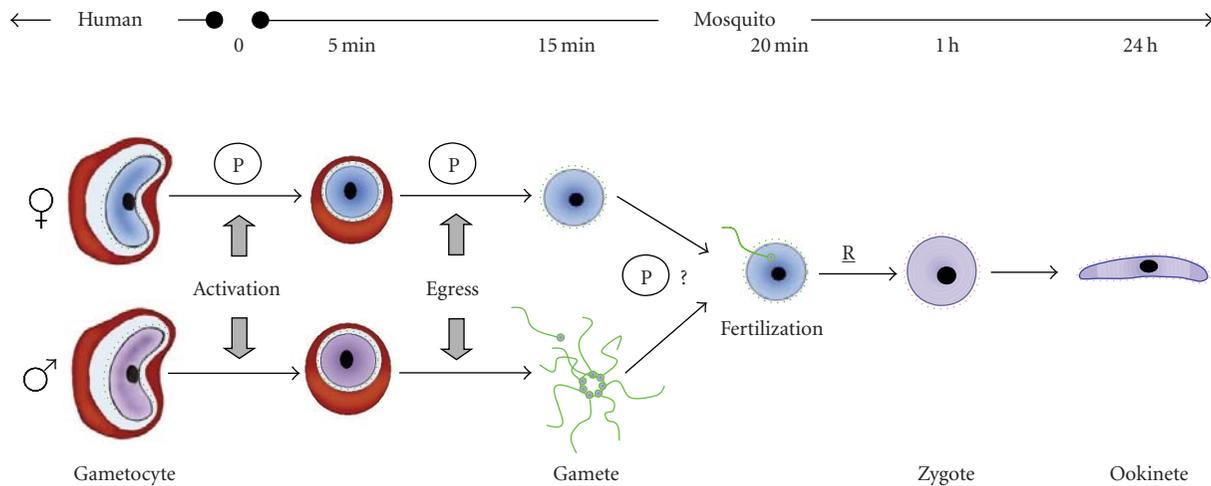


FIGURE 3: Morphological changes of malaria parasites during transmission from the human host to the mosquito vector. The intraerythrocytic gametocyte stages mature in the human host and are taken up by the blood-feeding female mosquito. By entering the mosquito midgut, the gametocytes become activated and round up, before emerging from the enveloping host erythrocyte. Proteases (P) are involved in these processes. During gametogenesis, the female macrogametocyte transforms into a macrogamete, while the activated male microgametocyte forms eight microgametes. Within approximately twenty-minute post-activation, the motile microgamete fertilizes the macrogamete and the resulting zygote transforms within a day into the infective ookinete. Two classes of sexual stage proteins are expressed in association with the parasite surface. A first class of proteins (shown in green) is expressed in the parasitophorous vacuole of the developing gametocyte, where some of them assemble to form adhesive multiprotein complexes. The proteins are later exposed on the surface of the newly emerged gametes, but expression ceases during fertilization. Expression of a second class of surface-associated proteins (shown in pink) is repressed in the gametocyte stage, but repression is released during fertilization (R) and protein expression persists to the ookinete stage.

therefore considered bottleneck stages of the parasite's life cycle [92].

6. Mating of Malaria Parasites

During exflagellation, the microgamete detaches from the residual body and is freely motile, moving via sinusoidal or helical waves [93]. It is not known whether the microgamete meets the macrogamete by coincidence, whether it actively scans the blood meal, or whether it migrates along a gradient of an attractant that is released by the macrogamete. Interestingly, we recently identified filamentous protrusions of the *P. falciparum* gamete surface, which form immediately upon activation and which appear to establish long-distance contacts between parasites in the mosquito midgut (G. Pradel, unpublished observations). These filaments fit the typical characteristics of so-called nanotubes, novel organelles that were recently described for a number of animal cells (reviewed in [95, 96]). It has been proposed that nanotube-like filaments can be formed by almost all cells serving as a medium for exploring the extracellular environment [94] and therefore are likely to represent ancient features of unicellular eukaryotes. Nanotubes were reported to have a function in communication between cells, including calcium signaling and organelle transfer [95, 96]. We therefore hypothesize that the "nanotubes" of malaria gametes might be tools to facilitate association within the midgut in order to increase the chance of parasite mating.

Once the microgamete adheres to a macrogamete, fertilization begins by fusion of the plasma membranes. Two recent studies on *P. berghei* described the identification of the microgamete protein GCS1 (generative cell specific 1), also termed HAP2, which enables gamete fusion, and disruption of the respective gene results in male sterility and blocked fertilization [97, 98]. GCS1/HAP2 is a conserved protein of algae and plants, where it is involved in pollen tube guidance and seed formation [99, 100], and was also identified in protozoan parasites [97, 98, 101]. Importantly, GCS1/HAP2 does not mediate the initial binding between the two mating partners, which appears to involve other adhesion proteins [98].

Cell fusion is followed by nuclear fusion, and over the next 3 hours, meiosis occurs and the zygote becomes tetraploid [102]. During the following 24 hours, the zygote transforms into the infective ookinete stage, thus marking the end of the malaria sexual phase. The ookinete is motile and possesses an apical complex which enables it to disrupt and traverse the midgut epithelium, before settling down between epithelium and basal lamina. Parasite tetraploidy persists throughout the ookinete stage until sporozoite budding in the oocyst restores the haploid state [102].

7. Concluding Remarks

Despite intense work on the sexual stages of malaria parasites, they represent the least understood stages of the

parasite's life cycle. Gametocyte differentiation and gametogenesis have mostly been studied in the human malaria pathogen *P. falciparum*, and for these sexual stages, a variety of proteins have been identified and characterized. On the other hand, the implication of malaria sexual stage proteins for malaria transmission was preferentially investigated in the murine *P. berghei* model, which is more easily accessible for genetic manipulations and transmission studies. Up to date it is challenging to combine information gained by both systems to receive the big picture on the malaria sexual phase. We expect that in the near future research on the sexual stages of malaria parasites will be dominated by two major tasks: (i) the big hunt for "the gene", which enables the blood stage parasite to enter the sexual pathway and (ii) the analysis of the molecular mechanisms and signaling events of sexual stage parasites during fertilization in the mosquito midgut.

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Review Article

Telomeric Heterochromatin in *Plasmodium falciparum*

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Until very recently, little was known about the chromatin structure of the telomeres and subtelomeric regions in *Plasmodium falciparum*. In yeast and *Drosophila melanogaster*, chromatin structure has long been known to be an important aspect in the regulation and functioning of these regions. Telomeres and subtelomeric regions are enriched in epigenetic marks that are specific to heterochromatin, such as methylation of lysine 9 of histone H3 and lysine 20 of histone H4. In *P. falciparum*, histone modifications and the presence of both the heterochromatin “writing” (PfSir2, PKMT) and “reading” (PfHP1) machinery at telomeric and subtelomeric regions indicate that these regions are likely to have heterochromatic structure that is epigenetically regulated. This structure may be important for telomere functions such as the silencing of the *var* gene family implicated in the cytoadherence and antigenic variation of these parasites.

1. Introduction

Plasmodium falciparum, a protozoan that is the causal agent of the most severe form of human malaria, has a complex life cycle with two different hosts: the *Anopheles* mosquito and humans. In order to complete its life cycle, *P. falciparum* invades different types of cells and self-propagates in very distinct environments in the mosquito (gut, hemolymph and salivary glands) as well as in the human host (skin, liver and erythrocytes). Each of these distinct environments exerts selective pressures related to morphological changes and forces *P. falciparum* to exhibit differential gene expression during its life cycle [1–3]. Transcriptome analyses have shown that the parasite displays tightly coordinated cascades of gene expression [2, 3]. However, the molecular mechanisms that drive this regulation have not yet been deciphered. Surprisingly, the *P. falciparum* genome encodes a relatively low number of transcription factors, although the basal core transcriptional machinery and protein-coding genes involved in nucleosome assembly and the regulation of chromatin structure are conserved [4–6]. To date, no

specific DNA-binding proteins have been identified other than PfTBP [7], PfMyb1 [8] and members of the ApiAp2 gene family [9]. These observations suggest that epigenetic mechanisms play a significant role in the control of gene expression in *P. falciparum* [4, 6, 10]. We define “epigenetic” as the situation in which the activation and silencing of individual genes are inherited in the absence of changes in the DNA sequence. With this definition in mind, we will begin by providing a brief overview of chromatin and telomere structure, followed by a summary of the proteins and epigenetic modifications involved in heterochromatin assembly in this parasite. Finally, we discuss the potential role of heterochromatin in the control of the *var* genes expression.

2. *P. falciparum* Chromosome Organization

The genome of *P. falciparum* is 22.8 Mb in total, organized in 14 chromosomes whose sizes range from 0.7 to 3.4 Mb [11]. Chromosome mapping has revealed that the chromosomes are compartmentalized, containing conserved regions at

their central domains and polymorphic regions at their terminal domains. Thus, the housekeeping genes are located within the central regions of the chromosomes, whereas the highly variable gene families responsible for the antigenic variation of the parasite are clustered towards the telomeres [12]. The chromosome ends are made up of telomeric repeats (GGGTTT/CA) organized in tandem, followed by an array of noncoding DNA elements at the subtelomeric regions (Figure 1(a)). These telomere-associated sequences (TAS) are species-specific and consist of a coding and a noncoding region. The non-coding region is composed of a mosaic of six different blocks of repetitive sequences located between the telomere and the coding regions. These elements are called “telomere associated repetitive elements” (TAREs) and span 20–40 kb (Figure 1(a)) [13, 14]. The six elements are positioned in the same relative order on all the chromosomes, while the size and the DNA sequence of each TARE are polymorphic [11, 13]. Adjacent to the non coding TAREs are members of multi-gene families that are involved in immune evasion and cytoadhesion (*var* genes) and putative variant antigens (*rif* and *stevor* genes) [11, 13].

3. Chromatin in *P. falciparum*

As in all eukaryotic organisms, chromatin in *Plasmodium* is organized into fundamental units called nucleosomes [15]. These units restrict the binding of transcription factors to their target sequences and regulate gene expression. *P. falciparum* contains genes coding for each of the four histones required for the assembly of nucleosomes: H2A, H2B, H3 and H4, as well as the histone variants H3.3, CenH3, H2AZ and H2Bv [16]. However, no gene encoding for histone H1 has been identified in this parasite to date, which is particularly significant since histone H1 is responsible for binding the DNA linker present between two nucleosomes and helping to compact the chromatin into the canonical 30 nm fiber.

It is feasible that chromatin structure might contribute to the regulation of gene expression in *P. falciparum* because the histones that make up the nucleosomes in their chromosomes have a primary structure very similar to those found in other eukaryotes.

In most organisms, histones H3 and H4 are among the most conserved proteins. Their amino terminal ends, which are necessary for the formation of the nucleosomal core, are fairly conserved in terms of their sequence, particularly at residues that are susceptible to specific covalent modifications [17]. The amino terminal ends of histones H3 and H4 of *P. falciparum* contain sites susceptible to post-translational changes (Figure 1(b)). Several modifications have been identified in *P. falciparum* by mass spectrometry analysis of histones, Western blot assays performed with antibodies specific to methylated and acetylated histones, and ChIP on ChIP assays. Those modifications are: acetylation of histone H3 at residues K9, K14, K18, and K27 [16, 18–20] and of histone H4 at K5, K8, K12 and K16 [16, 21]; methylation of histone H3 at K4, K9 and K36 [20, 22, 23] and histone H4 at K20, as well as the sumoylation of histone H4 [24]. Recently, a comprehensive mass spectrometry analysis of *P.*

falciparum histones identified 44 new post-transcriptional modification sites in these proteins, most of them associated with a transcriptionally permissive state (Figure 1(b)) [25].

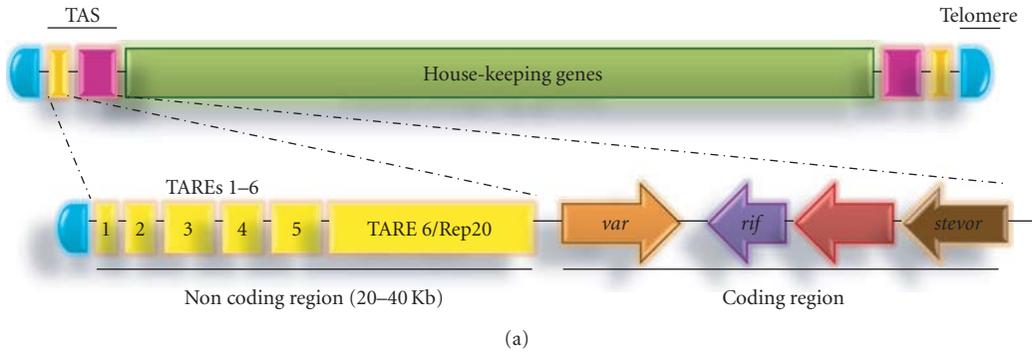
Additionally, both biochemical and molecular studies have demonstrated that this parasite contains chromatin-modifying enzymes, including histone deacetylases (PfHDAC1-Rpn3, PfHDAC2 and PfSIR2) and the PfGcn5 histone acetyl transferase (HAT) [26]. Bioinformatic analyses conducted in the last year have identified at least nine genes encoding histone lysine methyl transferases (HKMTs) in the *P. falciparum* genome (PfHKMT1–9) [22]. One methylase that belongs to the SET 8 family, displays H4K20 mono-, di-, and trimethylase activities [27]), and another HKMT (SET 3) is likely involved in the methylation of histone H3K9 [22]. The same study showed that the genome of *P. falciparum* also encodes several proteins that contain the JmjC domain, which removes methyl groups from histones (histone demethylases) [22]. However, SNFL2 is the only *P. falciparum* ATP-dependent chromatin-remodeling enzyme that has been characterized [28].

Although several proteins responsible for introducing histone modifications (writers) have been identified in *Plasmodium*, none of the proteins that recognize the modifications (readers) had been identified in the parasite until recently; the HP1 protein of *P. falciparum* (PfHP1) was identified [29]. PfHP1 contains domains that are characteristic of HP1 proteins, such as a chromo-domain (CD) and a chromo-shadow domain (CSD) [29]. As in higher eukaryotes, PfHP1 recognizes dimethylated or trimethylated H3K9 through its CD, and it can form homodimers, probably through its CSD [29]. These data suggest that this protein has an important role in the compaction of nucleosomes, activity required to form heterochromatin.

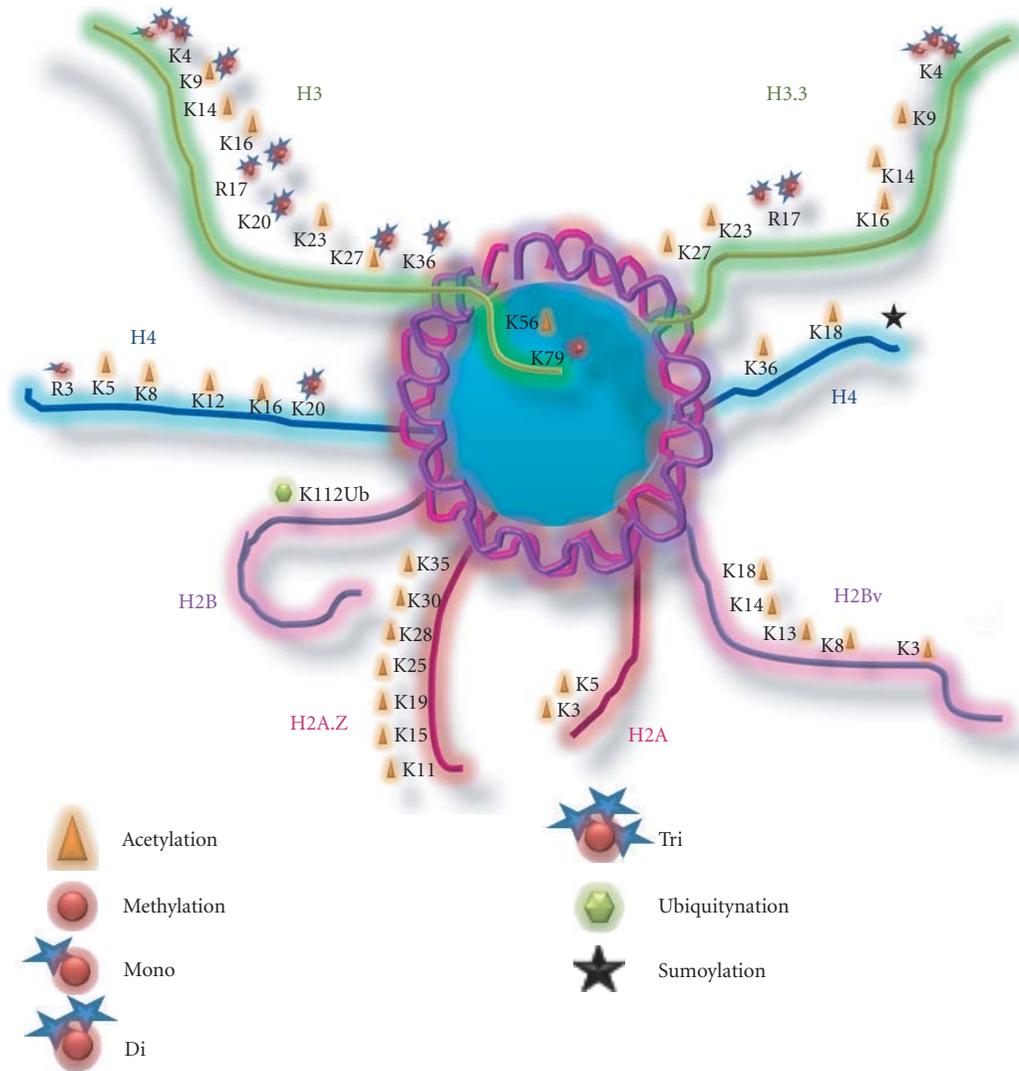
4. The Function of Post-Translational Modifications in Histones

The various chemical modifications that occur at the amino terminal of the histones affect chromatin architecture, that is, they help establish the functional and structural domains known as euchromatin and heterochromatin. Euchromatin, is open chromatin that allows transcription factors to access and transcriptionally activate their target genes. Euchromatin, which is maintained largely by housekeeping genes, is condensed during metaphase and decondensed during interphase. Heterochromatin differs from euchromatin in that it is condensed during interphase. It is often said to be “poor in genes” and mainly constituted for repetitive DNA sequences. Moreover, since it is highly condensed and inaccessible to transcription factors, heterochromatin is generally transcriptionally silent [30].

The formation and maintenance of heterochromatin depends on chromosomal localization, nuclear localization, and the presence and density of repeated DNA elements [31]. Chromosomal regions that contain a high density of repeated DNA elements organized in clusters, such as telomeres and centromeres, are the primary targets for the formation of heterochromatin. These regions remain condensed throughout the cell cycle and are known as “constitutive



(a)



(b)

FIGURE 1: A model of *P. falciparum* chromosome organization. (a) The *P. falciparum* haploid nuclear genome is organized into 14 linear chromosomes. Each chromosome is composed of an internal region in which housekeeping genes are located, and a chromosome end that displays a higher-order DNA organization common to all *P. falciparum* chromosomes. Downstream of telomeres resides at highly polymorphic TAS composed of a noncoding and a coding region. The noncoding region contains six TAREs, which are always positioned in the same order but are of variable length. TARE6, also known as Rep20, is responsible for most of the length polymorphism observed in the noncoding region of the TAS. The coding region contains several gene families encoding important virulence factors, such as the *var* and *rifin* genes. (b) A schematic representation of post-translational histone modifications in *P. falciparum*. The amino acids on the N-terminal tails of histone H2A, H2B, H3, H4 and the histone variants H2A.Z and H3.3 that are subject to different modifications are described in the figure.

heterochromatin". However, heterochromatin is also found at loci that are regulated during development; the chromatin state switches from compact (heterochromatin) to open (euchromatin) in response to cellular signals that regulate gene activity (e.g., inactivation of the X chromosome and the senescence-associated heterochromatin SAHF locus). This is known as "facultative heterochromatin" [32]. Facultative heterochromatin is interspersed with euchromatin and is organized in small domains along the chromosomes [30, 33–35]. The domains may contain promoter regions of individual genes or chromosomal domains that need to be silenced [36]. Facultative heterochromatin may be specific of cell type and/or of cell differentiation stage, and even more, appear just in one of two homologous chromosomes [30, 34, 35].

5. Post-Translational Histone Modifications and Their Role in Telomeric Heterochromatin Formation

Studies from yeast to mammals strongly suggest that histones and their modifications have an important role in the assembly of heterochromatin. The increase in the acetylation or methylation of specific histone residues (e.g., H3K9ac, H3K4me and H3K36me) invariably correlates with transcriptional activity; a decrease in acetylation levels is correlated with a repressed transcriptional state. Therefore, heterochromatin is associated with histone hypoacetylation [37]. Furthermore, the methylation of H3K9 or H4K20 and the sumoylation of the four histones are characteristic heterochromatin markers [38]. In general, the methylation of specific lysine residues in the amino terminal ends of the core histones is critical for the establishment, maintenance and silencing of chromatin domains in the chromosome centromeric and telomeric regions.

6. Heterochromatin Assembly Is an Orchestrated Mechanism

So far, we have described how epigenetic marks such as H3K9me3 and histone deacetylation are associated with transcriptional repression and occur frequently in histones at the telomeric and subtelomeric regions of linear chromosomes. We have also discussed how proteins such as histone methyltransferases and histone deacetylases are involved in the formation of a hypoacetylated state that correlates with increased levels of methylation, which in turn leads to the formation of a more compact chromatin structure. Finally, we have seen that in addition to these proteins that alter the amino ends of the histones, enzymes like protein PfHP1 are required to read these histone modifications and translate this information into biological processes in the cell. Thus, the recognition of histone methylation by PfHP1 and the resulting chromatin compaction leads to the silencing of gene expression. However, in order to ensure that all this machinery is in the right place, the appropriate region or regions for heterochromatin formation must be recognized in a sequence-specific manner. The current understanding in

the mechanisms to produce this telomeric heterochromatin will be described in the following sections.

7. Composition and Chromatin Structure of Telomeric Regions

Telomeres are DNA-protein complexes that stabilize the ends of linear chromosomes in eukaryotes [39]. Telomeres perform several essential functions, allowing the complete replication of chromosomes, providing a cap that protects chromosome ends from degradation by nucleases and preventing chromosome fusion. The absence of telomeres results in genetic instability and loss of cellular viability [39, 40]. The subtelomeric regions located next to telomeres are composed of repetitive DNA and contain few genes. The telomeric DNA consists of tandem short repeats of G-rich sequences (TTGGGG for *Tetrahymena* and TTAGGG in humans) [41]. The telomeres of *P. falciparum*, present at the ends of its 14 linear chromosomes, consist of the sequence GGGTT(T/C)A, which is highly conserved among the different species of *Plasmodium* [42]. The average telomere length varies among different species of the parasite. For example, it has been shown that the telomere regions of *P. falciparum* are 1.2 kb long, while those in *P. vivax* are 6.7 kb long. The relatively conserved telomere length among the different strains of *P. falciparum* can be considered specific to the species [42].

Differences in telomere length may be due to differences in the organization of chromatin in these regions. Micrococcal nuclease assays have established that the innermost part of telomeres in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have a nucleosomal organization that consists of three or four nucleosomes (Figure 2). The outermost part of the telomeres, known as the telosome, is not associated with nucleosomes [43]. Instead, this portion of the chromosome is bound by a large number of telomere-specific proteins that form the telosome complex. In *S. cerevisiae*, for example, multiprotein complexes such as Rap1, the Ku complex, Taz1, Rif 1, the Mlp complex, telomerase and the SIR silencing complex comprised of the proteins Sir2, Sir3 and Sir4 [44, 45] have been isolated and characterized. Telomeres in *P. falciparum* have an organization similar to that of the telomeres in *S. cerevisiae*, with an internal region associated with three to four nucleosomes and an outer region free of them [13]. Several proteins orthologous to those in the yeast telosome complexes, such as PfSir2 [21], PfOrc1 [46] and telomerase (PfTERT) [47], have also been found in this parasite. All these findings support the existence of a telosome complex in *P. falciparum* (Figure 2).

8. The Telosome Complex and Its Participation in the Assembly of Telomeric Heterochromatin

The telosome complex performs multiple functions, such as the protection of the terminal ends of linear chromosomes from degradation by exonucleases and prevention of recombination of heterologous telomeres. Telosomes also

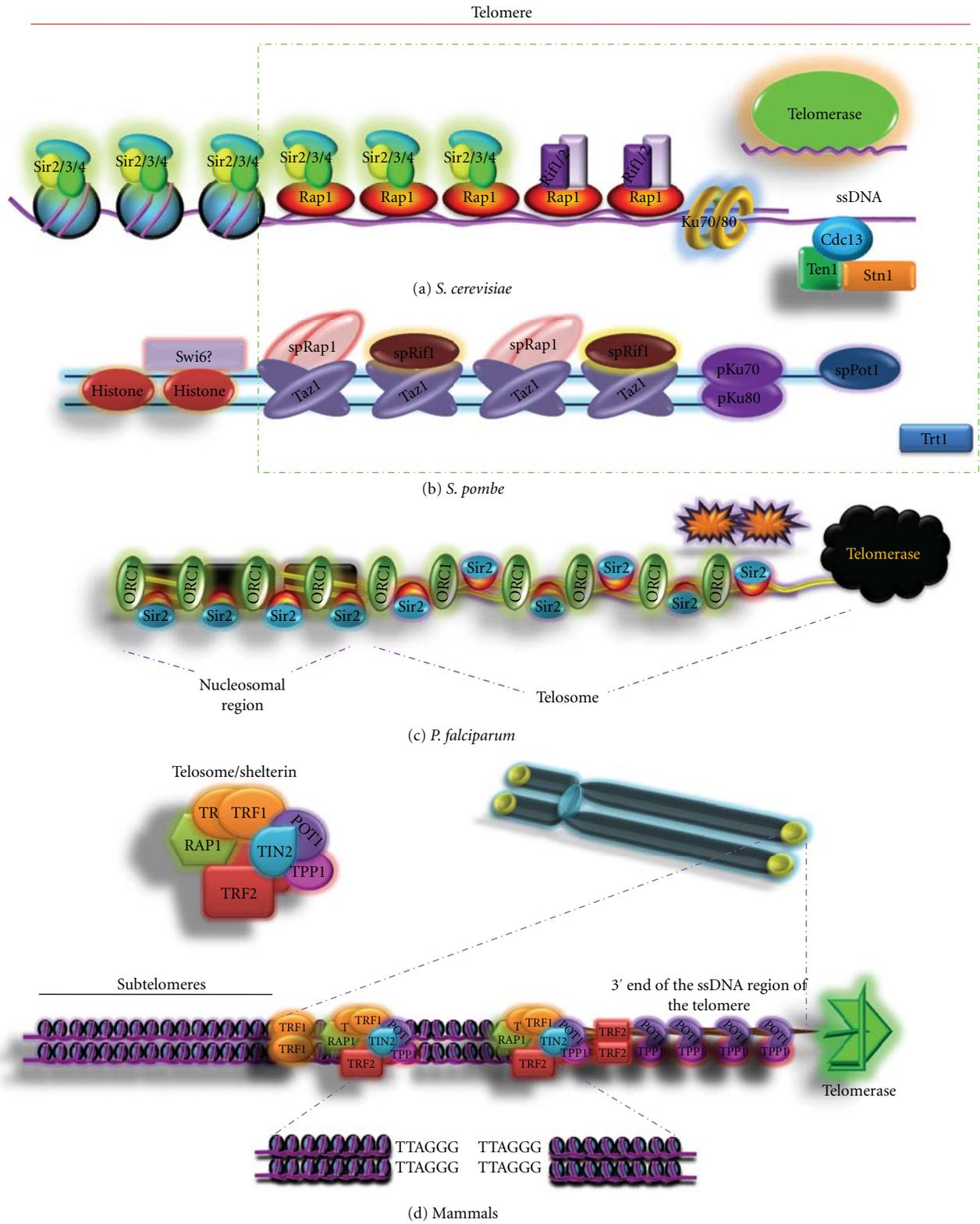


FIGURE 2: Heterochromatin in *S. cerevisiae*, *S. pombe*, *P. falciparum* and human.

participate in the replication of telomeric regions, help to anchor telomeres to the nuclear periphery, control the length of telomeres, influence the formation of telomeric clusters and heterochromatin, and affect the regulation of the expression of genes adjacent to subtelomeric and telomeric regions through a telomere position effect (TPE) [14, 44, 45, 48].

But how does the telosome complex participate in the formation of telomeric heterochromatin? In *S. cerevisiae*, one of the proteins that bind to the telosome is Rap1, which recognizes telomeric repeats through its two Myb domains [49]. Once Rap1 binds to telomeric DNA in a sequence-specific manner, it can recruit Sir2, Sir3, and Sir4 [50]. Once this silencing complex has been formed, the full SIR complex is able to spread along the adjacent sequences because of the high affinity that Sir3 and Sir4 have for hypoacetylated histones H3 and H4, thus generating heterochromatin structure (Figure 2(a)) [51–54]. The spreading of the SIR complex may require the active deacetylation of histone tails next to the SIR complex performed by Sir2, as spreading appears to be counteracted by the histone acetylase Sas2 [55].

The heterochromatin assembly machinery in *S. pombe* is more similar to that of mammals than that of *S. cerevisiae*. The assembly process begins when Taz1 protein binds to telomeric repeats and recruits the proteins spRap1 and spRif1. *S. pombe* does not have Sir3 and Sir4 orthologues, but rather the HP1 orthologue Swi6. This protein contains chromo and chromo-shadow domains, which are involved in the formation of protein complexes and association with chromatin. As in higher eukaryotes, the assembly of heterochromatin requires the action of a histone methyltransferase (called Clr4 in *S. pombe*), which methylates H3K9 to create a binding site for Swi6. In addition, the deacetylation of H3K14 seems to be important for the silencing and recruitment of Swi6 to heterochromatic regions. Once Swi6/HP1 has bound the chromatin, the methylation of histones continues, thus creating additional binding sites for Swi6 on adjacent nucleosomes. This allows the histone modifying enzymes and Swi6 to extend in *cis* beyond the site where the heterochromatin complex was nucleated (Figure 2(b)) [39].

The repeats present in the subtelomeric regions also contribute to the formation of telomeric heterochromatin through the formation of the RIST complex (RNA-induced initiation of transcriptional gene silencing). This transcriptional silencing machinery uses small non-coding RNAs (siRNA) to signal from regions where H3K9 is methylated as well as to assemble heterochromatin on the repetitive DNA [32]. The current model proposes that the methylation of H3K9, established by the RNA interference (RNAi) machinery in *trans* and/or by DNA binding factors, anchors the RIST complex to chromatin in a stable fashion. This enhances the ability of the RDRC complex (RNA-dependent RNA polymerase complex) and Dicer enzyme to process siRNA from nascent transcripts. The generated siRNA is responsible for directing histone-modifying enzymes such as the Clr4 methyltransferase toward the repetitive elements of DNA in order to methylate them. Then, the H3K9me3 epigenetic mark recruits chromo-domain-containing proteins such as

Chp1, Chp2 and Swi6. Chp1 is part of the RITS complex, so this recruitment establishes a new siRNA transcription process that will promote the recruitment of Clr4 in order to establish the structure of heterochromatin more efficiently [32].

Similar to yeast, in mammals a multiprotein complex known as shelterin binds to the telomeric repeats. The shelterin complex contains orthologues of the yeast proteins that bind both to the repeats present in double-stranded DNA and to a broken G end. The assembly of this heterochromatin begins when TRF1 and TRF2 bind to telomeric repeats. In this way, TRF1 interacts with TIN2 as well as with the proteins TANK1 and TANK2. It has been suggested that TRF1, and other proteins that interact with it, act as negative regulators of telomere length. On the other hand, TRF2 and POT1 appear to have additional functions in the protection of the telomeres, particularly in preventing the fusion of chromosome ends. Sirt1 and SUV39 (HMT) are enzymes responsible for the modulation of chromatin that accompanies the formation of facultative heterochromatin. SUV39H1 forms histone H3 that contains a trimethyl group at lysine 9 (H3K9me3) in heterochromatic regions. Sirt1 is a NAD-dependent deacetylase that removes acetyl groups from H4K16. Moreover, Sirt1 directly interacts with SUV39H1 and deacetylates HMT, which in turn increases the activity of HMT and thus H3K9me3 levels. Even when H3K9 has been methylated by the action of the histone methyltransferase, this change alone is not enough to establish highly condensed chromatin. In order to achieve this effect, it is necessary to recruit the non-histone HP1 protein [56]. HP1 binds to histone H3 when it recognizes di- or trimethylated lysine 9 through its chromo-domain, so HP1 colocalizes with Su(var)3–9 in the heterochromatin regions (Figure 2(d)) [39].

One question that arises is how HP1 contributes to the generation of highly condensed chromatin and the repression of gene expression? This mechanism is not yet fully understood, but it has been suggested that the recruitment of HMT can lead to H3K9 trimethylation, which in turn is recognized by the HP1 CD. The recruitment of HP1 through H3K9me3 could promote HP1 dimerization via its CSD, which would allow HP1 to spread over the subtelomeric region, resulting in a more compact chromatin structure [34]. It has been demonstrated recently that HP1 is not only associated with constitutive heterochromatin, but is also involved in the silencing of specific genes within euchromatin to form facultative heterochromatin [34, 57, 58].

9. Telomeric and Subtelomeric Assembly of Heterochromatin in *P. falciparum*

P. falciparum possesses short chromosomes, and until very recently, heterochromatin was not observed in this parasite. The repetitive structure of the subtelomeric DNA [13] and the association of telomeres in *clusters* at the nuclear periphery [59] suggested the existence of heterochromatin at the ends of the chromosomes. Moreover, chromosome-painting studies revealed that a pair of subtelomeric probes

separated by 60 to 80 kb colocalized at the same spot in the nucleus, while two similarly distant internal probes are seen as two distinct signals [21]. These data indicated that the regions proximal to the telomeres (known as telomere-associated repeat elements; TAREs, and coding region that containing the *var*, *rifin* and *stevor* genes) exist in a more condensed form than the inner regions of the chromosome. Consistent with this, electron-dense heterochromatin was observed at the periphery of *P. falciparum* nuclei in ultrathin sections [21]. All of these data support the existence of telomeric and subtelomeric heterochromatin in *P. falciparum*. Furthermore, ChIP assays demonstrated that in *Plasmodium*, the histone deacetylase PfSir2 can associate indirectly with telomeres and with all the subtelomeric repetitive elements until TARE6, also known as Rep20. Therefore, it is proposed that this protein may generate a heterochromatin gradient from telomeric regions to TARE6 [59].

Heterochromatin formation in *S. cerevisiae* depends only on the SIR silencing complex formed by Sir2, Sir3 and Sir4. Sir2 is the only member of this complex that has been identified and characterized in *P. falciparum* [21, 60]. A recent search for genes encoding Sir3 and Sir4 orthologues in *P. falciparum* had not succeed; instead, Orc1 (PfOrc1), whose amino end is highly similar to that of the Sir3 protein, was identified [46]. It has been demonstrated that the amino end of Orc1 is involved in the formation of heterochromatin at the *S. cerevisiae* mating locus [61], and that Orc1 is also part of the protein complex that participates in the assembly of telomeric heterochromatin in *Drosophila* and *Xenopus* [62–64]. All these data suggest that this protein is involved in the assembly of heterochromatin. Furthermore, immunofluorescence assays showed that PfOrc1 localizes in telomeric clusters. We have used EMSA and ChIP assays to demonstrate that PfORC1 can specifically interact with telomeres and subtelomeric elements similar to PfSir2, making PfORC1 a strong candidate for contributing to telomeric silencing in *P. falciparum* [46]. Another finding was that PfORC1 associates with TARE6 and that this association is not sequence-dependent but rather structure-dependent [46]. Our results lead us to speculate that *P. falciparum* chromosome ends may fold back on themselves, allowing telomeric chromatin to interact with subtelomeric heterochromatin. This represents the first evidence of a structure similar to a t-loop in *P. falciparum*, which may account for the stabilization of telomeric and subtelomeric chromatin at the nuclear periphery, as has been suggested for yeast. Interestingly, the PfORC1-DNA interaction is mediated by a putative leucine zipper DNA-binding motif present in the N-terminal region of the protein [46, 65]. We hypothesize that similar to Rap1 in yeast; ORC1 could recruit PfSir2 protein to the chromatin, since PfSir2 has no evident DNA-binding motifs. Although no interaction between PfORC1 and PfSir2 was observed in a yeast two-hybrid assays [66], PfORC1 may act as the initiator of heterochromatin assembly and be involved in the indirect recruitment of PfSir2 to the telomeric repeats [46]. Besides PfSir2 and PfOrc1, the nonhistone protein HP1 is also thought to be involved in heterochromatin formation in *P. falciparum*. Recently, also was demonstrated by ChIP assays

that PfHP1 protein binds strongly to the whole subtelomeric region [29]. Therefore, this protein together with PfSir2 and PfOrc1 may participate in the formation and establishment of telomeric and subtelomeric heterochromatin.

In order to explain the formation and assembly of heterochromatin in *P. falciparum*, a model that considers these data (analogous to the formation of telomeric heterochromatin in *Drosophila*) has been suggested. Initially, PfORC1, which possesses a DNA binding domain, could directly recognize the telomeric/subtelomeric repeats, as occurs at the HM loci in yeast, resulting in PfHP1 recruitment. ORC has been shown to be necessary for HP1 targeting to chromatin [67]. In parallel, another as yet unknown factor would recruit PfSir2 to the telomeric repeats, deacetylating H3K9 along the subtelomeric region. Once this is done, this residue would be accessible for methylation, probably by PfSet3 (a histone lysine methyltransferase). The recruitment of a histone lysine methyltransferase would lead to H3K9 trimethylation, which in turn is recognized by the chromodomain of PfHP1. PfHP1 recruitment through H3K9me3 could promote PfHP1 dimerization via its CSD, which would allow sequential PfHP1 spreading over the subtelomeric region, resulting in a more compact chromatin structure [29] (Figures 2(c) and 3).

10. The Var Genes as an Example of Transcriptional Silencing by TPE in *P. Falciparum*

Telomeric chromatin has several characteristics similar to those of centromeric heterochromatin for example, the silencing of nearby genes. The silencing of genes located near centromeres is known as a variegation effect (VE), while silencing of genes near the telomeres is known as TPE [52]. TPE is caused by the spread of heterochromatin in a Sir protein-dependent manner. Since its original characterization in yeast, numerous studies in various organisms have reported several protein modulators and effectors of TPE, as well as nuclear and chromosomal contexts that are required for this effect to take place [68].

The first evidence of transcriptional repression mediated by telomeres in *P. falciparum* arose from a study that compared the transcription of the gene HRP1 located 100 kb from the telomeric repeats on chromosome 2 with the transcription of the same gene relocated next to the telomere [69]. Quantitative RT-PCR analysis showed that the transcription of the HRP1 gene located next to the telomere was 50 times lower than the transcription of the same gene at an internal locus. These results suggested that HRP1 was silenced by the heterochromatin that had spread from the telomere to proximal regions. Therefore, it was proposed that telomeres alone are capable of producing a partial TPE effect for this gene [46].

To date, the *var* genes, which encode the PfEMP1 protein that plays essential roles in cytoadherence and antigenic variation in *P. falciparum*, are the only genes in this organism known to be regulated by chromatin remodeling. PfEMP1 proteins are encoded by a multigene family that consists

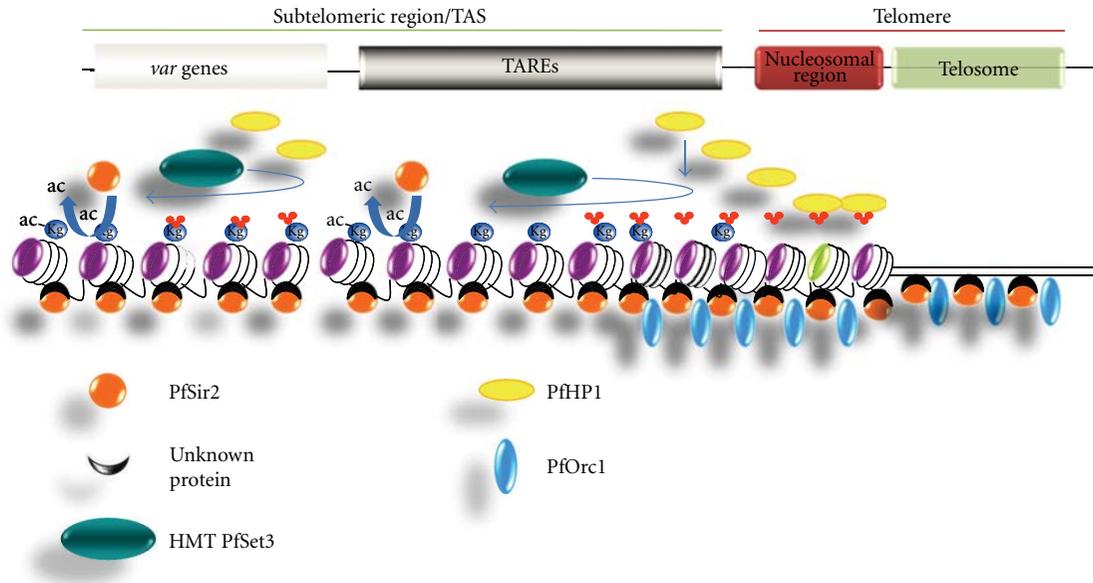


FIGURE 3: Hypothetical model for heterochromatin assembly at *P. falciparum* chromosome ends. This is a general view of the known chromatin components at *P. falciparum* subtelomeres. The spreading of heterochromatin along the different TAREs into adjacent coding regions probably involves PfHP1, PfSir2 and PfKMT1 in cooperation. The role of PfOrc1 in this process remains unknown.

of 60 *var* genes [70]. The *var* genes are regulated at the transcriptional level in such a way that only one *var* gene is transcribed at any given moment while the remaining 59 are transcriptionally silent [71]. Thus, the parasite must have some regulatory mechanisms that coordinate the expression of this gene family to ensure that only one gene is expressed in each organism. Evidence that the regulation of *var* genes occurs at the epigenetic level emerged from studies performed by Deistch KW and Voss T., who found that when the promoter region of a *var* gene (type Ups C or Ups B) was transfected transiently, it was then expressed in a constitutive rather than a stage-specific manner [72, 73]. This suggested that the chromosomal context of the *var* promoter is crucial for the silencing process, and that chromatin might play an important role in the regulation of the expression of this multigene family. The epigenetic control of *var* genes was clearly demonstrated in 2005 by Scherf and colleagues who showed that the PfSir2 protein is present at the perinuclear heterochromatin and telomeres. Moreover, super-shift and chromatin immunoprecipitation (ChIP) assays with the PfSir2 antibody showed that this protein is able to immunoprecipitate telomeres and TARE6 elements [21]. Later, using the same antibody (anti-PfSir 2), this protein was found associated with telomeric regions as well as the TARE1, 2, 3, and 6 elements, suggesting that PfSir 2 is capable of generating a heterochromatin gradient from the telomeres to TARE6, covering a distance of 50 kb. [46]. Some *var* genes are located adjacent to TAREs, which suggests the hypothesis that PfSir2 spreads towards the *var* loci and inhibits the expression of these subtelomeric *var* genes. Again, ChIP assays using the anti-PfSir2 antibody and two populations of parasites, each of which expressed a different *var* gene (FCR3-CSA, in which only the *var2CSA*

gene is active, and FCR3-CD36, in which only *var*-CD36 is transcribed), demonstrated that PfSir2 is associated with the Ups E promoter (which directs the gene expression of *var2CSA*) when it is transcriptionally inactive, but not when it is active. This experiment showed that PfSir2 is involved in the repression of the subtelomeric *var2CSA* gene through the extension of subtelomeric heterochromatin toward the gene in a mechanism similar to TPE. In addition, ChIP studies using antibodies against acetylated histone H4 demonstrated that the promoter of *var2CSA* associates with acetylated H4 only when it is transcriptionally active and not when it is repressed. All of these results suggest that the transcriptional activity of the subtelomeric gene *var2CSA* is reversibly regulated by epigenetic mechanisms through the acetylation and deacetylation of histones [21] (Figure 3).

These data were later confirmed with quantitative ChIP assays in the same parasite populations (FCR3-CSA, *var2CSA*-on; FCR3-CD36, *var2CSA*-off). The results of these experiments established that tri- and dimethylated H3K4 and acetylated H3K9 are enriched in the 5' UTR region of the active *var2CSA* gene during the ring stage; whereas H3K4me2 is highly represented at this locus during the trophozoite and schizont stages, when *var2CSA* is not actively transcribed. Thus, these modifications have proven to be a signal that allows the parasite to "know" which one of the *var* gene is going to be reactivated in the next intraerythrocytic cycle (poised state) [20]. The results also suggest that H3K4me2 may be contributing to the establishment of cell memory, thus helping to maintain the same pattern of monoallelic expression for several generations. Moreover, the same study showed that when the gene *var2CSA* is transcriptionally inactive, H3K9me3 is highly represented both in the 5' UTR region of this gene and throughout

its coding region. Therefore, the trimethylation of H3K9 reflects an inactive or repressed state of the *var2CSA* gene and acts antagonistically towards H3K9ac and H3K4me3 [20].

A complementary microarray study performed by Cowman's team, comparing global transcription in wild-type versus Δ Sir2 parasites, showed a significant increase in transcripts for a subset of *var* (*upsA* and *upsE*) and *rifin* genes. However, the *var* genes found at internal chromosomal loci, were similarly repressed in wild-type and Δ Sir2, which indicates that PfSir2 is not required to silence these *var* genes. This result is consistent with PfSir2 ChIP experiments performed in our lab in which PfSir2 was not found at the promoters of internal *var* genes. These data suggest that different mechanisms mediate the silencing of subtelomeric and central *var* genes [21, 74].

Recently, ChIP on ChIP assays had shown that H3K9me3 is highly enriched at subtelomeric regions associated with TAREs, subtelomeric *var* genes, *rifins* and *stevor* genes at the ends of all chromosomes, as well as in the central region of chromosomes 4, 6, 7, 8 and 12 where the central *var* genes are located. This mark is strongly associated with *var* genes that are transcriptionally inactive. These data reinforce the idea that the H3K9 trimethylation plays an important role in the transcriptional silencing of both the subtelomeric and the central *var* gene. Therefore, H3K9me3 is an epigenetic mark that seems to regulate the silencing of the *var* genes in *P. falciparum* [20].

11. Conclusions and Perspectives

The study of constitutive heterochromatin has gained interest as significant experimental evidence shows that this type of heterochromatin, which was long considered transcriptionally inactive, is sometimes transcribed in *S. pombe*, mice and humans [39, 75]. Moreover, non-coding RNAs appear to play a very important role in the assembly and formation of heterochromatin in these three organisms. On the other hand, the clear participation of telomeric heterochromatin in the regulation of gene expression in parasites, fungi and humans (as well as in the transcriptional activity in these regions) demands a review of the concept of facultative heterochromatin, which has been given only a structural role.

The initial evidence that constitutive heterochromatin might be transcribed in *P. falciparum* was provided when Deitsch and colleagues identified the first transcripts from the centromeric repeats of the parasite; this group also showed that non-coding RNA is associated with centromeric regions [76]. Meanwhile, no transcriptional activity has been reported in the telomeric or subtelomeric regions of this protozoan so far, despite the important role that these regions have in the silencing of subtelomeric type A and type E *var* genes. Telomeric heterochromatin could also participate in regulating the expression of other genes involved in clonal variation, such as *rifins* and *stevor*, all of which are located in subtelomeric regions. Therefore, in order to understand what controls the expression of the proteins most directly involved in the virulence of this parasite, it is of great importance to establish which ones are

the proteins and/or other elements involved in the assembly of heterochromatin in *P. falciparum*.

A better characterization of the heterochromatin and euchromatin in *P. falciparum* will allow us to further elucidate the regulation of the complex process of gene expression dynamics. Combining of ChIP technology with microarray analysis may give us the tools to understand how epigenetic factors are linked to different aspects of the parasite's chromosome biology, as well as generate potential avenues for malaria intervention strategies.

Acknowledgments

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Research Article

The Lipid Moiety of Haemozoin (Malaria Pigment) and *P. falciparum* Parasitised Red Blood Cells Bind Synthetic and Native Endothelin-1

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Endothelin1 (ET-1) is a 21-amino acid peptide produced by the vascular endothelium under hypoxia, that acts locally as regulator of vascular tone and inflammation. The role of ET-1 in *Plasmodium falciparum* malaria is unknown, although tissue hypoxia is frequent as a result of the cytoadherence of parasitized red blood cell (pRBC) to the microvasculature. Here, we show that both synthetic and endothelial-derived ET-1 are removed by parasitized RBC (D10 and W2 strains, chloroquine sensitive, and resistant, resp.) and native haemozoin (HZ, malaria pigment), but not by normal RBC, delipidized HZ, or synthetic beta-haematin (BH). The effect is dose dependent, selective for ET-1, but not for its precursor, big ET-1, and not due to the proteolysis of ET-1. The results indicate that ET-1 binds to the lipids moiety of HZ and membranes of infected RBCs. These findings may help understanding the consequences of parasite sequestration in severe malaria.

1. Introduction

Plasmodium falciparum (*Pf.*) infection may evolve into severe disease if untreated or inadequately treated, causing an estimated one million deaths annually [1]. The severe forms of malaria are characterized by the systemic release of inflammatory cytokines and the cytoadherence of *Pf.* parasitized red blood cells (pRBCs) to the vascular endothelium. This results in the sequestration of pRBCs in various organs, such as the brain, lung, and placenta, causing microcirculatory obstruction and subsequent tissue hypoxia, metabolic disturbances, and multiorgan failure [2–4].

Products of parasite origin, such as haemozoin (HZ, malaria pigment), contribute to the pathogenesis of severe

malaria by increasing the production of host inflammatory cytokines and the expression of adhesion molecules on the endothelium [4–9]. HZ, the detoxification product of haem, accumulates as insoluble crystals inside the food vacuole of intraerythrocytic parasites and, once released into the circulation, is phagocytised by host cells. Both native HZ and its synthetic analogue, β -haematin (BH), can modulate phagocyte and endothelial cell functions in vitro [10–12].

The sequestration of pRBC in the microvasculature may reduce local oxygen tension with increased risk of tissue hypoxia, hence inducing the transcriptionally regulated expression of vasoactive substances in order to modulate the vascular tone. One of these products is endothelin-1 (ET-1), a potent vasoconstrictor agent, which is produced

by the vascular endothelium in response to various stimuli including hypoxia and cytokines [13, 14]. ET-1 is a 21-amino acid long peptide derived from a larger precursor, pre-proET-1 that is cleaved first to big ET-1 (whose vasoconstrictor activity is approximately two orders of magnitude lower than that of ET-1 itself) and then converted to ET-1 by neutral metallo-endopeptidases known as endothelin-converting enzymes (ECEs). ET-1 exerts various important biological actions mediated by three receptor subtypes, ET-A, ET-B, and ET-C, which have been identified in various human tissues [15].

The expression and/or production of endothelins and their receptors is increased in several pathological conditions such as cardiovascular diseases, pulmonary hypertension, renal failure, and subarachnoid haemorrhage [16–18]. Elevated levels of ET-1 are also associated with serious infectious diseases, such as bacterial sepsis and trypanosomiasis [19–21].

The role of ET-1 in falciparum malaria is still controversial and largely unknown. It has been reported that severe malaria patients have increased plasma levels of big ET-1 which correlate with the levels of TNF- α , but not with parasitaemia, fever, or other features of severe infection [22]. Recently, children with malaria were shown to have significantly higher plasma levels of ET-1 compared to healthy controls. However, there was an apparent trend for those with severe malaria to have lower ET-1 levels compared to uncomplicated malaria patients, although a direct relationship with parasitaemia was not evident [23]. In the previous *in vitro* studies, we observed that pRBC down modulate the constitutive or hypoxia-induced ET-1 production by microvascular endothelial cells indicating that pRBC may induce local modifications of the vascular tone and the inflammatory response [24, 25]. To obtain further insights on the relevance of ET-1 modulation for severe malaria, we performed the experiments herewith presented to identify the parasite product(s) and the mechanism through which pRBC modulate the production and/or the activity of ET-1 under normoxic or hypoxic conditions.

2. Materials and Methods

2.1. Cells, Reagents, and Plasmodium falciparum Cultures. A long-term cell line of dermal microvascular endothelial cells (HMECs-1) immortalized by SV 40 large T antigen was kindly provided by Dr. F. Candal, the Center for Disease Control, Atlanta, GA, USA [26]. Cells were maintained in MCDB 131 medium (Invitrogen, Milan, Italy) supplemented with 10% fetal calf serum (HyClone), 10 ng/mL of epidermal growth factor (Chemicon), 1 μ g/mL of hydrocortisone, 2 mM glutamine, 100 units/mL of penicillin, 100 μ g/mL of streptomycin, and 20 mM Hepes buffer, pH 7.4 (EuroClone). Unless otherwise stated, all reagents were from Sigma Aldrich, Milan, Italy.

In vitro cultures of *Pf.* were adapted from Trager and Jensen [27]. A chloroquine (CQ)-sensitive *Pf.* strain (D10) and a CQ-resistant strain (W2) were maintained at 5% haematocrit (human type A RBC) at 37°C in RPMI

1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated A+ human plasma, 20 mM Hepes buffer pH 7.4 in a standard gas mixture consisting of 1% O₂, 5% CO₂, 94% N₂.

2.1.1. Preparation of HZ (Native and Delipidized) and β -Haematin (BH). To isolate HZ, pRBC (4–8% parasitaemia) were washed twice with serum-free culture medium, resuspended to 25% haematocrit, and fractionated on a discontinuous Percoll/4% sorbitol (wt/vol) gradient (0, 40, 80%) [28]. After centrifugation at 1075 g, HZ was collected at the top of the gradient, 0–40% interphase, washed three times with PBS, and stored at –20°C.

For the preparation of the delipidized HZ, native HZ was washed 3 times with Tris-HCl buffer (10 mM, pH 7.5) and the pellet extracted twice in a mixture of chloroform/methanol 2 : 1 (v/v) and twice in chloroform/methanol 1 : 2 (v/v). At the end, the pellet, containing the delipidized HZ was dried under a flux of nitrogen and resuspended in Tris-HCl buffer.

Synthetic malaria pigment, β -haematin (BH), was synthesized from haematin (Sigma, Milan, Italy) in methanol as described [29]. The haem content of a weighed amount of the native or delipidized HZ and BH dissolved in 0.1 M NaOH was determined by reading the absorbance at 385 nm ($\epsilon_{385\text{hematin}} = 6.1 \times 10^4 \text{ M cm}^{-1}$) (Soret band).

2.2. HMEC-1 Treatment. HMECs-1 were seeded in quadruplicate at 1.5×10^4 /well in 96 well flat bottom tissue culture clusters (Costar, 3596). After overnight incubation, monolayers were exposed to asynchronous *Pf.* cultures. pRBCs were added to the cells at 1% haematocrit with a parasitaemia ranging between 2 and 4%. Uninfected RBCs were used as controls in all experiments. In some experiments, HMECs-1 were exposed to HZ isolated from the pRBC cultures as described above.

HMECs-1 treated with pRBC or haemozoin were cultured for 24 hours at 37°C under normoxic (20% O₂, 5% CO₂, 75% N₂) or hypoxic conditions (1% O₂, 5% CO₂, 94% N₂) in sealed chambers.

2.3. Quantification of ET-1 and Big ET-1. At the end of each treatment, plates were centrifuged and ET-1 and big ET-1 peptides quantified in the supernatant using a specific ELISA test (PANTEC, Torino, Italy). ET-1 was also determined by a fluorimetric procedure according to Chaloin [30]. Fluorescence spectra were recorded in quartz cuvettes by a Perkin Elmer LS50 spectrofluorimeter at an excitation wavelength of 280 nm and the area of the peaks were calculated by the FLWinLab software. To correct for the interference on the intensity of the fluorescence caused by the release of small amounts of haemoglobin during incubation, control samples were prepared by diluting ET-1 in the supernatant recovered by centrifugation of a suspension of normal RBC in PBS.

2.4. RT-PCR Detection of ET-1 mRNA. For mRNA and RT-PCR analysis, HMEC-1 were seeded at 5×10^5 /well in 6 well

plates (Costar, 3516), treated with pRBC or control RBC, and incubated for 24 hours in normoxic or hypoxic conditions. At the end of the treatment cells were lysed in 1 mL Trizol Reagent (Invitrogen). Total RNA was isolated according to the manufacturer instructions. 1 μ g of total RNA was reverse-transcribed using Superscript First-Strand Synthesis System (Invitrogen, USA), according to the manufacturer's protocol. A portion of cDNA (0.2 μ g) was then used as the template in subsequent PCR amplifications in a total volume of 30 μ L containing 1X buffer (Promega), 2.0 mM MgCl₂ (Promega), 0.2 mM each dNTP, sense and antisense primers (0.2 μ M), and 1.25 Units of Taq thermostable polymerase (Promega). The sequences of ET-1 specific primers were: forward 5'-TGC TCC TGC TCG TCC CTG ATG GAT AAA GAG-3', reverse 5'-GGT CAC ATA ACG CTC TCT GGA GGG CTT-3' (462-bp fragment). G3PDH control primers were: forward 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3', reverse 5'-CAT GTG GGC CAT GAG GTC CAC-3' CAC (983-bp fragment). Amplification was performed for 30 cycles and the annealing temperature was 58°C. A negative control was included in each assay to confirm that only cDNA PCR products were detected and that none of the reagents was contaminated with cDNA of previous PCR products.

All PCR products were analyzed by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining.

2.5. Incubation of ET-1 and Big ET-1 Peptides with RBC of *P.f.* Cultures. Different concentrations of ET-1 or big ET-1 (5.0, 2.5, 1.2 fmol/mL) (Calbiochem Corp.) were added to pRBC suspension (2–5% parasitaemia, 2% haematocrit) in 100 μ L of HMEC-1 culture medium distributed in 96 well flat bottom microplates. Control unparasitized RBCs were maintained for 3–4 days before the experiment in the same conditions of pRBC (5% haematocrit at 37°C) and incubated with ET-1 or big ET-1 as described. After 2, 4, 6, 24 hours of incubation, plates were centrifuged and supernatants were collected and assayed for the presence of ET-1 or big ET-1. ET-1 (5 fmol/mL) was incubated for 24 hours with pRBC in the presence of the receptor ET-A or ET-B antagonists, alone or combined, (0.5–0.1 μ M) (JKC302 and BQ788, resp.) (Sigma) or a Protease Inhibitor Cocktail (used at a dilution of 1 : 200) (P1860 Sigma). ET-1 was then assayed in the supernatants.

Since parasite cultures are a heterogeneous population of cells made of pRBC and normal RBC of different density, the total RBC suspension was fractionated onto a Percoll/4% sorbitol (wt/vol) gradient [28]. This procedure allows the separation of the HZ containing fraction (top of the gradient), pRBC (density = 1.078), and three fractions (young, medium, and old) of unparasitized RBC. Each fraction was incubated with ET-1 (5–2.5 fmol/mL) for 24 hours after which the residual ET-1 was determined in the supernatant. Delipidized HZ, BH, or the supernatants of *P.f.* cultures were also employed.

When ET-1 was determined by fluorescence spectroscopy, normal uninfected RBC, pRBC (D10 strain, 2% haematocrit) were incubated in PBS in the presence of ET-1 (10 pmol/mL) for 18 hours, in 2 mL final volume.

Samples were then centrifuged at 600 \times g for 10 minutes and supernatants recovered for the fluorimetric analysis.

2.6. Statistical Analysis. All the assays were performed at least three times in quadruplicate and the results analyzed by the Student's paired *t*-test. Statistical significance was set at $P < .05$.

3. Results

3.1. Impaired Recovery of ET-1 Produced by HMEC-1 Cocultured with *P.f.* pRBC. The constitutive production of ET-1 by endothelial HMEC-1 cells is significantly increased by hypoxia. Coincubation of endothelial cells with pRBC (D10 and W2 strains at 2–4% parasitaemia), but not with normal uninfected RBC, induced a decrease in the levels of ET-1 detected in the supernatants; no differences were seen between the two strains used (Figure 1(a)). The decrease in ET-1 induced by D10 was dose-dependent (see Figure 1(b) and previous work [24]) and was not due to inhibition of ET-1 gene transcription or translation by pRBC since the level of ET-1 mRNA as well as that of big ET-1, the precursor of the active peptide ET-1, was not affected in either normoxic or hypoxic conditions (Figures 1(b) and 1(c)). We also excluded that pRBC may inhibit the processing of big ET-1 to ET-1 since no accumulation of big ET-1 was seen in the supernatants of endothelial cells incubated with pRBC.

3.2. Effect of pRBC on Synthetic ET-1 and Big ET-1. Having determined that pRBC did not interfere with the production of ET-1 by HMEC-1, we investigated whether pRBCs were able to bind or degrade synthetic ET-1. The first set of experiments was conducted by incubating different concentrations of commercially available, synthetic ET-1 peptide with pRBC for 24 hours and measuring the amount of residual ET-1 in the culture medium by ELISA. In the presence of pRBC (D10 strain, 2–4% parasitaemia), the levels of ET-1 in the extracellular medium were significantly reduced while big ET-1, used as control, was not affected (Figure 2(a)). Similar results were obtained with pRBC from the CQ resistant *P.f.* strain W2 (data not shown).

The removal of ET-1 by pRBC was a slow process, with a significant reduction of ET-1 (60–70% compared to control) occurring only after 24 hours (Figure 2(b)). ET-1 levels were not reduced following incubation of ET-1 with uninfected RBC (Figure 2(b)). The decrease of ET-1 in the presence of pRBC was confirmed using a fluorimetric method of quantification of ET-1; the data of a representative experiment are reported in Table 1. A good correlation ($r^2 = 0.939$) between the % of ET-1 recovery obtained by the two methods was achieved. The reduction of ET-1 after incubation with pRBC was not due to the enzymatic degradation of the peptide or to soluble factors released by pRBC as shown by the experiments conducted in the presence of protease inhibitors or supernatants from cultures of different *P.f.* strains (Table 2). This suggests that ET-1 was not degraded, but likely bound to pRBC membrane.

TABLE 1: Absorption of ET-1 by pRBC (D10 strain) or haemozoin: comparison between fluorimetric detection and ELISA assay.

Samples	Fluorescence Intensity (AUC)	ET-1 recovery		
		Fluorescence spectroscopy	ELISA	
		% control	pmol/mL	% control
Control ^a	41305		9.04	
Normal RBC	40503	98.1	8.69	96.1
pRBC (D10 1.6%)	32224	78.0	5.93	65.5
pRBC (D10 3.2%)	30784	74.5	5.03	55.6

Data of a representative experiment out of three experiments performed in the same conditions. ET-1 (10 pmol/mL) was incubated for 18 hours in PBS or in the presence of normal RBC or pRBC (D10 strain at 1.6% and 3.2% parasitaemia). ^aControl samples for fluorescence spectroscopy were prepared by diluting ET-1 in the supernatant recovered by centrifugation of a suspension of normal RBC incubated in PBS for the same length of time. Control samples for ELISA test were prepared by diluting ET-1 in PBS. At the end of the incubation, RBCs were centrifuged and supernatants used for ET-1 determination by both methods.

Data are expressed as arbitrary fluorescent units (AUC Area Under the Curve) or as pmol/mL.

TABLE 2: Effect of normal RBC, pRBC, or supernatants from *P. falciparum* cultures on the recovery of synthetic ET-1.

	% ET-1 recovery when incubated with:						
	Medium	Normal RBC	pRBC (D10)	pRBC (W2)	SN normal RBC	SN pRBC (D10)	SN pRBC (W2)
ET-1 ^a 5	100	91.96 ± 7.2	39.8 ± 8.3*	40.2 ± 7.5*	102 ± 5.4	98 ± 6.3	96 ± 7.8
+proteaseinhibitor	105.7 ± 2.1	101.7 ± 4.8	29.6 ± 7.3*	48.3 ± 11*	ND	ND	ND

^aET-1 (5 fmol/mL) was incubated for 24 hours in medium or in the presence of normal unparasitized RBC, pRBC from different *P. falciparum* strains or supernatants (SNs) from parasite cultures. ET-1 was also incubated with RBC or pRBC in the presence of a protease inhibitor cocktail. At the end of the incubation, ET-1 was detected by ELISA.

Data are expressed as % of ET-1 recovery respect to controls and represent the mean ± SD from three different experiments. **P* < .01 versus control.

To investigate whether ET-1 receptors were involved in the removal/binding of ET-1 by pRBC, the experiments were repeated using two receptor antagonists, JKC 302 or BQ788 which are specific for the ET-A or ET-B receptors, respectively. As shown in Figure 3, none of the receptor antagonists (used alone or combined) prevented the removal of ET-1 by pRBC suggesting that most of the binding was aspecific. Similar findings were obtained in binding experiments with radio-labelled ET-1 (¹²⁵I-ET-1) (data not shown).

Since parasite cultures are composed by pRBC, normal RBC of different age/density, parasite debris and free HZ, a Percoll fractionation of the culture was performed, as previously described [28]. Five different fractions were recovered: young, medium and old normal RBC, enriched pRBC (mainly at the trophozoite stage) and free HZ. Each fraction was then incubated with 2.5 fmol/mL of ET-1 for 24 hours. As shown in Figure 4, only the pRBC fraction and HZ (2 µg/mL) significantly bound ET-1. This demonstrates that the decrease in the content of ET-1 in HMEC-1 cultures treated with pRBC (Figure 1) was due to binding of the released peptide to pRBC and/or HZ.

Native HZ purified from parasite cultures is mainly made of crystals of dimers of Fe(III) protoporphyrin IX (haematin), but includes also proteins and lipids of parasite origin [31, 32]. Since data in the literature suggest that ET-1 interacts by electrostatic forces with neutral phospholipids, we investigated the role of the lipid fraction in ET-1 binding. To this purpose, the lipid fraction was removed from native HZ and the delipidized HZ assayed in the ET-1 adsorption

assay. The haem containing portion of HZ (beta haematin, BH) was used as control. The experiment was performed by incubating ET-1 (5 fmol/mL) with different concentrations of native HZ or delipidized HZ or BH. As shown in Figure 5(a) native HZ removed ET-1 in a dose-dependent manner, while delipidized HZ or BH had no effect at any of the doses employed, indicating that the lipid portion, but not the haem moiety of HZ was involved in ET-1 binding.

To verify whether HZ could bind ET-1 produced by endothelial cells, HMECs-1 were treated for 24 hours with different concentrations of native HZ. Similarly to pRBC, HZ reduced the ET-1 levels in a dose-dependent manner both in normoxic and hypoxic conditions (Figure 5(b)). At the highest dose used (50 µg/mL), the decrease of ET-1 by HZ was 87.1% and 83.6% in normoxic or hypoxic conditions, respectively. As expected, BH did not interfere with ET-1 production by HMEC-1 or recovery.

4. Discussion

The present study provides an evidence that the decrease in ET-1 caused by *P. falciparum*-parasitized RBCs is due by the aspecific binding of ET-1 to pRBCs. The reduction of ET-1 levels is specific for ET-1 (its precursor, big ET-1 is unaffected), is not caused by proteolytic degradation by soluble products or ET-1 binding to ET-A or B receptors [33, 34], or inhibition of endothelial cells functions by pRBCs [24]. The slow reaction kinetics (18–24 hours of incubation needed for a significant effect to occur) further support this hypothesis. The aspecific binding of ET-1 to pRBC involves

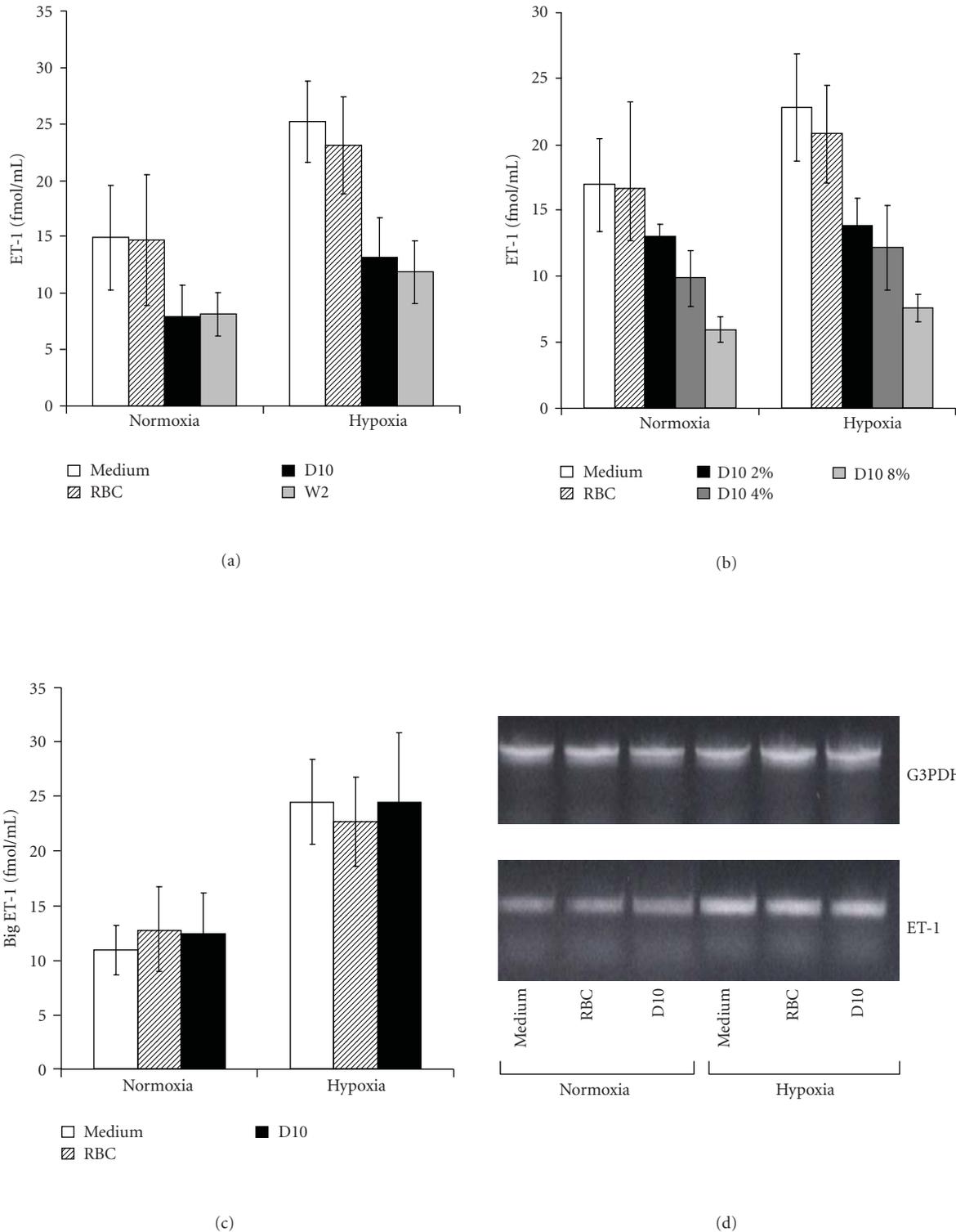


FIGURE 1: Effect of pRBC on the release of ET-1 and big ET-1 from human microvascular endothelial cells (HMECs-1). HMECs-1 were treated for 24 hours in the presence of normal RBC or pRBC (D10 or W2 strains) at 2–4% parasitaemia in (a), (c), (d) or at the indicated levels of parasitaemia (b) under normoxic or hypoxic conditions. Supernatants were then assayed for the presence of ET-1 (a, b) or big ET-1 (c) by ELISA. Results represent the mean \pm SD from five different experiments. (a) $P = .0014$ and $P = .0041$ D10 versus control in normoxia and hypoxia, respectively; $P = .0017$ and $P = .0052$ W2 versus control in normoxia and hypoxia, respectively; (b) $P = .014$ and $P = .033$: D10 8% and 4% versus control, in normoxia; $P = .006$, $P = .01$, $P = .027$: D10 8%, 4%, and 2% versus control in hypoxia. (d) RT-PCR analysis of ET-1. G₃ PDH served as control. The cells were lysed and mRNA extracted. The PCR products were separated through agarose gel electrophoresis and visualized by ethidium bromide. The reported data are representative of three independent experiments.

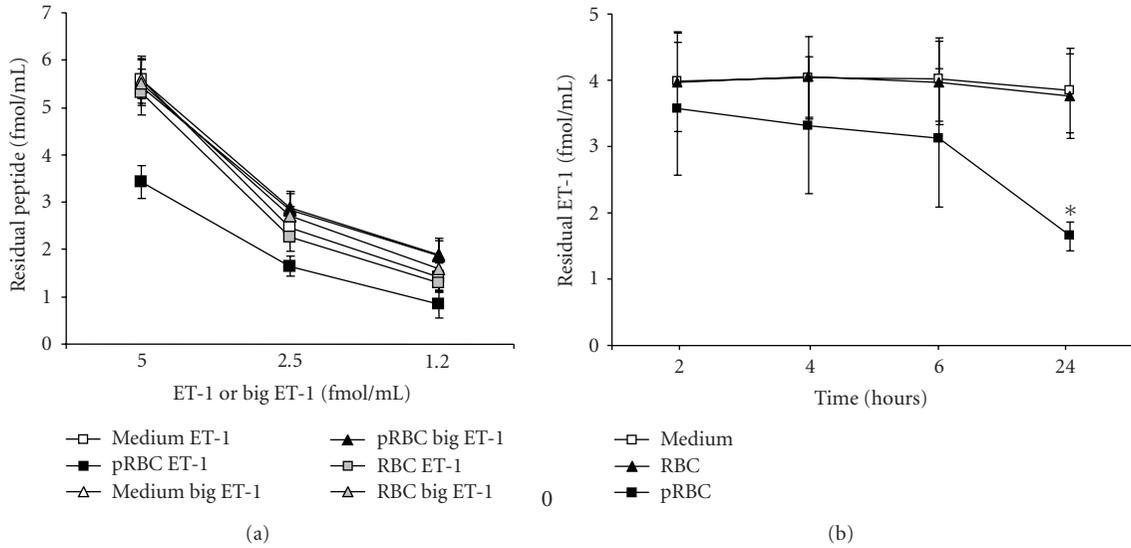


FIGURE 2: Effect of incubation of pRBC (D10 strain) or normal RBC on synthetic ET-1 and big Et-1. (a) Different concentrations of ET-1 (square) or big ET-1 (triangle) were incubated in medium (empty symbols) or in the presence of uninfected RBC (grey symbols) or pRBC (black symbols). After 24 hours of incubation, samples were collected and assayed for the presence of the residual ET-1 or big et-1 by ELISA. The results represent the mean \pm SD from three different experiments. (b) ET-1 (5 fmol/mL) was incubated in medium (empty square) or in the presence of pRBC (black square) or control RBC (black triangle) for 2, 4, 6, 24 hours. At the end of incubation, samples were collected and assayed for the presence of the residual ET-1. Results represent mean \pm SD from three different experiments (* $P = .029$ versus medium).

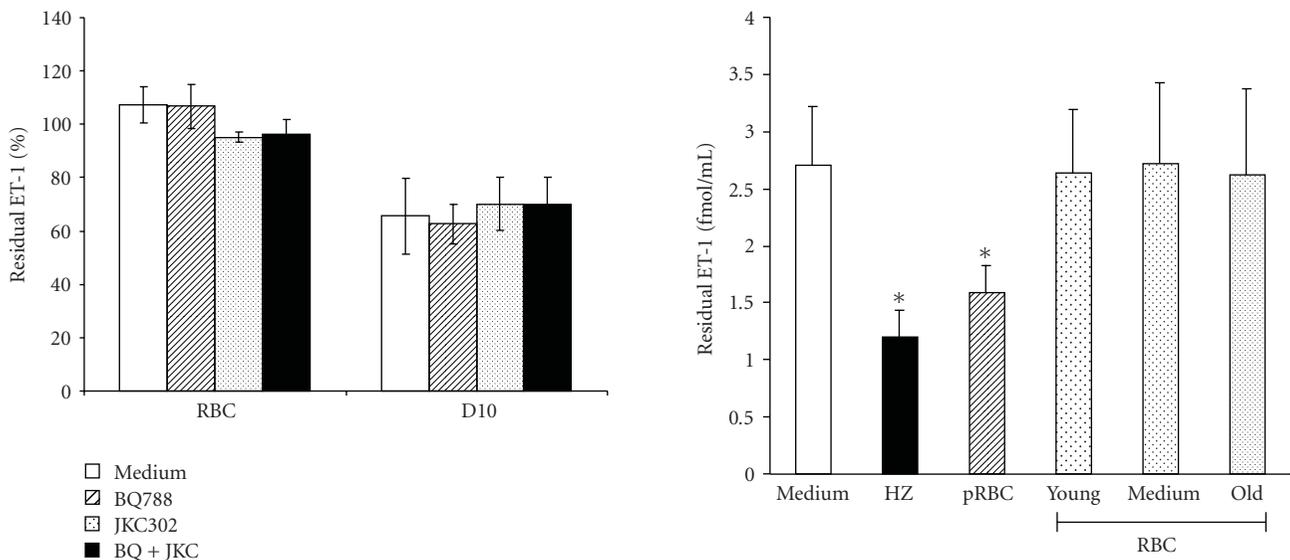


FIGURE 3: Failure of ET-1 receptor antagonists to inhibit ET-1 removal by pRBC ET-1 (5 fmol/mL) was incubated for 24 hours with normal RBC or pRBC (D10) in medium only (empty bars) or in the presence of JKC302, an ET-A receptor antagonist (striped bars) or BQ788, an ET-B receptor antagonist (dotted bars), or in the presence of JKC302 and BQ788 simultaneously. Data are expressed as % of ET-1 recovered in the supernatants and represent the mean \pm SD of three different experiments.

FIGURE 4: Fractions isolated by density gradient from the parasite culture have different ability to remove ET-1. Parasite culture (D10 strain) where density separated by Percoll/sorbitol gradient. Each fraction was incubated with synthetic ET-1 (2.5 fmol/mL) for 24 hours and, at end of the incubation, ET-1 was determined by ELISA. Recovery of ET-1 incubated in: medium (empty bar); RBC separated by different density and age (subdivided in young, medium and old) (dotted bars); pRBC at the trophozoite stage (4–8% parasitemia) (striped bar); fraction containing HZ (black bar). Results represent the mean \pm SD from three different experiments. $P = .007$ HZ versus control; $P = .038$ pRBC versus control.

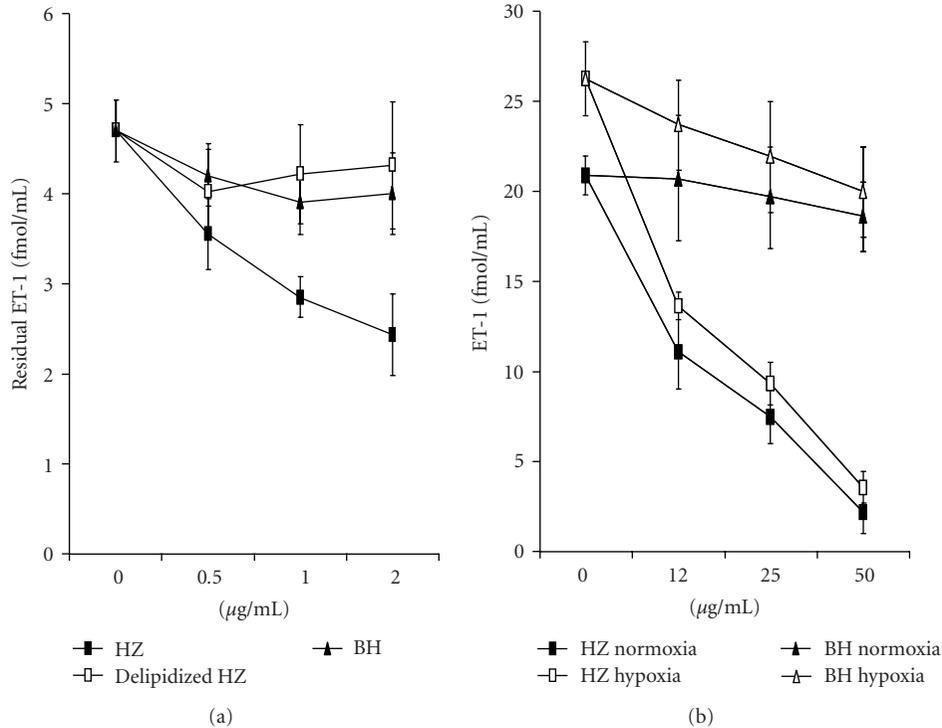


FIGURE 5: Effect of incubation of haemozoin with ET-1 synthetic or produced by HMEC-1. (a) ET-1 (5 fmol/mL) was incubated for 24 hours in the presence of different concentrations of native HZ (black square), delipidized HZ (empty square), or BH (triangle). After incubation the recovery of ET-1 was determined by ELISA. Results represent mean \pm SD from three different experiments. (b) HMECs-1 were treated for 24 hours in the presence of different concentration of native HZ (square), BH (triangle) under normoxic (black symbols), or hypoxic conditions (empty symbols). Supernatants were assayed for the presence of ET-1 by ELISA. Results represent mean \pm SD from three different experiments.

lipids; after density fractionation, only fractions enriched in pRBC and the HZ containing fraction can bind ET-1, but not normal RBCs, delipidized native HZ or synthetic BH. ET-1 can be adsorbed by neutral phospholipids via electrostatic interactions [30].

The process of HZ formation and the modified lipid composition of the membrane following malaria infection [28, 35] may explain why ET-1 does not bind to delipidized HZ or synthetic BH or normal RBCs.

Parasite neutral lipids have recently attracted considerable attention as the intracellular environment where crucial events of the parasite metabolism occur. The detoxification of haemoglobin-derived haem into HZ crystals seems to take place in neutral lipid nanospheres within the parasite food vacuole [36] and different neutral lipids associated with HZ have been identified by ESI-MS/MS. The most abundant are monostearic and monopalmitic glycerol, with a small amount of polar lipids such as phosphatidylcholine or phosphatidylethanolamine. These neutral lipids have been shown to promote HZ formation by increasing the solubility of haem monomers [37, 38]. The observation that ET-1 adsorbs to the native HZ, but not to the delipidated HZ or to BH, strongly argues in favour of the absorption of ET-1 to the HZ-associated neutral lipids. Likewise, it can

be speculated that ET-1 binds aspecifically to the lipids of the pRBC membrane. The lipid composition of pRBC membrane, in particular the phospholipid distribution and fatty acid pattern, is significantly modified by *P. falciparum* [28]. In particular, higher levels of the neutral sphingomyelin and lower levels of the acidic phosphatidylserine, phosphatidylinositol, and phosphatidylethanolamine compared to normal RBC of the same age/density have been described. [28, 36]. This phospholipid pattern accounts for the higher content of palmitic and stearic acid, two saturated fatty acids shown to be associated with native HZ [36]. Based on these findings we can speculate that the aspecific binding of ET-1 to pRBC could be mainly related to the increased fraction of stearic and palmitic acid due to the different phospholipid pattern of pRBC. Another parasite product, the glycosylphosphatidylinositol (GPI), that is known to modulate endothelial cells functions, would be a candidate for further ET-1 binding studies [39].

The conditions used in these experiments are biologically sound. pRBC concentrations were 2–4%, and hyperparasitaemia in falciparum malaria is defined as $\geq 4\%$ pRBCs in the peripheral blood (locally in the small vessels of the brain and other target organs it may be much higher). The HZ concentration of 2 $\mu\text{g/mL}$ is also relevant to biological

condition; in fact it has been calculated that ~3.9 million trophozoites contain 47 fg of haemozoin per cell with parasite at 32 hours in the life cycle [32]. Considering a 42% haematocrit, a 0.1% parasitaemia would be sufficient to release 2 µg/mL of haemozoin in vivo.

The clinical findings whereby ET-1 levels are unrelated to parasitaemia or severity of malaria [23] are not contradictory but simply hardly relevant to the events occurring in the small vessels of the target organs (brain, lungs, placenta). ET-1 action is mainly local [14]. In these vessels, the jamming of infected and uninfected RBCs will cause hypoxia which triggers the release of ET-1 by endothelial cells. However, accumulating pRBCs will increasingly be removing ET-1 and preventing it from exerting its vasoconstrictive action. The consequent vasodilation may ameliorate locally the blood flow but may also contribute to perivascular oedema that plays a crucial role in the pathogenesis of paediatric severe malaria and in fatal cases in adults [40, 41]. Cerebral oedema is a common finding with modern techniques such as the magnetic resonance imaging (MRI) (T. Taylor, personal communication). In conclusion, we have shown that ET-1, but not its precursor, big ET-1, is efficiently bound in vitro by pRBC or HZ through the lipids contained within the HZ crystals or present in the membrane of pRBC. In vivo, in the local microenvironment where pRBC sequester, high levels of ET-1 are likely to be produced, particularly in cerebral malaria or in the placenta of pregnant women with malaria. Binding to pRBC or HZ may neutralize ET-1 activity leading to reduced vasoconstriction and reduced inflammation. The extent of the decrease in ET-1 bioavailability will be different in different areas as function of parasite density and/or HZ release. As feed back effect, the endothelial cells will augment the production of nitric oxide (NO) which is reported to have beneficial effects in murine cerebral malaria [42]. The interaction between host, parasites, and treatment are complex. The mortality caused by severe falciparum malaria remains ~10–25% in spite of effective antimalarial treatment [43]. Reducing parasitaemia alone may not be enough to stop or reverse the pathological processes associated with malaria complications. This means that, even with reduced parasitaemia with antimalarials, adjunctive treatments need to be investigated to stop the progression of disease.

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Research Article

Tomatine Adjuvantation of Protective Immunity to a Major Pre-erythrocytic Vaccine Candidate of Malaria is Mediated via CD8⁺ T Cell Release of IFN- γ

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The glycoalkaloid tomatine, derived from the wild tomato, can act as a powerful adjuvant to elicit an antigen-specific cell-mediated immune response to the circumsporozoite (CS) protein, a major pre-erythrocytic stage malaria vaccine candidate antigen. Using a defined MHC-class-I-restricted CS epitope in a *Plasmodium berghei* rodent model, antigen-specific cytotoxic T lymphocyte activity and IFN- γ secretion ex vivo were both significantly enhanced compared to responses detected from similarly stimulated splenocytes from naive and tomatine-saline-immunized mice. Further, through lymphocyte depletion it is demonstrated that antigen-specific IFN- γ is produced exclusively by the CD8⁺ T cell subset. We conclude that the processing of the *P. berghei* CS peptide as an exogenous antigen and its presentation via MHC class I molecules to CD8⁺ T cells leads to an immune response that is an in vitro correlate of protection against pre-erythrocytic malaria. Further characterization of tomatine as an adjuvant in malaria vaccine development is indicated.

1. Introduction

Adjuvants are immunogenic compounds that, when combined with an antigen, potentiate an antigen-specific immune response. Adjuvants may not only boost the response of an immunologically weak antigen but also influence the type of immune response elicited [1]. While it has been known for many years that formulating antigen(s) with adjuvants may potentiate the magnitude of vaccine-elicited immune response, traditionally accepted adjuvants such as alum and various oil-in-water emulsions have failed to induce cytotoxic T lymphocyte (CTL) responses. However, the ability of new generation adjuvants to deliver soluble protein to the major histocompatibility complex (MHC) class I processing pathway, thereby inducing antigen-specific CTL responses, has been recognized recently [1, 2]. The development of tomatine as an immunostimulating agent was initiated as a direct response to the hypothesis that

reagents that were capable of delivering soluble protein into the class I pathway would induce antigen-specific CTL responses, and thus were likely to be powerful adjuvants [3].

The adjuvant tomatine is based upon the glycoalkaloid lycopersicon (C₅₀H₈₃NO₂₁), which is derived from the leaves and unripe fruit of the wilt-resistant wild tomato species *Lycopersicon pimpinellifolium*. This compound has been shown to have membrane-disrupting qualities [4, 5], similar in character to that of saponins which have long been established as potent immunostimulators [6]. In its naturally occurring form tomatine is known to be a primary toxicity-based plant defense mechanism against viral and bacterial pathogens; furthermore, it prevents infestation by arthropods and discourages ingestion (of unripe tomatoes) by vertebrates [7]. In spite of this, tomatine is safe and well tolerated in mice as it does not elicit haemolytic activity, granuloma formation, or tissue damage at the site of inoculation. However, mononuclear cells infiltrate within

24 hours post immunization, indicating the recruitment of immunological mediators [8]. The adjuvant-antigen preparation consists of a colloidal suspension of solid-state aggregates (0.1–2 μm) containing the antigen, tomatine and cholesterol [8].

The malignant tertian malaria of humans, *Plasmodium falciparum*, kills 2–3 million people and causes a further 500 million clinical infections annually. Due to increased resistance of the parasite to available drugs and of the mosquito vector to insecticide treatment there is a pressing need for novel control measures, one of which is an antimalarial vaccine [9]. For prophylactic purposes, a pre-erythrocytic vaccine is required as it aims to prevent or reduce the acquisition of clinical infection. By preventing either invasion of hepatocytes by sporozoites or pre-erythrocytic stage development within hepatocytes, a vaccine targeting the liver would preclude both the progression of disease, since clinical symptoms of malaria manifest only during the subsequent erythrocytic stage, and parasite transmission, since no sexual stages would develop [10]. This would benefit individuals who either are malaria naive or have lost their previously acquired immunity. It would also enhance the naturally acquired protective immune response of individuals resident in malaria-endemic countries that is achieved upon prolonged exposure, in order to either prevent blood stage infection or to reduce the numbers of parasites that emerge from the liver [11].

Preclinical vaccine trials may be conducted in murine malaria models to validate candidate antigens prior to testing in humans. One such antigen that has been studied extensively is the immunodominant circumsporozoite (CS) protein that is the major surface protein of sporozoites, the hepatocyte-invasive stage of the *Plasmodium* life cycle [12]. Passive transfer of a CTL clone recognizing *P. berghei* CS peptide SYIPSAEKI (aa 252–260) derived from mice immunized with irradiated *P. berghei* sporozoites conferred a high degree of protection to mice against homologous sporozoite challenge [13]. Furthermore, vaccination with this defined *P. berghei* CS peptide conferred protection against homologous challenge in mice which express MHC class I molecules of the H2-k^d haplotype when CS peptide-specific CD8⁺ T cells were elicited [14]. As both the target antigen and the protective immune response to it in this malaria model are characterized this provides a powerful tool for vaccinologists, as the potential of novel adjuvants to elicit an antigen-specific MHC-class-I-restricted CTL response may be assessed.

The objective of this study was to exploit the ability of the novel adjuvant tomatine to potentiate a CTL response against the *P. berghei* CS peptide in order to identify cytokine production of defined lymphocyte populations. This would have the dual effect of both helping to evaluate further the mechanism of action of tomatine as a vaccine adjuvant and to elucidate the mechanism of protective immunity against pre-erythrocytic malaria. The most successful vaccine against human malaria to date, protecting six of seven volunteers, contains regions of CS protein that stimulate peptide-specific CD8⁺ T cell responsiveness [15, 16].

2. Materials and Methods

2.1. Preparation of Tomatine-Antigen Vaccine. Tomatine was prepared with the *P. berghei* CS peptide by minutesor modification of a protocol described in detail previously [8, 17]. Briefly, the adjuvant comprised two mixtures, A and B, which were formulated as follows. *Mixture A.* 25 mg tomatine (Fluka, Gillingham, UK), 125 mg octylglucopyranoside (Sigma, Poole, UK) and 3.1 mg phosphatidylethanolamine (PE) (Fluka) were added to 4 mL sterile saline, vortexed and heated to 60°C until a clear solution was obtained, which was then allowed to cool to room temperature. *Mixture B.* 6.25 mg cholesterol (Sigma), 125 mg octylglucopyranoside and 3.1 mg PE were added to 3 mL sterile saline, the solution then prepared as for mixture A and allowed to cool to 37°C. *P. berghei* CS peptide SYIPSAEKI (aa 252–260) was prepared in-house by solid phase chemical synthesis and confirmed as 75% full-length product by high-performance liquid chromatography and mass spectrophotometry. 2.5 mg peptide was dissolved in 3 mL sterile saline and added to mixture A, vortexed and incubated at 37°C for 10 minutes, after which mixture B was added, vortexed and incubated at 37°C for a further 30 minutes. After vortexing, the completed formulation was placed at 37°C for 24 hours after which the resultant cloudy solution was dialyzed against sterile saline using a 10000 MW cutoff membrane (Slide-a-lyzer, Pierce, Chester, UK) to remove any unassociated octylglucopyranoside. A tomatine-saline adjuvant control was prepared by an identical method but without the addition of the CS peptide.

2.2. Immunization. Three experimental groups comprised naive mice or mice immunized with a preparation of either tomatine-*P. berghei* CS peptide or tomatine-saline. BALB/c (H2^d) inbred strain mice (Harlan Olac, Bicester, UK) were used when 6–8 weeks old. Female mice (four per group) were injected subcutaneously in the scruff of the neck with 200 μL of the adjuvant-antigen preparation (50 μg peptide/mouse) on day 0 and then again 28 days later.

2.3. Preparation of Splenic Lymphocytes. Mice were sacrificed on day 42 postprimary immunization (14 days after boosting). Spleens were aseptically removed and single cell suspensions in RPMI 1640 (Gibco, Paisley, UK) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (complete medium) prepared using a 20 μm sieve from each mouse individually as described previously [18, 19]. Erythrocytes were lysed with 0.17 M Tris-buffered ammonium chloride and membrane debris was removed by filtration through sterile gauze. Viability determined by trypan blue exclusion was routinely >95%.

2.4. Lymphocyte Subset Depletion. Lymphocyte subset depletion was performed by immunomagnetic cell sorting to >98% purity on splenocytes from mice immunized with tomatine-*P. berghei* CS peptide following ex vivo restimulation with homologous peptide (25 $\mu\text{g}/\text{mL}$), as previously

TABLE 1: Cytolytic activity of splenocytes from mice immunized with adjuvant-antigen preparations of tomatine-*P. berghei* CS peptide or tomatine-saline following ex vivo restimulation with *P. berghei* CS peptide (25 µg/mL). Control cytolytic activity of similarly stimulated splenocytes from naive mice is also shown. Data represent percentage specific lysis of P815 target cells loaded with *P. berghei* CS peptide or with no peptide (medium control) over a range of effector: target cell ratios. Data shown represent the mean of four mice, assayed individually in triplicate, from one of four similar experiments. Cytolytic activity following CS peptide stimulation (shown in bold): **P* < .025 versus tomatine-saline group, *P* < .05 versus naive group, ***P* < .05 versus tomatine-saline group, determined by the Mann-Whitney *U*-test. The positive control cytolytic T cell clone CS.C7 specific for *P. berghei* CS peptide elicited 81.2 and 53.0% P815 cell lysis at an effector: target ratio of 100:1 and 50:1, respectively.

Effector: target cell ratio	Percentage target cell lysis			
	Naive	Tomatine saline	Tomatine-CS peptide	Medium
100:1	6.8 (0.9)	3.7 (0.2)	19.2 (3.6)*	2.5 (0.1)
50:1	6.1 (0.3)	4.8 (0.9)	16.4 (1.4)*	4.3 (0.1)
25:1	5.1 (0.5)	3.0 (1.0)	9.7 (0.2)**	4.3 (0.5)
1:1	7.7 (0.8)	4.0 (0.2)	2.5 (0.8)	4.8 (0.5)

TABLE 2: Type 1 cytokine production by splenocytes from mice immunized with adjuvant-antigen preparations of tomatine-*P. berghei* CS peptide or tomatine-saline following ex vivo restimulation with *P. berghei* CS peptide (25 µg/mL). Control cytokine production of similarly stimulated splenocytes from naive mice is also shown. Data represent fold increases in secretion of each cytokine over that of splenocytes from identically immunized mice within each group but which were not restimulated ex vivo (absolute levels shown in italics). Data shown represent the mean of four mice, assayed individually in triplicate, from one of four similar experiments. IFN-γ production (shown in bold): **P* < .05 versus tomatine-saline group; *P* < .04 versus naive group, determined by the Mann-Whitney *U*-test.

Cytokine	Naive	Tomatine saline	Tomatine-CS peptide	Unstimulated cells (pg/mL)
IL-12	1.02 (0.32)	0.92 (0.44)	1.22 (0.27)	<i>97.84 (21.22)</i>
IFN-γ	0.88 (0.38)	1.48 (0.40)	3.48 (0.64)*	<i>153.17 (32.74)</i>
TNF-α	1.66 (0.61)	1.54 (0.41)	1.28 (0.71)	<i>39.08 (5.61)</i>

described [20, 21]. Lymphocytes were prepared from single-cell suspensions of spleen cells by Ficoll gradient centrifugation (Lympholyte-Mammal; Tebu-Bio, Peterborough, UK). Immunomagnetic cell sorting was performed for B, T, CD4⁺ and CD8⁺ T cells using Dynal mouse cell negative isolation kits (Invitrogen, Oxford, UK), following manufacturer's instructions for binding, washing and elution. The specificity of each depletion treatment was assessed by flow cytometry. Cells were incubated with rat IgG_{2b} monoclonal antibody (mAb) specific for murine CD20 (pan-B), CD3 (pan-T), CD4, and CD8 (AbD Serotec, Oxford, UK). Negative controls of cells incubated with normal rat serum in place of a primary mAb and of untreated cells were included. FITC-conjugated goat anti-rat IgG (Sigma) was used as the secondary mAb. Labelled cells were analyzed on a FACSCalibur flow cytometer (Becton-Dickinson, Oxford, UK) after correction for nonspecific fluorescence of controls and exclusion of dead cells and granulocytes on the basis of forward and right-angle light scatter.

2.5. Measurement of Cytolytic Activity. To assay for a CTL response, the cultured spleen cells were used as effector cells against P815 target cells labelled with ⁵¹Cr (Amersham Int., Little Chalfont, UK). P815 cells, which express H2-k^d molecules [13], were loaded with *P. berghei* CS peptide. A cytolytic T cell clone (CS.C7) specific for this peptide [13] was similarly assayed as a positive control. Three aliquots of 1 × 10⁶ cells were each labelled with 1 mCi ⁵¹Cr in 1 mL. Five micrograms CS peptide or 5 mL saline was added to the aliquots. The cells were then incubated for 1 hour at 37°C,

after which they were washed twice with 10 mL complete medium. Effector and target cells were incubated at ratios of 100:1–1:1 in V-bottomed microtitre plates (Gibco) for 4 hours at 37°C. The cells were pelleted by centrifugation at 200 × *g* for 2 minutes and 100 µL of the supernatant was added to 100 µL of scintillant (Optiphase HiSafe 3, Wallac, Milton Keynes, UK) in a 96-well plate (Isoplate, Wallac), mixed well and ⁵¹Cr activity was measured on a beta counter (1450 Microbeta, Wallac). Cytolytic activity was calculated using the following formula to determine target cell lysis:

$$\% \text{ lysis} = \frac{(\text{test release} - \text{spontaneous release})}{(\text{maximum release} - \text{spontaneous release})}$$

2.6. In Vitro Restimulation for Cytokine Production. To generate cytokine-containing supernatants, spleen cell suspensions were adjusted to a final concentration of 5 × 10⁶/mL in complete medium and 100 µL aliquots placed in 96-well flat-bottom tissue culture plates (Nunc, Roskilde, Denmark), to which were added 100 µL volumes of complete medium alone, or containing final concentrations of one of the following: 25 µg/mL *P. berghei* CS peptide, 1 µg/mL concanavalin A (Con A; Sigma), 25 µg/mL *Escherichia coli* lipopolysaccharide (LPS; Sigma) [20]. Cultures were incubated for 6 days (37°C, 5% CO₂); supernatants were removed, centrifuged at 300 × *g* for 5 minutes, and stored at –20°C until assayed.

2.7. Cytokine Measurement. Levels of the type 1 cytokines IL-12, IFN-γ and TNF-α were quantified by two-site sandwich enzyme-linked immunosorbent assay (ELISA) [22], using

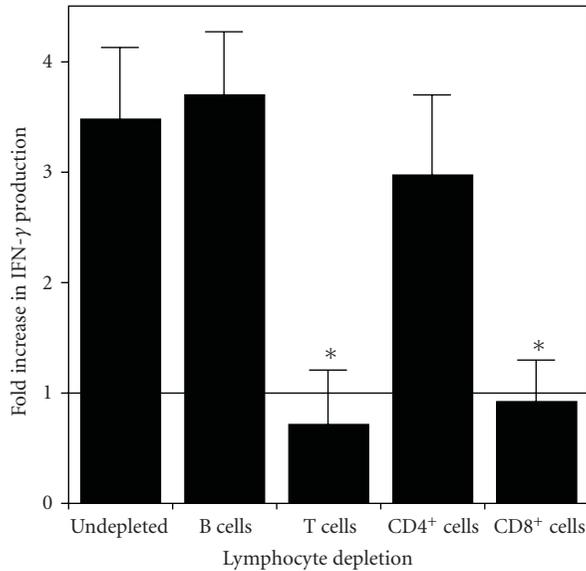


FIGURE 1: IFN- γ production by splenocytes from mice immunized with tomatine-*P. berghei* CS peptide following ex vivo restimulation with *P. berghei* CS peptide (25 μ g/mL). Lymphocyte subset depletion was performed by immunomagnetic cell sorting to >98% purity. Data are fold increases in cytokine secretion over that of splenocytes from identically immunized mice not restimulated ex vivo (by convention, value = 1.0, depicted as a horizontal line). Data shown represent the mean of four mice, assayed individually in triplicate, from one of four similar experiments. * $P < .05$ versus other groups, determined by the one-way ANOVA test.

DuoSeTTM matched antibodies (Genzyme, West Malling, UK) and following manufacturer's instructions. Reactivity was visualised using 3,3',5,5'-tetramethylbenzidine (TMB) in 0.05 M phosphate-citrate buffer and 0.014% (v/v) hydrogen peroxide (Sigma) as substrate. Optical densities were determined at 450 nm using an Emax plate reader (Molecular Devices, Crawley, UK). Recombinant murine cytokines (Genzyme) were used for calibration. Control samples of spleen cell culture supernatants from naive, nonimmunized mice, derived under identical conditions to experimental samples, showed low background cytokine levels to specific antigen (<5 ng/mL; SI \ll 2). The minimum level of detection for each cytokine was between 30–60 pg/mL.

3. Results

3.1. Cytolytic Activity of Splenocytes after Stimulation with *P. berghei* CS Peptide. The cytolytic activity of splenocytes was assessed by coinoculation with P815 target cells loaded with peptide. In the presence of homologous peptide (25 μ g/mL), cells derived from *P. berghei* CS-immunized mice produced significantly raised peptide-specific CTL activity at 100–50 : 1 effector: target ratios compared to splenocytes from either tomatine-saline immunized mice ($P < .025$) or from naive controls ($P < .05$) (Table 1). At a 25:1 effector: target ratio peptide-specific CTL activity was significantly greater

for splenocytes from tomatine-CS peptide-immunized mice than for splenocytes from mice immunized with tomatine-saline ($P < .05$) but not from naive controls ($P > .05$) (Table 1). There was no significant difference between groups at a 1 : 1 effector: target ratio ($P > .05$).

3.2. Cytokine Production from Splenocytes After Stimulation with *P. berghei* CS Peptide. Following restimulation ex vivo with *P. berghei* CS peptide (25 μ g/mL), splenocytes from mice immunized with tomatine-CS peptide showed significantly upregulated production of IFN- γ when compared to splenocytes from either tomatine-saline-immunized mice ($P < .05$) or from naive controls ($P < .04$) (Table 2). Production of the other type 1 cytokines measured, IL-12 and TNF- α , was similar for all three experimental groups ($P > .05$). The relatively low antigenicity of tomatine on its own was exemplified by the similar production of each of IL-12, IFN- γ and TNF- α by splenocytes from tomatine-saline-immunized mice compared to controls ($P < .05$) (Table 2). This therefore demonstrated the capacity of tomatine to act as an adjuvant for delivery of *P. berghei* CS peptide, notably for the induction of the pronounced production of antigen-specific IFN- γ .

In the presence of homologous peptide (25 μ g/mL), splenocytes from tomatine-CS peptide-immunized mice did not elicit a type 2-specific cytokine profile when compared to the response of similarly stimulated splenocytes from the adjuvant control and naive mice. IL-4 and IL-10 were measured in all supernatants but production of each was below the level of detection of the respective ELISA. Stimulation with the mitogens Con A or LPS induced splenocytes from each experimental group to produce predominantly type 1 and type 2 responses, respectively, (data not shown).

3.3. IFN- γ Production by Lymphocyte Subsets After Stimulation with *P. berghei* CS Peptide. Following restimulation ex vivo with *P. berghei* CS peptide (25 μ g/mL), splenocytes were depleted of B, T, CD4⁺ and CD8⁺ T cell fractions by immunomagnetic cell sorting. In each case contamination with residual cells was <2% of the undepleted population, as determined by flow cytometry. Splenocytes depleted of either B cells or CD4⁺ T cells produced similar levels of IFN- γ to undepleted splenocytes ($P > .05$). In contrast, depletion of either T cells or the CD8⁺ T cell subset ablated the IFN- γ response to the CS peptide ($P < .05$) (Figure 1).

4. Discussion

The purpose of an adjuvant is to elicit an appropriate and effective immune response against the antigen(s) with which it is administered. Protective antigens generally require a specific type of response to be induced if the immunized host is to combat effectively a challenge infection. Studies in various murine malaria models have shown that animals immunized with radiation-attenuated sporozoites develop parasite-specific CD8⁺ T cells and that depletion of such cells abrogates protection [23–25]. Moreover, this has been

corroborated by the recent development of genetically-attenuated sporozoites [26].

In the present study, immunization of mice with the defined *P. berghei* CS peptide SYIPSAEKI (aa 252–260) was used to examine the capacity of the novel adjuvant tomatine to potentiate antigen-specific cellular immune responses to pre-erythrocytic malaria. When restimulated with homologous peptide ex vivo, splenocytes derived from mice immunized with the tomatine-CS peptide vaccine elicited a peptide-specific CTL response and upregulated production of the type 1 cytokine IFN- γ . This supported our hypothesis that immunization with the tomatine-CS peptide vaccine might elicit a CD8⁺ T cell response if this peptide were presented in association with MHC class I molecules.

The pronounced production of IFN- γ by splenocytes from tomatine-*P. berghei* CS peptide-immunized mice upon CS peptide stimulation has particular significance, since this is recognized as a major host defence mechanism against liver stage malaria [10, 23]. The CD8⁺ T cell epitope for BALB/c mice within the *P. berghei* CS peptide appeared to elicit IFN- γ production by tomatine-CS peptide-immunized splenocytes ex vivo since when the cells were depleted of specific lymphocyte fractions, removal of CD8⁺ T cells ablated the IFN- γ response.

The pivotal importance of IFN- γ to the immune response to pre-erythrocytic malaria is well established. Recombinant IFN- γ inhibits the in vitro development of intrahepatic parasites [27, 28], while *anti*-IFN- γ mAb treatment abrogates protection in mice immunized with radiation-attenuated sporozoites [23, 29]. Administration of IFN- γ or IL-12 protects mice [27, 30] and monkeys [31, 32] against pre-erythrocytic malaria, and in mice the protection is reduced when the synthesis of nitric oxide (NO) is inhibited [30]. These findings suggest that CD8⁺ T cells may additionally perform a noncytolytic role in this protective immunity. Following induction by IL-12, CD8⁺ T cells produce IFN- γ which stimulates the production of inducible NO that subsequently mediates the elimination of the liver stage parasite [33, 34]. Indeed, perforin-deficient, CD95- and CD95L-mutant mice immunized with irradiation-attenuated sporozoites were each shown to be protected against a *P. berghei* challenge infection [35], indicating that this response alone is protective. It is probable therefore that sensitization of a CD8⁺ T cell population to produce high levels of IFN- γ promotes the induction of both cytolytic and noncytolytic mechanisms of protective immunity [12, 36].

5. Conclusions

The release of IFN- γ by CD8⁺ T cells is considered a critical component of immunity induced by liver stage malaria [36], and successful vaccination of humans with vaccines designed to elicit protective immunity will require induction of specific CD8⁺ T cells that home to the liver [37]. In this context, our findings validate the use of tomatine to potentiate a cellular immune response to antigenic stimulus by testing in an important biologically relevant system [38, 39]. Specifically, the processing of the *P. berghei* CS peptide

as an exogenous antigen and its presentation via MHC class I molecules to CD8⁺ T cells led to IFN- γ secretion that is an in vitro correlate of protection against pre-erythrocytic malaria [10, 40]. This was confirmed by the protective capacity of the tomatine-CS peptide combination upon in vivo immunization [19]. These findings merit further work to optimize the use of tomatine as an adjuvant in malaria vaccine development. Future studies are required to understand the mechanism by which tomatine generates antigen-specific CTL when formulated with soluble protein. In particular, the processing pathways used to load the MHC class I molecules and which antigen-presenting cells are involved are being investigated.

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Research Article

Pretreatment with Cry1Ac Protoxin Modulates the Immune Response, and Increases the Survival of *Plasmodium*-Infected CBA/Ca Mice

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Malaria is a major global health problem that kills 1-2 million people each year. Despite exhaustive research, naturally acquired immunity is poorly understood. Cry1A proteins are potent immunogens with adjuvant properties and are able to induce strong cellular and humoral responses. In fact, it has been shown that administration of Cry1Ac protoxin alone or with amoebic lysates induces protection against the lethal infection caused by the protozoa *Naegleria fowleri*. In this work, we studied whether Cry1Ac is able to activate the innate immune response to induce protection against *Plasmodium berghei* ANKA (lethal) and *P. chabaudi* AS (nonlethal) parasites in CBA/Ca mice. Treatment with Cry1Ac induced protection against both *Plasmodium* species in terms of reduced parasitaemia, longer survival time, modulation of pro- and anti-inflammatory cytokines, and increased levels of specific antibodies against *Plasmodium*. Understanding how to boost innate immunity to *Plasmodium* infection should lead to immunologically based intervention strategies.

1. Introduction

Each year, malaria infects approximately 500 million people and kills one to two million people, mainly children below the age of five years [1]. Despite decades of research on the subject, naturally acquired immunity to *Plasmodium* is still poorly understood [2–4]. There are reports about the immunosuppressive effects of *Plasmodium* infection in humans [5] and in animal models [6]. It is believed that the initial interaction of the parasitised red blood cells with the host immune system is one of the most important factors in determining the nature of the subsequent innate and acquired response, and in determining whether or not severe pathology, such as cerebral malaria, severe anaemia, or cachexia, results [7–9].

On the other hand, insecticidal proteinaceous crystals called Cry proteins are produced as protoxins by *Bacillus*

thuringiensis (Bt) during sporulation. Upon ingestion, crystalline protoxins are solubilised and proteolytically activated by midgut proteases of susceptible insects. The activated toxin, which is not toxic to vertebrates, binds to specific receptors on the brush-border membrane surface of the midgut epithelium of the insect, inducing the formation of pores and eventually leading to insect mortality [10]. In particular, Cry1Ac is a pore-forming protein that is specifically toxic to lepidopteran insect larvae and acts by binding to the cell-surface receptor aminopeptidase N in the *Manduca sexta* midgut via the sugar N-acetyl-D-galactosamine (GalNAc) [11, 12].

Although most studies on Cry proteins have been performed with regard to their toxicity in insects, we have described that recombinant Cry1Ac protoxin from *Bacillus thuringiensis* is a potent mucosal and systemic immunogen with adjuvant properties [13, 14].

In addition, we have shown that recombinant Cry1A toxins possess the ability to induce serum and mucosal specific antibody responses as well as to modulate IgG subclasses due to their strong immunogenic properties [14, 15]. Furthermore, it has been demonstrated that Cry proteins from *B. thuringiensis* can induce strong cellular immune responses. In particular, we have described that these toxins are able to promote IFN- γ responses [16]. In malaria infections, an initial IFN- γ response, mainly produced by NK cells, is implicated in the activation of macrophages, which leads to parasite elimination [17, 18]. In a previous study, we found that administration of the immunogenic protein with adjuvant properties, Cry1Ac protoxin alone or with amoebic lysates, markedly increased protective immunity against experimental *N. fowleri* meningoencephalitis in mice [13]. In this work, we determined the ability of Cry1Ac protoxin to activate the innate immune response. So we tested whether the pretreatment with the protein alone improved the resistance of mice to *Plasmodium chabaudi* AS and *P. berghei* ANKA experimental infections.

2. Materials and Methods

2.1. Mice and Parasites. CBA/Ca mice were kindly donated by Dr. W Jarra (National Institute for Medical Research, London). The mice were bred, fed, and maintained in a specific, pathogen-free environment at the FES Zaragoza, Universidad Nacional Autónoma de México animal house facility in accordance with the institutional and national official guideline NOM-062-ZOO-1999 for use and care of laboratory animals.

P. chabaudi AS and *P. berghei* ANKA were donated by Dr. William Jarra (National Institute for Medical Research, London).

2.2. Infection and Treatment. Batches of 6 to 8 sex- and age-matched (6–8 weeks) CBA/Ca mice were treated weekly with Cry1Ac protoxin (5 μ g/mouse i.p.) or with vehicle (PBS) during four weeks. One day after the last treatment, mice were inoculated intravenously with either 5×10^4 *P. chabaudi* AS- or 5×10^4 *P. berghei* ANKA-parasitised erythrocytes. On the days indicated, mice were sacrificed under ether anaesthesia. As controls, a parallel batch of noninfected mice was divided into two groups and treated with PBS or Cry1Ac at the same dose. Data presented are representative of two separate experiments.

2.3. Blood Sampling. Parasitaemias were evaluated daily by examination of Giemsa-stained blood smears. Numeration of the parasitaemia was performed under oil, using a Zeiss Standard 20 microscope (Carl Zeiss Ltd., Welwyn Garden City). Parasitaemias of 0.5% and above were determined by counting the number of parasitised erythrocytes present in a total of 200 red blood cells. Lower levels of parasitaemia were assessed by counting the number of parasitised erythrocytes present in 50 fields. The course of infection in each group is shown as the geometric mean of the percentage of parasitaemia.

2.4. Recombinant Cry1Ac *Escherichia coli* JM103 (pOS9300). The recombinant Cry1Ac *E. coli* JM103 (pOS9300) strain was kindly donated by Dr. Dean, from Ohio State University. The bacteria were grown in Luria-Bertani medium containing 50 μ g/mL of ampicillin, and Cry1Ac production was induced with 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) [19]. Recombinant Cry1Ac was purified from the IPTG-induced *E. coli* JM103 (pOS9300) cultures as follows. Cell pellets harvested by centrifugation were suspended in 50 mM Tris-HCl, 50 mM EDTA (pH 8) (TE buffer) and sonicated (Fisher Sonic Dismembrator Model 300) three times for 5 minutes on ice. Inclusion bodies were collected by centrifugation at $10\,000 \times g$ for 10 minutes, and pellets were washed twice with TE buffer, twice with 0.5 M NaCl, and once with 0.5 M NaCl-1% Triton X-100, once with 0.5 M NaCl, once with cold distilled water and were finally solubilised in CBP buffer (0.1 M Na₂CO₃, 1% 2-mercaptoethanol [pH 9.6]). Particulate material was discarded by centrifugation at $10\,000 \times g$ for 10 min, and the purified solubilised protoxin was stored at 4°C and examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined using the Bradford method [20]. The presence of endotoxin contamination in the Cry1Ac protoxin preparations was tested using the E-toxate, Part 1 kit (Sigma), which has a sensitivity limit of 0.05–0.1 endotoxin units (EU)/mL, following the manufacturer's instructions. Endotoxin levels in the purified Cry1Ac protoxin preparations were below 0.1 EU/mL. These preparations were further treated with an excess of a polymyxin B resin (Bio-Rad, Hercules CA, USA) to remove any possible remnants of endotoxin.

2.5. Cytokine mRNA Expression. Groups of CBA/Ca mice were treated weekly with Cry1Ac protoxin or with PBS for four weeks. Twenty-four hours after the last inoculation, mice were infected with either *P. chabaudi* AS or *P. berghei* ANKA. On the days indicated, three mice of each group were sacrificed under ether anaesthesia, and spleen mRNA was extracted using Trizol (Invitrogen, Carlsbad, CA, USA). DNA was digested with DNase I (Invitrogen) according to the manufacturer's instructions, and RNA was quantified spectrophotometrically at 260 nm. Next, 1.5 μ g of RNA were retrotranscribed using 1.5 μ g of oligo dT (Invitrogen), 0.5 mM dNTPs (Pharmacia, Uppsala, Sweden), 40 U RNase inhibitor, and 200 U MMLV-RT (Invitrogen). Next, 1 μ L of the resulting cDNA was used to amplify IFN- γ and TGF- β by PCR. Each sample was amplified in duplicate using a previously described method [21].

Each set of primers as well as the cDNA concentration was optimized for a number of cycles to obtain amplicons in the linear phase of amplification. The following gene-specific primer sequences were used: (IFN- γ) forward: 5' TGC ATC TTG GCT TTG CAG CTC TTC CTC ATG GC 3', reverse 5' TGG ACC TGT GGG TTG TTG ACC TCA TTG GC 3'; (TGF- β) forward 5' GAC CGC AAC AAC GCC ATC TA 3' reverse 5' GGC GTA TCA GTG GGG GTC AG 3'; (β -actin) forward 5' GTG GGC CGC TCT AGG CAC CAA 3', reverse 5' CTC TTT GAT GTC ACG

CAC GAT TTC 3'. PCR reactions were performed in a total volume of 20 μ L. Amplification was carried out in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1 mg/mL gelatin, 200 mM of each dNTP, 2 mM MgCl₂, 100 nM of each primer, 0.5 U Ampli Taq polymerase (Applied Biosystems, Branchburg, NJ, USA), and 15 ng of cDNA. The β -actin gene and either IFN- γ or TGF- β were then simultaneously amplified in a single tube. After 27–29 cycles, the PCR products were separated on 5% polyacrylamide gels and stained with ethidium bromide. Each band was analysed by densitometry, and the results are shown as the relation of the absorbance of the corresponding cytokine to that of β -actin in the same sample.

2.6. Cytokine Serum Measurement. On days indicated, mice from both the *P. chabaudi* AS- and the *P. berghei* ANKA-infected groups were sacrificed under ether anaesthesia. Immediately, blood from the heart was extracted and then centrifuged at 2000 \times g at 4°C for 15 min. The serum was removed and aliquoted into two tubes and snap frozen at -70°C until used.

The levels of the cytokines interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interferon- γ (IFN- γ), and tumour necrosis factor- α (TNF- α) in the serum samples were measured using a cytometric bead array performed according to the manufacturer's instructions (BD Mouse Th1/Th2 Cytokine CBA Kit Biosciences-Pharmingen, Heidelberg, Germany) with the following modifications. We performed all steps in microtubes, and we started the standard curve at a concentration of 0.625 pg/mL. The sensitivity achieved with these minor adaptations to the protocol was 0.9 \pm 0.05 pg/mL while the variation inter-assay was approximately 5%.

2.7. Measurement of Protective Antibodies. Antibody-specific responses were evaluated using a previously described method [21].

A lysate of pRBC was used as the capture antigen; it was prepared as follows. *P. chabaudi* AS- or *P. berghei* ANKA-infected mice (25% parasitaemia) were bled into PBS-heparin at 4°C to provide parasitised erythrocytes. The blood was passed through a CF11 cellulose powder (Whatman, Maidstone, UK) column to remove leukocytes and then washed three times with PBS by centrifugation at 750 \times g for 15 min at 4°C. The final cell pellet was resuspended to 5 mL in PBS, and 3 μ L of 10% (w/v) saponin in PBS was added to lyse the erythrocyte membranes. After centrifugation at 18,000 \times g for 5 min at 4°C, the supernatant was removed, the pellets were resuspended to 3 mL in PBS, and the cells were lysed by ultrasonication for 3 seconds with 21% amplitude (Ultrasonic Processor Model GE750, USA). The protein concentration of the lysates was determined using a Bio-Rad commercial reagent, and the lysate was diluted in carbonate-buffered solution to give a coating concentration of 10 μ g/mL. A volume of 100 μ L/well was applied to flat-bottomed 96-well ELISA plates (Corning USA). First, the plates were washed with 0.05% (v/v) Tween 20 in PBS, and then the excess binding sites were blocked using a solution of 3% skim milk in PBS for 2 hours at 37°C. The plates were

then incubated with test sera in duplicate for 1 h at 37°C and diluted to 1/20 in PBS. Plates were washed extensively before detection of parasite-specific Abs using goat antimouse horseradish peroxidase-conjugated monoclonal Abs (mAb) specific to IgG1, IgG2a, IgG2b, IgG3, total IgG, or IgM (Zymed, San Francisco California, USA) diluted in 0.02% skim milk, 0.05% Tween 20 in PBS to previously calibrated dilutions, which were applied to plates for 1 h at 37°C. Plates were washed before incubation with a streptavidin peroxidase solution (diluted 1 : 3000 in 0.05% Tween 20 in PBS) before the final wash. Plates were developed with ortho phenylenediamine at 0.4 mg/mL in citrate buffer (pH 5) with 0.03% of hydrogen peroxide as a substrate and incubated in the dark at room temperature for 20 min. Absorbance was determined at 492 nm by measurement of optical density (OD) using a Stat-Fax 2100 microplate reader (Awareness Technology Inc, USA). No standard of known concentration for each Ig isotype was available. Hence results were expressed directly as OD 492 nm values and compared to an internal standard of normal CBA mouse serum obtained from eight- to ten-week-old naive female mice. This internal standard provided a background value of nonspecific responsiveness to the lysate used.

2.8. Statistical Analysis. Statistical analysis was performed with the Stat Graphs software (version 5.1). Differences between groups were tested for statistical significance by nonparametric analysis of variance (Kruskal-Wallis). A *P* value <.05 was considered significant. All data are expressed as the mean \pm S.D. Each experiment was performed in duplicate.

3. Results

3.1. Cry1Ac Treatment Decreases Parasitaemia in CBA/Ca Mice Infected with Plasmodium chabaudi AS or P. berghei ANKA. Groups of CBA/Ca mice were injected once weekly for four weeks with Cry1Ac protoxin or PBS as described in the Materials and Methods. One day after the last injection, mice were intravenously infected either with *P. chabaudi* AS or with *P. berghei* ANKA. Mice treated with Cry1Ac protoxin prior to *P. chabaudi* AS infection developed a moderate parasitaemia that increased from day 6 postinfection (PI) to reach a peak of 27% at day 10 PI. Parasitaemia resolved spontaneously and was cleared by day 15 PI. In contrast, control mice treated with vehicle (PBS) developed higher parasitaemias from day 6 to 15 PI (significantly (*P* < .05) from days 8 to 11 PI) compared to mice treated with Cry1Ac protoxin. Parasitaemia reached a peak of 40% at day 10, and the parasite was completely cleared at day 16 PI, one day later than in the group of mice treated with Cry1Ac (Figure 1(a)).

In contrast, infection with *P. berghei* ANKA was lethal. In control CBA/Ca mice treated with vehicle (PBS), parasitaemia increased from day 5 PI to reach a peak of 23% at day 8 PI. There was a slight decrease in parasitaemia at day 9, and then mice started to die on day 10 PI with parasitaemias around 20%, confirming the reported lethality of this strain [22, 23]. Infected mice previously treated with

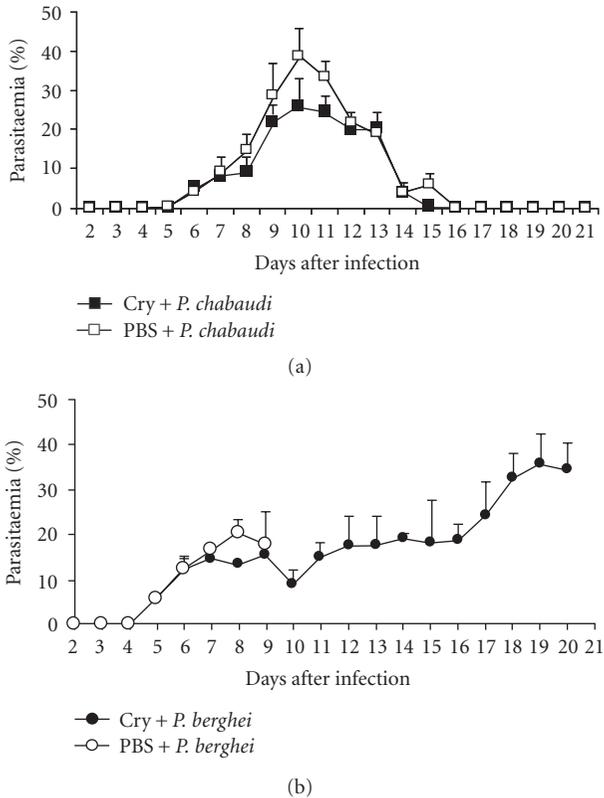


FIGURE 1: Effect of Cry1Ac on parasitaemia in CBA/Ca mice. Groups of eight mice were treated with Cry1Ac protoxin once a week for four weeks. One day after the last injection, mice were infected with *P. chabaudi* AS (a) or *P. berghei* ANKA (b). Groups of control mice were treated with PBS. Data are representative of two separate experiments.

the protoxin Cry1Ac developed lower levels of parasitaemia than PBS-treated mice from days 7 to 9 PI; the number of parasites in their blood decreased on day 10 PI and then started to rise slowly. Half of this group of mice survived until day 20 PI with parasitaemias of approximately 40% (Figures 1(b) and 2).

3.2. Cry1Ac Protoxin Increases Survival in Plasmodium-Infected Mice. Despite the fact that *Plasmodium chabaudi* AS is not considered to be lethal, infection with this parasite could be fatal for 10% to 20% of CBA/Ca mice. Interestingly, mice treated with Cry1Ac and infected with *P. chabaudi* AS had a survival rate of 100% compared to mice treated with PBS, which had a survival rate of 80%. On the other hand, mice treated with Cry1Ac and infected with the lethal parasite *P. berghei* ANKA showed an increased survival of 12 days compared to control mice treated with PBS, which died at day 9 PI (Figure 2).

3.3. Cry1Ac Protoxin Modulates Cytokine mRNA Expression in Splenocytes from Malaria-Infected Mice. RT-PCR analysis was performed to determine the levels of cytokine mRNA expression in splenocytes from infected and uninfected mice.

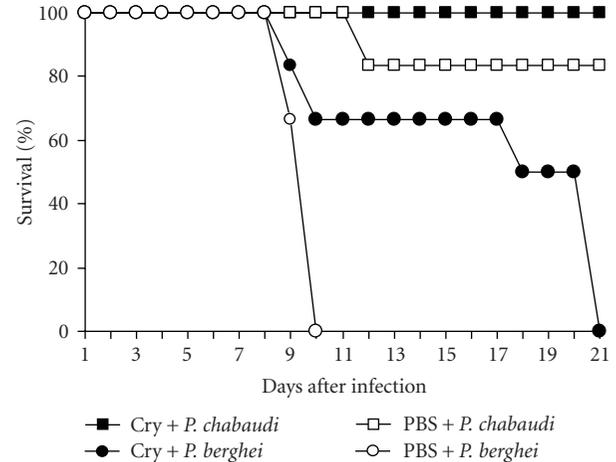


FIGURE 2: Effect of Cry1Ac on the survival of *Plasmodium*-infected mice. Cry1Ac pretreated mice ($n = 8$) were infected with *P. chabaudi* AS or *P. berghei* ANKA. Survival was recorded until day 21 PI, at which time all of the *P. berghei* ANKA-infected mice had died. Data are representative of two separate experiments.

Cells were obtained at days 4 and 8 PI with *P. berghei* ANKA and at days 8 and 18 PI with *P. chabaudi* AS. The levels of cytokine mRNA expression were normalised to β -actin mRNA levels, which was used as an internal standard. Moderate, constitutive mRNA expression of IFN- γ and TGF- β was detected in control mice receiving the vehicle, while in uninfected mice treated with Cry1Ac, similar levels of these cytokines were recorded (Figure 3).

In *P. berghei* ANKA-infected mice that previously had been administered Cry1Ac protoxin, increased levels of IFN- γ mRNA expression were detected at days 4 and 8 PI compared to infected mice receiving just the vehicle or uninfected mice. At day 4 PI, similar levels of TGF- β were found in both infected groups (slightly higher than those in uninfected mice). In contrast, at day 8 PI, the levels of TGF- β mRNA were lower in mice pretreated with Cry1Ac protoxin than in those receiving just the vehicle (Figures 3(a) and 3(c)).

In control mice infected with *P. chabaudi* AS, the levels of IFN- γ mRNA at days 8 and 18 PI were higher than those in mice pretreated with Cry1Ac protoxin, which exhibited IFN- γ levels akin to those in uninfected mice. On the other hand, the mRNA levels of TGF- β recorded in *P. chabaudi* AS-infected mice did not change significantly either by infection or by pretreatment with Cry1Ac protoxin.

3.4. Cry1Ac Modifies Cytokine Levels in Sera from Plasmodium-Infected Mice. The levels of cytokines (IFN- γ and IL-4) in serum samples were measured with a cytometric bead array. Samples were obtained at days 4 and 8 or 8 and 18 postinfection with *P. berghei* ANKA and *P. chabaudi* AS, respectively.

The levels of IFN- γ were significantly increased at day 8 PI with *P. berghei* ANKA in mice pretreated with Cry1Ac, confirming the RT-PCR results. At day 4 postchallenge,

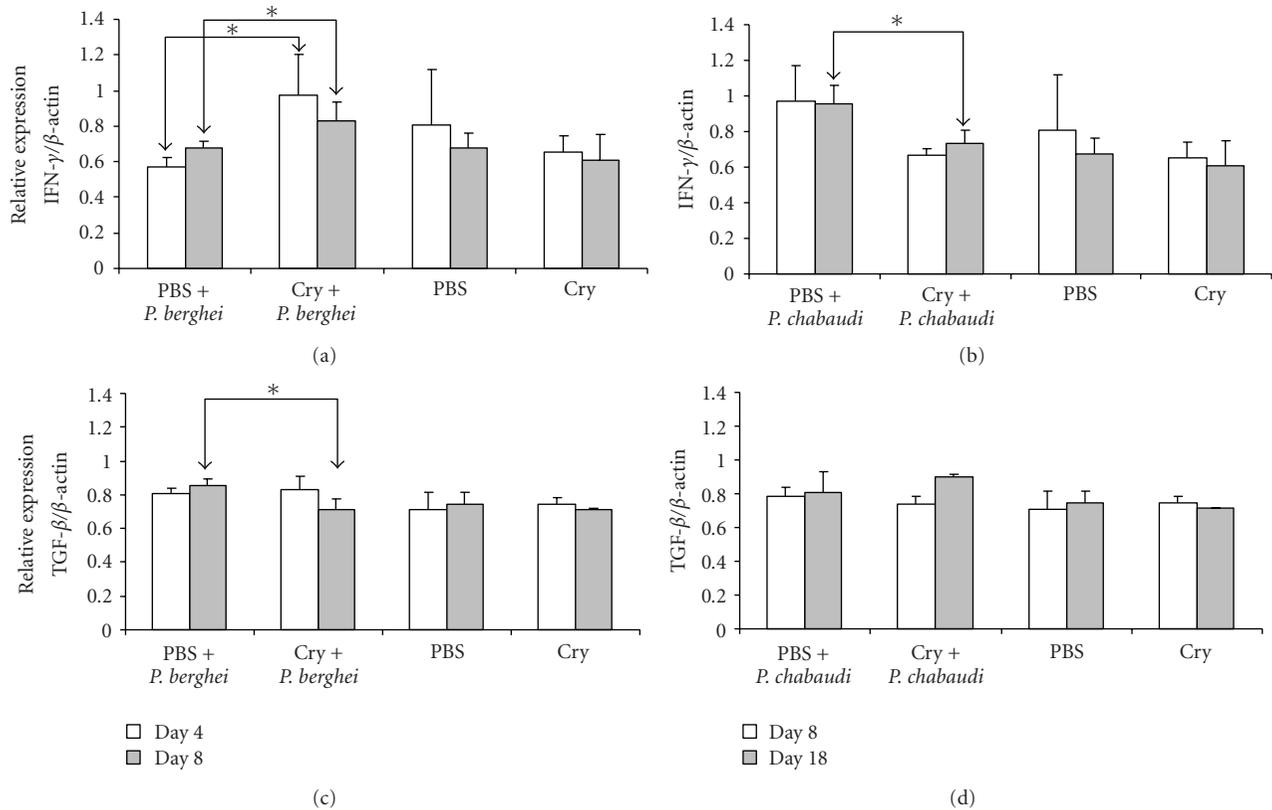


FIGURE 3: Effect of Cry1Ac on the mRNA expression of IFN- γ (a and b) and TGF- β (c and d) in mice infected with *P. chabaudi* AS or *P. berghei* ANKA. Mice were treated with Cry1Ac protoxin or PBS as a control. One day after the last injection, mice were infected with *P. berghei* ANKA or *P. chabaudi* AS. On days 4 and 8 PI (a and c) or days 8 and 18 PI (b and d), three mice from each group were killed, and their splenic mRNA was extracted and retrotranscribed. The cDNA obtained was used to amplify the β -actin gene and either IFN- γ or TGF- β by PCR in the same tube. PCR products were separated on 5% polyacrylamide gels. Bands were analysed by densitometry, and the results are shown as the absorbance of the corresponding cytokine divided by the absorbance of β -actin. Data are representative of two separate experiments. Asterisks indicate statistically significant differences between the indicated groups.

the IFN- γ mRNA levels recorded were low and akin to infected control mice, although they are greater than the levels in uninfected control mice. The levels of IL-4 were also low and did not vary significantly as a result of infection with *P. berghei* ANKA or administration of Cry1Ac protein (Figure 4). In contrast, following infection with *P. chabaudi* AS, the levels of IFN- γ were higher in control mice than in those pretreated with Cry1Ac protoxin. The IFN- γ levels induced after *P. chabaudi* AS infection were considerably lower compared to those elicited following *P. berghei* ANKA infection, but they were still higher than those present in uninfected mice.

In control *P. chabaudi* AS-infected mice, the levels of IL-4 increased at day 18 PI, while in mice pretreated with Cry1Ac, the levels of this cytokine did not change (Figure 4). Levels of IL-2, IL-5 and TNF- α were not modified by any of these treatments (results not shown).

3.5. Protoxin Cry1Ac Increases the Levels of Specific Antibodies for *P. berghei* ANKA and *P. chabaudi* AS in Infected Mice. Specific anti-*P. berghei* ANKA antibodies were induced in sera from mice infected with the parasite (at days 4 and 8

PI), while sera from control uninfected mice, which were untreated or received PBS or Cry1Ac alone, did not have detectable anti-*P. berghei* ANKA antibodies (Figure 5).

The treatment with protoxin Cry1Ac before infection increased the levels of IgG1, IgG2a, IgG2b, and IgM in *P. berghei* ANKA-infected mice. Interestingly, this increase was only detected on day 4 PI compared to mice receiving the vehicle alone. At day 8 PI, similar levels of IgG and IgM responses were induced in both experimental groups (Figure 5).

In infected mice pretreated with Cry1Ac, the specific anti-*P. chabaudi* AS IgG response was significantly higher on days 8 and 18 than that elicited in infected mice pretreated with the vehicle (Figure 6). Regarding the analyses of the different IgG subclasses, specific responses at day 4 PI of the four IgG subclasses (IgG1, IgG2a, IgG2b, and IgG3) were also significantly higher in the group receiving Cry1Ac before the infection compared to the group receiving the vehicle alone (Figure 5). However, at day 8 PI, the IgG2b responses detected were higher in the vehicle group with respect to the Cry1Ac group, while the IgG responses of the rest of the isotypes recorded were similar between the two groups.

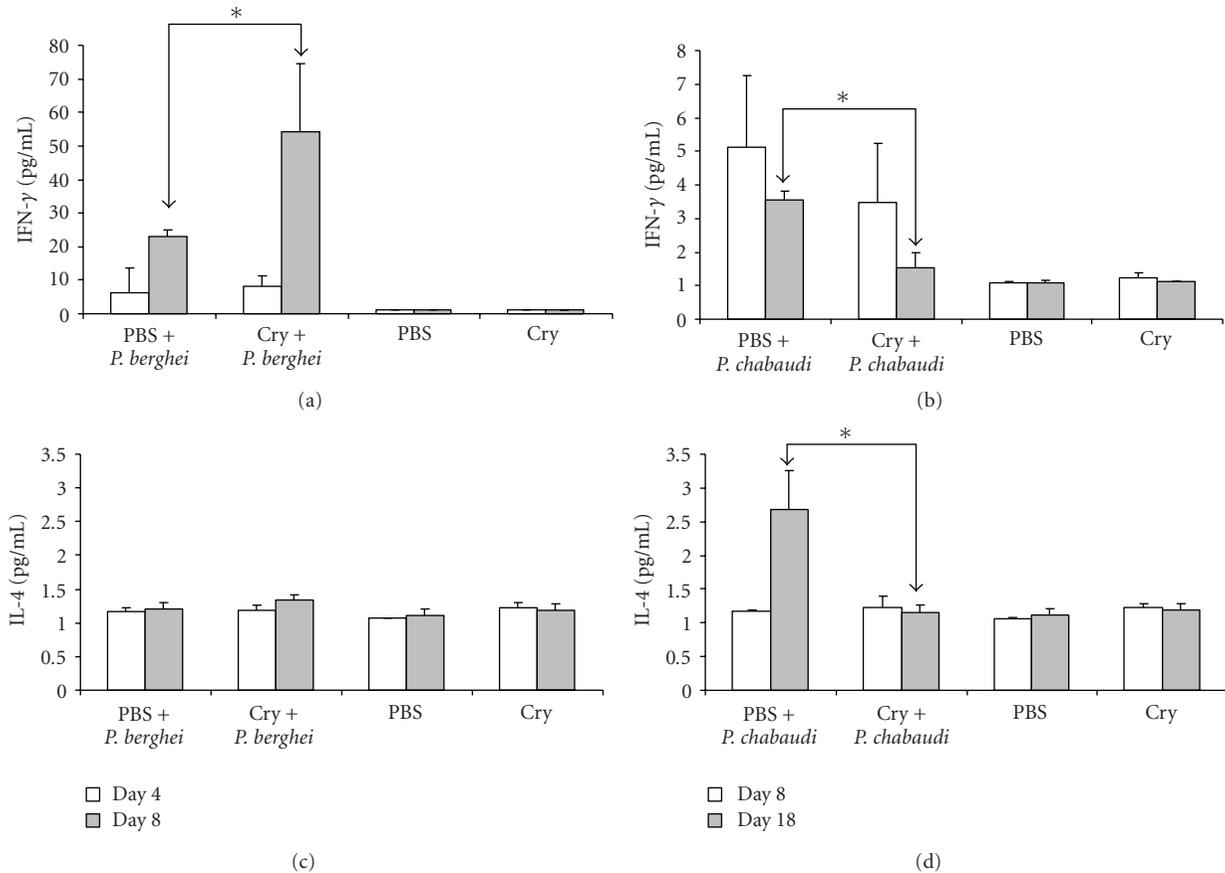


FIGURE 4: Effect of Cry1Ac on serum cytokines in *Plasmodium*-infected mice. Mice were treated with Cry1Ac protoxin or PBS once weekly for four weeks. One day after the last injection, mice were infected with *P. berghei* ANKA or *P. chabaudi* AS. On days 4 and 8 PI (a and c) or days 8 and 18 PI (b and d), three mice from each group were killed, their sera was separated from peripheral blood and the levels of the cytokines IFN- γ (a and b) and IL-4 (c and d) were measured by cytometric bead array. Data are representative of two separate experiments. Asterisks indicate statistically significant differences between the indicated groups.

The IgM-specific response at day 8 PI was significantly higher in the group that received Cry1Ac than in the group treated with vehicle.

The specific antibody responses recorded in mice infected with *P. berghei* ANKA were lower in relation to those elicited in mice infected with *P. chabaudi* AS. This result was expected since the latter group of mice had a longer period of antigenic stimulation. In sera from uninfected mice receiving either the vehicle (PBS) or Cry1Ac, specific anti-*P. berghei* ANKA antibodies were not detected.

4. Discussion

Our results demonstrate that administration of the Cry1Ac protoxin from *B. thuringiensis* induces protection against the malaria parasite when it is administered in CBA/Ca mice before infection with *P. chabaudi* AS and induces a longer survival time in *P. berghei* ANKA-infected mice (Figures 1 and 2). Protection was shown by lower levels of parasitaemia (first peak) in groups of mice infected with either *P. chabaudi* AS or *P. berghei* ANKA compared to

control mice. In addition, Cry1Ac protoxin modulated the mRNA expression of proinflammatory cytokines, such as IFN- γ and TGF- β , and increased the levels of IgG and IgM in both *P. berghei* ANKA- and *P. chabaudi* AS-infected mice.

Due to the different courses of parasitaemia between CBA/Ca mice infected with the lethal *P. berghei* ANKA, which killed all of the mice around day 9 PI, and the non-lethal *P. chabaudi* AS, the samples were analysed at different days (4 and 8 versus 8 and 18, resp.) to get the best comparison between both infections.

We evaluated the effect of Cry1Ac protoxin against *Plasmodium* infection because we have previously described that this protein may be a valuable tool for the improvement of mucosal vaccines; when Cry1Ac protoxin is coadministered as an adjuvant, it increases protective immunity against experimental *Naegleria fowleri* meningoencephalitis, an acute fulminant infection initiated at the nasal mucosa, in mice [13]. Interestingly, intranasal administration of Cry1Ac alone also had protective effects against *N. fowleri* infection, as this treatment increased

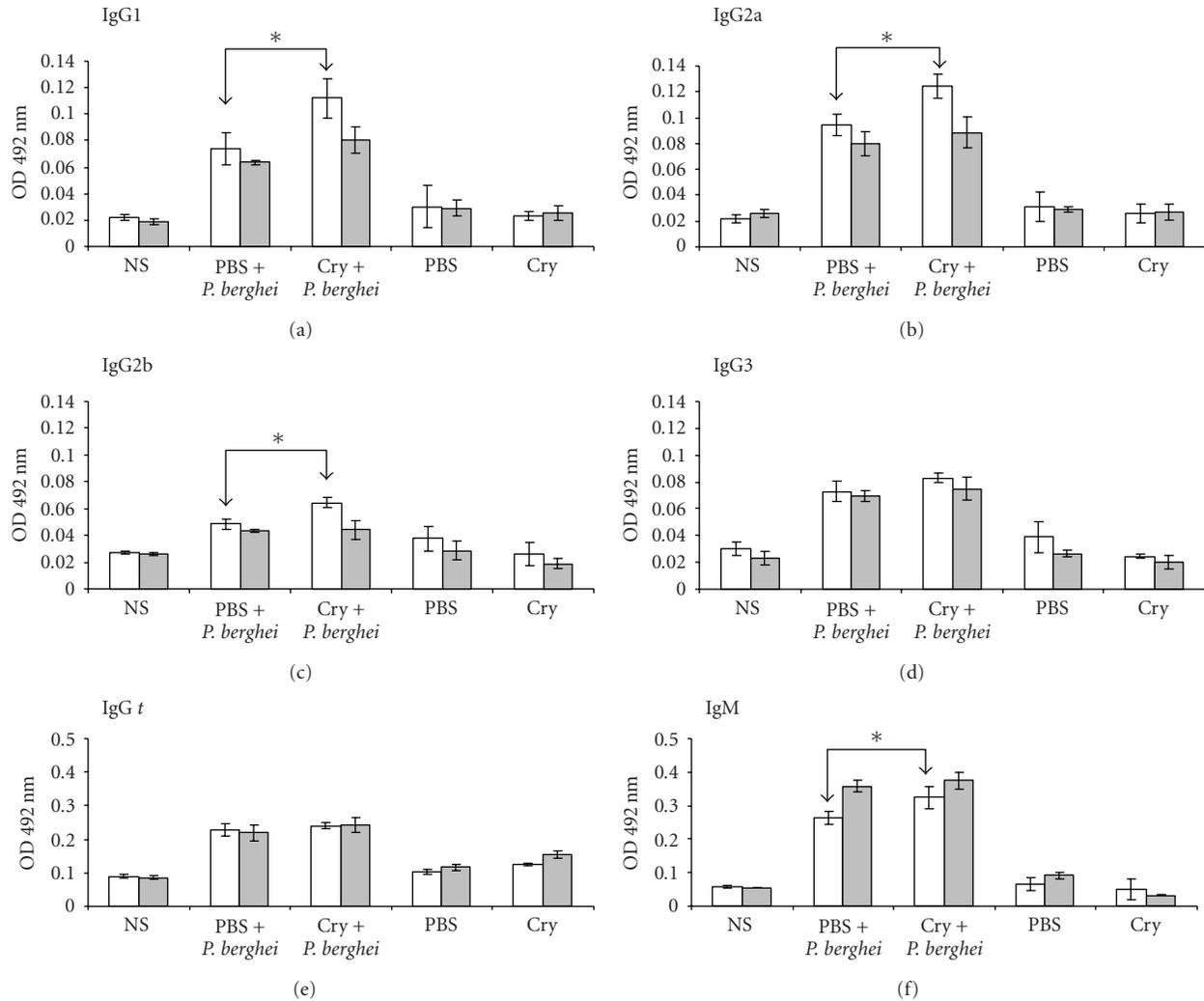


FIGURE 5: Parasite-specific antibody responses in mice treated with Cry1Ac and infected with *P. berghei* ANKA. Mice were treated with Cry1Ac protoxin or PBS as described previously and were infected with *P. berghei* ANKA. At days 4 (white bars) and 8 (grey bars) PI, the sera of three mice from each group were collected and measured by ELISA for antibody levels. The results are expressed as the mean OD value \pm SD ($n = 3$). Control absorbance values were provided by normal mouse serum (NS) obtained from age- and sex-matched CBA/Ca mice. Data are representative of two separate experiments. Asterisks indicate statistically significant differences between the indicated groups.

survival, as immunization with amoebal lysates alone did, suggesting that Cry1Ac protoxin may boost innate immunity.

In addition, it has been reported that Cry1Ac protoxin enhances the respiratory burst of human monocytes and neutrophils [24]. Accordingly, our unpublished results suggest that Cry1Ac activates mouse macrophages, inducing the expression of the costimulatory molecules B7-1 and B7-2 and the production of some proinflammatory cytokines (IFN- γ and MCP-1), but further studies are required to elucidate the mechanisms involved.

Despite the fact that Cry proteins are not toxic to vertebrates and Cry1Ac is known to form pores exclusively in the midgut epithelial cells of lepidopteran insect, the existence of an unknown receptor in mammals has been suggested, because Cry1Ac protoxin binds to brush border membrane vesicles prepared from mouse small intestine in vitro [14]. The nature of the molecules interacting with Cry

proteins in mammalian enterocytes seems to be different than the receptor glycoproteins described in insects, such as the 120-kDa aminopeptidase N [11] and the 210-kDa cadherin-like glycoprotein (Bt-R1) [25] because binding to its receptor was not inhibited by GalNAc, mannose, or biotin.

On the other hand, it has been shown that IFN- γ is able to activate macrophages, which are responsible for elimination of the malaria parasite [26–28]. Our results show that treatment with Cry1Ac protoxin decreased parasitaemia in mice infected with *P. berghei* ANKA at day 8 PI. At that time, downregulation of TGF- β expression and an increase in both mRNA expression and serum levels of IFN- γ were found (Figures 3 and 4). This finding is consistent with a previous report showing that protective immunity was associated with a decrease in TGF- β and a concomitant increase in IFN- γ production in *P. yoelii* 17XL-infected mice [29]. In addition, it has been shown

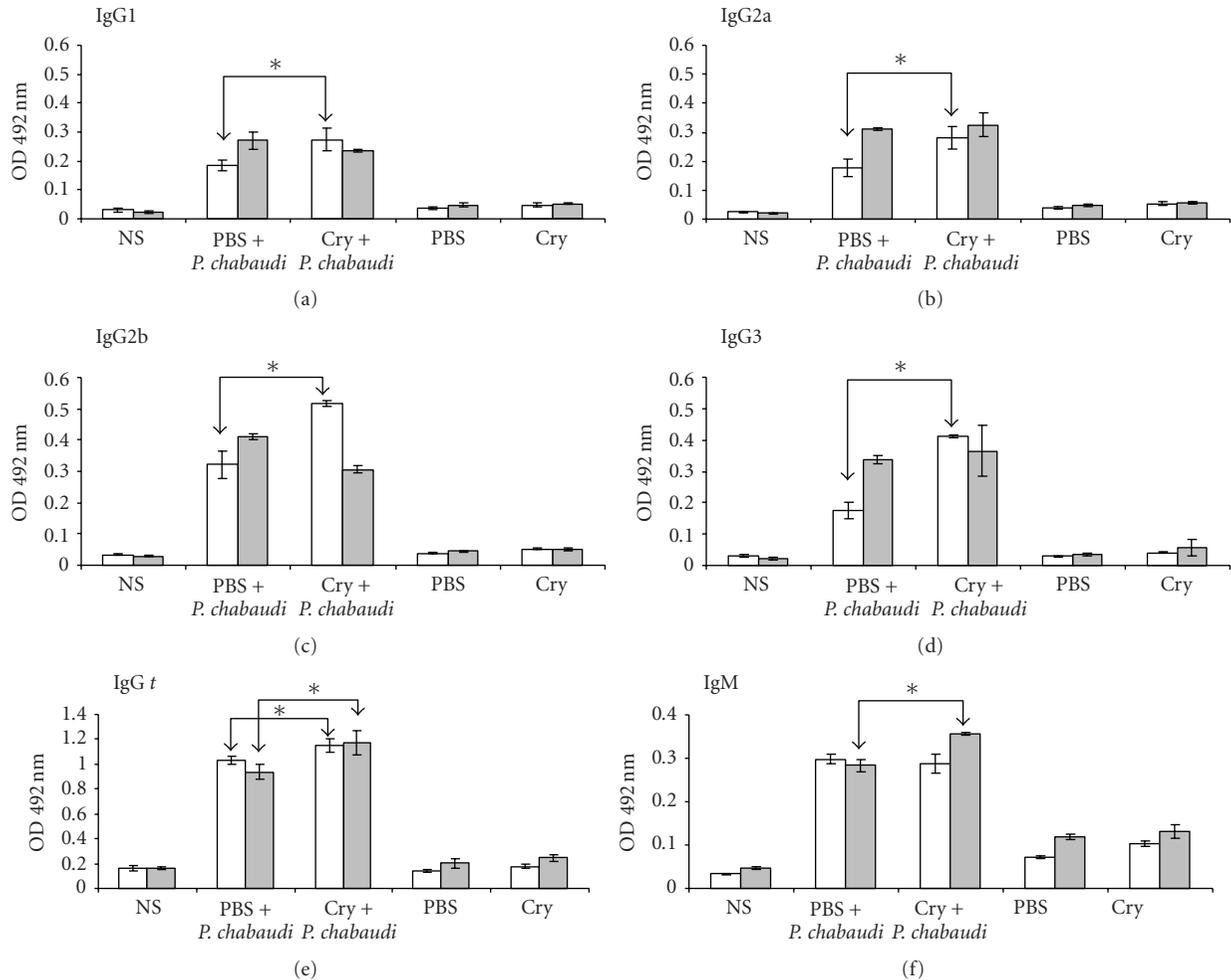


FIGURE 6: Parasite-specific antibody responses in mice treated with Cry1Ac and infected with *P. chabaudi* AS. Mice were treated with Cry1Ac protoxin or PBS as described previously and were infected with *P. chabaudi* AS. At days 8 (white bars) and 18 (grey bars) PI, the sera of three mice from each group were collected and measured by ELISA for antibody levels. The results are expressed as the mean OD value \pm SD ($n = 3$). Control absorbance values were provided by normal mouse serum (NS) obtained from age- and sex-matched CBA/Ca mice. Data are representative of two separate experiments. Asterisks indicate statistically significant differences between the indicated groups.

that upregulation of IFN- γ in *P. berghei* ANKA-infected mice leads to the activation of macrophage, which increases parasite elimination [30]. However, mice infected with *P. berghei* ANKA in our study were not fully protected, and all of them died at day 21 PI. A possible explanation for this finding is that the effect of Cry1Ac protoxin is limited; it could promote a decrease in parasitaemia in mice at day 8 PI, which, in turn, diminishes the antigenic stimulation and downregulates the immunopathology. As the parasite was not completely eliminated, it started to proliferate again, and by day 11, PI Cry1Ac protoxin no longer had an effect.

On the other hand, clearance of *P. chabaudi* in mice depends first on their ability to mount an early proinflammatory cytokine response and second on their ability to downregulate the inflammatory response before the onset of the immunopathology [31]. In our study, mice treated with protoxin Cry1Ac and infected with *P. chabaudi* AS more efficiently eliminated parasitaemia, despite the fact that this

group demonstrated a decrease in IFN- γ mRNA expression that correlated with decreased serum levels of this cytokine compared to mice treated with PBS (Figures 3 and 4). This fact could be related to the upregulation of TGF- β , which has been shown to play a double role in malaria infections; it is able to lead pro- and anti-inflammatory responses that may downregulate the production of IFN- γ [29]. It is also possible that the improved parasite elimination in the mice treated with Cry1Ac could be related to higher levels of IgG and IgM antibodies, which could be associated with better parasite elimination due to an increase in phagocytosis [32–36].

Treatment with protoxin Cry1Ac before *Plasmodium* infection induced a stronger antibody response in CBA/Ca mice to both *Plasmodium berghei* ANKA and *P. chabaudi* AS infections compared to control mice treated with PBS (Figures 5 and 6). Currently, the reason for this increase is not clear; however, this finding could be explained by a cross-reaction resulting from molecular similarities between

epitopes of *Plasmodium* and the Cry1Ac protoxin. We have performed ELISA assays in which the Cry1Ac protoxin or *P. berghei* ANKA antigen was bound to the plate and several dilutions of *P. berghei* ANKA immune mice serum were tested. In these assays, immune sera to *P. berghei* ANKA or *P. chabaudi* AS recognised Cry1Ac protoxin and developed higher OD values compared to normal mouse serum (data not shown), strongly suggesting that *P. berghei* ANKA and Cry1Ac share common antigens. However, further study is required to characterise the precise antigens involved.

Another possible mechanism, in which the pretreatment with Cry1Ac may increase survival in *Plasmodium*-infected mice and may increase both the levels of *Plasmodium*-specific antibodies elicited after the challenge and the levels of IFN- γ , may involve the activation of innate immune cells, such as antigen presenting cells, that permit a faster establishment of adaptive immune responses. In agreement with this proposal, our unpublished data indicate that Cry1Ac protoxin activates mouse macrophages, inducing the expression of the costimulatory molecules B7-1 and B7-2 and the production of proinflammatory cytokines.

The isotypes of protective antibodies in *Plasmodium* infections are under debate. In general, it is accepted that IgG1 and IgG2a are protective [37–40]. However, in some reports, IgG2b or IgG3 are also mentioned as being protective [41, 42]. In mice, it is well known that IFN- γ , the principal Th1 effector cytokine, regulates the production of the opsonising or cytophilic isotype IgG2a [43], which is in accordance with our results in *P. berghei*-infected mice, and that IL-4 is central to the synthesis of IgG1, while TGF- β is involved in the synthesis of IgG2b [38]. In our study, we found that pretreatment with Cry1Ac protoxin significantly increases the IgG subclasses assessed and IgM in *P. chabaudi* AS- or *P. berghei* ANKA-infected mice, which could be associated with parasite clearance because it has been shown that antibody responses play a critical role in immune protection against *Plasmodium* in asexual blood stages. This role has been demonstrated by passive transfer experiments using sera or purified immunoglobulins from adults residing in areas with hyperendemic malaria [44, 45]. However, the mechanisms by which malaria-specific antibodies interfere with the development and/or multiplication of the asexual stages of human *Plasmodia* are still unclear. It has been postulated that antibodies inhibit parasite growth in cooperation either with monocytes or neutrophils via antibody-dependent cellular inhibition [46, 47] or by immunophagocytosis through Fc receptors expressed on the cell surface after binding their parasite target [48]. In addition, a correlation between immune protection and the ability of serum to mediate opsonisation of infected erythrocytes has been described [49]. Further studies are required to clarify the mechanisms involved in boosting the innate immunity against malaria.

Like most adjuvants Cry1Ac protoxin might exert its activity by activating innate immune cells. The data presented in this study suggest that pretreatment with this protein could lead to design prophylactic strategies to improve

resistance against malaria infections. However, further studies are required to clarify the mechanisms involved in boosting the innate immunity against *Plasmodium* infection.

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Research Article

Excreted/Secreted Proteins from Trypanosome Procyclic Strains

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Trypanosoma secretome was shown to be involved in parasite virulence and is suspected of interfering in parasite life-cycle steps such as establishment in the *Glossina* midgut, metacyclogenesis. Therefore, we attempted to identify the proteins secreted by procyclic strains of *T. brucei gambiense* and *T. brucei brucei*, responsible for human and animal trypanosomiasis, respectively. Using mass spectrometry, 427 and 483 nonredundant proteins were characterized in *T. brucei brucei* and *T. brucei gambiense* secretomes, respectively; 35% and 42% of the corresponding secretome proteins were specifically secreted by *T. brucei brucei* and *T. brucei gambiense*, respectively, while 279 proteins were common to both subspecies. The proteins were assigned to 12 functional classes. Special attention was paid to the most abundant proteases (14 families) because of their potential implication in the infection process and nutrient supply. The presence of proteins usually secreted via an exosome pathway suggests that this type of process is involved in trypanosome ESP secretion. The overall results provide leads for further research to develop novel tools for blocking trypanosome transmission.

1. Introduction

Tsetse flies are vectors of African trypanosomes, the causative agents of sleeping sickness in humans and nagana in animals. Following a long period of increasing prevalence, human African trypanosomiasis now seems to be decreasing [1]. However, this debilitating disease still affects a wide range of people in sub-Saharan Africa [2] and is invariably fatal if untreated. Nagana is estimated to cost African agriculture US \$4.5 billion per year [3]. Until now, drugs have been unsatisfactory, some being toxic and all difficult to administer [1]. Furthermore, resistance to drugs is increasing [4]. Therefore, the search for novel strategies must continue and among them are alternative vector-based strategies [5]. These strategies require a clear and full understanding of the various steps and mechanisms involved in the transmission of the parasite. To be transmitted, trypanosomes must undergo cycles of development of varying complexity within the tsetse fly, transforming from bloodstream forms to

procyclic nonmammalian infective forms in the fly midgut (establishment). To complete their life cycle, *Trypanosoma brucei brucei* and the causative agents of human African trypanosomiasis, *Trypanosoma brucei rhodesiense*, and *Trypanosoma brucei gambiense* must migrate from the midgut to the salivary glands where they transform into infective metacyclic forms. The fly's ability to acquire the parasite, favor its maturation, and transmit it to a mammalian host is known as vector competence, which depends on complex interactions between *Glossina*, the parasite, and the environment. The factors involved in establishment are largely unknown and those involved in maturation are unclear. Nevertheless, among factors involved in vector competence appear to be the sex of the fly [6, 7], the trypanosome genotype [8], the tsetse intestinal lectin [9, 10], and the tsetse immune responses [11]. More recently, antioxidants have been shown to greatly increase midgut trypanosome infection rates in tsetse [12], suggesting that oxidative stress plays a role in the refractoriness of tsetse to trypanosome

infection. Moreover, a NO signal has been suggested to be required to promote the trypanosome migration to the salivary glands and its subsequent maturation into mammalian infective forms [13]. Different *Glossina* species exhibit large differences in vector competence [14, 15]. Nevertheless, vectorial competence of a given *Glossina* species could also be influenced by the nature of the parasite species [16]. Moreover, differences in the establishment and maturation rates have even been observed for different genotypes belonging to a given parasite subspecies [17]. These observations demonstrate the complexity of the mechanisms governing parasite development in the tsetse fly. Given that knowledge on the infection process that remains limited, further studies are required to characterize the overall *Glossina*-parasite molecular interactions.

The identification of the proteins secreted by the insect procyclic parasite may contribute to the increasing of the understanding of the infection process and lead to the identification of potential targets for drug and/or vaccine design.

In this respect, we have investigated the excreted/secreted proteins (ESPs) produced by two procyclic *Trypanosoma brucei* parasite subspecies (*T. brucei brucei* and *T. brucei gambiense*) and compared their respective secretomes.

2. Materials and Methods

2.1. Procyclic Parasite Cultures. STIB 215 [18] and Biyamina [19, 20] procyclic forms of *Trypanosoma brucei brucei* and *Trypanosoma brucei gambiense*, respectively, were grown at 25°C, in Cunningham's medium supplemented with 20% fetal calf serum. When the cultures displayed logarithmic growth, procyclic parasites were washed four times with phosphate buffer saline (PBS) and spun for 10 minutes for 2600 g at 25°C.

2.2. Excreted/Secreted Protein (ESP) Production. The parasites were resuspended at a concentration of $2 \cdot 10^8$ cells/mL in a secretion buffer [21] and incubated for 2 hours at 25°C for ESP production. During the incubation process, the parasite cell viability was controlled every 15 minutes by flow cytometry using the DNA intercalant propidium iodide (IP) procedure. Briefly, $2 \cdot 10^6$ parasites were incubated with 1 µg IP in 1 mL PBS as recommended by the manufacturer (Immunotech, Marseille, France). The cells were immediately analyzed with a FACScan flow cytometer (Becton Dickinson, Ivry, France) using an argon-ion laser. Cellular integrity was assessed by microscopic examination. After the 2-hour incubation, the secretion of ESPs was stopped by centrifugation of the parasites, 2600 g for 10 minutes at 4°C. The supernatant was collected and filtered on 0.2-µm filter and immediately mixed with protease inhibitors. The ESPs were then concentrated by ultrafiltration on a PM – 10 (10 KDa cut-off) membrane (Amicon) for further protein electrophoretic separation (SDS-PAGE). The protein concentration was determined by the Bradford dye binding procedure (Bio-Rad), and 300 µg of ESPs were further separated using SDS-PAGE.

2.3. Total Proteome Preparation. Parasite pellets were resuspended at a concentration of $2 \cdot 10^8$ cells/mL of laemmli buffer. The protein concentration was determined, and 100 µg of proteins of the total proteomes were further separated using SDS-PAGE.

2.4. One-Dimensional Electrophoretic Analysis. Proteins from the different samples (ESPs and total proteome) were heated at 100°C for 2 minutes and spun for 5 minutes for 14.000 g prior to separation using one-dimensional SDS-PAGE. Proteins were separated on 24 × 18 cm Tricine/SDS/urea-polyacrylamide gels (12% acrylamide) [22]. After migration, the gels were fixed, and the proteins were visualized using coomassie brilliant blue R-250. Pictures of the gels were taken with an Amersham Biosciences Personal Densitometer (Bio-Rad).

2.5. Protein Identification by Mass Spectrometry. Sixty four protein spots were excised manually from the one-dimensional gels of ESPs, washed, digested with trypsin, and extracted with formic acid. Protein digests were analyzed using either a triple-quadrupole mass spectrometer (Q-TRAP 4000; Applied Biosystems), coupled to a nanochromatography system (Dionex) or an ion trap mass spectrometer (Esquire HCT; Bruker), and interfaced with an HPLC-Chip system (Agilent). MS/MS data were searched against NCBI and *Trypanosoma brucei* databases using Mascot software. Raw data were analyzed using Data Analysis software (Bruker) to generate a peak list for searching a *Trypanosoma* database extracted from the Sanger Institute. The Mascot (v2.2) search engine was used with the following parameters: one missed cleavage allowed for trypsin, carboxymethylation of cyst as fixed modification, methionine oxidation as variable modification, and a 0.6-Da tolerance range for mass accuracy in MS/MS. At least one matching sequence of tags of high quality was needed for positive identification of proteins. Potential false-positive identifications have been addressed as described by Elias et al., (2005) [23], using identical search parameters against a database in which the sequences have been reversed. We set a false discovery rate (FDR) of 1%. When the Mascot peptide score was below (and even above) the Mascot peptide score indicated for an FDR of 1%, a systematic manual validation was done with stringent parameters (at least 6 y or b ions, at least 4 consecutive ions, and peptidic sequence formed of more than 7 amino acids). The proteins were classified according to MapMan (<http://mapman.gabipd.org/>).

3. Results

The main objective of the study was a comprehensive characterization of the proteins secreted by two different strains of *T. brucei* in procyclic form, *T. brucei gambiense*/Biyamina strain (whose bloodstream form is pathogenic for humans) and *T. brucei brucei*/Stib 215 strains (whose bloodstream form is pathogenic for animals). A three-step investigation was conducted and consisted in the following: (1) one-dimensional electrophoresis profiling of the secreted proteins

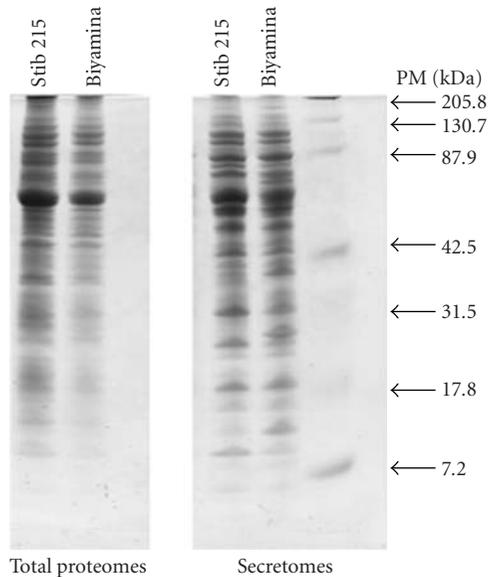


FIGURE 1: Protein profile of two different *T. brucei* strains. Coomassie blue-stained SDS-PAGE showing (from right to left) the marker (PM), secretome, and proteome from two procyclic *Trypanosoma* strains: Biyamina and Stib 215.

and comparison with the corresponding proteomes, (2) the identification of the secreted proteins by mass spectrometry and distribution into functional classes, and (3) a cross-comparative analysis between (a) the secretomes of the two strains (procyclic forms) and (b) the secretome of the procyclic forms and the proteome.

3.1. One-Dimensional Electrophoresis Protein Profiling of the Secretomes and Proteomes. The profiles generated by the electrophoretic separation of the secretome proteins from the two procyclic parasite strains (Biyamina and Stib 215) and their corresponding proteomes are shown in Figure 1. A high number of bands were separated, corresponding to proteins with molecular weights ranging from 7.2 to 205.8 kDa.

Clear differences were noted between the electrophoretic profiles of the proteome of the two procyclic strains and their corresponding secretome profiles, in terms of both proteic band intensity and presence or absence of several protein bands. Furthermore, since over the entire 2-hour secretion process (a) the parasites' viability remained constant and was greater than 98% and (b) the incubated trypanosomes presented normal morphology and motility, all these data indicate that the secretome resulted from an active export of proteins from living trypanosomes and not from cellular lysis.

The electrophoretic profiles of the proteome of the two procyclic strains show that they have many protein bands in common (32–34 visible bands); some differences in intensity were noted after coomassie staining.

Visual observation of the 1D gels showed similarities as well as some quantitative and qualitative differences between the secretome profiles of the two parasite subspecies.

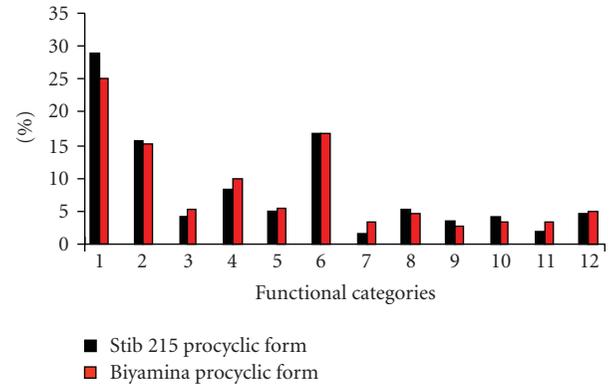


FIGURE 2: Classification of *T. brucei* proteins from two different procyclic strains into functional categories. Proteins from the two strains (Biyamina and Stib 215) were classified into 12 functional categories. The *x*-axis shows the following categories: (1) unassigned function, (2) folding and degradation, (3) nucleotide metabolism, (4) carbohydrate metabolism, (5) amino acid metabolism, (6) protein synthesis, (7) signaling, (8) cell cycle and organization, (9) lipid and cofactor, (10) transport, (11) redox, and (12) RNA/DNA metabolism. The *y*-axis shows the percentage of each category for each strain.

3.2. Identification of the Secreted Proteins and Grouping into Functional Categories. Secreted proteins from procyclic strains were fractionated by 1D SDS-PAGE, and 64 spots were selected all along the 1D gel for MS/MS analysis. This resulted in the identification of 427 secreted proteins from the Stib 215 (*T. brucei brucei*) strain and 483 proteins from Biyamina (*T. brucei gambiense*). Supplementary table 1 (in supplementary material available online at doi:10.1155/2010/212817) lists the proteins identified. They were classified into 12 main functional categories (Figure 2 and Supplementary table 1). Both of the procyclic strains' secretomes, the proteins associated with unassigned functions, those associated with (un)folding and degradation processes, and protein synthesis were quantitatively the largest categories, with a total of 60% of the secreted proteins (Figure 2). Moreover, lipid and cofactor, transport, RNA/DNA metabolism, cell cycle and organization, nucleotide metabolism, and amino acid metabolism contribute approximately 26% of the secreted proteins, and finally carbohydrate metabolism accounts for about 9% of the secreted proteins. Globally, the importance (expressed as the percentage of the proteins concerned) of each functional class is similar for both procyclic strains, except for the redox and signaling classes that are under-represented in the *T. brucei brucei* strain (Stib 215) secretome as compared with the *T. brucei gambiense* strain (Biyamina) (Figure 2).

3.3. Overlap between the Secretomes of the Two Procyclic Parasites. Comparing the secretomes of two procyclic strains (Figure 3) showed 279 common secreted proteins (57.8% and 65.3% of the Biyamina and the Stib 215 ESPs, resp.), 204 (42.2%) were specifically secreted by *T. brucei gambiense* (Biyamina), and 148 (34.7%) were secreted by *T. brucei*

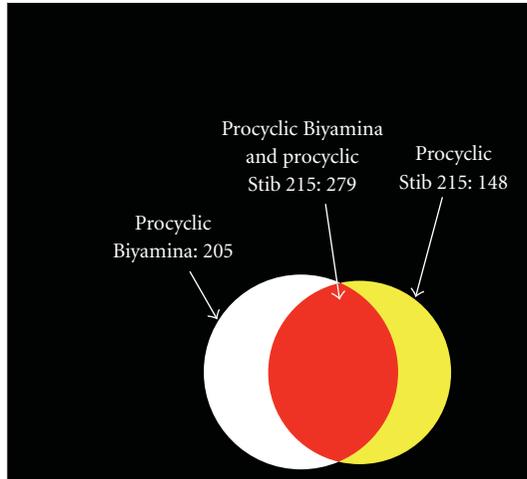


FIGURE 3: Overlap between secretomes of Biyamina and Stib procyclic strains. Proteins found in the analysis of Biyamina and Stib secretomes were compared. The red domain represents the proteins common to the two strains (279 proteins).

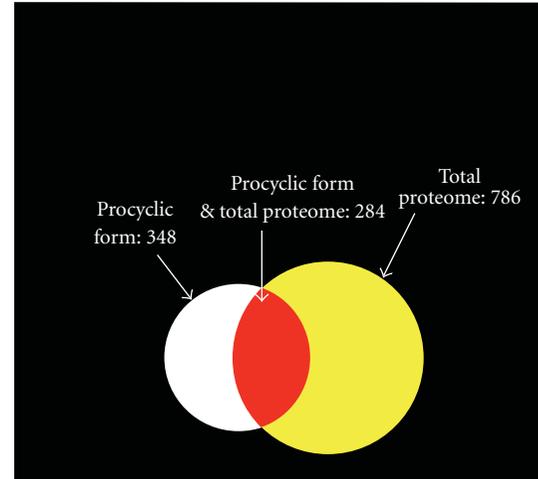


FIGURE 4: Overlap between all the proteins from procyclic secretomes (Biyamina and Stib) and total proteome [59]. Proteins found in the analysis of secretomes and *T. brucei* total proteome were compared. The red domain represents the proteins common in the two proteomes (284 proteins).

TABLE 1: Peptidase families found in the secretome of procyclic forms of parasites.

	Stib 215	Biyamina	Common
Metallopeptidase, family M16	+		
Cysteine peptidase, family C65	+		
Serine peptidase, family S10		+	
Major surface protease gp63, family M8		+	
Cysteine peptidase, family C1		+	
Metallopeptidase, family M32		+	
Cysteine peptidase, family C2			+
Serine peptidase, family S9A			+
Aminotripeptidase, family S8			+
Metallopeptidase, family M3			+
Metallopeptidase, family M24			+
Metallopeptidase, family M17			+
Metallopeptidase, family M1			+
Peptidase (M20/M25/M40 Family)			+

brucei (Stib 215). The proteins specifically secreted by either Stib 215 or Biyamina were represented in all 12 functional categories previously identified. However, the (un)folding and degradation protein class was particularly valuable because it grouped 14 different families of peptidases; four of which were specifically secreted by the *T. brucei gambiense* procyclic strain (Biyamina) and two others by *T. brucei brucei* (Stib 215). The other eight secreted peptidase families were common to both strains (Table 1). Surprisingly, we also identified a serine protease inhibitor among the proteins secreted by both strains.

More than one-third of the proteins (39.2%) specifically secreted by Stib 215 procyclic strain corresponds to hypothetical proteins, although they account for only 27.8% in Biyamina. Of major interest was the observation that some proteins, such as calreticulin and IgE-dependent histamine-releasing factor which belong to the class of signaling proteins, were specifically secreted only by the Biyamina strain; this strain also secreted higher levels (2.9%) of proteins belonging to the redox class.

3.4. Overlap between the Secretome of Procyclic Parasites and Total Proteome. Finally, only 45% of the proteins secreted by the procyclic strains were common to the proteins from the procyclic total proteome (Figure 4). This data also corroborates that the secretome results from an active protein exportation from living cells.

4. Discussion

The life cycle of *Trypanosoma* is complex and requires the expression of specialized proteins for the development of the parasite in both invertebrate and vertebrate host environments, to escape host immune responses.

For successful *Glossina* infection, the parasite has to adapt to its novel environment; it must switch both from a homoiothermic to a poikilothermic host and from a mammal to an insect. Furthermore, it must develop new weapons or choose among its panel of weapons to fight a novel type of host defense.

In vertebrate hosts, trypanosome bloodstream forms are protected by the variant surface glycoprotein (VSG), which prevents their recognition by the host immune system. When the parasites are taken up by the insect in the blood meal, they must confront the insect's immune system, mainly

composed of midgut proteases, physical barriers such as peritrophic matrix [24], reactive oxygen species [25, 26], and antimicrobial peptides [27]. A number of studies have revealed that the procyclins, surface glycoproteins expressed by trypanosome procyclic forms, may protect these forms against tsetse midgut proteases [28, 29]. However, there are yet no examples of direct interactions between tsetse fly and trypanosome molecules. The purpose of this study was to identify the whole excreted/secreted proteins in two trypanosome procyclic forms to better understand the interactions between the trypanosome and tsetse fly in terms of establishment in the midgut or vectorial competence and to search for new tools for vector control strategies.

We successfully used 1D electrophoresis and MS/MS to establish the first extensive protein map of the secretome of the procyclic form of the parasite using stringent mass spectrometry criteria to validate potential candidates (False Discovery Rate (FDR) < 1%). The substantial differences between the set of proteins secreted by both trypanosome strains and those of their respective total proteomes demonstrate that the proteins identified in the secretome are indeed secreted and are not an artifact resulting from possible cell lysis during the secretion step. Sequencing 1D gel showed that a large number of proteins were specifically secreted by both Biyamina (*T. brucei gambiense*) and Stib 215 (*T. brucei brucei*) (42.4% and 34.6%, resp.). The differences in the protein secretion profiles between the two parasite subspecies may reflect their genetic differences and could possibly be related to differences in *Glossina* vector competence [14, 15, 30–34].

To better understand the possible role of the secreted proteins, they were assigned to functional groups. The groups of unfolding and degradation proteins, protein synthesis, and proteins with no assigned functions were the most numerous.

Unknown or hypothetical proteins are a source of candidates that could be used to explore novel functions.

We did not expect to find so many different peptidase families among the unfolding and degradation classes of secreted proteins. Members of serine, cysteine proteinases, and metallopeptidases were identified. The group of proteases deserves the greatest attention because these enzymes cover a large panel of physiological and pathological functions. Consequently, representatives of this group are known to be virulence factors, to favor parasite invasion and its growth in the hostile host environment, to make it possible to escape the host immune defenses, and/or, finally, to produce nutrients by hydrolyzing host proteins. Some parasites such as *Plasmodium falciparum* produce a diversified panel of secreted and surface-bound proteases [35]. In a variety of animals, including humans [36] as well as arthropods [37], the blood clotting system plays an important role in the immobilization of invading parasites, and in preventing their dispersal from the site of initial invasion. Proteolytic degradation on the blood clots would allow escape from this confinement. How the parasite proteases process to inactivate or cause the inappropriate expression of host immune defense systems is a major question. In

addition to their direct role as virulence factors, secreted proteases are involved in various housekeeping functions during parasitic infection. As shown in *Porphyromonas gingivalis*, the proteolytic degradation of host proteins is able to produce nutritive elements for parasite growth [38]. Virulence has correlated positively with the expression of secreted proteases in a variety of systems where virulence can be modified genetically or in response to environmental cues. As a result, strains of *Leishmania mexicana*, engineered by targeted gene disruption of the genes encoding a pair of cysteine proteases, showed attenuated virulence [39]. The virulence of laboratory strains and clinical isolates of *Entamoeba histolytica* correlates well with the level of activity of a secreted cysteine protease [40]. In addition, inhibitors of serine proteases have been shown to block the entry of merozoites of *Plasmodium* into erythrocytes [41].

Surprisingly, an inhibitor of serine proteases (ecotin) has been discovered in the secretion products of trypanosome strains. The wide distribution of serine protease inhibitors, and their ability to regulate a variety of divergent proteinase-dependent physiological functions, shows that they are closely involved in a host of biological processes [42]. In several systems, serine protease inhibitors from viruses have been implicated in pathogen escape from the host immune system [43]. In *Brugia malayi*, cysteine-protease inhibitors that can inhibit host proteases involved in antigen processing have been discovered [44].

Besides contributing to protect the parasite against the host's defense mechanisms, many proteases show the capacity to induce perturbations in the host physiology. In addition to their protein degrading activity, proteases perform highly specific processing tasks that can affect protein structure, function, life span, and localization. By limited and specific cleavage, proteases can act as switches, turning protein activity on or off; they can also modulate protein function in more complex ways, regulating vital processes. Metallopeptidase of the M32 family has been found in the secretome of procyclic parasite. This family is absent in eukaryotic genomes other than trypanosomatids [45]. Consequently, proteases constitute both promising candidates for developing diagnostic tools and attractive drug targets to fight trypanosomes and subsequently to control sleeping sickness.

Proteins involved in signaling make up another group of proteins identified in the secretome of procyclic trypanosome, but even this is not a major class; some proteins, such as calreticulin, could play physiopathological roles. Autoantibodies against calreticulin are found in the sera of human hosts in a number of parasitic diseases [46] and it was suggested that the parasite-derived calreticulin could trigger an inappropriate immune response against self-antigens through molecular mimicry [47].

The class of proteins involved in protein synthesis is quantitatively (17%) well represented in the secretome. The role of such secreted proteins in the tsetse fly midgut is currently unknown. However, some proteins, such as elongation factor-1, were suggested to be a virulence factor in *Leishmania* [48].

We did not expect to find so many representatives of the carbohydrate metabolism protein class (9.79% in the *T. brucei gambiense* secretome and 8.27% in the *T. brucei brucei* secretome). Several papers have reported on the ability of the procyclic cell to adapt its metabolism to different environments. Because tsetse hemolymph contains abundant supplies of amino acids, and because the tsetse fly itself uses proline as a major carbon source during flight [49], it has long been speculated that in situ, the procyclic form of trypanosomes, might use proline. Accordingly, proline is a key component in the culture media currently used to cultivate the parasite. In contrast, another study has shown that, when grown in high glucose-concentrated media, the procyclic cells are susceptible to glycolytic inhibitors and to the downregulation of genes encoding glycolytic enzymes. This confirms the flexibility of the trypanosome metabolism and its adaptability to diverse growth conditions. Cells grown under chemostat conditions were shown to regulate their glucose metabolism according to the availability of proline [50]. Since the buffer we used to stimulate protein secretion contained sugar, it was not surprising to find a high level of enzymes involved in carbohydrate metabolism. Besides, procyclic forms establish mainly in the vector's midgut where sugar may be abundantly available immediately after a blood meal. Nevertheless, two major questions remain unresolved because glycolytic enzymes are expected to be intracellular enzymes. (1) Why is there such a high level of carbohydrate metabolism enzymes secreted? (2) What is the role of such enzymes when secreted in the fly's midgut?

The redox-protein class of secreted proteins was poorly represented. This finding was also unexpected because in the flies' midgut, reactive oxygen species are generated during immune responses or because of the abundance of heme molecules freed from the digested blood meal [51]. In addition, some of these proteins may play roles other than the detoxification of reactive oxygen. Therefore, a superoxide dismutase (SOD) has been shown to be secreted by *T. cruzi*; it was suggested that this enzyme was involved in both the parasite defense mechanism and the establishment of the parasite in the host [52, 53]. In another context, SOD was considered to participate in the immune escape in filarial infections [54, 55].

Finally, in the procyclic parasite secretome, we have identified 13 (GAPDH, clathrin heavy chain, Rab protein, ubiquitin, 14-3-3 proteins, cyclophilin, enolase, hsp70, actin, cofilin, tubulin α and β , and histone) out of the 22 proteins commonly associated with the exosome secretion pathway in various organisms [56]. Moreover, we have also identified translationally controlled tumor protein (TCTP) in this secretome. This protein was shown to be present in exosomes [57, 58].

These results suggest that the secretome proteins may be secreted via an exosome pathway that has never been described until now in trypanosomes. This also suggests that exosome-directed transmission-blocking vaccines could be developed and are capable of suppressing the capacity of the parasite to infect the vector and consequently preventing the spread of the parasite in human populations.

5. Conclusions

To conclude, the *Trypanosoma* parasite is a multistage organism that evolves in tsetse flies and vertebrates. Designing drugs that persistently interrupt the life cycle of this parasite requires a comprehensive understanding of its biology and the mechanism involving the vector-parasite interactions. The primary goal of our proteomic investigation was to identify secretome proteins of procyclic parasites to improve the knowledge of these interactions. The secretome encompasses a spectrum of proteins that may be required for every facet of the parasite's lifestyle, from the modification of the physiological environment to the immune escape. This may open leads to initiate novel strategies for controlling the parasites and sleeping sickness.

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Methodology Report

An Experimental Approach for the Identification of Conserved Secreted Proteins in Trypanosomatids

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Extracellular factors produced by *Leishmania spp.*, *Trypanosoma cruzi*, and *Trypanosoma brucei* are important in the host-parasite relationship. Here, we describe a genome-based approach to identify putative extracellular proteins conserved among trypanosomatids that are likely involved in the classical secretory pathway. Potentially secreted proteins were identified by bioinformatic analysis of the *T. cruzi* genome. A subset of thirteen genes encoding unknown proteins with orthologs containing a signal peptide sequence in *L. infantum*, *L. major*, and *T. brucei* were transfected into *L. infantum*. Tagged proteins detected in the extracellular medium confirmed computer predictions in about 25% of the hits. Secretion was confirmed for two *L. infantum* orthologs proteins using the same experimental system. Infectivity studies of transgenic *Leishmania* parasites suggest that one of the secreted proteins increases parasite replication inside macrophages. This methodology can identify conserved secreted proteins involved in the classical secretory pathway, and they may represent potential virulence factors in trypanosomatids.

1. Introduction

The Trypanosomatidae comprise a large group of parasitic protozoa, some of which cause important diseases in humans. The organisms that have been most extensively studied are *Trypanosoma brucei*, the causative agent of African sleeping sickness, *T. cruzi*, responsible for Chagas disease in South America, and *Leishmania spp.*, which causes Leishmaniasis in Asia, South America, and Mediterranean countries [1]. In order to complete their life cycle, these parasites have to adapt and develop in an insect vector and in a vertebrate host. These single-celled organisms have developed several strategies to modify their surrounding environment, modulate host immune responses, or interfere with the host's anti-microbial activity. Materials secreted by the parasite are involved in these processes helping the parasite survive in an environment more favorable for its own development [2–5]. In addition, previous studies indicate that trypanosomatid secreted factors elicit strong

immunity and protection against infection in mice and dogs [6, 7]. Thus secreted factors could be a source of antigens for vaccine development, as demonstrated in the pathogen *Mycobacterium tuberculosis* [8]. Nevertheless, all trypanosomatid secreted factors involved in virulence and/or representing putative vaccine targets are not currently known.

The availability of three draft trypanosomatid genome sequences provides valuable data for protein-mining using bioinformatic tools, especially for the localization or prediction of function for hypothetical proteins. Given that a significant number of trypanosomatid protein-coding genes are annotated as hypothetical, additional studies are needed to ascertain their function.

In the present study, we designed an experimental genome-based approach to identify potentially secreted proteins that are conserved among the three main trypanosomatid pathogens and involved in the classical secretory pathway. We hypothesized that a phylogenetic conservation

among *Leishmania*, *T. cruzi*, and *T. brucei* would indicate evolutionary selection for this family of proteins and suggest an important role for such secreted proteins in the biology of these parasites. Our results demonstrate that the bioinformatic analysis, combined with the functional tests, provides a useful and reliable method for the identification of novel secreted proteins, representing potential virulence factors in trypanosomatids. The results are also discussed in relation to the relative importance of the classical and nonclassical secretory pathways for the release of proteins into the extracellular environment.

2. Materials and Methods

2.1. In Silico Sequence Analysis. Release V 5.0 of the *T. cruzi* genome was extracted from the integrated *T. cruzi* genome resource TcruziDB (<http://tcruziidb.org/tcruziidb/>). Protein sequences that do not bear an initial methionine amino acid were removed manually. Proteins belonging to large families of surface molecules, which include trans-sialidases, mucins, gp63s, and mucin-associated surface proteins, were also discarded. Finally ORFs encoding proteins bearing a molecular weight (MW) above 90 kDa were also eliminated. The software SignalP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) was used to predict the presence of a signal peptide and a cleavage site in amino acid sequences [9]. Protein sequences having a signal peptide probability greater than 0.8 associated with a cleavage site probability greater than 0.7 were analyzed for the presence of orthologs in the related *Trypanosoma brucei* and *Leishmania major* parasite genomes predicted by Jaccard COG clustering in Gene DB (<http://www.genedb.org/>). Most of these orthologs were confirmed in TriTrypDB (<http://tritrypdb.org/tritrypdb/>) using OrthoMCL and genomic context analysis (gene synteny). The TMHMM 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) and the DAS-TMfilter (<http://mendel.imp.ac.at/DAS/source.html>) servers were used for the prediction of transmembrane helices in protein sequences.

2.2. Parasite Strains and Cultures. The *T. cruzi* TcY7 (or Y cl7) clone derived from the Y strain [10] was used throughout this study. Epimastigotes were grown in liver infusion tryptose (LIT) medium supplemented with 10% FCS at 28°C in standard conditions [11] and harvested during the logarithmic growth phase. Metacyclic trypomastigotes, obtained from the differentiation of late stationary phase epimastigotes, were used to initiate infection of mouse fibroblasts (L929). Trypomastigotes and amastigotes were produced and harvested as previously described [12]. Pellets for RNA purification were processed immediately in lysis buffer. The wild-type (WT) promastigote clone from *L. infantum* (MHOM/MA/67/ITMAP-263) was maintained at 26°C by weekly subpassages in SDM 79 medium supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, and 100 µg/mL streptomycin [13].

2.3. Reverse Transcription and PCR Amplifications. Total RNA was extracted from epimastigotes, amastigotes, and

trypomastigotes with the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturers' instructions, and treated with DNase I (DNA-free kit, Ambion Inc., Austin, Texas). Reverse transcription was performed for 1 µg of total RNA using random hexamers and Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. The cDNA (4 µL of 1/10 dilutions) from each stage was amplified by PCR in a 20 µL reaction volume using 10 µL of 2X PCR Master Mix 1X (Promega, Madison, Wisconsin) and 0.5 µM gene-specific forward and internal reverse primers (listed in Table 1) using the following cycling conditions: 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C to 58°C (according to the primer pair) for 30 seconds, 72°C for 45 seconds, and a final elongation at 72°C for 5 minutes. Amplicons were electrophoresed on 2% agarose gels stained with ethidium bromide.

2.4. Cloning and Sequencing. The encoding genes selected by in silico analysis were cloned into the pTEX expression vector, carrying the Neomycin resistance gene (NEO) [14]. Full length ORFs were amplified from genomic DNA with specific reverse and forward primers, including different restriction sites and a 6-Histidine-Tag in the C-terminal region (listed in Tables 1 and 3). PCR reactions were carried out in 20 µL using 0.5 µM of each primer, 0.2 mM dNTP, 0.4 U of Phusion high-fidelity polymerase (Finnzymes, Espoo, Finland), and the following cycling conditions: 98°C for 30 seconds followed by 25 cycles of 98°C for 10 seconds, 64°C to 68°C for 15 seconds, 72°C for 25 to 60 seconds (according to gene size), and a 72°C elongation for 5 minutes. Digested and purified fragments were inserted into the dephosphorylated pTEX vector digested with the corresponding restriction enzymes. Cloned sequences were confirmed by restriction digestion and sequencing. Large-scale preparations of the different constructs were performed using the Plasmid Midi kit (Promega).

2.5. Transfection Procedures. Promastigotes of the *Leishmania infantum* clone were electroporated as described elsewhere [15]. Briefly, promastigotes were washed twice with HEPES-NaCl buffer saline (21 mM HEPES, 5 mM KCl, 0.7 mM NaH₂PO₄, 137 mM NaCl), resuspended at 10⁸ cells/mL in HEPES-NaCl electroporation buffer (pH 7.2) supplemented with 6 mM glucose and cooled on ice for 10 minutes. Cells (10⁸) were combined with 15 µg of vector, left on ice for 10 additional minutes, and electroporated using an Easyject Plus (Eurogentec, Seraing, Belgium) apparatus set at 450 V and 450 µF, for one pulse. The cells were left on ice for a further 10 minutes and transferred to 4 mL of growth medium. The antibiotic G-418 (20 µg/mL) was added 24 hours later, and parasites were subcultured at a dilution of 1/10 in 5 mL SDM in the presence of 20 µg/mL G418. Drug-resistant cells were observed 15–20 days later. Parasites were grown in the presence of gradually increasing concentrations of G418 and were routinely maintained in SDM containing 150 µg/mL of G418.

TABLE 1: Primer pairs designed to amplify the genes encoding the 13 *T. cruzi* putative secreted proteins conserved in trypanosomatids and *T. cruzi* Tubulin (negative control).

<i>T. cruzi</i> GeneDB Gene ID No.	Primer sequences ^(a)	F/Rint and F/R product sizes (bp)	MW (kDa) ^(b)
Tc00.1047053506417.30	F CATGAGCTTACTAGTATGTTGTCT CTGGCAGAAGTGTGT Rint ACGGTGCCCAAAGGCGTGTA R CACACGGAAGCTTTCAATGATGATGAT GATGATGGCGACCAAACCTAGCCATAAG	311 705	25.7
Tc00.1047053506155.99	F CTGGGGGAATTCATGCGGTGG ATTTTTTGTACTTGCC Rint CCGATACGTCCACCACCCCTC R CGTCGGAAGCTTCTAGTGATGGTGGTG GTGATGGACAAGTTCGTGGCATGTAATTG	336 735	28.1
Tc00.1047053506467.29	F ACACGGACTAGTATGATTG TATGAATGGAATTTCTGAG Rint CTAATAGTCCGAAGTCGTTGCG R CTACACAAGCTTTTAGTGATGGTGATG GTGATGGCTGCGCCTCCACACCGTGC	309 1065	39.7
Tc00.1047053511901.30	F CTGATAGGCACTAGTATGTTTC CGGCGCAGGAATTCCT Rint CCCCTTTCAGGTGACCATTACAAGAG R GCCGTCAAGCTTTTAGTGGTGGTGGTG GTGATGCTCCGCTCCCAACTCAAACGA	316 1041	39.8
Tc00.1047053511871.30	F CTGATAGGCACTAGTATGCGTC GCACTTATTTGTCTG Rint CTCTCCAACCTCGTACGGCGA R CTGCAGGCAAGCTTCTAATGGTGGTGATG GTGATGTTATGATACCGGCATCAAGTCCCC	305 1269	47.0
Tc00.1047053505789.10	F CGCACTCACTAGTATGCCCTCT GGCAAAGCAACTG Rint TCACTGCTCCGCCCTGGTTTC R CGCTCCCTCGAGTTAGTGGTGATGGTGGTG ATGGGCAGCATTACCGACCCTGA	308 1488	53.6
Tc00.1047053509669.70	F GCTCAGCCAAGCTTATGCG CACTTCTTCTGCCGTGT Rint ATCGGGGAGTTTTGTGCAGGTTGAG R GTGGTCTTCTCGAGTTAGTGATGGTGGTG GTGATGGTGCAGCTTAATGCTCGCGTATA	322 1944	73.1
Tc00.1047053507765.20	F CTGCCAGTACTAGTATGT CTGCTAAAGCCTCACGGC Rint TCCAGGTAGTCACCCATTCCGTG R CTCAGCCAAGCTTTTAATGATGATGATG ATGGTGTGCTGCTCGCCTCACAGTGCT	318 1521	57.2
Tc00.1047053510101.470	F CTGCGCTGGACTAGTATG TCTGTTAAAGCCTCACGGCG Rint CCATTCGGTGACCGCCGTAGAC R CTCGGTAAAGCTTTTAATGATGATGATG ATGGTGTGCTGCTCGCCTCACAGTGCT	302 1518	57.3
Tc00.1047053510443.30	F CTCGCTGGAATTCATGCGGTGG GTGATAGTTGTATTTGC Rint CGCCAACAACGTAGTTGCCAAG R ACGGACCTCGAGTTAGTGATGGTGGTG GTGATGCTTGTGAGTTTGAGCGGCG	313 612	23.0

TABLE 1: Continued.

<i>T. cruzi</i> GeneDB Gene ID No.	Primer sequences ^(a)	F/Rint and F/R product sizes (bp)	MW (kDa) ^(b)
Tc00.1047053509799.50	F CGCGGGACTAGTATGAAACAA AAAATGCGACGCAAATTG Rint GTGAGGATGGGGAACCAAAAGAGTC R CAGCCAAGCTTCTAGTGATGGTGATGATG ATGGACATTCTTCTCTTTGTAAAGTAG	297 687	26.5
Tc00.1047053509835.30	F CGCGGCACTAGTATGTATT CATGTTTGTGCTGAGGC Rint GCAGCAACGGCAACAAAGAGC R CATGGCAAGCTTTTAGTGATGGTGGTG GTGATGCTCCTCT CTGGGTTTCCTTCG	324 2031	71.6
Tc00.1047053509999.10	F CGCGCCACTAGTATGTACG TCGTGCTTTTTTTCGTTT Rint CGCATATTTCCGCTCCGTTCC R AGCAGTCCAAGCTTTTAGTGATGGTG ATGATGATGGCCGACCAGCGCTCCAGAA	305 1227	46.6
Tc00.1047053506563.40 ^(c)	F GGGTGCCACTAGTATGCGT GAGATTGTGTGCGTTCAG Rint GGGCGGAAGATCTGCCCGTATG R AGCGCTCAAGCTTTTAGTGATGGTGGTG GTGATGGTACTGCTCCTCCTCGTCAACT	259 1329	49.6

F: Forward primer including the start codon; Rint: Internal reverse primer for RT-PCR; R: Reverse primer used for the amplification of the full-length ORF.

^(a)Restriction sites used for cloning in the pTEX vector in italics and the His-Tag sequence in bold.

^(b)Expected molecular weight of the proteins (deduced from amino acid sequences).

^(c)Beta-tubulin gene.

2.6. PCR Amplifications in Transfected Parasites. PCR amplifications were carried out to check for the presence of the NEO gene and the corresponding gene in transfected parasites. A fragment of 800bp corresponding to the NEO gene was amplified with specific forward and reverse primers (F5' ATGATTGAACAAGATGGATTGCACGCA-GG 3', R5' TCAGAAGAAGCTCGTCAAGAA 3'). Full length ORFs of the specific genes were amplified with primers listed in Table 1. PCR reactions were carried out in a 20 μ L reaction volume using 10 μ L of Master Mix 1X (Promega, Madison, Wisconsin) and 0.5 μ M NEO and gene-specific forward and reverse primers using the following cycling conditions: 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds, 55°C to 58°C (according to the primer pair) for 30 seconds, 72°C for 45 seconds to 2 minutes (according to gene size), and a final elongation at 72°C for 5 minutes.

2.7. Production of Cell-Free Culture Supernatants. To analyze the presence of secreted proteins in the supernatant, 1×10^9 *L. infantum* promastigotes from log-phase culture were collected by centrifugation, washed twice in HEPES-NaCl buffer, resuspended in 40 mL of HEPES-NaCl (pH 7.2), 11 mM glucose, 200 μ g/mL G-418, and incubated for 6 hours at 27°C. Parasite viability was then assessed as previously described [16] and harvested by centrifugation at 2,100 g for 10 minutes at 4°C. The parasite pellet was stored at -80°C for subsequent SDS-PAGE analysis and the supernatant was filtered through a low retention 0.45 μ m PVDF filter membrane (Millipore, Boston, Massachusetts). After addition

of protease inhibitor cocktail (Sigma-Aldrich) the filtrate was concentrated up to 80-fold using an Ultra-Centrifugal Filter device, according to manufacturers' instructions (Amicon Bioseparations, MilliporeCorp). The concentrated cell-free culture supernatant was frozen and stored at -80°C.

2.8. Production of Parasite Lysates. Cell pellets of wild-type and episomally transfected *L. infantum* promastigotes were resuspended in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% Sodium deoxycholate and 0.1% SDS), incubated on ice for 30 minutes and sonicated three times for 20 seconds. The soluble phase was recovered by centrifugation at 10,000 g for 30 minutes (4°C) and the protein concentration was determined using a Bradford protein assay (Bio-Rad Laboratories, Hercules, California).

2.9. Gel Electrophoresis and Western Blot Analysis. Proteins from parasite lysates (35 μ g) or from 80 \times concentrated cell-free supernatants (2 μ g) were separated on an NuPAGE Bis-Tris gel (4%–12%) in MOPS-SDS running buffer (Invitrogen) under reducing conditions (50 mM DTT) and transferred to a PVDF membrane (Hybond-P, Amersham). The membrane was rinsed twice in TBS and incubated for 1 hour in the anti-His HRP conjugate blocking buffer (Qiagen). The membrane was then incubated in 1/3000 anti-His HRP conjugate antibody (Qiagen) for 1 hour and washed seven times for 5 minutes in TBS-T buffer (TBS-0.5 % Tween 20). Signals were detected by chemiluminescence emission using

the ECL Plus Western blotting detection system and ECL Hyperfilms (GE Healthcare, UK).

2.10. Generation of Bioluminescent *L. Infantum* Promastigotes and In Vitro Infection of Human Macrophages. A homologous episomal expression system was devised to further examine the infection in vitro of two secreted proteins from *L. infantum*. The vector pSP- α HYG α LUC [17] carrying the firefly-luciferase gene was used to cotransfect *L. infantum* promastigotes overexpressing the secreted proteins LinJ19.0410 (ortholog of Tc00.1047053505789.10) or LinJ36.5780 (ortholog of Tc00.1047053506155.99). Recombinant parasites were selected for their growth in increasing concentrations of Hygromycin (up to 300 μ g/mL) over a period of several weeks. Promastigotes transfected with the pTEX vector alone and the pSP- α HYG α LUC were used as controls for infection experiments. The survival of transfected parasites was evaluated within human leukemia monocyte cells (THP-1 cells). THP-1 cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 100 IU of penicillin/mL, and 100 μ g of streptomycin/mL. THP-1 cells in the log-phase of growth were differentiated into macrophages by incubation for 2 days in a medium containing 20 ng/mL of phorbol myristate acetate (Sigma-Aldrich). THP-1 cells treated with PMA were washed with prewarmed medium and then infected with stationary-phase promastigotes of transfected-*Leishmania* in a 24-well plate at a parasite/macrophage ratio of 10 : 1 for 4 hours at 37°C with 5% CO₂. Noninternalized parasites were removed. After different incubation periods (24 hours to 96 hours) Luciferase activity was determined using the Steady Glo reagent (Promega, Madison,WI), according to the manufacturers' instructions. After 5 minutes, the plate was read using a Multilabel Counter VICTOR² model 1420 (Perkin Elmer). Results are expressed as the mean of RLU (Relative Luminescence Units) activity of three independent experiments, each performed in triplicate. Statistical significance was analyzed by the Mann-Whitney U test.

3. Results

3.1. Bioinformatic Selection for Secreted Proteins in Trypanosomatids. The preliminary analysis of the 19613 *T. cruzi* putative proteins from the CL-Brener genome was performed to discard potential uncompleted sequences. A total of 1796 sequences were removed manually since they did not bear an initial methionine amino acid. The remaining 17817 (90.8%) protein coding sequences were kept for subsequent analysis. House-keeping genes and sequences belonging to large gene families, like the trans-sialidases, mucins, Mucin-Associated Surface Proteins (MASPs), were discarded given that the main goal was to identify novel secreted proteins. Finally, sequences encoding proteins with a molecular weight above 90 kDa were eliminated, in order to facilitate subsequent gene cloning. The remaining coding sequences were screened for the presence of both the signal peptide and the peptidase cleavage site with a probability of 0.8 and 0.7, respectively. A total of 216 sequences were obtained by using these criteria. Among them, 91 (42%)

were annotated as “hypothetical proteins, conserved” in the data bank. The final criterion for selected proteins likely to be secreted by the classical eukaryotic pathway was the presence of the signal peptide and the signal peptidase site in orthologs of the related parasites: *Leishmania major*, *L. infantum*, and *T. brucei*. Among the 91 sequences, only 45 showed orthologs with the signal peptide criteria. The 13 proteins bearing the highest probability for the presence of the signal peptide were selected (Table 2) for confirmation of extracellular localization. Among the 13 selected genes, Tc00.1047053505789.10 and Tc00.1047053509835.30 are homologous genes and members of a multigene family (Table 2). These genes show about 40% identity at the protein level and possess the same predicted orthologs in the GeneDB server. Nevertheless in the TritypDB resource the prediction of orthologs is different (see Table 2). This could be explained by the use of different algorithms (Jaccard cog clustering or OrthoMCL) for the prediction of orthologous groups [18]. The beta-tubulin *T. cruzi* gene (GeneID Tc00.1047053506563) was added to our sample as a potential negative control for protein secretion. Among the 13 selected genes, 7 genes were predicted to have transmembrane domains (Table 2). These genes were included for the functional test because previous studies identified extracellular proteins with putative transmembrane domains as constituents of the secretome of different pathogens, including *L. donovani* [19, 20]. Furthermore, in the protozoa *Toxoplasma gondii*, the dense granule protein GRA5 is a transmembrane protein that bears a signal peptide and is secreted as a soluble protein into the vacuolar space, before being inserted into the parasitophorous vacuole membrane. Based on these studies, we included genes with potential transmembrane domains to test whether the presence of these domains represents a useful criterion for identifying extracellular secreted proteins.

3.2. Transcription of the Selected Genes in the Different Forms of *T. cruzi* Life Cycle. Reverse transcription-PCR (RT-PCR) was performed for the 13 in silico selected genes in the different developmental stages of *T. cruzi* in order to verify transcription. The beta-tubulin *T. cruzi* gene (GeneID Tc00.1047053506563), constitutively expressed in all the three stages of *T. cruzi*, was used as a positive control for RNA quality. RT-PCR was positive for all genes in the infective trypomastigote and amastigote forms. Two genes (Gene ID Tc00.1047053511901.30 and Tc00.1047053509999.10) were negative for the amplification of cDNA in the noninfective epimastigote form (Figure 1) suggesting a possible stage-specific expression of these genes. Nevertheless, since trypanosomatid gene expression is almost exclusively regulated posttranscriptionally, further studies at the protein level have to confirm these observations.

3.3. Experimental Approach for the Detection of Secreted Proteins. A functional test was set up to confirm the presence of selected proteins in the extracellular environment by detection of target proteins in cell-free supernatants. The 13 selected encoding genes of *T. cruzi* and the gene encoding the beta-tubulin (negative control) were amplified from genomic

TABLE 2: *T. cruzi* genes selected by in silico analysis and predicted properties of the encoded hypothetical proteins.

<i>T. cruzi</i> GeneDB Gene ID No.	Orthologs		Probability		TM ^(d)	GPI ^(e)	Conserved domains (and E-value) ^(f)
	<i>L. major</i> and <i>T. brucei</i> Gene ID No. ^(a)		SPP ^(b)	CSP ^(c)			
Tc00.1047053506417.30	LmjF22.0225	Tb927.8.2180*	0.937	0.917	1	Yes	None
Tc00.1047053506155.99	LmjF36.5220	Tb11.01.2470	0.984	0.962	0	No	Glucosidase II beta subunit-like (E = 1.6 e-13)
Tc00.1047053506467.29	LmjF26.2000	Tb09.160.1070	0.811	0.771	0	Yes	Methyltransferase domain (E = 4.3 e-4)
Tc00.1047053511901.30	LmjF24.2160	Tb927.8.6080	0.989	0.898	0	No	Glycerophosphoryl diester phosphodiesterase (E = 5.7 e-8)
Tc00.1047053511871.30	LmjF25.1010*	Tb927.3.950*	0.979	0.958	0	No	2OG-Fe(II) oxygenase (E = 1.7 e-13)
Tc00.1047053505789.10 ^(g)	LmjF19.0540*	Tb927.8.6700 ^(h)	1.000	0.768	5	No	Lipocalin signature (E = 0.0)
Tc00.1047053509669.70	LmjF19.0570* LmjF29.1600	Tb11.39.0005 ⁽ⁱ⁾ Tb927.3.4190	0.999	0.980	9	No	Endomembrane protein 70 (E = 0.0)
Tc00.1047053507765.20	LmjF11.0720	Tb11.02.4400	0.993	0.986	0	No	None
Tc00.1047053510101.470	LmjF11.0720	Tb11.02.4400	0.931	0.919	0	No	None
Tc00.1047053510443.30	LmjF30.3150	Tb927.6.4500	0.903	0.838	1	No	Translocon-associated protein beta (TRAPB) (E = 4.1 e-4)
Tc00.1047053509799.50	LmjF36.5570	Tb10.6k15.1130	0.981	0.931	1	No	None
Tc00.1047053509835.30 ^(g)	LmjF19.0540* LmjF19.0570*	Tb927.8.6700 ^(h) Tb11.39.0005 ⁽ⁱ⁾	0.866	0.803	5	No	Heavy-metal-associated domain, Heavy metal transport/detoxification (E = 0.0)
Tc00.1047053509999.10	LmjF29.1200*	Tb927.3.3820	1.000	0.952	3	No	None

^(a) *L. major* and *T. brucei* gene ID No. of putative orthologs from GeneDB.

*Nonsynthetic predicted orthologs in the TriTrypDB server.

^(b) SPP Signal peptide probability predicted by SignalP 3.0.

^(c) CSP Maximal cleavage site probability predicted by SignalP 3.0.

^(d) TM Number of transmembrane domains (other than peptide signal sequence) predicted by TMHMM 2.0.

^(e) GPI Identification of GPI-anchor signal by GPI-SOM.

^(f) Conserved domains (and corresponding E-value) from InterPro, PROSITE or Pfam.

^(g) Tc00.1047053505789.10 and Tc00.1047053509835.30 are homologous genes presenting 45% identity at the protein level. These genes are members of a multigene family including Tc00.1047053505789.20, Tc00.1047053509441.10, Tc00.1047053510063.30, Tc00.1047053510065.10, and Tc00.1047053504235.9.

^(h) Tb927.8.6700, Tb927.8.6710, Tb927.8.6720, and Tb927.8.6730 represent the paralogs/orthologs of Tc00.1047053505789.10, Tc00.1047053505789.20, Tc00.1047053509441.10, Tc00.1047053510063.30, and Tc00.1047053510065.10 in the TriTrypDB server.

⁽ⁱ⁾ Tb11.39.0005 represents the predicted ortholog of Tc00.1047053509835.30 and Tc00.1047053504235.9 in the TriTrypDB server.

DNA. A sequence encoding a 6xHis-Tag was added at the C-terminal end of each encoded gene, to allow subsequent detection of the protein in total parasite protein extracts or in concentrated cell-free supernatants (CCFSs). Amplified PCR products were cloned into the pTEX shuttle vector widely used for expression in trypanosomatids [14]. Transformation and selection of *T. cruzi* is not as easy to perform as for *Leishmania*, mainly due to longer periods required for selecting drug-resistant parasites. Since we aimed to develop a fast and reliable approach to identify trypanosomatid conserved secreted proteins, we used the related *Leishmania* parasite as the recipient organism for the experimental validation of our selected proteins. Thus, *L. infantum* promastigotes

were separately transformed with pTEX carrying one of the 14 selected *T. cruzi* genes (including the beta-tubulin gene), and the recombinant parasites were selected for resistance to Geneticin G418. Each parasite population was checked for the presence of both the NEO^R gene and the selected gene whose secretion was to be analyzed. A specific 800 bp fragment, indicative of the presence of the NEO^R gene, was detected in the transfected promastigotes and not in wild-type parasites (Figure 2(a)). Moreover, the presence of each candidate gene in recombinant parasites was confirmed using specific primers designed from *T. cruzi* gene sequences (Figure 2(b)). PCRs performed on wild type *Leishmania* were negative, demonstrating that the amplification was

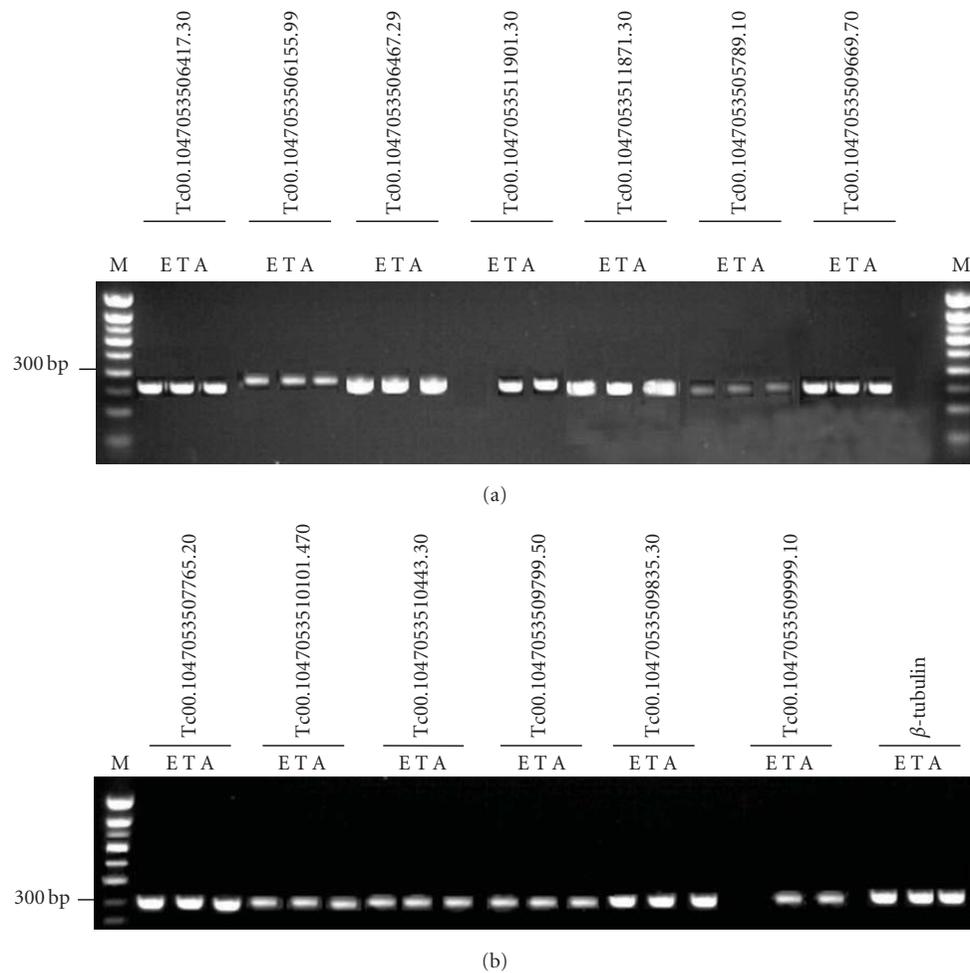


FIGURE 1: Amplification of the cDNAs encoding the potentially secreted proteins in the different stages of *T. cruzi*: RT-PCR analysis of total RNA from *T. cruzi* (clone derived from the Y strain) epimastigotes (E), trypomastigotes (T), and amastigotes (A). cDNA was amplified using gene-specific PCR primers (Listed in Table 1). Gene ID and expected lengths of cDNA are listed in order in Table 1. M: Molecular marker: Smart Ladder SF.

genus specific although the genes are conserved in both trypanosomatids (data not shown). The expression of these genes was screened using an antibody directed against the His-Tag carried by the recombinant proteins (Figure 3(a)). Western blot analysis demonstrated that (i) it was possible to easily and specifically detect the 6xHis tag protein in the extract derived from recombinant parasites, (ii) recombinant *Leishmania* expressed a relatively high level of *T. cruzi* protein, and (iii) the molecular weight of the detected tagged protein corresponded to the expected MW (see Table 1).

Subsequently, an approach was set up to detect recombinant proteins in cell-free supernatants. In order to limit potential contamination by proteins derived from dying organisms, incubation in serum-free mediums was restricted to 6 hours, and the viability of parasite populations was checked before and after this incubation period. Parasites and cell-free supernatants were collected if the viability of the cell population was above 98%. Western Blot analysis of the concentrated cell-free supernatants revealed that among the 14 proteins, only 3 were actively

secreted (Tc00.1047053506155.99, Tc00.1047053505789.10, and Tc00.1047053509999.10) (Figure 3(b)). These proteins represent genuine secreted material, since (i) the overexpression of the beta-tubulin gene does not induce translocation of the beta-tubulin protein into the extracellular space (difference between Lys and CCFS in Figure 3(b)), and (ii) the detection of the tagged protein in the cell-free supernatant is not related to the level of its expression by *Leishmania* (low abundance of Tc00.1047053506155.99 in Figure 3(b)). As expected, a slight molecular weight difference was observed between the tagged protein detected into the whole soluble extract and that detected in the cell-free supernatant that could be explained by the loss of the Signal Peptide (Figure 3(b)). As anticipated, no cross-reactive band was detected in wild-type parasites (Figure 3(b)). To confirm that the secretion observed was not related to the heterologous expression system, two *Leishmania* genes (Gene ID LinJ19.0410 and LinJ36.5780) corresponding to the orthologs of Tc00.1047053505789.10 and Tc00.1047053506155.99 genes were selected to validate

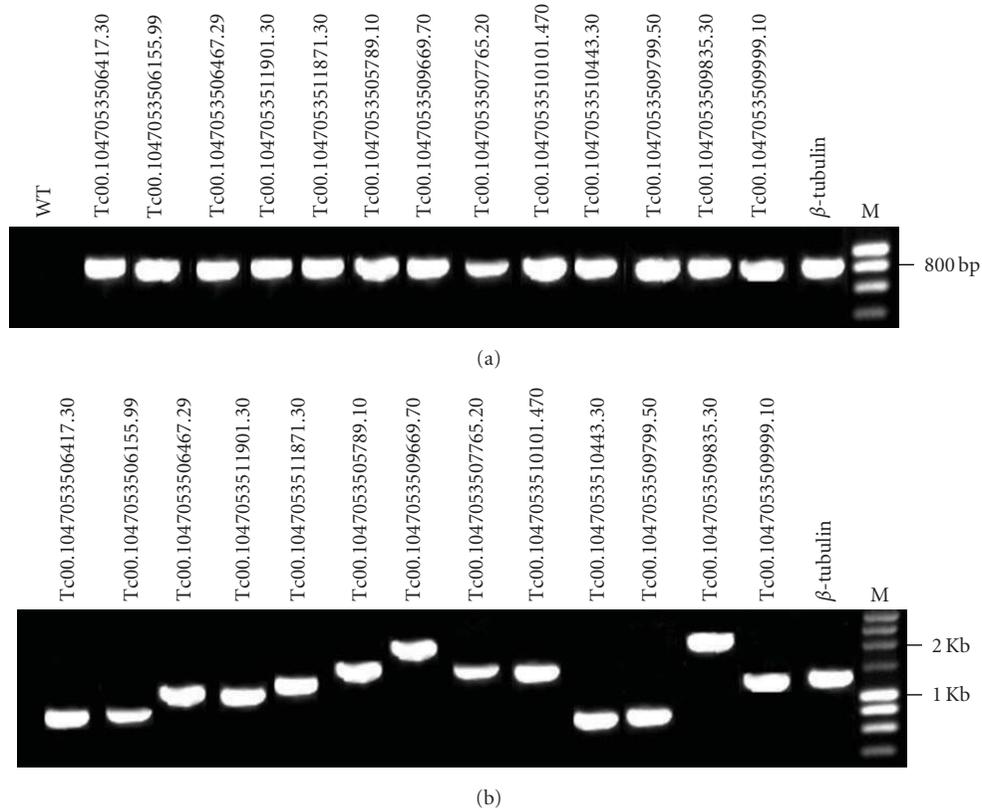


FIGURE 2: PCR analyses in episomally transfected *L. infantum* promastigotes. (a) Amplification of NEO gene fragment in *L. infantum* episomally transfected promastigotes. (b) Amplification of full length transfected genes in *L. infantum* promastigotes. Specific forward and Reverse PCR primers and gene lengths are listed in order in Table 1. WT: Wild Type Parasites. M: Molecular marker: Smart Ladder SL.

our approach. By using the same protocol as above, *Leishmania* expressing the 6xHis tagged proteins were generated (See Table 3). As expected, the presence of the tagged protein in the extracellular medium was only detected in the episomally transfected parasites (Figure 4). Together, these results indicate that this approach allows the identification of new and genuinely extracellular proteins involved in the endoplasmic reticulum/Golgi-dependent secretory pathway.

3.4. Expression of Secreted Proteins Increases Ability of Recombinant *Leishmania* Parasites to Infect and Survive inside Macrophages In Vitro. We attempted to determine whether the expression of *Leishmania*-secreted proteins could interfere with the capacity of recombinant parasites to replicate within human macrophages in vitro. Both confirmed secreted proteins (LinJ19.0410 and LinJ36.5780) from *L. infantum* were tested by using the luciferase reporter system in transfected parasites overexpressing these proteins. We used bioluminescence as a quantitative indicator of the viability and multiplication of parasites. The number of promastigotes cells and luciferase activity were linearly correlated for the different recombinant parasites before macrophage infection (data not shown). Results of in vitro infection showed that overexpression of secreted protein LinJ19.0410 (ortholog of Tc00.1047053505789.10) increases the capacity of *Leishmania* to survive in THP-1 differentiated

macrophages as early as 24 hours postinfection (Figure 5). Furthermore, a statistically significant increase in luciferase activity of recombinant parasites expressing LinJ19.0410 was maintained throughout the experiments ($P < .05$). This effect was not observed in parasites overexpressing LinJ36.5780 (ortholog of Tc00.1047053506155.99) where infectivity levels were similar to the control parasites transfected with the pTEX vector alone and the pSP- α HYG α LUC (Figure 5).

4. Discussion

In trypanosomatids the secretion process is not fully understood and various pathways including classical and nonclassical mechanisms may contribute to the formation of the “extracellular proteome.” Thus the individual identification of secreted materials would enhance efforts towards understanding mechanisms of protein secretion in these medically important parasites. In an attempt to provide a new approach to analyse the large number of hypothetical conserved proteins in trypanosomatids, we developed an experimental approach to identify hypothetical extracellular proteins likely to be involved in the classical pathway. We combined a web-based bioinformatics approach that used the Signal P 3.0 program, one of the most accurate predictors for the presence of a signal peptide sequence [21], with

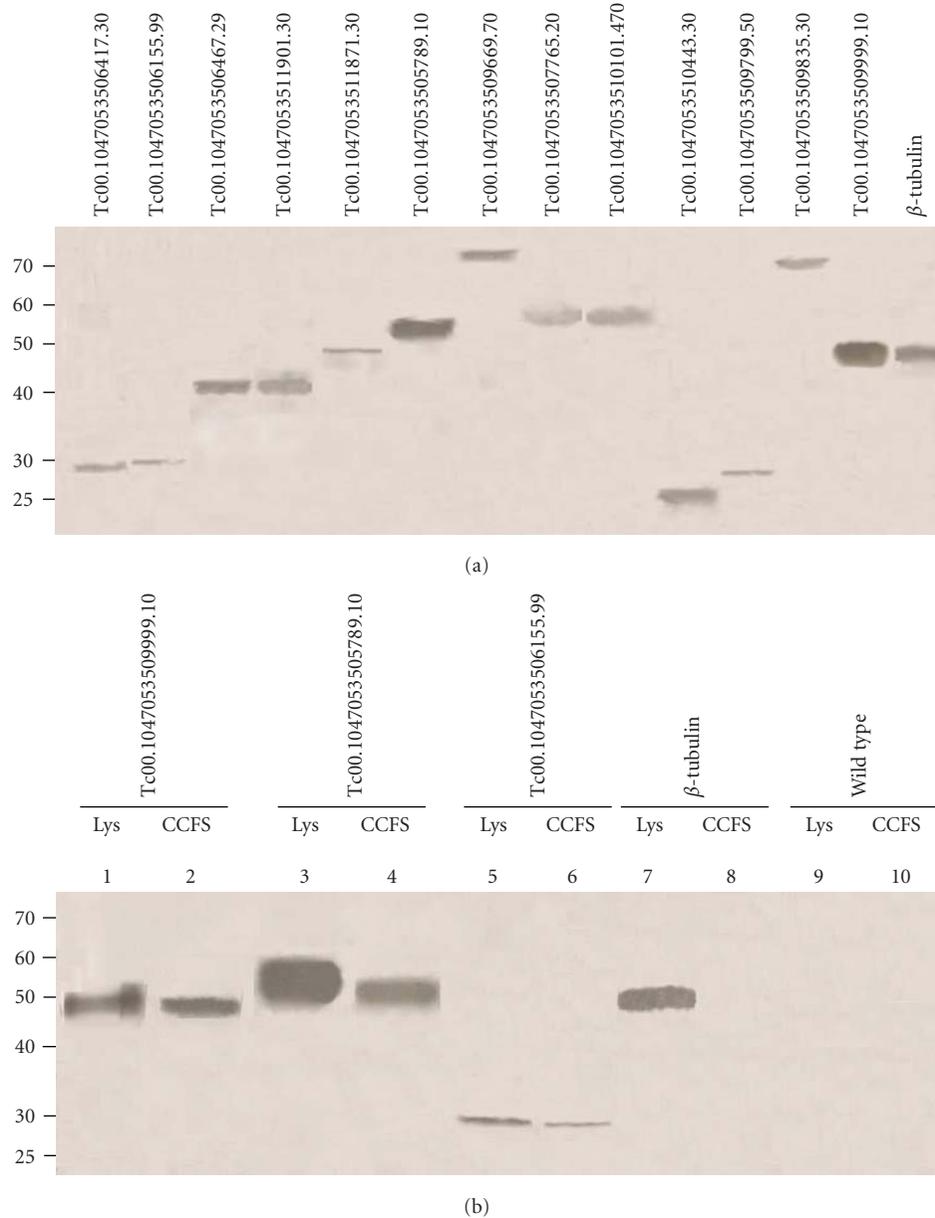


FIGURE 3: Protein expression in *L. infantum* episomally transfected promastigotes during the exponential phase of development (a) Western blot analysis of His-tagged proteins detected in whole cell lysate. Equal amounts of total protein ($35 \mu\text{g}$) were resolved by electrophoresis in 4–12% gradient gels (Invitrogen), blotted, and developed with anti-HisTag antibody followed by ECL (Amersham). Gene ID and the theoretical molecular weight of detected proteins are listed in order in Table 1. (b) Identification of secreted proteins in whole cell lysate (Lys) and concentrated cell-free culture supernatant (CCFS) obtained from promastigotes incubated for 6 hours in serum-free medium. Note the absence of β Tubulin in the concentrated supernatant of Line 8. Nontransfected *L. infantum* promastigotes (Wild Type) were used as a negative control. Protein molecular mass standards in kDa are shown on the left of each panel.

a functional test that takes advantage of the relative ease to genetically transform *Leishmania*. We assumed that a phylogenetic conservation among *Leishmania*, *T. cruzi*, and *T. brucei* would point to evolutionary selection for this family of proteins and indicate an important role for these proteins in the biology of these parasites. Using these criteria we selected a pool of 13 trypanosomatid-conserved hypothetical proteins from the *T. cruzi* database for which secretion was tested.

Identification of secreted proteins has been hampered in trypanosomatids due to the difficulty in distinguishing genuine secretions from molecules released by lysed, dead, or dying organisms. Additionally, the in vitro growth of trypanosomatid developmental stages that occur in mammals is impossible or laborious.

Characterization by screening cDNA libraries with sera raised against culture medium supernatants has been performed for the identification of extracellular proteins

TABLE 3: Gene ID of *L. infantum* orthologous genes and primers used for cloning.

Gene ID	Primer sequences ^(a)	F/R product sizes (bp)	MW(kDa) ^(b)
LinJ19.0410 ^(c)	F CATGACCACTAGTATGGCCAAAACAGCGCTTCTC R GCAGTCCAAGCTTTTAGTGATGGTGATGATGATG AGGTGTTCTCAGGGGTGACGA	1590	58,4
LinJ36.5780 ^(d)	F CATGCTCGACTAGTATGGGGTGCCGCAGTAGCTG R GCAGTCCAAGCTTTAATGATGATGGTGATGATG ATCATCCAACATCTGGCACCGC	738	28

F: Forward primer including the start codon; R: Reverse primer used for the amplification of the full-length ORF.

^(a) Restriction sites used for cloning in the pTEX vector in italics and the His-Tag sequence in bold.

^(b) Expected molecular weight of the proteins.

^(c) Ortholog of LmjF19.0540 and Tc00.1047053505789.10.

^(d) Ortholog of LmjF36.5220 and Tc00.1047053506155.99.

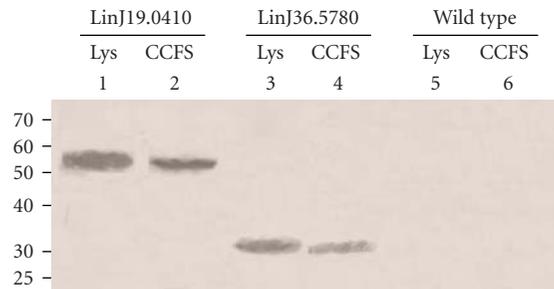


FIGURE 4: Homologous expression of secreted proteins in *L. infantum* episomally transfected promastigotes. *L. infantum* promastigotes were transfected with genes LinJ19.0410 and LinJ36.5780 corresponding to secreted proteins Tc00.1047053505789.10 and Tc00.1047053506155.99, respectively. Cell whole lysate (Lys) and concentrated cell-free culture supernatant (CCFS) and electrophoresis procedure were as in Figure 3. Tagged proteins were detected only in recombinant parasites transfected with LinJ19.0410 (58 kDa) (Line 1 and 2) and LinJ36.5780 (28 kDa) (Line 3 and 4). Nontransfected *L. infantum* promastigotes (Wild Type) were used as negative controls (Line 5 and 6). Protein molecular mass standards in kDa are shown on the left.

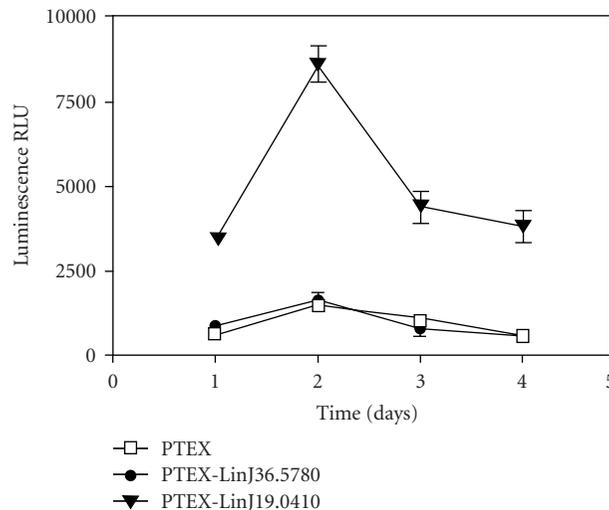


FIGURE 5: Bioluminescence activity of intracellular *Leishmania* expressing episomal luciferase from infected macrophages in vitro. Recombinant *L. infantum* promastigotes overexpressing the secreted proteins pTEX-LinJ19.0410 (▼) or pTEX-LinJ36.5780 (●) were cotransfected with the pSP-YαHYGROαLUC carrying the firefly-luciferase gene. Survival of luciferase-expressing parasites was monitored in infected human monocyte cell line THP-1 differentiated into macrophages as indicated in the Methods section. Promastigotes transfected with the pTEX vector alone and the pSP-αHYGαLUC (□) were used as control for infection experiments. RLUs (Relative Luminescence Units) were measured at various time points post infection using the Steady Glo reagent. Results are expressed as the mean of three independent experiments, each carried out in triplicate.

[22, 23]. However, using this approach proteins with a low abundance or that are poorly immunogenic are likely to be missed. A more exhaustive approach relying on a highly sensitive methodology, like the quantitative mass spectrometry, was recently used to analyze the protein content of the whole conditioned medium from stationary-phase *L. donovani* promastigotes [19]. Nevertheless, proteins produced at low abundance and that are mainly exported to the extracellular compartment are likely to be missed, since the method relies on the comparison of conditioned medium versus cell-associated proteins. Indeed proteins that are well known to be extracellular, such as chitinase and SAcP (secreted acid phosphatase), were not identified with the SILAC-based approach [19]. The method we designed allowed us to identify three new trypanosomatid conserved proteins, likely to be secreted through the classical secretory pathway. We were confident that the proteins detected in the cell-free supernatant were genuinely secreted since (i) we were not able to detect the beta-tubulin-tagged protein in the cell-free supernatant after overexpression of the gene, (ii) the 6-hour incubation time avoided excessive cell death and contamination by proteins released from dead parasites, (iii) in the extracellular environment we detected orthologous *Leishmania* proteins (LinJ19.0410, LinJ36.5780) suggesting that secretion in this protein family is likely to be evolutionary conserved, and (iv) we detected no relation between the amount of secreted protein and its intracellular expression in the transfected parasites, demonstrating that the translocation of the his-tagged protein into the cell supernatant is not related to the methodology we used.

A recent study involving conditioned medium derived from stationary-phase *Leishmania* promastigotes [19] demonstrated that the extracellular proteome was mainly composed of proteins derived from different microvesicles. The parasite growth phase studied by these authors contains the infectious metacyclic parasites and also a high percentage of dying parasites in apoptosis [24]. Consequently, the analysis of extracellular material revealed that the main contributors to the “extracellular proteome” are vesicles likely to be “apoptotic vesicles” or “blebs” [25]. This work leads to the general conclusion that 98% of the proteins of the *Leishmania* secretome lacked a targeting signal, indicating that nonclassical secretion pathways are likely to be the dominant way by which *Leishmania* export proteins [19]. The proteomic characterization of the released/secreted proteins of *L. braziliensis* promastigotes showed that about 5% of the identified proteins possess a putative N-Signal peptide [26], indicating that protein export may depend on unconventional pathways as suggested in *L. donovani*. However, some evidence strongly supports the notion that the classical secretory pathway is operational in trypanosomatids and contributes to the composition of the parasite’s secretome. For example, the screening of an *L. major* cDNA library with antiserum raised against culture supernatant from stationary-phase promastigotes led to the detection of 8 proteins bearing a potential signal peptide among the 33 genes identified [23]. Moreover some of the well-known *Leishmania* proteins

found in the extracellular environment have a signal peptide for secretion, such as gp63 or chitinase [3, 27]. In the current work we deliberately decided to experimentally validate the secretion of our candidate proteins during the exponential growth phase of *Leishmania* in order to avoid contamination of the cell-free supernatant by apoptotic vesicles or exosomes. In these conditions we did not detect the secretion of tubulin, even in an overexpression model of *Leishmania* transfectants, while tubulin was identified with a significant score in the *Leishmania* secretome studied by the proteomic analysis performed during the stationary-phase [19]. Therefore, our results suggest that tubulin might be associated with exosomes and/or apoptotic vesicles generated by promastigotes in the stationary-phase of growth. We suggest that the composition of the parasite “extracellular proteome” is variable and depends both on the parasite stage under consideration and on the relative contribution of the various pathways operating in protein secretion. Further studies are required to highlight the importance of both classical and nonclassical secretory pathways in different developmental stages of trypanosomatids. Since we selected and tested secretion for several genes in the trypanosomatid genomes, our methodology provides a potential tool for genomewide screening to identify extracellular proteins. Furthermore, our methodology may complement other strategies, such as the SILAC approach, for the identification of proteins missed when using proteomic-based approaches.

Regarding proteins not detected in the extracellular environment, it is important to point out that proteins bearing a signal peptide are not only targets for secretion but also for transfer to specific organelles, for example, lysosomes or the cell surface. Thus, these proteins could be retained in specific organelles within the parasite or attach to the cell surface via a GPI-anchor. In this light, analysis of the 13 proteins with GPI-SOM [28] suggests that 2 out of 13 proteins (Tc00.1047053506417.30 and Tc00.1047053506467.29) have a predicted glycosyl-inositol phosphate (GPI) anchor signature sequences. Hence, the presence of the GPI anchored domain may explain the absence of these proteins in the culture supernatant of recombinant parasites.

An unexpected finding in our results was the prediction of transmembrane helices in two of our secreted proteins (Tc00.1047053505789.10 and Tc00.1047053509999.10). We included proteins with transmembrane domains to test whether their presence in protein sequences is a suitable criterion for the prediction of extracellular secreted proteins. Previous studies suggested that the presence of transmembrane helices in protein sequences is not a suitable criterion for discarding potentially secreted proteins [19, 20]. In this regard our results provide further evidence that empirical studies are required to verify bioinformatic predictions since two of the extracellular proteins bear transmembrane domains. However, another speculative explanation for the presence of transmembrane domains is the potential insertion of secreted materials into membranes after secretion, as demonstrated for the pathogen *Toxoplasma gondii* [29, 30].

The secreted protein Tc00.1047053505789.10 contains a lipocalin signature. Lipocalins are a widely distributed group of mostly extracellular proteins, several of which have been implicated in the regulation of host immune responses, such as, alpha-1-microglobulin and alpha-1-acid glycoprotein [31]. Despite a well-conserved tertiary structure of lipocalins, the pairwise sequence identity within this family is low, often below 30%, [32]. This may explain the absence of a predicted lipocalin signature in the *Leishmania spp.* or *T. brucei* orthologs. Remarkably, the protein encoded by Tc00.1047053509835.30 (homolog of Tc00.1047053505789.10) lacks a lipocalin signature. Furthermore, this protein was not detected as extracellular in our experimental conditions. Although these genes belong to a multigene family (see Table 2) and possess about 40% identity at the protein level, our findings suggest a different cellular localization and/or function for the corresponding proteins. A clear assignment of the protein Tc00.1047053505789.10 to the lipocalin family would only be possible after NMR or X-ray crystallography structure analysis. Further studies are needed to ascertain if this large and diverse group of proteins is present in trypanosomatids and plays a role in the transmission process. A Glucosidase II beta subunit-like protein domain has also been detected in the protein Tc00.1047053506155.99. Although mostly localized to the ER, Glucosidase II was found in endocytic structures beneath the plasma membrane and has been associated with the protein-tyrosine phosphatase CD45 [33]. There is also evidence that in some cell types Glucosidase II beta is capable of being trafficked to the cell surface [34].

Having demonstrated that our methodology is reliable for the identification of extracellular secreted proteins in trypanosomatids, we tested the hypothesis that recombinant *Leishmania* parasites carrying extra copies of *Leishmania* secreted proteins may interfere with their survival or infectivity towards human monocyte-derived macrophages in vitro. Results of these assays showed a significant survival advantage to *Leishmania* parasites overexpressing the gene LinJ19.0410 (Ortholog of Tc00.1047053505789.10) suggesting that this protein is involved in a process increasing survival and/or replication of the parasite inside its target cell. Given that *Leishmania* and *T. cruzi* do not target the same host cells and follow different cell invasion processes, further experiments in the *T. cruzi* homologous system are needed to address whether the identified secreted protein Tc00.1047053505789.10 from *T. cruzi* is also involved in host cell invasion and/or replication. Current in vitro and in vivo studies are in progress to characterize this protein which represents a potential conserved virulence factor in trypanosomatids.

5. Conclusions

In conclusion, our results show that the bioinformatics method combined with the functional tests, provides a fast and reliable method for the identification of novel extracellular secreted proteins involved in the classical secretory pathway and represents potential virulence factors in trypanosomatids.

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Research Article

Characterization of Major Surface Protease Homologues of *Trypanosoma congolense*

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Trypanosomes encode a family of proteins known as Major Surface Metalloproteases (MSPs). We have identified six putative MSPs encoded within the partially sequenced *T. congolense* genome. Phylogenetic analysis indicates that *T. congolense* MSPs belong to five subfamilies that are conserved among African trypanosome species. Molecular modeling, based on the known structure of *Leishmania Major* GP63, reveals subfamily-specific structural variations around the putative active site despite conservation of overall structure, suggesting that each MSP subfamily has evolved to recognize distinct substrates. We have cloned and purified a protein encoding the amino-terminal domain of the *T. congolense* homologue TcoMSP-D (most closely related to *Leishmania* GP63). We detect TcoMSP-D in the serum of *T. congolense*-infected mice. Mice immunized with the amino-terminal domain of TcoMSP-D generate a persisting IgG1 antibody response. Surprisingly, a low-dose challenge of immunized mice with *T. congolense* significantly increases susceptibility to infection, indicating that immunity to TcoMSP-D is a factor affecting virulence.

1. Introduction

African trypanosomes cause devastating disease within humans and livestock. *Trypanosoma brucei gambiense* and *T. b. rhodesiense* cause sleeping sickness in humans. African trypanosomiasis in domestic animals is caused by infections with *T. congolense*, *T. vivax*, or *T. b. brucei*, of which *T. congolense* is the most important pathogen for livestock [1]. Antiparasite drugs are available but far from ideal [2]. Vaccination has not been effective as the periodic switching among genes encoding for the immunodominant variable surface glycoprotein (VSG) coat of the parasite precludes use of this surface molecule as a candidate [3–6].

Given the variability in the VSG coat we are examining the feasibility of generating pre-existing immunity to invariant surface proteins or secreted virulence factors as a means of controlling trypanosome infection. Our model organism is *T. congolense*, a causative agent of the chronic wasting

disease of livestock, Nagana. *T. congolense* remains in the blood stream throughout the infection. Thus generation of a protective immune response to surface-exposed or secreted virulence factors of the blood stream form should have the potential to prevent or limit disease.

Among the African trypanosomes, *T. brucei* has previously been demonstrated to encode Major Surface Proteases (MSPs) with homology to MSPs of *Leishmania* [7]. In addition, the blood stream form *T. brucei* has been shown to be killed in vitro by a peptidomimetic inhibitor that specifically blocks *L. major* MSP activity [8]. Together, these observations suggest that at least one MSP homologue is expressed and essential for survival in the blood stream form of *T. brucei*. Based on this, and the findings that in *Leishmania* this family of proteins is important for virulence and is surface-exposed [9], we were encouraged to pursue whether *T. congolense* also encoded MSP homologues, and whether immunity to a member(s) of this family could attenuate *T. congolense* infection.

To date, there have been no reports of MSP homologues found in *T. congolense*. Fortunately, the African trypanosome sequencing project undertaken by the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk/>) has now generated a significant amount of sequence information for *T. congolense*, though it has yet to be annotated. We performed searches of the available *T. congolense* sequence for predicted homologues to known MSPs of *T. brucei* and *Leishmania sp.* We found six potential MSP encoding ORFs. Two of the potential MSPs are >95% identical whereas the remaining four are <40% identical to each other, suggesting that we had tentatively identified five subfamilies of MSPs within *T. congolense*. A phylogenetic analysis between the *T. congolense* MSPs and homologues found in other African and new world trypanosomes revealed that they indeed are representatives of five subfamilies that are shared among African trypanosomes. We subsequently cloned and expressed a region encoding the amino-terminal domain of the putative MSP ORF from *T. congolense* whose subfamily was found to group the closest with MSPs from *Leishmania sp.* Antisera against this domain revealed one major band in a Western blot of lysates of blood stream forms of *T. congolense*, strongly suggesting that this subfamily is expressed during infection. We describe the immunization with this purified partial protein and its effects on a subcutaneous infection of mice with a low dose of *T. congolense*.

2. Materials and Methods

2.1. Parasites. *T. congolense*, Trans Mara strain, variant antigenic types TC13 and TC14 were used in this study. The origin of the parasite strain [10] and clone TC13 [11] has been described previously. *T. congolense* clone TC14 was obtained from a C57BL/6 mouse infected with 10^3 *T. congolense* clone TC13. It was cloned from a blood sample collected 14 days after the infection. The variant surface glycoprotein of clone TC14 is different from that of clone TC13.

2.2. Sequence Analysis and Molecular Modeling. *T. congolense* DNA sequences encoding potential MSP homologues were identified using TBLASTN via the Trypanosoma Blast Server web page (<http://www.sanger.ac.uk/cgi-bin/blast/submitblast/t.brucei/>), using the published amino acid sequences for *T. brucei* MSP-A, MSP-B, and MSP-C [7]. Potential open reading frames were identified using ORF Finder on the NCBI server (<http://www.ncbi.nlm.nih.gov/projects/gorf/>) and MSP family homology confirmed by independent BLAST analysis of each identified ORF against the NCBI database. ORFs showing homology to annotated MSPs from the NCBI database were collected. The closest homologues to these *T. congolense* putative MSPs in other individual *Trypanosoma* species were identified using TBLASTN via the Trypanosoma Blast server for *T. b. gambiense* and *T. vivax* and via the NCBI Blast Server (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for *T. brucei* TREU927, *T. b. rhodesiense*, and *T. cruzi*, as were homologues

in *Leishmania* (taxid:5658). Full amino acid sequences for each protein analyzed are provided in the Supplementary Material available online at doi: 10.1155/2010/418157. Protein sequence alignments were generated and prepared for presentation using ClustalX ver 2.0.7 [12]. Phylogenetic trees were generated by ClustalX using the bootstrapped N-J method with 1000 iterations. The unrelated *E. coli* protease HflB was included as an outgroup for alignment. Trees were prepared for presentation using PhyloDraw ver 0.8 as radial trees with terminal leaf extension for clarity of labeling, and Njplot ver 2.3 for display of bootstrap values (Supplementary Figure 1S). Three-dimensional structures for the predicted amino acid sequences of the putative MSPs were generated via the web-based server 3D-JIGSAW (<http://bmm.cancerresearchuk.org/~3djigsaw/>) [13], using the provided default settings. Figures were generated from the resulting structural files using PyMol1.1 [14]. Predicted amino acid sequences for the MSPs were submitted to PredGPI (<http://gpcr2.biocomp.unibo.it/predgpi/pred.htm>) [15] to predict the presence of a potential GPI anchor site and to Signal 3.0 Server (<http://www.cbs.dtu.dk/services/SignalP/>) [16] to predict the presence of a signal peptide indicative of secretion.

2.3. Cloning, Expression, and Purification of the Putative TcoMSP-D. The DNA sequence encoding the amino-terminal domain of the MSP homologue from clone TC13 (predicted residues 14–248), which we named TcoMSP-D (see below), was amplified via PCR from *T. congolense* chromosomal DNA. The primers used for PCR were as follows: amino end, 5' CTGGGTACCGCAACAGTTTTGCTGCTGAC; carboxyl end, 5' GTGCTCGAGTTAGCCACATGTTCACTAAGG. Restriction enzyme recognition sites for KpnI (amino end primer) and XhoI (carboxyl end primer) used for directional cloning are underlined. The resulting PCR product was digested with KpnI and XhoI and ligated into similarly digested pET30a expression vector (Novagen). The protein construct is expressed under the control of the T7 promoter and contains 39 additional residues (MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTA) including a six residue histidine tag at the N-terminal. The plasmid was transformed into competent cells of the *E. coli* BL21(DE3) strain for protein expression. The amino-terminal domain of TcoMSP-C was similarly prepared but will be described in full elsewhere (H. Bull, G. Wei, V. Marcoux, H. Tabel unpublished).

Protein expression was induced by adding 0.4 mM isopropyl 1-thio- β -D-galactopyranoside when the cells reached an OD₆₀₀ of 0.5–0.7 absorbance units, followed by a 2-3-hour incubation period at 37°C. The cells were then harvested by centrifugation, and the resulting pellet suspended in buffer A prior to lyses by French press. The resulting lysate was centrifuged at 15,000 g for 20 minutes and supernatant and pellet assayed by SDS PAGE for the presence of the expressed protein. The protein was found to remain with the cell debris pellet, likely as an inclusion body. The pellet was dissolved in 6M guanidine hydrochloride and the his-tagged protein was

subsequently purified using nickel affinity chromatography in the presence of 6M guanidine hydrochloride throughout. The elution buffer was exchanged through extensive dialysis with PBS at pH 7.4. The protein yield was 2 mg mL⁻¹ with an estimated 95% purity as determined by SDS-polyacrylamide gel electrophoresis and staining with coomassie brilliant blue.

2.4. Immunization and Antibody Production. Each of two female eight-week-old New Zealand White rabbits was injected subcutaneously at multiple sites with 1 mg purified amino-terminal domain of TcoMSP-D emulsified in TiterMax Gold adjuvant (Sigma chemicals). Rabbits were boosted at four weeks with identically prepared inoculums. Blood was collected at eight weeks, and separated serum was stored at -20°C without further purification. Groups of 5 six- to eight-week-old female BALB/c AnNCrIbR (BALB/c) mice were injected subcutaneously (s.c.) with TiterMax alone (Control), 5 µg, or 50 µg of N-terminal fragment of TcoMSP-D in TiterMax at 4 different sites (25 µL/site). Blood was collected at two, four, and six weeks and checked for serum antibody titers to purified amino-terminal domain of TcoMSP-D by ELISA. Rabbits and mice were kept in polycarbonate cages on sawdust and allowed free access to food and water throughout the experiments, according to the recommendation of the Canadian Council of Animal Care. All animal experiments were conducted in accordance with the standards of the University Committee on Animal Care and Supply of the University of Saskatchewan (Protocol numbers 20070043 and 19920139).

2.5. Western Analysis. Purified blood-stream *T. congolense* TC13 [17] was solubilized in 2X-SDS loading buffer (95°C for two minutes) and the equivalent of 5 million parasites per lane were separated by SDS PAGE in a 12% slab gel. Separated proteins were transferred to a Hybond-P (GE Healthcare) nylon membrane using a Minitrans-Blot cell (Bio-Rad) at 100 V for 1 hour. After transfer, membranes were blocked for one hour with TBS/0.2% Tween-20/2.5% skim milk, followed by one-hour incubation in the presence of immune sera (1/500 dilution for rabbit anti-amino-terminal domain of TcoMSP-D). Bound antibody was detected by incubation with HRP-conjugated secondary goat anti-rabbit antibody at 1/500 in TBS/0.2% Tween-20/2.5% skim milk. For detection of TcoMSP-D in serum of infected BALB/c mice the equivalent of 0.5 µL of cell-free serum was loaded per lane of a 12% SDS PAGE gel and detected as above.

2.6. ELISA for TcoMSP-D Amino-Terminal Domain-Specific Antibodies. Immulon 4HBX (Thermo) ELISA plates were coated overnight at 4°C with purified TcoMSP-D amino-terminal domain (1 µg/well in 100 µL of coating buffer). The plates were washed twice with PBS/Tween-20 (PBST) and blocked with 5% skim milk in PBST, 200 µL/well, for 2 hours at 37°C. After 4 washes, twofold serial diluted normal or test mouse sera were carried out in antibody diluents (PBST containing 2.5% skim milk), 100 µL/well. The plates were

incubated for 2 hours at 37°C and then washed four times. HRP-conjugated goat antimouse IgG1 or IgG2a (Southern Biotechnology Associates) in antibody diluents was added and incubated for one hour at 37°C. After six washes with PBST, color development was achieved by adding 100 µL of TMB peroxidase substrate (KPL) and incubating the plates at room temperature in the dark for 10 to 20 minutes. The reaction was stopped by adding 50 µL of 1 M H₂SO₄ to each well. OD450 was read in a microtiter plate ELISA reader.

2.7. Mouse Infection Challenge. Mice immunized as described above were infected with 2 × 10³ *T. congolense* clone TC14 s.c. in a hind footpad (i.f.p.). A drop of blood was taken from the tail vein of each infected mouse. The parasitemia was estimated by counting the number of parasites present in at least 10 fields at X400 magnification by phase-contrast microscopy. A count of 256 parasites per field is equivalent to ~10⁹ parasites/mL [18]. The survival time was defined as the number of days post infection during which the infected mice remained alive. Moribund mice were euthanized.

2.8. Statistical Analysis. Data are represented as means ± SE. Significance of differences was determined by ANOVA using StatView SE1988 Software (Abacus Concepts) or a log-rank test for curve comparison using a GraphPad Prism computer program (BD Biosciences).

3. Results

3.1. Identifying MSP Homologues in *T. congolense*. To identify potential MSP homologues encoded by *T. congolense*, we performed a TBLASTN analysis against the partially completed and nonannotated *T. congolense* genome sequencing database currently being compiled by the Wellcome Trust Sanger Institute (<http://www.sanger.ac.uk/>). We searched for proteins with significant homology to previously characterize MSP-A, MSP-B, and MSP-C from *T. brucei* [7] as well as to the highly conserved nine amino acid motif thought to be important in Zn²⁺ binding at the catalytic active site. Both approaches yielded similar results. Our searches revealed a number of DNA sequence contigs that potentially encode MSP homologues. Each contig was examined for potential open reading frames (ORFs). The predicted protein sequence from each detected ORF was independently subjected to a BLAST homology search of the NCBI public databases to reveal those with high homology to previously identified members of the MSP family in related species. Using this approach we have identified six full length ORFs encoding putative MSP homologues in *T. congolense*. Four of the six full-length homologues are notably diverse in their predicted amino acid sequences (Figure 1). On the basis of phylogenetic analysis described in detail below we have named the putative MSPs TcoMSP-A, TcoMSP-B1, TcoMSP-B2, TcoMSP-C, TcoMSP-D, and TcoMSP-E. Interestingly, the two members showing high homology to each other are located adjacent to each other on the same DNA contig, perhaps suggesting

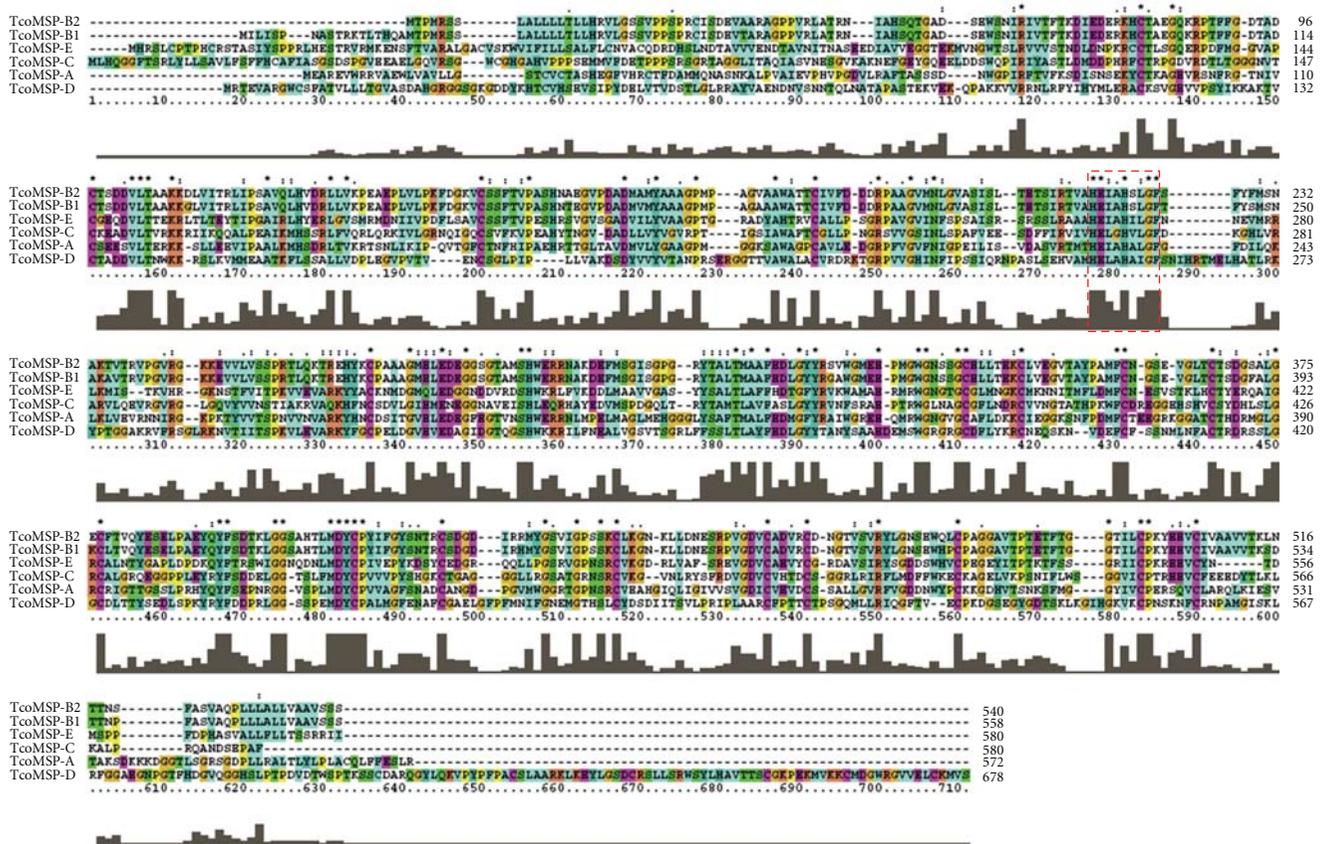


FIGURE 1: Multiple sequence alignment of *T. congolense* MSP homologues. The predicted amino acid sequences for each *T. congolense* MSP homologue were aligned to each other using the ClustalW alignment tool. Output was directly generated using ClustalX for Windows. The dashed red box indicates a conserved motif shown to be essential for Zn^{2+} binding in related proteins.

a tandem array with multiple members of this particular subfamily. Notably, others have found a family of MSP-B homologues to be arranged as a tandem array in *T. brucei* [19]. The remaining four genes identified are not flanked by MSP homologues within their respective assembled contigs.

The alignment shown in Figure 1 reveals that there is considerable variation among the proteins, with extensive variation at both the amino and carboxyl ends. Remarkably however, there are 18 conserved cysteine residues interspersed throughout the proteins as well as a lesser number of conserved glycine and proline residues. This finding, in conjunction with the conservation of the putative active site, strongly suggests that this family of diverse proteins has retained a common set of folds, and thus an overall conservation of tertiary structure.

Given the potential retention of tertiary structure, we attempted molecular modeling of the putative MSPs using the 3DjigSaw protein modeling server (<http://bmm.cancerresearchuk.org/~3djigsaw/>). The program was able to generate predicted structure files for each MSP. The first column of Figure 2 presents a cartoon model for each predicted structure as well as the solved structure for *L. major* MSP GP63 (1LML.pdb) [20]. Despite some individual loop deviations the overall three domain core structure is remarkably well retained through all six structures. The

overlays between each *T. congolense* homologue and 1LML structure in the center column reveal that the key folds around the putative active site (centered around the zinc ion shown in grey) are very highly conserved. This supports the interpretation that these proteins are homologues of *L. major* MSP GP63, which is a virulence factor [9]. However, the third column reveals key topological and surface charge differences between GP63 and between each of the predicted *T. congolense* MSPs around the putative active site. If these proteins retain protease activity, it is evident that they are very likely to have distinctly different substrate specificities.

3.2. Identifying Subfamilies among MSP Homologues. Given the observed diversity between the putative MSPs, we were interested to determine if they were representative members of conserved subfamilies in African trypanosomes, or if they were specific to *T. congolense*. To address this question, we queried the databases for predicted proteins showing the highest homology to each *T. congolense* MSP independently. To ensure sufficient depth to our analysis, we retained the predicted amino acid sequences for the three most homologous to each *T. congolense* MSP from each of *T. brucei* TREU 927, *T. brucei* rhodesiense, *T. brucei* gambiense, and *T. vivax* as well as from the Central and South

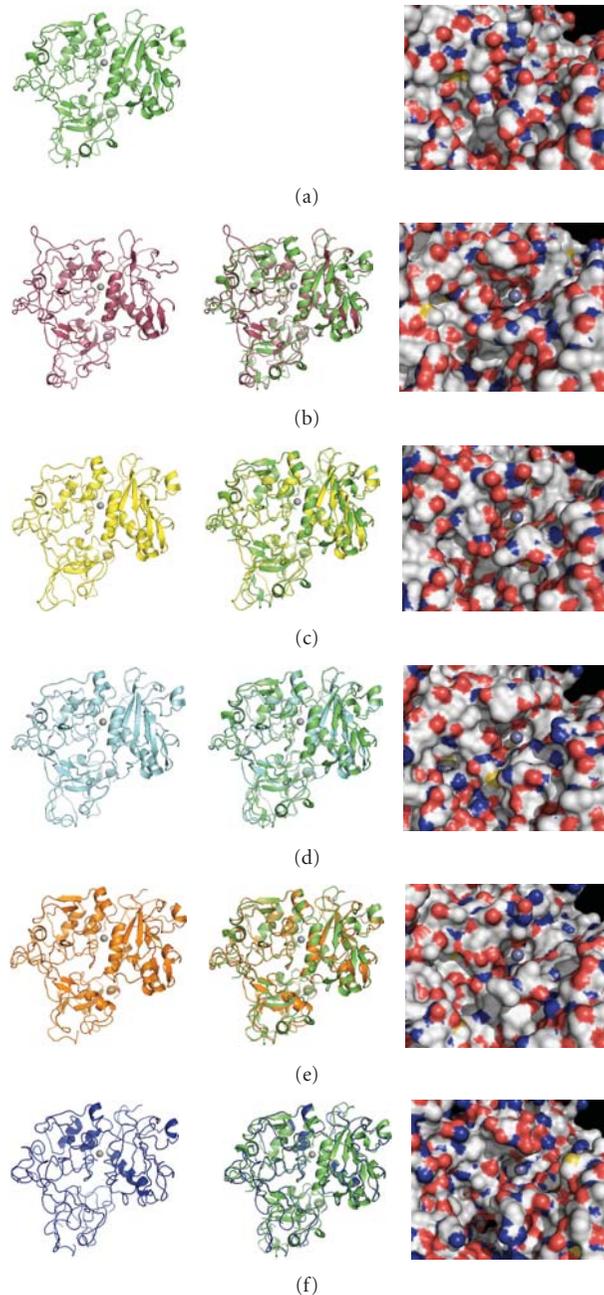


FIGURE 2: Predicted structures of *T. congolense* MSP homologues. Predicted amino acid sequences for each homologue were submitted for molecular modeling to the 3D-JigSaw comparative modeling web server (<http://bmm.cancerresearchuk.org/~3djigsaw/>). The solved *Leishmania* GP63 crystal structure 1LML.pdb was chosen by the program (independently of the user) as the best fit as a modeling template for each protein. The resulting generated pbd structure files were visualized using PyMOL Molecular Graphics System Version 1.1r1. Individual structures on the left side of the figure are as follows: (a) *Leishmania* GP63 (1LML.pdb), (b) TcoMSP-D, (c) TcoMSP-B1, (d) TcoMSP-A, (e) TcoMSP-C, and (f) TcoMSP-E. The center column depicts an overlay of that structure with the structure of *Leishmania* GP63. The third column depicts a surface representation of each predicted structure and *Leishmania* GP63 on the face containing the putative active site. Red-orange regions denote oxygen atoms, blue regions denote nitrogen atoms, green denotes carbon and hydrogen atoms, and yellow denotes sulfur atoms. The light grey sphere indicates the position of the Zn^{2+} ion in the solved *Leishmania* GP63 structure. It has been modeled into the predicted structures as an aid to orientation.

American trypanosome species *T. cruzi*. We also performed queries against the related *Leishmania* taxid. In cases where individual *T. congolense* MSPs retrieved the identical protein from a queried species, duplicates were removed. The amino

acid sequences described for *T. brucei* MSP-A, MSP-B, and MSP-C were also included for comparison [7]. Figure 3 shows the radial phylogenetic tree generated from comparison of this resulting group of 55 predicted protein sequences.

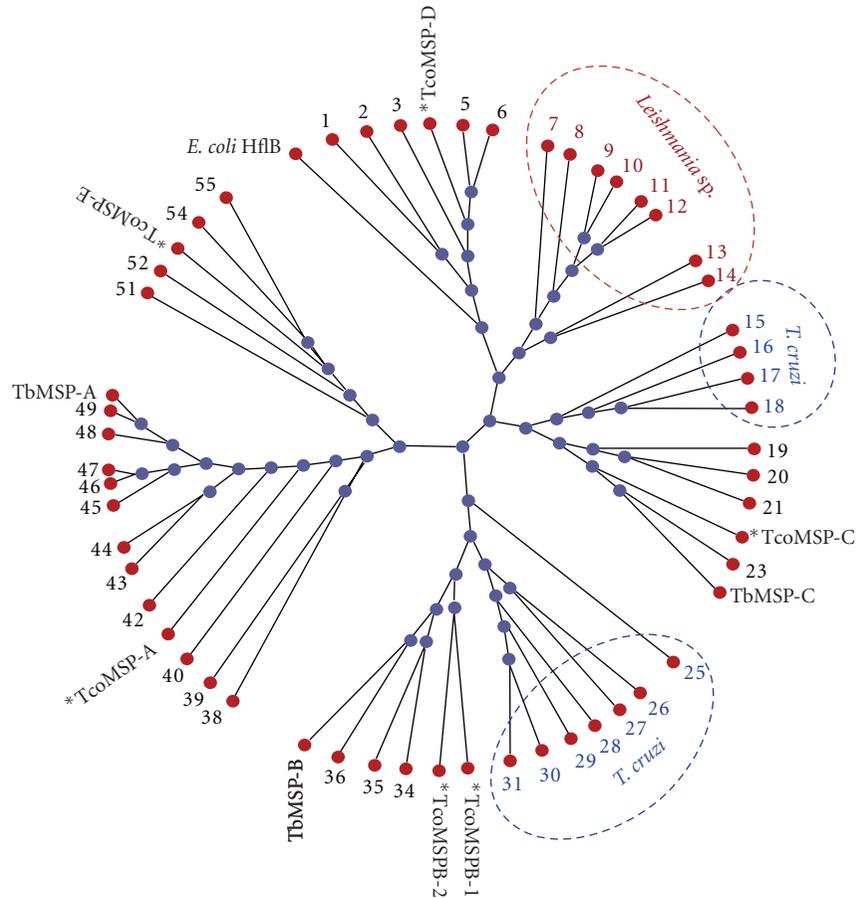


FIGURE 3: Clustal-W generated dendrogram comparing *T. congolense* putative MSPs with predicted homologues from *T. brucei* TREU927, *T. brucei gambiense*, *T. vivax*, *T. brucei rhodesiense*, *T. cruzi*, and *Leishmania* sp. (taxid 5658). Predicted protein sequences were obtained in a TBLASTN search against the translated genome database for each species indicated. The top three homologues were obtained for each *T. congolense* putative MSP independently for each species indicated. Redundant retrievals were eliminated. Protein sequence alignments were generated and prepared for presentation using ClustalX ver 2.0.7 [12]. Phylogenetic trees were generated by ClustalX using the bootstrapped N-J method with 1000 iterations. The unrelated *E. coli* protease HflB was included as an outgroup for alignment. Trees were prepared for presentation using PhyloDraw ver 0.8 as radial trees. Note that the terminal arms have been extended for clarity of presentation and labeling and therefore should not be considered in interpreting phylogenetic distance. *T. congolense* putative MSP proteins are indicated in bold lettering. The *T. brucei* homologues previously characterized by LaCount et al. [7] are indicated for comparison. Nodes populated solely from individual proteins from *T. cruzi* and *Leishmania* sp. are indicated by blue and red dashed circles, respectively. The individual putative proteins represented by numbers in the tree are listed by number in Table 1. Full amino acid sequences for the proteins represented here are available in the supplementary information as is a phylogenetic tree with bootstrap values and genetic distance indicated.

A number of interesting features are revealed by the analysis. It is evident that indeed the six *T. congolense* MSPs we have identified do fall into five discrete subfamilies. The previously described *T. brucei* MSP-A, -B, and -C fall into discrete subfamilies and colocalize with TcoMSP-A, TcoMSP-B1 and 2, and TcoMSP-C, respectively. Notably, TcoMSP-D and TcoMSP-E each map to individual subfamilies that are clearly distinct from those containing *T. brucei* MSP-A, -B, and -C. This finding indicates that the African trypanosomes share, at a minimum, five distinct subfamilies of MSPs, each with closer homologues in related species than within their own species. This is expected for proteins that evolved and were selected in a common ancestor prior to speciation. This also very likely implies subfamily specific functions/targets.

In an attempt to further classify the MSPs examined in Figure 3, we submitted each of the proteins to predictions of secretion signals (signal peptides) and GPI anchors. Table 1 lists each of the MSP proteins analyzed and indicates the presence or absence of predicted signal peptides and GPI anchor signals. The homologues examined fall into three categories. Most (32 of the 55) are predicted to have a signal peptide and a moderate or high probability of having a GPI anchor. 19 are predicted to have a signal peptide and not have a GPI anchor, suggesting that they are either actively secreted, or anchored by another mechanism. Interestingly, the remaining four homologues are not predicted to encode either a signal peptide or a GPI anchor. If the motif predictions reflect biological function, this indicates that this

subset of “Major Surface Proteases” may actually function within the cytoplasm.

Predictions of localization for each of the *T. congolense* MSPs can be made based on these motif patterns. TcoMSP-D and neighboring proteins 3–7 have strong signal peptide motifs and are not predicted to be GPI anchored. This suggests that these proteins are actively secreted. Interestingly, TcoMSP-C and its two closest predicted neighbors, including the previously described TbMSP-C protein, are also predicted to be actively secreted. Conversely, TcoMSP-B-1 and TcoMSP-B-2 and their MSP-B grouping homologues are predicted to be exported and surface linked via a GPI anchor. This prediction is in agreement with the known function of TbMSP-B in life-cycle specific cleavage of VSG from procyclic *T. brucei* [19]. It is very probable that each member of this subfamily provides the same function in each of the species represented. Of note, the *T. cruzi* specific subgrouping of MSPs (proteins 25–31) also universally encodes both signal peptides and GPI anchors. This commonality with the TbMSP-B subfamily is surprising given that *T. cruzi* does not express VSG but rather produces a variable mucin coat [21]. It is possible that this subgroup of *T. cruzi* MSPs plays an analogous role to TbMSP-B in the *T. cruzi* life cycle, perhaps in shedding bloodstream-expressed mucins. Similarly to the MSP-B subfamily, TcoMSP-A and the grouping homologues from 42–50 also are predicted to be exported and surface linked via a GPI anchor. The role and targets of the MSP-A homologues have yet to be elucidated. Unexpectedly, TcoMSP-E, and three out of four of its closest homologues appear to lack both a leading transport signal sequence and a GPI anchor motif indicating that they are retained within the cytoplasm.

The *Leishmania* MSPs are all predicted to encode a signal sequence but are mixed with regard to encoding predicted GPI anchors. It is possible that some MSP members failed to be recognized as having GPI anchors when in fact they do, as others have found that some variants of *Leishmania* MSP contain a variant GPI anchor signal [22]. Alternatively, it is likely that some subsets of *Leishmania* MSPs are actively secreted [23].

3.3. Cloning, Expression, and Purification of Amino-Terminal Domain of TcoMSP-D and Generation of Specific Antisera. Given the conservation of the MSP-D subfamily, even among the new world trypanosome *T. cruzi*, the known role of *Leishmania* MSPs as virulence factors, and the possibility that TcoMSP-D may be secreted, we elected to further examine TcoMSP-D as a potential target for attempted attenuation of *T. congolense* infection. Indeed, the three MSP-D homologues encoded by *T. congolense*, *T. brucei* TREU97, and *T. gambiense* display a remarkably high level of identity (Figure 4). It is very probable that these homologues target the identical substrate in vivo. It is therefore likely that conditions or treatments that are found to disrupt TcoMSP-D function will also prove effective against homologues in these related species.

The amino-terminal domain from the ORF predicted to encode TcoMSP-D was amplified by PCR and cloned into the pET30a expression vector such that the expressed

protein would encode a poly-histidine (6-his) tag on the amino end. The recombinant protein was purified following induction from the expression vector via nickel affinity chromatography. Purified protein was used to inoculate rabbits for polyclonal antibody production using a standard immunization protocol. Rabbit antiserum was used as the primary antibody for Western analysis of whole cell lysates of blood stream forms of *T. congolense* separated by SDS PAGE. Figure 5(b) reveals that the rabbit antiserum raised against the purified TcoMSP-D amino-terminal domain reacts with a protein with an apparent molecular weight of about 40,000 daltons, which is considerably less than the predicted molecular weight of 74,538 daltons for the full length nascent form. Not visible on the scanned image but visible to the naked eye are two faint larger molecular weight bands of about 50,000 and 70,000 daltons. The relative intensities of the observed bands are consistent both with rapid and significant posttranslational processing and with degradation of the full length protein, neither of which can be conclusively proven from these data. We have independently raised antiserum against the amino-terminal domain of TcoMSP-C and find that this serum detects a single protein of approximately 120,000 daltons in lysates of blood-form *T. congolense* (Bull, Wei, and Tabel, unpublished) suggesting that there is little cross-reaction between expressed MSPs in *T. congolense* (consistent with the low level of identity among the putative MSP members (Figure 1)), and that more than one subfamily is expressed in the blood stream form.

In an attempt to resolve the localization of TcoMSP-D we tried to visualize TcoMSP-D on the surface of fixed blood stream forms of the parasite with the use of fluorescent antibodies. The attempts by both direct microscopic examination and by FACS were unsuccessful. However, we did detect TcoMSP-D in the serum of BALB/c mice following intraperitoneal infection with 10^3 *T. congolense*, clone TC13. No antigen was found in normal serum or serum collected at three days postinfection (DPI) when the parasitemia was less than 10^5 parasites per mL of blood. TcoMSP-D was detected as a strong double band in serum at six DPI (Figure 5(a)) when the parasitemia was 5×10^8 parasites per mL. The TcoMSP-D detected in the serum of infected mice had apparent molecular weights of about 40,000 and 43,000 daltons. While this result is in agreement with the prediction that TcoMSP-D is secreted and further processed and not surface-bound, we cannot rule out the possibility that the observed protein is simply a degradation product released from in vivo disrupted trypanosomes, or during serum preparation. The relative abundance of the detected protein band(s) in the $0.2 \mu\text{L}$ serum sample (equivalent to 1×10^5 parasites) versus the obviously reduced amount seen in 5×10^6 lysed purified blood-stream parasites suggests that during the course of infection TcoMSP-D accumulates in the blood to a level in excess of 50-fold greater than can be accounted for by intact parasites. This indicates either that TcoMSP-D is being actively secreted and is stable or that in vivo destruction of the parasites by day six is very prominent and released TcoMSP-D accumulates. Future studies will be required to determine whether the detected form of the protein has any function.

TABLE 1: Major surface protease homologues.

Number	Species	Identifier*	Probability of a signal peptide	Probability of a GPI anchor
1	<i>T. cruzi</i>	<u>XM_808234</u>	High (29-30)	moderate
2	<i>T. cruzi</i>	<u>XM_806897</u>	Not probable	not probable
3	<i>T. vivax</i>	<u>1037d01.p1k</u>	High (36-37)	not probable
4	<i>T. congolense</i>	TcoMSP-D	High (23-24)	not probable
5	<i>T.b. gambiense</i>	<u>11_v2.orf-3</u>	High (23-24)	not probable
6	<i>T. brucei</i> TREU927	<u>XM_823726</u>	High (23-24)	not probable
7	<i>L. brazilliensis</i>	<u>XM_001562766</u>	High (30-31)	not probable
8	<i>L. guyanensis</i>	<u>LEIGP63X</u>	High (41-42)	High
9	<i>L. mexicana</i>	<u>X64394</u>	High (41-42)	not probable
10	<i>L. donovani</i>	<u>AJ495007</u>	High (41-42)	not probable
11	<i>L. major</i>	<u>XM_001681325</u>	High (41-42)	High
12	<i>L. major</i>	<u>XM_001681324</u>	High (41-42)	High
13	<i>L. major</i>	<u>XM_001684283</u>	High (24-25)	High
14	<i>L. infantum</i>	<u>XM_001470056</u>	High (24-25)	High
15	<i>T. cruzi</i>	<u>XM_802134</u>	High (28-29)	not probable
16	<i>T. cruzi</i>	<u>XM_807916</u>	High (30-31)	not probable
17	<i>T. cruzi</i>	<u>XM_801120</u>	High (29-30)	not probable
18	<i>T. cruzi</i>	<u>XM_799438</u>	High (29-30)	not probable
19	<i>T. vivax</i>	942f04.q1k	High (30-31)	low
20	<i>T. vivax</i>	1924b07.q1k	High (36-37)	High
21	<i>T. vivax</i>	1240d01.p1k	High (36-37)	High
22	<i>T. congolense</i>	TcoMSP-C	High (30-31)	not probable
23	<i>T.b. gambiense</i>	10_v2	High (24-25)	not probable
24	<i>T.b. TREU927</i>	TbMSP-C= <u>XM_817402</u>	High (24-25)	not probable
25	<i>T. cruzi</i>	<u>XM_799266</u>	High (27-28)	High
26	<i>T. cruzi</i>	<u>XM_802023</u>	High (59-60)	moderate
27	<i>T. cruzi</i>	<u>XM_798784</u>	High (59-60)	moderate
28	<i>T. cruzi</i>	<u>XM_815532</u>	High (22-23)	High
29	<i>T. cruzi</i>	<u>XM_815930</u>	Poor (24-25)	moderate
30	<i>T. cruzi</i>	<u>XM_812094</u>	High (20-21)	High
31	<i>T. cruzi</i>	<u>XM_798806</u>	High (22-23)	High
32	<i>T. congolense</i>	TcoMSP-B1	High (21-22)	High
33	<i>T. congolense</i>	TcoMSP-B2	High (21-22)	High
34	<i>T. brucei</i> TREU927	<u>XM_841902</u>	High (58-59)	High
35	<i>T.b. gambiense</i>	08_v2	High (58-59)	High
36	<i>T. brucei</i> TREU927	<u>XM_841905</u>	High (64-65)	moderate
37	<i>T. brucei</i> TREU927	TbMSP-B= <u>XM_841904</u>	High (64-65)	moderate
38	<i>T. vivax</i>	1393e12.p1k	High (20-21)	not probable
39	<i>T. vivax</i>	797h07.q1k	High (22-23)	not probable
40	<i>T. vivax</i>	899g05.p1k	High (19-20)	not probable
41	<i>T. congolense</i>	TcoMSP-A	High (27-28)	moderate
42	<i>T. brucei</i> TREU927	<u>XM_823758</u>	High (26-27)	High
43	<i>T.b. gambiense</i>	1167d01.p1k	High (26-27)	High
44	<i>T.b. rhodesiense</i>	<u>MSP-A2</u>	High (26-27)	High
45	<i>T. brucei</i> TREU927	<u>XM_823757</u>	High (26-27)	High
46	<i>T. b. gambiense</i>	11_v2-orf2	High (26-27)	High
47	<i>T.b. gambiense</i>	2537b02.p1k	High (26-27)	High
48	<i>T. brucei</i> TREU927	<u>XM_823755</u>	High (26-27)	High

TABLE 1: Continued.

Number	Species	Identifier*	Probability of a signal peptide	Probability of a GPI anchor
49	<i>T.b. rhodesiense</i>	<u>MSP-A1</u>	High (26-27)	High
50	<i>T.b. rhodesiense</i>	TbMSP-A= <u>U86345</u>	High (26-27)	High
51	<i>T. vivax</i>	1124g05.p1k	High (28-29)	low
52	<i>T. vivax</i>	1764g03.p1k	High (23-24)	moderate
53	<i>T. congolense</i>	TcoMSP-E	not probable	not probable
54	<i>T. brucei TREU927</i>	<u>XM.823843</u>	not probable	not probable
55	<i>T.b. gambiense</i>	11_v2-orf1	not probable	low

* See supplementary data for full protein sequences.

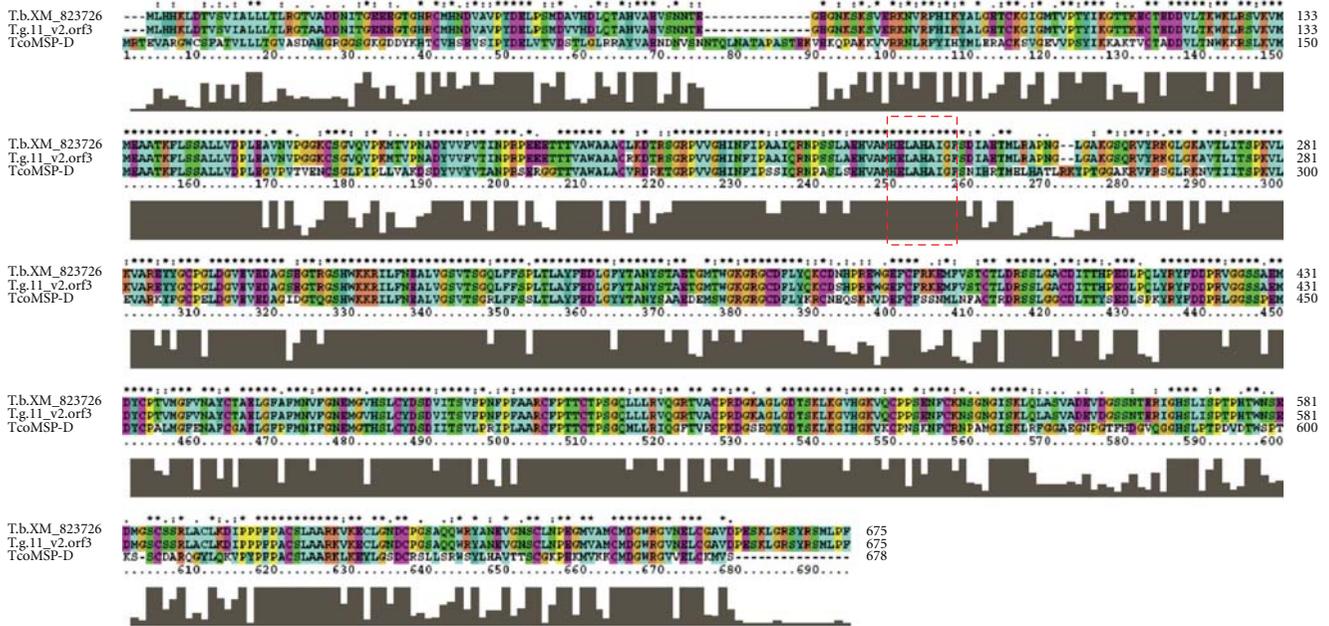


FIGURE 4: Multiple sequence alignment of MSP putative functional homologues; and *T. congolense* TcoMSP-D, *T. brucei* TREU927 XM.823726, *T. gambiense* chromosome 11 MSP ORF3. The predicted amino acid sequences for each homologue were aligned using the ClustalW alignment tool. Output was directly generated using ClustalX for Windows. The dashed red box indicates a conserved motif shown to be essential for Zn²⁺ binding in related proteins.

3.4. Effect of TcoMSP-D Immunization on Infection by *T. congolense*. We wished to address whether prior immunization of mice with recombinant TcoMSP-D amino-terminal domain provides protection against a *T. congolense* infection. BALB/c mice were immunized with purified protein in adjuvant and sera were collected at various times post immunization. Figure 6(a) shows that immunization generated a strong IgG1 antibody response to TcoMSP-D amino-terminal domain as measured by ELISA. There was also a detectable but modest IgG2a antibody response (Figure 6(b)). When we challenged the immunized mice with a dose of 10⁴ *T. congolense* TC14 or higher, all control mice as well as all mice immunized with the amino-terminal domain of TcoMSP-D developed parasitemia (data not shown). Thus, at high-dose challenge the immunization had no observable effect.

To determine if immunized mice had gained increased protection to a lower dose challenge, we challenged immunized and control (adjuvant only) BALB/c mice with a subcutaneous low-dose infection in the hind footpad. This route and challenge size was chosen as it provides a border line infection probability. The results of a typical challenge assay are shown in Figure 7. BALB/c mice that had received adjuvant only and were infected with 2 × 10³ *T. congolense* clone TC14 did not develop parasitemia (Figure 7(a)) and remained entirely healthy until the end of the observation period of 30 days (Figure 7(d)). More than 50% of the mice immunized with either 5 μg or 50 μg of the amino-terminal domain of TcoMSP-D, however, developed parasitemia (Figures 7(b) and 7(c)) and had a mean 50% survival time of only 11 days (Figure 7(d)). Thus unexpectedly, immunization with TcoMSP-D amino-terminal domain results in

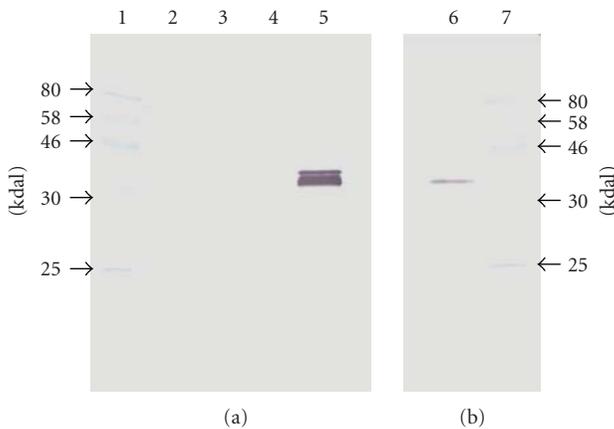


FIGURE 5: Demonstration of TcoMSP-D in whole cell lysates of *T. congolense* and in cell-free serum of infected mice. Cell-free serum (equivalent to 0.2 μ L of undiluted serum) from control and infected mice and *T. congolense* whole cell lysates were separated by SDS PAGE and transferred to nylon membrane for Western analysis. The membrane was probed with rabbit α -TcoMSP-D (NH3 domain) at 1/500 dilution and visualized with goat α -rabbit HRP conjugated secondary antibody at 1/500 dilution. Lane 1: molecular weight standards; lane 2: normal mouse serum; lane 3: serum from *T. congolense*-infected mouse at 3 days post infection; lane 4: empty lane; lane 5: serum from *T. congolense*-infected mouse at 6 days post infection; lane 6: lysate from equivalent of 5 million blood-form *T. congolense* parasites; lane 7: molecular weight standards.

a significant increase in susceptibility to infection with *T. congolense*.

4. Discussion

Kinetoplastid protozoa have been known to encode a conserved family of zinc metalloproteases on their surface, collectively called Major Surface Proteases (MSPs) [7, 9]. In vitro treatment of *T. brucei* with a peptidomimetic inhibitor, which has been demonstrated to inhibit a purified predominant *L. major* MSP in vitro, also results in active killing of *T. brucei* [8]. This finding strongly suggests that at least one MSP protein in *T. brucei* plays a vital role in cell survival. To date, there has not been any report of a member of this family of proteins in *T. congolense*. Given the expected conservation of this family of potentially surface-exposed proteins in *T. congolense*, and the potential for targeting this group of proteins as invariant surface antigens and key virulence factors, we have searched for potential homologues predicted to be encoded within the partially completed *T. congolense* genomic DNA sequence. We were successful in identifying six potential homologues. The large degree of variation among all but two of the predicted proteins suggested to us that these homologues were likely to have differing roles in the *T. congolense* life cycle. To date, three subtypes of MSPs have been described in the related *T. brucei*. These have been named TbMSP-A, TbMSP-B, and TbMSP-C [7]. Of these only TbMSP-B has been characterized to a level whereby its expression pattern and target substrate have been

demonstrated, indicating that there is still very much to learn about the roles these proteins play in trypanosome biology [7, 19]. As a starting point to understanding the observed divergence among the *T. congolense* MSPs we performed a phylogenetic analysis including the closest homologues from related trypanosomes and *Leishmania* sp. Our finding of five conserved subfamilies among the African trypanosomes clearly indicates that we have identified diverse, conserved functional subfamilies within the MSP family of proteins. Not surprisingly, the TbMSP-A, -B, -C proteins each fall in a separate subfamily. In addition, we have identified two novel subfamilies which we have named MSP-D and MSP-E. We believe this to be a major finding. However it is important to stress that, as was the case for the initial report of *T. brucei* MSP subfamilies [7], additional subfamilies may become apparent as additional complete genomic sequences become available. Indeed, *T. congolense* may likely be shown to harbor additional MSPs that have yet to be sequenced.

Notably, the less related New World trypanosome *T. cruzi* has members in the MSP-D (proteins 1 and 2 in Figure 3 and Table 1) and MSP-C (proteins 15–18 in Figure 3 and Table 1) subfamilies as well as a grouping of six related proteins in what appears to be their own subfamily that maps near the MSP-B subfamily (Figure 3). This may suggest that the MSP-D and MSP-C subfamilies are more ancient than, for example, the MSP-A subfamily, or alternatively, that this subfamily was lost in the *T. cruzi* species after species separation. As a consequence, the MSP-A subfamily may be uniquely required for an aspect of the African trypanosome life cycle that is not shared with *T. cruzi*. Similarly, even though the protein homologues most similar to each individual *T. congolense* MSP were independently obtained from the database, the entirety of unique *Leishmania* homologues groups together in the phylogenetic tree. This indicates that each is more similar to other *Leishmania* proteins than to any *Trypanosome* protein included in the analysis. This may indicate separation of *Leishmania* from trypanosomes prior to the generation of the subfamilies or species-specific loss of all but one of the subfamilies in *Leishmania* species. Notably, *Leishmania* MSPs have recently been shown to fall into three distinct clades as well [24]. It may be of interest to determine if the homologues retrieved here are restricted to one clade or represent multiple clades. Of particular interest is the finding that the *Leishmania* MSP subfamily branches out closest to the MSP-D subfamily, which, as argued above, may be one of the more ancient subfamilies.

To test the potential usefulness of immunization against members of a given MSP subfamily, we chose to concentrate on TcoMSP-D. This member had a number of perceived characteristics that attracted our attention. The MSP-D subfamily falls in a node that is closest to the MSPs of *Leishmania*. A *Leishmania* MSP, called GP63 or leishmanolysin, is an important virulence factor that has been demonstrated, among other functions, to protect *L. major* against complement-mediated lysis [25]. GP63 can cleave complement component C3 and in particular cleave C3b into iC3b [25], which, in turn, prevents complement-mediated lysis of *L. major* but favors its CR3-mediated phagocytosis by macrophages [25].

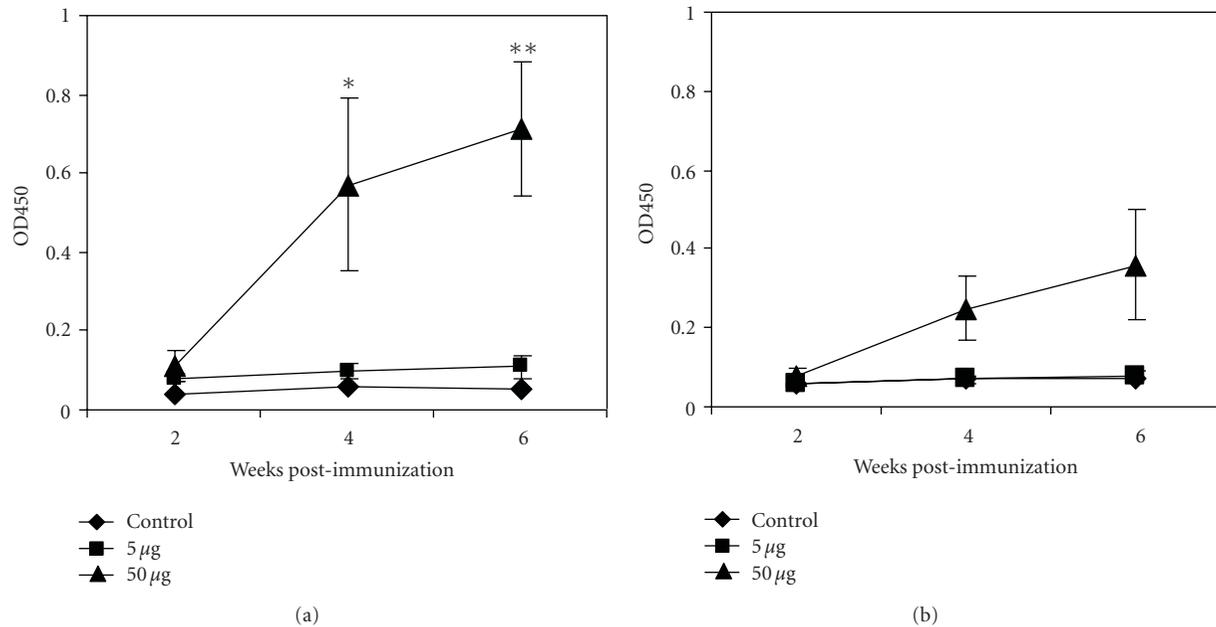


FIGURE 6: Immunization with a high dose (50 µg/mouse) of TcoMSP-D resulting in an increased antigen specific IgG1 antibody response. Groups of 5 BALB/c mice were immunized subcutaneously with TiterMax alone (Control), 5 µg, or 50 µg of antigen TcoMSP-D (amino-terminal domain) in TiterMax at 4 different sites (25 µL/site). Serum was collected at weeks 2, 4, and 6 postimmunization. Sera IgG1 (a) and IgG2a (b) specific for TcoMSP-D were determined by ELISA. The 50 µg group showed a significantly higher IgG1 antibody titer than the control group at week 4 and week 6 postimmunization. * $P \leq .05$; ** $P \leq .005$.

T. congolense infections lead to profound and persistent hypocomplementemia in cattle [26], sheep [27], and mice [28]. Homogenates of *T. congolense*, and particulate as well as supernatant fractions thereof, activate bovine complement via the alternative pathway [10]. Binding of IgM antibodies to the variant surface glycoprotein of *T. congolense* activates complement, mediates binding of C3b to the trypanosomes, and leads to phagocytosis of *T. congolense* by macrophages via complement receptor 3 (CR3) [29]. Lysis of *T. congolense* by antibody and complement is inefficient [30, 31]. Since we found that TcoMSP-D appears to be most closely related to *Leishmania* GP63 (Figure 3), we are considering that TcoMSP-D may have enzyme activities similar to those of *Leishmania* GP63. Our demonstration that TcoMSP-D accumulates in mouse serum to greater than 50-fold higher levels than can be accounted for by intact live trypanosomes by day 6 post infection (Figure 5) is consistent with an extracellular role. It is intriguing to speculate that the inefficiency of complement-mediated lysis of *T. congolense* might be mediated by the effects of TcoMSP-D. Definitive testing of this hypothesis will require purification of the active form of the protein.

Our finding that immunization against TcoMSP-D amino-terminal domain significantly enhanced the infectivity of a low dose of *T. congolense* was unanticipated. We expected that the binding of the antibody to the MSP would inhibit the function of this MSP and either would lead to an attenuated infection, or would have no detectable effect. The demonstration of an effect of enhanced infectivity (Figure 7) indicates that immune responses against this MSP protein

can somehow downregulate the resistance to infection. CD4⁺ CD25⁺ Foxp3⁺ T regulatory cells are involved in immune suppression in *T. congolense* infections [32]. When mice were infected subcutaneously with a low dose of *T. congolense* clone TC13 (10²), they controlled the infection but showed enhanced susceptibility upon subcutaneous challenge with a different clone of *T. congolense* (Wei and Tabel, unpublished). Though this finding very closely mirrors the increased susceptibility seen in TcoMSP-D immunized mice, we presently have no information on the potential mechanism(s) responsible. Perhaps significantly, the amino-terminal domain of TcoMSP-D, used for immunization, did not include the potential catalytic site (Figure 1). How could one envision an increase of infectivity of *T. congolense* by a specific antibody response to the N-terminal fragment of the putative enzyme? It is conceivable that, rather than neutralizing enzyme activity, the binding of antibodies to the N-terminal portion of the putative enzyme might actually stimulate its activity, as has been demonstrated for antibody binding to horse radish peroxidase [33]. The propeptide of *Leishmania* GP63 inhibits the activation of the proenzyme [34]. It is conceivable that the antibody, when binding to the N-terminal domain of the putative proenzyme form of TcoMSP-D, might alter the structure of the protein in such a way that it is able to exert enzyme activity.

In summary, we have cloned, expressed, and purified a partial protein encoding the amino-terminal domain of TcoMSP-D, the *T. congolense* MSP homologue found to be most closely related to *Leishmania* GP63. Whatever

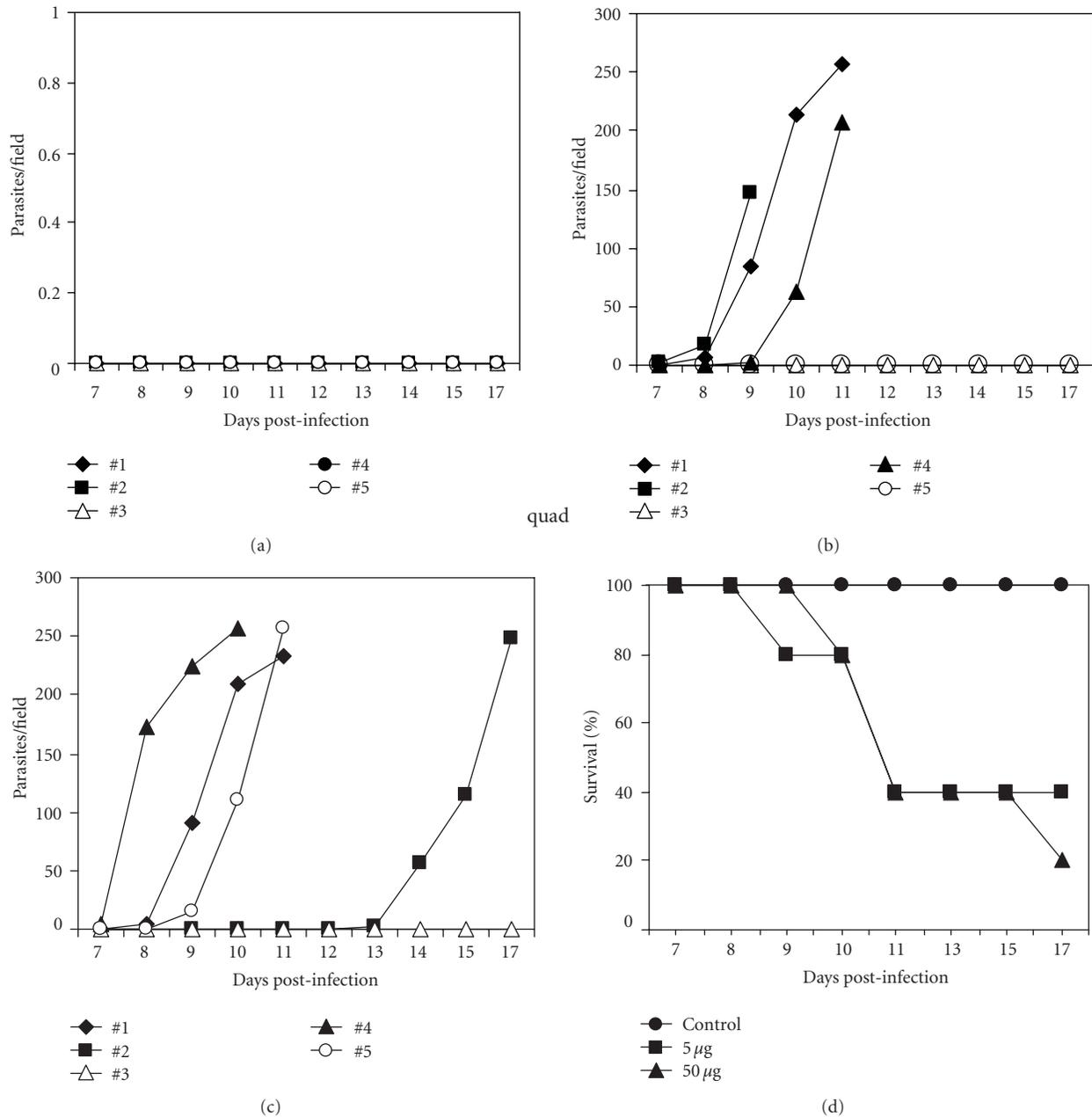


FIGURE 7: Immunization with TcoMSP-D amino-terminal domain resulted in increased susceptibility to infection: enhanced parasitemia and shorter survival time. Groups of 5 BALB/c mice were injected subcutaneously (s.c.) with TiterMax alone (Control), 5 µg, or 50 µg of the amino-terminal domain of TcoMSP-D in TiterMax at 4 different sites (25 µL/site). Six weeks postimmunization, all mice were infected with 2×10^3 *T. congolense* clone TC14 s.c. in a hind footpad. Parasitemia of the individual mice in each group [(a), control; (b), 5 µg; (c), 50 µg] and survival (d) were monitored after the infection. Mice which did not show any detectable parasitemia lived normally without any sign of disease until the termination of the experiment on day 30 postinfection. The results are representative of two different experiments.

the mechanism might be, our observations have led us to conclude that TcoMSP-D is a virulence factor of *T. congolense*.

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Review Article

Gene Expression in Trypanosomatid Parasites

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The parasites *Leishmania* spp., *Trypanosoma brucei*, and *Trypanosoma cruzi* are the trypanosomatid protozoa that cause the deadly human diseases leishmaniasis, African sleeping sickness, and Chagas disease, respectively. These organisms possess unique mechanisms for gene expression such as constitutive polycistronic transcription of protein-coding genes and trans-splicing. Little is known about either the DNA sequences or the proteins that are involved in the initiation and termination of transcription in trypanosomatids. *In silico* analyses of the genome databases of these parasites led to the identification of a small number of proteins involved in gene expression. However, functional studies have revealed that trypanosomatids have more general transcription factors than originally estimated. Many posttranslational histone modifications, histone variants, and chromatin modifying enzymes have been identified in trypanosomatids, and recent genome-wide studies showed that epigenetic regulation might play a very important role in gene expression in this group of parasites. Here, we review and comment on the most recent findings related to transcription initiation and termination in trypanosomatid protozoa.

1. Introduction

The process by which an RNA molecule is synthesized from a DNA template is known as transcription. All cells must constantly produce RNA molecules that are directly or indirectly involved in life processes like reproduction, growth, repair, and regulation of metabolism. Eukaryotic cells have three distinct classes of nuclear RNA polymerases (Pol): Pol I, II, and III. Each class of polymerase is responsible for the synthesis of a different kind of RNA. Pol I is involved in the production of 18S, 5.8S and 28S ribosomal RNAs (rRNAs), and Pol II participates in the generation of messenger RNAs (mRNAs) and most of the small nuclear RNAs (snRNAs). Pol III synthesizes small essential RNAs, such as transfer RNAs (tRNAs), 5S rRNA and some snRNAs. Most organisms control the expression of their genes at the level of transcription initiation. However, the regulation of gene expression can also be achieved during either transcription elongation (at the level of chromatin

structure), RNA processing, RNA stability or transport, or translation. A large number of transcription factors help the RNA polymerases produce RNA. Studies on gene expression in eukaryotes have focused mainly on animals, fungi and plants; whereas a relatively small amount of information is available for parasitic protozoa.

The flagellated protozoa *Leishmania*, *Trypanosoma brucei* and *Trypanosoma cruzi* are trypanosomatid parasites (from the order Kinetoplastida) that produce devastating human diseases. Together, these pathogens cause millions of deaths in developing countries (in the tropical and subtropical regions of the world). They exhibit complex life cycles, with different developmental stages that alternate between vertebrate and invertebrate hosts. *Leishmania* species cause a spectrum of diseases, known as leishmaniasis, which range from self-resolving skin ulcers to lethal infections of the internal organs [1]. The World Health Organization (WHO) has estimated that there are over two million new cases of leishmaniasis each year in the world, with 367 million

people at risk. The infection with *Leishmania* starts with the introduction of the infective form, the metacyclic promastigote, into the skin by the bite of an infected sandfly. Once inside the mammalian host, the infective promastigotes invade the macrophages and differentiate into amastigotes, which are the proliferative forms within the vertebrate host. In the insect vector, the parasite replicates as a non-infective procyclic promastigote [2]. *T. brucei*, the African trypanosome, is the causative agent of sleeping sickness in humans and nagana in animals. Approximately 500,000 people, in the least developed countries of Central Africa, are affected by the disease every year. The parasite is transmitted among mammalian hosts by the tsetse fly. The procyclic form of *T. brucei* multiplies in the gut of the insect vector and differentiates into a bloodstream form that is found in the blood and tissue fluids of mammalian hosts. *T. brucei*, unlike *T. cruzi* and *Leishmania*, does not present any intracellular forms [3]. *T. cruzi* is the etiological agent of Chagas disease, which affects several million people in Latin America. It is normally transmitted by reduviid insects via the vector feces. The parasite replicates as an epimastigote in the midgut of the insect, and transforms into an infective metacyclic trypanomastigote in the hindgut. Amastigotes are the proliferative form in the vertebrate host [4]. Trypanosomatids have also attracted the attention of molecular biologists because they possess unique mechanisms for gene expression, such as polycistronic transcription, trans-splicing, the involvement of Pol I in the synthesis of mRNA and RNA editing [5–10]. This work will review the current knowledge on transcription initiation and termination in trypanosomatids. Recent findings regarding the identification of the proteins involved in transcription and epigenetic regulation will be discussed.

2. Organization of the Nuclear Genome

The 32.8 megabases (Mb) of DNA constituting the nuclear genome from *L. major* is distributed among 36 relatively small chromosomes that range from 0.28 to 2.8 Mb in size [11, 12]. *T. cruzi* possesses a genome of 60.3 Mb organized into 41 small chromosomes [13, 14], whereas *T. brucei* (genome of 26 Mb) has 11 large chromosomes [15, 16]. The genomes of trypanosomatids are organized into large polycistronic gene clusters (PGCs), that is, tens-to-hundreds of protein-coding genes arranged sequentially on the same strand of DNA (Figure 1). This unusual gene organization was first observed on *L. major* chromosome 1 (the first entirely sequenced chromosome in trypanosomatids), which contains 85 genes organized into two divergent PGCs, with the first 32 genes clustered on the bottom strand and the remaining 53 genes grouped on the top strand [17]. The publication of the complete genomes for *L. major* [12], *T. brucei* [14] and *T. cruzi* [16], showed that the majority of genes in all the trypanosomatid chromosomes are organized into large PGCs. Tandem arrays of rRNA genes are present between PGCs. Most tRNA genes are organized into clusters of 2 to 10 genes, on either top or bottom strand, which may contain other Pol III-transcribed genes; most of the clusters

are located at the boundaries of PGCs [12, 18]. In contrast to other organisms, the distribution of tRNA genes in the genomes of *L. major* and *T. brucei* does not seem to be totally random, as these genes are confined to a subset of chromosomes [12, 18]. The 5S rRNA genes in *T. brucei* and *T. cruzi* are organized into tandem arrays [19, 20], whereas in *L. major* they are dispersed throughout the genome and are always associated with tRNA genes [12]. Despite the fact that these species diverged more than 200 million years ago, the strong conservation of gene order (synteny) observed in the genomes of trypanosomatids for protein-coding genes is remarkable [21]. In contrast, the majority of the tRNA clusters do not show synteny [18]. Also, the vast majority of protein-coding genes in trypanosomatids lack introns; in fact, cis-splicing has only been demonstrated for the gene encoding the poly(A) polymerase [22]. Similarly, only one isotype of tRNA genes, tRNA-Tyr, contains an intron in trypanosomatids [18, 23]. These organisms are diploid, even though some chromosomes are aneuploid [24]. In addition, the ends of the chromosomes in trypanosomatids contain the telomeric repeat GGGTTA, while the subtelomeric regions are composed of variable repetitive elements, which are responsible for a major part of the size polymorphisms observed between homologous chromosomes [25, 26].

3. Processing of mRNA

Unlike the majority of eukaryotic organisms, transcription in trypanosomatids is polycistronic (Figure 1) [27–29]. Most chromosomes contain at least two PGCs, which can be either divergently transcribed (towards the telomeres) or convergently transcribed (away from the telomeres). Genes from a polycistronic unit in trypanosomatids generally do not code for functionally related proteins [7]. This is entirely different from how operons function in bacteria and nematodes. Mature nuclear mRNAs are generated from primary transcripts by trans-splicing and polyadenylation (Figure 1) [6]. Trans-splicing is a process that adds a capped 39-nucleotide miniexon or spliced leader (SL) to the 5' termini of the mRNAs [30, 31]. Like cis-splicing, trans-splicing occurs via two transesterification reactions, but it involves the formation of a Y structure instead of a lariat intermediate [32]. An AG dinucleotide at the 3' splice site and an upstream pyrimidine-rich region are the most conserved sequences required for this process [33–35]. The trans-splicing and polyadenylation of adjacent genes are apparently linked, as the selection of a splice site for a gene influences the choice of a polyadenylation site for the upstream gene [36].

All the genes that are part of a PGC are transcribed at the same level, as a consequence of polycistronic transcription. However, the mature mRNAs of adjacent genes might show very different concentrations and/or stage-specific expression. This is because gene expression in trypanosomatids is mainly regulated posttranscriptionally at the level of mRNA processing and stability [6, 37]. Sequences in the 3' untranslated region (3'-UTR) of an mRNA play a key role in gene expression. For example, the

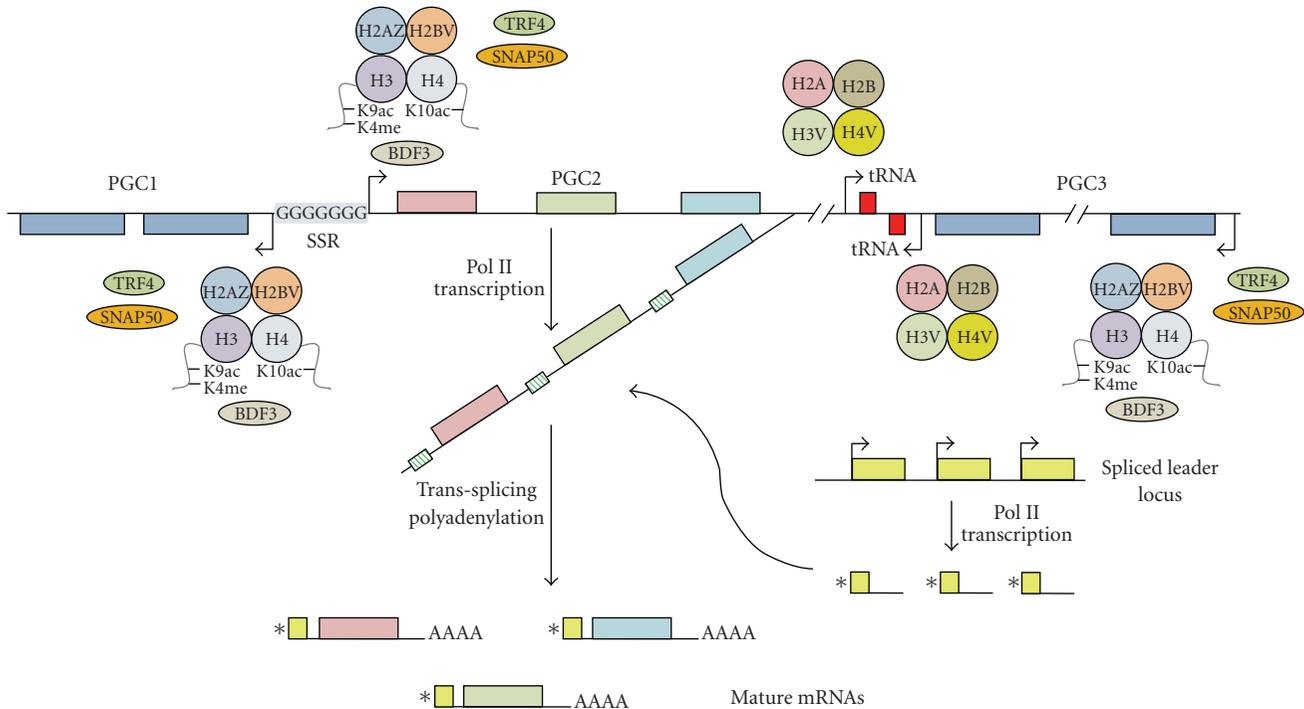


FIGURE 1: Transcription and processing of mRNAs in trypanosomatids. The top part of the figure represents a hypothetical chromosome with three polycistronic gene clusters (PGC1-3). Pol II transcription initiates upstream of the first gene of the PGCs (arrows) [29, 38]. The G-run usually present at divergent strand-switch regions (SSR) is indicated. Nucleosomes located at the vicinity of transcription initiation regions contain histone variants H2AZ and H2BV [39]. The N-terminal tail of histone H3 in such nucleosomes are acetylated at K9/K14 (labeled as K9ac in the figure) and tri-methylated at K4 (K4me) [40, 41]. The N-terminal tail of histone H4 is acetylated at K10 (K10ac) [39], and at K5/K8/K12/K16 [41] (not shown in the figure). The bromodomain factor BDF3 [39], and transcription factors TRF4 and SNAP50 [40] also bind at transcription initiation regions. Pol II transcription of some PGCs terminates near tRNA genes (convergent strand-switch region between PGC2 and PGC3) [38], in regions of DNA that contain nucleosomes with histone variants H3V and H4V [39]. Transcription of a PGC produces a primary transcript (shown only for PGC2) that is processed by trans-splicing and polyadenylation to generate the mature mRNAs. By trans-splicing, a capped SL RNA (yellow box) is added to the 5' end of every mRNA. In the spliced leader locus (located in a different chromosome) each gene possesses a Pol II promoter region (arrows). The cap in the SL RNA is indicated by an asterisk at the 5' end of the RNA. The polycistronic transcript contains pyrimidine-rich regions (indicated by a striped box in the intergenic regions) that are needed for both trans-splicing and polyadenylation. The pyrimidine-rich regions are also present in the DNA, but they are not shown to simplify the figure. The four As located at the 3' end of the mature mRNAs represent the poly-A tail.

3'-UTR from the amastin mRNA in *L. infantum* has a 450 bp region that confers amastigote-specific gene expression by a mechanism that increases the mRNA translation [42]. Also, the mRNA of the phosphoglycerate kinase PGKB in *T. brucei* contains a regulatory AU-rich element in the 3'-UTR that destabilizes the mRNA in bloodstream forms but not in procyclic forms [43]. Moreover, the 3'-UTR region of the EP procyclin mRNA contains 16-mer and 26-mer elements that contribute to mRNA stability and translation efficiency [44, 45]. Interestingly, a growing number of reports have shown that mRNA and protein abundance do not always correlate, and that translational and post-translational control play an important role in trypanosomatids [46–48].

4. Promoter Regions

4.1. Initiation of Transcription by Pol II. Precise transcription initiation of eukaryotic genes is controlled by a segment of

DNA, the promoter region, which includes the transcription start site (TSS, +1) and the immediate flanking sequences. Promoter regions for Pol II typically comprise about 40 bases and contain functional subregions called core promoter elements. These elements include the TATA box, the initiator (Inr) and the downstream promoter element (DPE). The core promoter elements direct the recruitment and assembly of the preinitiation complex (PIC), which is composed of Pol II and the general transcription factors TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH [49, 50].

Presently, the only Pol II promoter that has been extensively characterized on trypanosomatids is the one driving the expression of the SL RNA [51–53]. In *Leishmania tarentolae* it consists of two domains: the –60 element (from –67 to –58, relative to the TSS) and the –30 element (from –41 to –31) (Figure 2). In *Leptomonas seymouri* (a trypanosomatid that infects insects), an initiator sequence at the TSS is additionally required for Pol II to synthesize the SL RNA.

Identification of the Pol II sequences that direct the expression of protein-coding genes in trypanosomatids has proven to be an elusive aim, complicated by factors such as relatively low transcriptional activity and rapid processing of the primary transcripts. However, a run-on analysis of chromosome 1 from *L. major* showed that Pol II transcription of the entire chromosome initiates in the strand-switch region (between the two divergent PGCs) and proceeds bidirectionally towards the telomeres [29]. Several TSSs were mapped for both PGCs within a <100-bp region that contains long G-tracts (or C-tracts), but do not contain a TATA box or any other typical Pol II core promoter elements (Figure 2). Thus, as opposed to the case in most eukaryotes, where each gene possesses its own promoter, a single region seems to drive the expression of the entire chromosome 1 in *L. major*. Similar studies performed on chromosome 3 from *L. major* confirmed that Pol II transcription initiates bidirectionally within a divergent strand-switch region, and close to the “right” telomere upstream of a 30-gene cluster [38]. Supporting these observations, a recent ChIP-chip study in *L. major* showed that H3 histones acetylated at K9/K14, a marker for sites of active transcription initiation in other eukaryotes, are found at all divergent strand-switch regions in the parasite [40]. Moreover, peaks for two transcription factors, TRF4 and SNAP50, were also associated with divergent strand-switch regions [40]. Also, histone modifications linked to active genes in other organisms were found to be enriched at divergent strand-switch regions in *T. cruzi* and *T. brucei* [39, 41]. Strand-switch regions in *T. brucei* also contain histone variants H2AZ and H2BV, which are associated with transcription initiation (Figure 1) [39].

As mentioned above, most of our knowledge regarding the transcription initiation process comes from promoters that have a TATA-box or other core promoter elements, which direct the positioning of the preinitiation complex and initiate transcription from a single nucleotide. However, recent genome-scale computational analyses have shown that ~80% of human protein-coding genes are driven by TATA-less promoters [54, 55] that possess several transcription initiation sites spread over 50–100 bp [56, 57]. While promoters with a TATA box and a unique TSS are generally related to tissue-specific expression, TATA-less promoters usually occur within a CpG island and drive expression of ubiquitously expressed genes. *In vitro* transcription analyses showed that transcription from TATA-less promoters does not require the complete TFIID (TBP and about 15 TAF subunits), and instead requires only TBP [55, 58]. It is possible that other components of the PIC are not required either. This indicates that TATA-less promoters may need simpler initiation complexes. Also, recent studies indicate that bidirectional promoters are common in the human genome [59]. They normally lack TATA box sequences, contain several TSSs and have a high GC content. There is evidence of GC anisotropy (more guanines on the plus strand) in the region around the major TSSs; it is believed that the predominance of guanines on the plus strand could contribute to promoter orientation [56, 60]. Therefore, these genome-wide studies indicate that TATA-less promoters in humans and other

mammals share several characteristics with the strand-switch region from *L. major* chromosome 1 (and probably with all the strand-switch regions in trypanosomatids): they lack a TATA box and have an array of closely located TSSs that span over 50–100 bp; they contain G and C tracts that might direct bidirectional transcription; and they might direct constitutive transcription. Interestingly, some genes with TATA-less promoters in human and mouse have weak TSSs scattered over the majority of exons and 3'-UTRs [56]. The function of the resulting RNAs is uncertain at present. Likewise, the transcriptional analysis of *L. major* chromosome 1 indicated that low levels of nonspecific transcription (10 times lower than transcription starting at the strand-switch area) probably take place all along the chromosome [29].

4.2. Pol III Promoters. An unusual feature of Pol III promoter regions is that most of them require sequence elements located downstream of the TSS, within the transcribed region [61]. The majority of Pol III promoters fall into one of three different categories, depending on the location and type of cis-acting elements. Type I promoters, characteristic of 5S rRNA genes, consist of three internal domains: Box A, an intermediate element (IE), and box C. These elements span a region of approximately 50 bp starting at position +45. Type II promoters are present on tRNA genes and consist of two conserved internal elements: boxes A and B. While box A is normally positioned close to the TSS, the location of box B is variable, partly because some tRNAs have short introns within their coding regions. Type III promoters, characteristic of U6 snRNA genes, consist of elements that reside exclusively upstream of the coding sequence. They contain a TATA box near position -30, a proximal sequence element (PSE) at position -60, and a distal sequence element (DSE) further upstream of the TSS [62, 63].

Pol III synthesizes all the snRNAs in trypanosomatids, in addition to the 5S rRNA, 7SL RNA and tRNAs. The snRNA- and 7SL-gene promoters have been characterized in *T. brucei* and *Leptomonas* [64, 65]. Interestingly, these genes have a divergently oriented tRNA gene in their 5'-flanking region, and boxes A and B from the neighboring tRNA genes are essential for expression of the 7SL RNA and snRNAs [66] (Figure 2). Some of the snRNA genes also require intragenic regulatory elements to achieve an optimal level of expression. Boxes A and B are also needed for expression of the tRNA itself [64]. Gel-retardation assays on *T. brucei* tRNA genes showed the specific binding of nuclear proteins to both boxes [67]. 5S rRNA genes in trypanosomatids contain boxes A and C and IE sequences (Figure 2) [19, 20, 68], but they have not been functionally characterized.

4.3. Transcription by Pol I. In most eukaryotes, the genes encoding the 18S, 5.8S, and 28S rRNA molecules occur as tandem repeats that are clustered at one or several loci. rDNA repeat units are separated from each other by an intergenic spacer [63]. Each cell may contain 100 to several thousand rDNA units. Transcription of the rDNA unit by

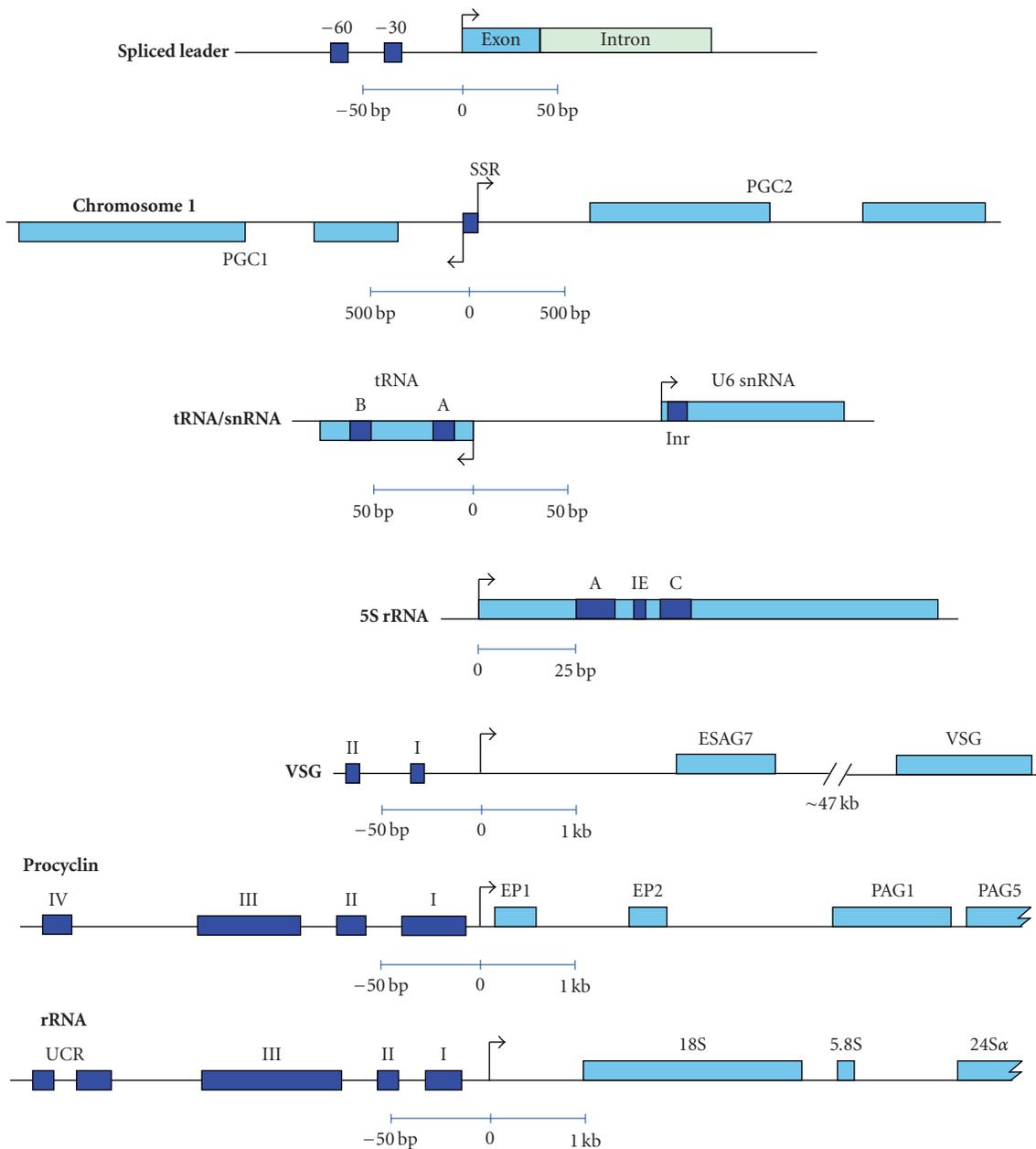


FIGURE 2: Schematic representation of promoter regions in trypanosomatids. Transcription start sites are indicated by arrows. DNA sequence elements required for transcription initiation are denoted by dark blue boxes. Coding regions are shown as light blue boxes. The spliced leader RNA and chromosome 1 (transcribed by Pol II) correspond to *L. tarentolae* and *L. major*, respectively. The tRNA/U6snRNA locus and the 5S rRNA (transcribed by Pol III) correspond to *T. brucei*. The VSG, procyclin and rRNA transcription units (transcribed by Pol I) also correspond to *T. brucei*. The spliced leader RNA promoter contains the -30 and -60 elements [51]. In the divergent strand-switch region (SSR) from chromosome 1 several TSSs were mapped, in a ~ 70 bp region, for both PGCs [29]. The tRNA gene possesses boxes A and B and the U6 snRNA an initiator element (Inr) [66]. The 5S rRNA gene contains boxes A and C and an intermediate element (IE). Domains I and II regulate transcription of the VSG expression site [69]. The VSG gene is located ~ 47 kb downstream of the expression-site-associated-gene 7 (ESAG7). The procyclin promoter consists of four domains (I–IV) [70]. Procyclin genes EP1 and EP2 are followed by procyclin-associated genes (PAGs) 1 and 5. The rRNA promoter contains a bipartite core element (domains I and II), a distal element (domain III) and an upstream control region (UCR) [71]. Coding regions of the 18S, 5.8S and 24S α rRNA genes are shown. Maps are drawn to scale. Please note that the scales are different upstream and downstream of the TSS in the VSG, procyclin and rRNA transcription units.

Pol I produces a large precursor (35–45S) that includes both internal and external transcribed spacers. Subsequent elimination of the transcribed spacers produces the mature rRNA molecules. The precursors of rRNA are synthesized in the fibrillar centers of the nucleoli [72]. In spite of the sequence divergence, promoters in most species have a common structural organization, because they contain two essential domains: an upstream domain located at the 5' boundary near position –150 and a core promoter domain near the site of transcription initiation at +1. Maintenance of the correct spacing between the two domains is critical [72].

A distinctive property of rRNA genes in trypanosomatids is the fragmentation of the 28S-like rRNA into multiple independent molecules: 24S α , 24S β , S1, S2, S4 and S6 [73–75]. In *L. major* there are two copies of the S6 gene per rRNA repeat [76]. The *T. brucei* rRNA promoter contains a bipartite core element (domains I and II in Figure 2) and a distal element (domain III), which resembles the typical eukaryotic Pol I promoter [77]; the promoter also contains an upstream control region (UCR) that extends to roughly –250, but it only has a minor influence on transcription efficiency [69, 71]. In *Leishmania* and *T. cruzi* the rRNA promoters are apparently smaller, as they lack upstream control elements [78–83]. Interestingly, a down regulating region of about 200 bp was located downstream of the TSS in the rRNA genes from *T. cruzi* [84].

Pol I not only produces rRNA in *T. brucei*, but also has the remarkable capacity to synthesize the mRNAs of two of the most abundant proteins in the parasite: the variant surface glycoproteins (VSG) and the EP/GPEET procyclins [85, 86]. VSGs are expressed in the bloodstream form of the parasite, and they participate in the process of antigenic variation, a survival strategy that allows the parasite to escape host cell immunological attacks [87, 88]. *T. brucei* has ~1000 VSG genes, but only the one located at the active expression site (ES) is expressed. VSG switching occurs by turning off the active ES and turning on another one (*in situ* switch), or by replacing the VSG gene within the active ES. The replacement of the VSG gene can be achieved by copying a new VSG gene into the active ES by duplicative gene conversion, or by reciprocal translocations between two expression sites [87–91]. It was recently demonstrated that antigenic switching by gene conversion is triggered by a DNA double-stranded break within the 70-bp repeats located upstream of the transcribed VSG gene [92].

The VSG coat is replaced by a new surface coat composed of EP/GPEET procyclins when bloodstream forms differentiate into the procyclic forms found in the tsetse fly gut [93]. The expressed VSG gene is located at the end of a ~50 kb polycistronic unit that contains up to twelve genes known as the expression-site-associated-genes (ESAGs) [5, 94] (Figure 2). The procyclin genes are organized into 5–10 kb polycistronic transcription units on chromosomes VI and X. Each procyclin locus has two procyclin genes followed by several procyclin-associated genes (PAGs) [95] (Figure 2). The promoter regions of the genes encoding procyclins are very similar to the rRNA gene promoter in *T. brucei*, as they are composed of four domains that extend to nucleotide –246 (Figure 2) [70, 77, 96]. In contrast, the VSG promoter

contains only a bipartite core domain that extends to position –67 (Figure 2) [69, 97, 98]. Further work is required to comprehend the functional implications of the differences among Pol I promoters in *T. brucei*.

5. RNA Polymerase Subunits and Transcription Factors

In *S. cerevisiae*, Pol II contains 12 subunits, whereas Pol I and Pol III are composed of 14 and 17 subunits, respectively [49, 63]. Five of the subunits are common to all three RNA polymerases (ABC27, ABC23, ABC14.5, ABC10 α and ABC10 β) while two are shared between Pol I and Pol III (AC40 and AC19), with homologues in Pol II (B44 and B12.5, resp.). Another five are homologous subunits (A190/B220/C160, A135/B150/C128, A43/B16/C25, A14/B32/C17, and A12/B12.6/C11). Five subunits are exclusive to Pol III (C82, C53, C37, C34 and C31), and two subunits are Pol I-specific (A49 and A34) [61, 62, 99].

All of the common and most of the homologous RNA polymerase subunits were identified in trypanosomatids by *in silico* analysis; however, some of the specific subunits were not found [12]. Interestingly, trypanosomatids have two or three different genes encoding ABC10 β , ABC23, and ABC27. The sequences of such genes are widely divergent, which suggests that the subunits they encode may not be common to all three RNA polymerases, as they are in other eukaryotes [12]. In fact, Pol II transcription complexes isolated by the TAP-tag protocol in *T. brucei* and *L. major* contained only one of the isoforms of ABC10 β , ABC23 and ABC27 (as discussed below, isoforms ABC10 β z, ABC23z and ABC27z are part of Pol I) [100, 101]. The other Pol II subunits (B220, B150, B44, B32, B16, ABC14.5, B12.6, B12.5 and ABC10 α) were identified in trypanosomatids by either *in silico* analysis or biochemical characterization [100–102]. Another difference between trypanosomatids and other eukaryotes is that the C-terminal domain (CTD) on the largest subunit (B220) of Pol II in trypanosomatids does not contain the characteristic heptapeptide repeats [103] that are phosphorylated at specific amino acids and play important roles in the regulation of transcriptional initiation, elongation and termination in yeast and vertebrates [49].

A few general transcription factors have been identified in trypanosomatids [104, 105]. While some of them show clear sequence identity to their orthologs in yeast and vertebrates, others present a very low degree of similarity. Regarding Pol II transcription, several general transcription factors that participate in SL RNA synthesis have been identified in *T. brucei*. These include the TBP-related protein 4 (TRF4) [106–108], a very divergent TFIIB ortholog [109, 110] and SNAPc [107, 111]. In humans, SNAPc is essential for the synthesis of small nuclear RNAs, transcribed by either Pol II or Pol III. In *T. brucei*, SNAPc binds to the upstream domain of the SL RNA gene promoter and consists of three subunits (SNAP50, SNAP2 and SNAP3) [107]. Other proteins that have been identified as part of the Pol II complex that transcribes the SL RNA gene, are the two subunits of TFIIA [107, 108] and TFIIF [112, 113].

In *T. brucei*, TFIIF consists of nine different subunits, including two essential trypanosomatid-specific subunits [114]. Complex PBP2 is also required for transcription of the SL RNA in *Leptomonas* [115]. Thus, these recent findings indicate that trypanosomatids possess more general transcription factors than initially estimated from *in silico* studies.

In *L. major*, Pol III transcription complexes were purified using the TAP-tag procedure with ABC23 as the target [101]. Analysis of the purified complexes revealed 12 Pol III subunits: C160, C128, C82, C53, C37, C34, C17, AC40, AC19, ABC27, ABC23 and ABC14.5. The rest of the 17 subunits, with the exception of C31, have been identified by *in silico* analysis. Eight Pol II subunits were also co-purified with TAP-tagged ABC23. However, no single Pol I-specific subunit co-eluted with ABC23, which showed that this isoform of ABC23 is restricted to Pol II and Pol III, while the other isoform (ABC23z) is limited to Pol I. This result was confirmed in *T. brucei* by co-immunoprecipitation experiments [116]. Other proteins that were purified with TAP-tagged ABC23 are: two RNA binding proteins, a putative transcription factor, the splicing factor PTSR-1, four helicases and several proteins of unknown function [101]. BRF1 and B'', two subunits of the Pol III transcription factor TFIIB, have also been identified in trypanosomatids [12]. However, neither TFIIA nor TFIIC have been found in this group of parasites. Interestingly, ChIP-chip studies indicated that TRF4 and SNAP50 bind to all tRNA, snRNA and 5S rRNA gene clusters in *L. major* [40].

Analysis of the Pol I complex in *T. brucei* led to the identification of ten subunits: A190, A135, A12, ABC23z, ABC27z, ABC14.5, ABC10βz, ABC10α, AC19 and AC40. These subunits were identified by *in silico* analysis and by TAP-tagging A12 [117], A190 [116] and ABC23z [118]. It was recently shown that B16 (RBP7), a Pol II-specific subunit, is also associated with Pol I in *T. brucei* [119]. Also, a novel trypanosomatid-specific Pol I subunit (p31) was identified in *T. brucei* [118].

Several transcription factors take part in the synthesis of rRNAs in vertebrates and yeast, including UBF and SL1, which interact with each other in the promoter region to allow the binding of Pol I to the initiation complex. UBF is not present in yeast, which have a different factor called UAF. Another protein involved in Pol I transcription initiation is RRN3 (also known as TIF-IA) [63, 72]. None of these transcription factors have been identified in trypanosomatids. However, a new Pol I protein, named class I transcription factor A (CITFA), was recently purified and characterized in *T. brucei* [120]. It consists of a dynein light chain and six proteins that are conserved only among trypanosomatids. CITFA specifically binds to VSG, procyclin and rRNA promoters [120]. Unexpectedly, it was demonstrated in *L. major* that TRF4 and SNAP50 bind to the rRNA coding regions, but not to the promoter sequences [40].

6. Transcription Termination

Termination of transcription is a process that has received little attention in trypanosomatids. It has been reported that

a T tract, similar to the T-rich regions that are involved in Pol III transcription termination, located downstream of the SL RNA gene directs Pol II transcription termination. At least six Ts are required for efficient termination *in vivo* in *L. tarentolae* [121]; the mature 3' end of the SL RNA is generated by nucleolytic processing. Interestingly, Pol II transcription of protein-coding genes does not stop at T-rich sequences, as such sequences are very common to intergenic regions in PGCs. Therefore, there must be functional differences between the Pol II complexes that synthesize the SL RNA and those that transcribe protein-coding genes; alternatively, epigenetic regulation might cause the observed differences. Termination of transcription has also been analyzed on chromosome 3 from *L. major*, which contains two convergent PGCs separated by a tRNA gene [122]. Nuclear run-on and RT-PCR assays indicated that Pol II-mediated transcription of both PGCs terminates within the tRNA-gene region [38]. The presence of a termination region between two convergent PGCs on *L. major* chromosome 3 suggests that *Leishmania*, like yeast, may require the separation of adjacent Pol II transcription units by proper termination signals to avoid polymerase collisions [123]. Because several convergent PGCs in trypanosomatids are separated by tRNA genes (or other genes transcribed by Pol III) [18], the involvement of tRNA genes in transcription termination may not be exclusive to *L. major* chromosome 3. Interestingly, the tRNA gene region on chromosome 3 can also terminate Pol I and Pol III transcription [38]. A ChIP-seq study showed that histone variants H3V and H4V are present at convergent strand-switch regions and other parts of the *T. brucei* genome where transcription probably ends, suggesting that chromatin structure plays a significant role in transcription termination in trypanosomatids [39].

In most cases, Pol III ends transcription at simple clusters of four or more T residues, normally flanked by G+C-rich sequences [124]. In human and mice, tRNA genes need four Ts to end transcription, whereas in *S. pombe* and *S. cerevisiae* tRNA genes require six and seven Ts, respectively [125, 126]. For a particular species, termination efficiency tends to increase with the length of the T run. The proteins that stimulate Pol III transcription termination in human cells include Nuclear Factor 1 (NF1), Positive Factor 4 (PC4), and the La antigen, an UUU-OH-terminus-binding protein [61]. Subunit TFIIC2 of TFIIC participates in transcription termination and in transcription reinitiation. Moreover, it has been reported that Pol III subunits C11, C37 and C53 form a subcomplex that is also involved in transcription termination and reinitiation [61].

In trypanosomatids, as in higher eukaryotes, T runs function as Pol III termination signals. It was reported that transcription of the *T. brucei* U2 snRNA terminates at several Ts located downstream of the gene [127]. A cluster of Ts of variable length was found on every single tRNA gene in trypanosomatids. The distance between the end of the gene and the run of Ts varies from zero to seven bases [18]. In *L. major*, the average length of the T run is 4.87 bases, with a minimum of four and a maximum of nine Ts. In *T. brucei* the mean T-run length is 4.89 bases (ranging from four to ten Ts). The stretches of Ts are longer in tRNA genes from *T. cruzi*

(mean length of 6.56 bases), with two genes showing a T run of 16 residues [18]. In *L. major*, it has been shown that Pol III transcription of the tRNA gene located on chromosome 3 terminates within a tract of four Ts [38]. To date, no single protein involved in Pol III transcription termination has been identified in trypanosomatids.

In eukaryotic cells, transcription termination elements for Pol I are located downstream of the 28S rRNA gene and upstream of the transcription start site. In mammals, factor TTF-I binds the termination elements at the 3' end of the transcribed region, forcing Pol I to pause, and cooperates with the transcript-release factor PTRF in conjunction with a T-rich DNA region to induce transcription termination [72, 128]. Regarding termination of transcription in rRNA genes in trypanosomatids, in *L. infantum* it was found that transcription ceases downstream of the 3' end of the rDNA unit in an area that contains short sequences with the potential to form stem-loop structures, which are reminiscent of the bacterial rho-independent transcriptional terminators [129]. *L. major* rRNA genes contain a similar sequence in a region where run-on assays indicated that transcription terminates [76]. In the GPEET/procyclic locus from *T. brucei*, three sequence elements that are located downstream of the last gene of the cluster act synergistically to terminate Pol I transcription in an orientation-dependent way [130]. Also, it was recently reported that Pol I transcription of the EP/procyclic locus ends within the PAG1 gene (Figure 2). Transient and stable transfections showed that sequence elements on both strands of the gene can inhibit Pol I transcription [131].

7. Transcription of Transposable Elements

Although trypanosomatids do not seem to contain DNA transposons, analysis of their genomes confirmed the presence of abundant long terminal repeat (LTR) and non-LTR retrotransposons [14, 132]. They account for ~5% and 2% of the *T. cruzi* and *T. brucei* genomes, respectively. In *T. cruzi*, one of the most abundant retrotransposons is *L1Tc*, which encodes a protein that contains several domains, including reverse transcriptase, endonuclease, RNase H and DNA binding [133]. Interestingly, it was shown that the first 77 bp of *L1Tc* behave as a promoter region that activates transcription of the retrotransposon [134]. Run-on experiments indicated that transcription of *L1Tc* is driven by Pol II. It is worth noting that the 77 bp region is also present in other transposable elements in trypanosomatids [14]. SLACS is a retrotransposon from *T. brucei* that integrates exclusively at nucleotide 11 of the SL RNA gene [135]. It was reported that transcription of SLACS starts at the +1 nucleotide of the interrupted SL RNA gene, but in this case transcription is directed by the upstream SL RNA promoter (carried out by Pol II) [136].

In contrast to *T. cruzi* and *T. brucei*, *Leishmania* species do not contain active retrotransposons. However, they have remnants of extinct *ingi/L1Tc*-like retrotransposons called DIREs [137]. Recently, two new families of degenerated retrotransposons were identified in *Leishmania*: SIDER1 and SIDER2 [138, 139]. These sequences are predominantly located

within the 3'-UTR of *Leishmania* mRNAs. It was shown that SIDER2 acts as an instability element, since SIDER2-containing mRNAs are generally expressed at lower levels compared to the non-SIDER2 mRNAs [138]. Interestingly, a significant number of SIDER elements map to divergent strand-switch regions [140], which may contain Pol II transcription initiation sites (see Section 4.1). Thus, it is feasible that SIDER sequences participate in the control of transcription in *Leishmania* [140]. Supporting this possibility, a large fraction of binding sites for transcription factors are embedded in distinctive families of transposable elements in mammals [141, 142].

8. Epigenetics

Nuclear DNA in eukaryotic cells is organized in a complex DNA-protein structure called chromatin. The fundamental subunit of chromatin is the nucleosome core, composed of an octamer of small, basic proteins named histones around which ~147 bp of DNA are wrapped. The histone octamer consists of two copies each of H3, H4, H2A and H2B, known as the core histones. A different histone, H1, binds to the "linker DNA" region (~80 bp) between two nucleosomes, and helps to stabilize the chromatin. The nucleosomal array, which imparts about a sevenfold condensation of the DNA molecule, is compacted another sixfold into a 30-nm chromatin fiber. Dynamic changes in chromatin structure play a very important role in the regulation of DNA-dependent processes such as transcription. Several mechanisms regulate chromatin structure, including: nucleosome remodeling by ATP-driven complexes, covalent modifications of the N-terminal tails of the core histones, replacement of one or more of the core histones by their variants, and nucleosome eviction [143, 144]. In general, the nucleosome works as a transcriptional repressor that prevents the binding of transcription factors to promoter regions. However, correctly positioned nucleosomes can bring remote DNA sequences into close proximity to activate transcription [145]. At least eight different types of modifications have been found on histones [146]. Small covalent modifications such as acetylation and methylation have been extensively studied; acetylation is almost invariably associated with transcription activation, while methylation can be related to either activation or repression [146]. Histone variants, which are non-allelic isoforms of canonical histones, can be incorporated into nucleosomes. As a result, the structure and function of the nucleosome are modified [147]. The combination of histone modifications and histone variants generates a vast variety of nucleosomes.

Little is known about chromatin structure and epigenetic regulation in trypanosomatids [148–150]. These organisms have several copies of the genes encoding histones H1, H2A, H2B, H3, and H4 [12]. Histones in trypanosomatids are, however, extremely divergent from those found in other organisms. Nevertheless, as in other organisms, nucleosomes constitute the basic structural unit of chromatin in trypanosomatids [151, 152]. Micrococcal nuclease (MNase) digestions of chromatin showed the typical nucleosome ladder, with a monomer of ~200 bp in trypanosomes

[153, 154] and *Leishmania* [155, 156]. Also, regular arrays of nucleosomes have been observed by electronic microscopy in this group of parasites [157]. Interestingly, chromosomes in trypanosomatids do not condensate during mitosis; actually, chromatin does not even fold into the 30-nm fibers that are commonly found in higher eukaryotes [157].

Nucleosomal ladders analyzed by Southern-blot, with different regions of the SL RNA gene (transcribed by Pol II) from *L. tarentolae*, indicated that the promoter and transcribed regions are not organized into nucleosomes [158]. However, a consistently positioned nucleosome was found within the non-transcribed intergenic region [158]. Similar experiments performed in *L. major* indicated that while the promoter region of the rRNA unit is devoid of nucleosomes, the rRNA genes are packed into nucleosomes (Vizuet-de-Rueda and Martínez-Calvillo, unpublished results). Also, tRNA and 5S rRNA genes, which contain internal Pol III promoters, showed marked smearings in the MNase digestion profiles, suggesting an “open” structure of chromatin. Protein-coding genes and intergenic regions presented a nucleosomal organization (Vizuet-de-Rueda and Martínez-Calvillo, unpublished results). Thus, the presence and position of nucleosomes along DNA sequences most likely play an important role in controlling transcription initiation by the three different types of RNA polymerases in trypanosomatids, as has been reported in other eukaryotes.

A substantial number of post-translational modifications in histones have been recently identified in trypanosomes. These include several modifications commonly found in eukaryotes, such as acetylation and methylation at several lysine residues on histone H4 [159, 160]. N-methylalanine is a novel modification found at the N-termini of histones H2A, H2B, and H4 in *T. brucei* [161]. The functional relevance of this and other modifications has yet to be determined. *In silico* analysis indicates the presence of a number of enzymes implicated in histone modifications in trypanosomatids, such as acetyltransferases, methyltransferases and histone deacetylases [12]. In *T. brucei*, histone acetyltransferase 2 (HAT2) is required for H4-K10 acetylation [162], whereas HAT3 is responsible for acetylation at H4-K4 [163]. Histone modifications create a “histone code” that is “read” by effector complexes that will mediate subsequent functional outcomes. Components of the effector complexes possess different domains to bind to specific modified histones, including bromodomains, chromodomains and SANT domains [164]. Although several proteins with such domains are present in trypanosomatids, only the Bromodomain Factor 2 (BDF2) from *T. cruzi* has been proven to associate with acetylated histones [165]. Many histone variants have been identified in trypanosomatids [39]; these include H2AZ [166], which has a role in the maintenance of silent chromatin boundaries in higher eukaryotes.

A recent report revealed that acetylated (H3-K9/K14 and H4-K5/K8/K12/K16) and methylated (H3-K4) histones are enriched at divergent strand-switch regions in the *T. cruzi* genome [41]. A genome-wide study showed the presence of H3 histone acetylated at K9/K14 at the origins of polycistronic transcription in *L. major*, together with TRF4 and SNAP50 (Figure 1) [40]. A very small number

of peaks (184) from the acetylated histone were found in the complete genome. While most of them were present at divergent strand-switch regions, some (54) were located at chromosome ends and within PGCs. Sixteen peaks were found in the vicinity of clusters of tRNA and snRNA genes [40]. Similarly, a ChIP-seq analysis in *T. brucei* demonstrated that histone H4 acetylated at K10, histone variants H2AZ and H2BV, and the bromodomain factor BDF3 are enriched at probable Pol II TSSs (Figure 1) [39]. As in *L. major*, most peaks were found upstream of PGCs, but 61 of them were located within PGCs (some peaks were associated with tRNA genes). It was demonstrated that H2AZ/H2BV-containing nucleosomes are less stable than canonical nucleosomes [39]. Thus, posttranslational histone modifications and histone variants might generate an open chromatin structure that is required for initiating transcription in trypanosomatids [167, 168]. Additionally, it was reported that histone variants H3V and H4V, only found in trypanosomatids presently, are enriched in regions where transcription seems to end [39].

9. Concluding Remarks

At the present time, we have limited knowledge about the DNA sequences and proteins that participate in transcription initiation in trypanosomatids. A better understanding of the way gene expression is regulated in these parasites will help us comprehend a number of important processes, such as differentiation, virulence and antigenic variation. It may also help in discovering key targets to control the infections caused by these organisms [9]. Moreover, the study of transcription and epigenetic regulation in early-branching eukaryotes, like trypanosomatids, will help us comprehend the evolution of the transcription machinery and the histone code [167]. Several lines of evidence indicate that transcription of protein-coding genes in trypanosomatids initiates upstream of the characteristic PGCs. Interestingly, the transcription initiation region from *L. major* chromosome 1 shares several features with TATA-less promoters from humans and other mammals. Likewise, recent functional studies have indicated that there are more general transcription factors in trypanosomatids than originally estimated. Thus, transcription of protein-coding genes in trypanosomatids might not be as atypical as initially thought. The identification of histone modifications and histone variants at the origins of polycistronic transcription revealed that chromatin structure plays an important role in transcription initiation in trypanosomatids, as it does in other organisms. Similarly, termination of transcription is likely to be influenced by chromatin-mediated epigenetic regulation. In the near future, genome-wide studies may provide more interesting data that will help us understand the complex mechanisms of transcription initiation and termination in this remarkable group of eukaryotes.

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Review Article

Regulation of Gene Expression in Protozoa Parasites

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Infections with protozoa parasites are associated with high burdens of morbidity and mortality across the developing world. Despite extensive efforts to control the transmission of these parasites, the spread of populations resistant to drugs and the lack of effective vaccines against them contribute to their persistence as major public health problems. Parasites should perform a strict control on the expression of genes involved in their pathogenicity, differentiation, immune evasion, or drug resistance, and the comprehension of the mechanisms implicated in that control could help to develop novel therapeutic strategies. However, until now these mechanisms are poorly understood in protozoa. Recent investigations into gene expression in protozoa parasites suggest that they possess many of the canonical machineries employed by higher eukaryotes for the control of gene expression at transcriptional, posttranscriptional, and epigenetic levels, but they also contain exclusive mechanisms. Here, we review the current understanding about the regulation of gene expression in *Plasmodium sp.*, Trypanosomatids, *Entamoeba histolytica* and *Trichomonas vaginalis*.

1. Introduction

For all cells, regulation of gene expression is a fundamental mechanism for development, homeostasis, and adaptation to the environment. In eukaryotes, every step in the process of gene expression is subject to dynamic regulation, including structural changes of chromatin, transcription of DNA into RNA, processing of the transcript, its transport to cytoplasm, and translation of messenger RNA (mRNA) into protein.

Activation of gene expression requires that cells alleviate nucleosome-mediated repression by means of activator proteins that modify chromatin structure. The activation process displaces or remodels chromatin and opens up regions of the DNA for the binding of regulatory proteins. Chromatin associated with transcription (active chromatin) is generally associated with a range of histone modifications including H3 acetylation of lysine 9 (H3K9) and H3 methylation of lysines 4, 36, and 79 (H3K4me, H3K36me, and H3K79me), whereas heterochromatin (inactive chromatin) appears to be

marked by H3 methylation of lysines 9 and 27 (H3K9me and H3K27me) as well as H4 lysine 20 (H4K20me) [1].

Transcription is the process by which an RNA molecule is synthesized from a DNA template. This process can be divided into three discrete steps: initiation, RNA chain elongation, and termination. Although any step of this process may be controlled, transcription initiation is the stage that usually is most highly regulated. Transcription initiation requires that a complex of proteins called general transcription factors bind to DNA through elements named promoters. The promoters for protein-coding genes consist of core and proximal promoter elements located from 20 to 3000 base pairs upstream from the transcription start site, depending on the organism. In metazoan, the core promoter contains one or typically more sequence motifs, including the TATA box, Inr, TFIIB recognition element (BRE), and downstream core promoter element (DPE) [2]. Together, these DNA elements recruit components of the transcription preinitiation complex (PIC) that facilitate the positioning

and assembly of the RNA polymerase II (RNA pol II) on the promoter.

Thus, transcription initiation is mediated by the concerted action of transcription factors along with the RNA pol II transcriptional machinery, a diversity of coregulators that bridge the DNA-binding factors to the transcriptional machinery, different chromatin remodeling factors that change the nucleosome structure, and a group of enzymes that catalyze covalent modification of histones and other proteins [3].

The synthesis of mRNAs is carried out by the RNA polymerase, which associates transiently not only with the template, but also with many different proteins, including general transcription factors. Finally, the chain of mRNA that results from the direct transcription, called the primary transcript, undergoes modification, sometimes quite extensively, before it can be translated by ribosomes into protein.

Infections with protozoan parasites cause high mortality and morbidity in developing countries and cause an increasing threat to human health. The lack of vaccines against most of the major parasitic diseases has made chemotherapy the only option for treatment. However, resistance to a large number of antiparasitic drugs currently in use causes major health problems. A better understanding to the molecular mechanisms that control the expression of parasitic genes involved in transmission success, pathogenicity, immune evasion, and drug resistance may help to develop novel therapeutic strategies. The progress of DNA transfection techniques and the associated tools have now boosted functional analyses in these microbial eukaryotes. In addition, the sequencing of their genomes and the microarray assays offer the opportunity to undertake comparative genomics of the genes involved in regulation of gene expression. Here, we review the general concepts that have emerged with regards to gene expression regulation in *Plasmodium sp.*, Trypanosomatids, *Entamoeba histolytica*, and *Trichomonas vaginalis*.

2. Regulation of Gene Expression in *Plasmodium sp.*

Human malaria is caused by the infection of four species of *Plasmodium*, *P. ovale*, *P. vivax*, *P. malariae*, and *P. falciparum*, which are transmitted by the bites of female *Anopheles* mosquitoes. Malaria affects upwards 400 million persons worldwide and produces over 2.5 million deaths annually [4].

The life cycle of *Plasmodium* consists of multiple rounds of asexual replication in the human and both asexual and sexual reproductions in mosquitoes. During the different stages, the parasite undergoes a series of morphological changes needed to infect to and replicate in different organs and cells of both mosquitoes and humans. The morphological transformations carried out by this parasite during its life cycle imply a high degree of regulation of gene expression. In addition, the immune evasion and drug-resistance mechanisms also depend upon finely tuned and accurate control of mRNA transcription.

2.1. Genomes. The *Plasmodium* genomes are estimated to contain 23–27 million bases, 14 chromosomes, and approximately 5,500 genes, including many members of multigene families likely to be associated with immune evasion and antigenic variation [5–8]. *Plasmodium* genomes have a high A/T content (*P. falciparum* 79.6%, *P. vivax* 67.7%), and possibly those genes that are exceptionally rich in A/T content may be more recombinogenic and more likely to be involved in immune evasion [9]. About 77% of the genes are conserved across the different species of *Plasmodium*. However, there are some differences among species; for example, in *P. falciparum* many of the multigene families involved in immune evasion are located near the ends of chromosomes and are often transcriptionally silent, whereas members of multigene families in *P. knowlesi*, primarily a monkey parasite, are spread across the chromosomes and are not strictly subtelomeric [8].

2.2. Transcriptomes. RNA expression profiles throughout the life cycle of *P. falciparum* and *P. berghei*, a parasite of mice, have been analyzed [10–12]. These studies showed that most of the predicted ORFs are transcribed during the intraerythrocytic stage [10, 11]. Additionally, this strategy has shown that approximately 200 genes are specifically transcribed in gametocytes, 41 transcripts are specific to sporozoites, whereas 20% of the predicted ORFs were characterized as specific to the intraerythrocytic stage [11]. The analysis of transcription during the intraerythrocytic stage at one-hour time intervals showed a clustering of genes based on temporal transcript accumulation [10]; each cluster contains genes related by either function or cellular process. An equivalent analysis including sporozoite and gametocyte samples similarly described the coaccumulation of mRNA from genes functionally related [11]. Thus, the timing of expression for the majority of the clusters correlates with a known physiological demand for that process at that time, suggesting a “just-in-time” mode of control, whereby genes are only activated as their biological function becomes necessary to the parasite, after which the genes are downregulated.

Subsequent studies performed to find a correlation between mRNA and protein accumulation throughout the life cycle showed a significant delay between the maximum detection of transcript and protein abundance [12, 13]. These data, in concert with initial analyses of the parasite genome that showed a relative absence of transcription factors [14, 15], suggested a more predominant role for posttranscriptional events in the control of gene expression. However, recent data shows a more complex mechanism of control of gene expression in this parasite.

2.3. General Transcription. In *Plasmodium*, the transcription of protein-coding genes is generally monocistronic, although in *P. falciparum* there was found a bicistronic mRNA for the *maebl* gene, encoding an erythrocyte-binding ligand, along with a putative mitochondrial ATP synthase (PF11_0485) gene [16]. Transcription of mRNA is carried out by an RNA pol II sensitive to low concentrations of α -amanitin,

and searches of the genome found homologs of all 12 subunits of RNA pol II and many, but not all, of the general transcription factors (GTFs) [15, 17]. Interestingly, many of the GTFs that still remained unidentified, such as TAF3, TAF4, TAF6, TAF8, TAF9, TAF11, and TAF13 contain histone fold domains (HFD). In other eukaryotes, these HFD-containing subunits form heterodimers with TFIID and mediate promoter recognition. The absence of the HFD-containing subunits suggests an unusual architecture of the TFIID complex in this parasite.

The TATA-binding protein (TBP) of *P. falciparum* (PfTBP) has low identity (42%) at the primary sequence level to the archetypal yeast homologue, but it contains most residues known to be involved in DNA binding [18]. The TATA box elements recognized by PfTBP are located further upstream from transcription start sites than those of other eukaryotes [19], suggesting a scanning mechanism for the accurate recognition of the transcription initiation site. However, the vast majority of *P. falciparum* genes contain multiple transcription sites, and initiation occurs mainly at adenine nucleotides [20]. This flexibility in the transcription initiation may be explained by the proportional increase in TATA-box-like sequences found in AT-rich upstream sequences.

Another difference in gene transcription in *Plasmodium* with respect to the classical model of higher eukaryotes is that during the intraerythrocytic phase of the parasite, the preinitiation complex containing PfTBP and PfTFIIE is pre-assembled on promoters of all intraerythrocytic-expressed genes, independent of their transcriptional activity [21].

2.4. Cis-Regulatory Elements and Transcription Factors. Several studies using upstream regions in transfection systems to drive expression of reporter genes have shown that *Plasmodium* promoters contain *cis*-elements several hundred bases upstream of the transcriptional start site that can either activate or repress transcription [22–27]. These studies also have identified DNA regions that may be involved in the temporal control of transcription activity. All the identified *cis*-elements share no similarity to transcription factor binding sites in other eukaryotes, and most of them contain homopolymeric (dA : dT) tracts that appeared in a statistically significant bias over that expected by chance.

An alternative approach used to identify putative *cis*-regulatory elements in *Plasmodium* has been through *in silico* analysis. The aligning of upstream regions of 18 heat-shock genes of *P. falciparum* and the *hsp86* sequences from six other *Plasmodium* species revealed the presence of a GC-rich sequence ([A/G]NGGGG[C/A]) called the G-box, which is present in multiple copies upstream of several heat-shock genes of different *Plasmodium* species [28].

Other studies based on the correlation of mRNA expression and on the conservation of *cis*-acting sequences among divergent species have been performed and putative regulatory elements have been found [29, 30]. A novel data-mining algorithm called OPI (ontology-based pattern identification) was utilized to generate highly associated clusters of potentially co-regulated genes [31]. Using this strategy, 34 putative regulatory motifs were found. These motifs are

located upstream of co-transcribed genes encoding proteins involved in a wide variety of cellular functions including development, cell invasion and antigenic variation [32]. For example, an OPI cluster of 246 gametocyte-associated genes revealed a palindromic 10-nucleotide sequence enriched in sexual stage-associated promoters [31]. In addition, a sequence found upstream of the gene encoding the rhoptry-associated protein 3 (RAP3) that contains two PfM18.1 motifs (ATGCA[N₆]GTGCA) showed specific binding to nuclear extracts in Electrophoretic Mobility Shift Assays (EMSA) [32].

Recently, by the use of three different motif-discovery programs, four motifs (G-rich, C-rich, CACA and TGTCG) were identified [33]. These motifs are over-represented in the upstream regions from genes of 13 clusters expressed in the intraerythrocytic cycle of *P. falciparum* [33]. These motifs showed similar positions relative to the translational start site, suggesting that they have an important role in expression regulation [33]. However, the transcription factors that bind these motifs remain uncharacterized.

Initially, the use of hidden Markov models (HMM) using 51 motifs commonly distributed in eukaryotic transcription factors to search for similar proteins in the *P. falciparum* genome revealed that these proteins represent only 1.3% of the genome, whereas in other eukaryotes they correspond to 5.7% [15]. However, the identification of an expanded family of apicomplexan-specific transcription factors (ApiAP2) containing at least one DNA binding domain called AP2 indicate that regulation at transcription initiation level may be also a critical function in the control of gene expression in *Plasmodium* [34]. Each member of the 26 proteins of the ApiAP2 family contains at least one copy of a small (≈ 60 amino acids) domain related to DNA-binding domains found in the plant *Apetala2*/ethylene response transcription factors of plants [34].

The screening of a protein binding array containing all variations of 10-mer DNA sequences identified putative palindromic *cis*-acting sequences for two transcription factors containing AP2 DNA-binding domains (PF14.0633 and PFF0200c) [35]. The analysis of the transcription profile during intraerythrocytic development showed that after expression of PFF0200c, a transcriptional activation occurs for the majority of the genes that contain the *cis*-acting sequence recognized by PFF0200c in their upstream regions [35], indicating an important role of this transcription factor in parasite development.

Recently, another transcription factor of *P. berghei* containing an AP2 DNA-binding domain (AP2-O) was characterized [27]. The AP2-O mRNA is synthesized by intraerythrocytic female gametocytes and translated during the ookinete development in the mosquito. The transcription factor specifically binds to a six-base sequence (TAGCTA) located within a short 100–400 bp region from the transcription start site of a set of genes implicated in the midgut invasion [27]. The amino acid sequence of the AP2 domain of AP2-O is highly conserved among several *Plasmodium* species, and the same *cis*-acting element was observed in the promoters of orthologous genes of *P. falciparum* and *P. vivax* implicated in midgut invasion [27].

Other transcription factors identified in *Plasmodium* are: a homologue of Myb1 (PfMyb1) [36], and two non-sequence specific transcriptional activators which contain the high mobility group box (HMGB) motif [37, 38]. The knockdown of *Pfmyb1* expression inhibits intraerythrocytic development and produces differential expression of several genes containing homologous sequences to Myb-regulatory elements in their 5' flanking sequences [36]. The two transcription factors containing the HMG motif are expressed during asexual (*Pfhmgb1*) and sexual (*Pfhmgb2*) stages of parasite development. Knockout of the *hmgb2* homologue in *P. yoelii* induced altered levels of a number of gametocyte-specific transcripts and a significant reduction in oocyst development, but gametogenesis and exflagellation did occur [38].

2.5. Chromatin Structure. The epigenetic control of gene expression plays an important role in *P. falciparum*. Much of the work in this parasite has focused on the mechanisms that control the mutually exclusive expression of the *var* gene family.

Plasmodium chromosomes, like those of nearly all eukaryotes, are tightly packed in nucleosomes [39]. *P. falciparum* contains each of the four core histones required for nucleosome assembly: H2A, H2B, H3 and H4 as well as the histone variants H2AZ, H2Bv, H3.3 and cenH3 [40]. No gene encoding the linker histone H1 has been identified in *Plasmodium*. Genes for histone modification enzymes, including histone deacetylases and a GCN5-like histone acetyl transferase, have been identified in *P. falciparum* [41, 42]. In accordance, multiple modifications were observed in the histones of this parasite [40], indicating that in *Plasmodium*, as in higher eukaryotes, different modifications in histone tails work in concert to generate a transcriptional state.

Studies combining chromatin immunoprecipitation (ChIP) with microarrays (ChIP-on-chip) in *P. falciparum* showed a positive correlation between a greater acetylation of H3K9 on individual genes and the levels of gene expression [43]. In agreement, inhibition of activity of PfGCN5, the histone acetyl transferase that directs acetylation of H3K9, results in decreased H3K9 acetylation, a decrease in transcriptional activity of a subset of genes and a delaying progress through intraerythrocytic development [44, 45]. In contrast, clusters of silenced genes involved in virulence of *P. falciparum* are located in heterochromatin, and H3K9m3 is specifically associated with *var* gene families clustered on subtelomeric and some chromosome internal regions localized to the nuclear periphery [46]. Furthermore, disruption of the histone deacetylase homologue to Sir2 (PfSir2) causes changes in H3K9me3 that are associated with disrupted monoallelic transcription [46–48], suggesting that PfSir2 is required for proper silencing. An orthologue of the HP1 protein, which participates in the formation of highly condensed chromatin by recognizing H3K9m2 and H3K9m3, was recently characterized in *P. falciparum* [49]. The recombinant chromodomain of PfHP1 binds to H3K9me2 and H3K9me3, but not to H3K4m or to unmodified H3 and in vitro forms stable homodimers. ChIP

assays showed that PfHP1 is linked to heterochromatin of subtelomeric non-coding repeat regions [49]. Interestingly, the presence of PfHP1 is directly linked to low expression of target genes. Particularly, most of the genes down-regulated upon PfHP1 overexpression are members of variegated gene families [49]. All these results show the relevance of the PfHP1 in epigenetic regulation of exported virulence factors and phenotypic variation, and suggest that in *P. falciparum* are conserved the elementary components of the histone code for the regulation of gene expression.

In order to avoid the splenic clearance of infected erythrocytes, *P. falciparum* express a protein (PfEMP-1) on the surface of the infected erythrocytes that allows their adherence to host endothelium. The parasite genome contains approximately 60 polymorphic *var* genes, each encoding a different form of PfEMP-1. Immune evasion through antigenic variation depends on the ability of the parasite to exclusively express only a single *var* gene at a time, and to periodically switch expression to alternative *var* gene variants, thus altering the antigenic properties of the infected cell and avoiding recognition of antibodies directed against previously expressed forms of PfEMP-1 [50]. The mutually exclusive expression of *var* genes and antigenic variation result from a combination of in situ activation and reversible gene silencing. Fluorescent in situ hybridization (FISH) experiments showed that *var* genes present in the genome are grouped in 6–8 perinuclear clusters [51–53]. The subtelomeric heterochromatic regions of *P. falciparum* with silent *var* genes are associated with high levels of H3K9me3 [46]. In contrast, the active *var* gene, regardless of its location on the chromosome, is localized in a euchromatic subdomain into the normally heterochromatic nuclear periphery [51, 52, 54], and it is enriched with the activation mark H3K9ac [47, 55]. In addition, the H3K4me3 modification is a predominant mark in the active *var* gene, but during the later stages of the life cycle, where no *var* genes are expressed (poised state), the promoter of the most recently active *var* is enriched with dimethylated H3K4 [55].

The presence of high levels of an unregulated episomal *var* promoter results in a downregulation of the active *var* gene, and when episomes are removed the parasites displayed random *var* gene activation [56]. These results suggest that in *P. falciparum* an active transcription of the variant *var* gene expressed in a population is necessary for the maintenance of the cellular memory through numerous cell cycles.

Similar to *var* genes, subtelomeric gene families of *P. falciparum* such as *rif*, *stevor* or *Pfmc-2tm* code for proteins that showed clonal variation in their expression, and a comparable epigenetic program of control has been proposed for their expression [57].

2.6. Post-Transcriptional Regulation. Translational repression is other mechanism used by *Plasmodium* to regulate its gene expression. A comparison of the gametocytes transcriptome with the proteomes of gametocytes and ookinetes identified nine genes for which transcripts accumulate in gametocytes but are not translated until the ookinete stage [12]. A subsequent study revealed that these transcripts were

highly abundant in the cytoplasm of female gametocytes and distributed as discrete punctuated compartments, similar to the eukaryotic P bodies. Analysis of the 3' UTR from these transcripts revealed the presence of a UUGUU motif, a known *cis*-acting sequence for Puf binding proteins involved in translational repression [58, 59]. In addition, two Puf proteins from *P. falciparum* are expressed in gametocytes and ookinetes and exhibit binding to UUGUU sequences [59].

The analysis of the proteome of *P. berghei* female gametocytes identified an abundantly expressed member of the DEAD-box RNA helicases termed DOZI (development of zygote inhibited), also implicated in translational repression via their RNA binding activity [60]. DOZI interacts with some *Plasmodium* mRNAs and disruption of its encoding gene results in a downregulation of approximately 370 genes, including some predicted to be important in the early ookinete motility, and in the abort of the development of fertilized female gametocytes [61]. Some other transcripts contain an iron-responsive element—a stem-loop structure formed at their 5' and 3' UTRs that binds an iron regulatory protein (PfiRPa) to inhibit translation or to modulate the mRNA stability [62].

A variant of PfEMP-1(VAR2CSA) is only expressed in the presence of a placenta, suggesting that its expression is repressed in men, children or nonpregnant women. Recently was shown that the gene encoding VAR2CSA contains a small upstream open reading frame that acts to repress translation of the resulting mRNA. The mechanism underlying this translational repression is reversible, allowing high levels of protein translation in the presence of placenta [63].

3. Regulation of Gene Expression in Trypanosomatids

Members of the Trypanosomatidae family constitute a fascinating group of flagellated protozoa. Collectively, these pathogens cause millions of deaths in tropical and subtropical regions of the world. African trypanosomes, transmitted by tsetse flies, are responsible for sleeping sickness in humans and nagana disease in cattle. The human disease takes two forms, depending on the parasite involved. *Trypanosoma brucei gambiense* is found in west and central Africa and represents more than 90% of reported cases of sleeping sickness, causing a chronic infection, whereas *Trypanosoma brucei rhodesiense* is found in eastern and southern Africa, represents less than 10% of reported cases, and causes an acute infection [64]. It was estimated that 300,000 individuals were infected in 2000 [65]. However, the access to diagnosis and treatment in many countries where African trypanosomiasis was endemic resulted in a reduction of 68% in the total number of new cases reported between 1995 and 2006 [64]. *Trypanosoma cruzi* transmitted by triatomine bugs is the causative organism of Chagas's disease, which is endemic to several regions in Latin America. It is estimated that around 75 million people live in risk areas and 13 million people are currently infected in Central and South America. The global incidence of the disease is considered to be 300,000 new cases per year [66]. *Leishmania* is a protozoan

parasite which alternates life-forms between an intracellular amastigote stage residing in vertebrate macrophages and an extracellular promastigote stage living in the digestive tract of sandflies. Leishmanial infections have diverse clinical manifestations, including cutaneous (CL), mucocutaneous (MCL), diffuse cutaneous (DCL), visceral (VL or kala-azar), postkala-azar dermal leishmaniasis (PKDL) and recidivans (LR) [67]. Leishmaniasis is a public health problem in at least 88 countries, including some of the poorest in the world [68]. The estimated global prevalence of all forms of the disease is 12 million, with 1.5–2 million added cases annually of CL and 500,000 of VL [69].

3.1. Genomes. The genome of *T. brucei* is 26-megabases and contains 9068 predicted genes, including approximately 900 pseudogenes and approximately 1,700 *T. brucei*-specific genes [70]. Large subtelomeric arrays contain an archive of 806 variant surface glycoprotein (VSG) genes used by the parasite to evade the mammalian immune system [70].

The *T. cruzi* genome size is 60.3 Mb organized in 41 chromosomes [71]. The diploid genome contains 22,570 protein-coding genes, of which 12,570 represent allelic pairs. The protein-coding genes are generally arranged in long clusters of tens-to-hundreds of genes on the same DNA strand. Putative function could be assigned to 50.8% of the predicted protein-coding genes [72]. At least 50% of the *T. cruzi* genome is repetitive sequence, consisting mostly of large gene families of surface proteins, retrotransposons and subtelomeric repeats [72]. The largest gene families encode surface proteins like mucin-associated surface proteins (MASPs), members of the trans-sialidase (TS) superfamily, the surface glycoprotein gp63 protease and some hypothetical proteins [72].

The *Leishmania* genome size is ~34 Mb and the chromosomes range in size from 0.3 to 2.5 Mb [73, 74]. The karyotype is conserved among *Leishmania* species (albeit with considerable size polymorphism) and the genes are syntenic (with conservation of gene order) [73–76], except that the Old World species have 36 chromosomes [73] and the New World species have 35 (*L. braziliensis*) or 34 (*L. mexicana*) [75].

A comparison of metabolic pathways encoded by the genomes of *T. brucei*, *T. cruzi*, and *Leishmania major* reveals the least overall metabolic capability in *T. brucei* and the greatest in *L. major* [70].

3.2. Transcriptomes. A microarray analysis comprising 21,024 different PCR products of *T. brucei* was utilized for the identification of genes specifically expressed in human bloodstream and in insect procyclic stages [77]. Approximately 2% of the genomic fragments exhibited significant differences between the transcript levels in the bloodstream and procyclic forms. Of 33 clones that showed overexpression in bloodstream forms, 15 contained sequences similar to those of VSG expression sites and at least six others appeared non-protein-coding. Of 29 procyclic-specific clones, at least eight appeared not to be protein-coding [77]. Other studies of the transcriptome changes

in *T. brucei* utilized a targeted oligonucleotide microarray, representing the strongly developmentally-regulated membrane trafficking system and approximately 10% of the *T. brucei* genome [78]. Results showed that 6% of the gene cohort is developmentally regulated, including several small GTPases, SNAREs, vesicle coat factors and protein kinases. Therefore, substantial differentiation-dependent remodeling of the trypanosome transcriptome is associated with membrane transport. Recently Jensen et al. [79] using microarrays that contain multiple copies of multiple probes for each gene showed that approximately one-fourth of genes display differences in mRNA levels, suggesting that despite the lack of gene regulation at the level of transcription initiation, this parasite perform an extensive regulation of mRNA abundance associated with different growth stages. However, while trypanosomes regulate mRNA abundance to effect the major changes accompanying differentiation, a given differentiated state appears transcriptionally inflexible because specific gene overexpression, knockdown, altered culture conditions or chemical stress do not provoke detectable changes to steady-state mRNA levels [78].

A *T. cruzi* DNA microarray was used to compare the transcript profiles of six human isolates: three from asymptomatic and three from cardiac patients [80]. Seven signals were expressed differentially between the two classes of isolates, but the approximately 30-fold greater signal in cardiac strains for ND7 was the most pronounced of the group. The ND7 gene from asymptomatic isolates showed a deletion of 455 bp from nt 222 to nt 677 relative to ND7 of the CL Brener reference strain. The ND7 deletion produces a truncated product that could impair the function of mitochondrial complex I [80]. These results suggested that ND7 constitutes a valuable target for the differential diagnosis of the infective *T. cruzi* strain [80]. Recently, Minning et al. [81] observed that transcript abundance is also an important level of gene expression regulation in *T. cruzi*. The microarray analysis of gene expression during the *T. cruzi* life-cycle showed that relative transcript abundances for over 50% of the genes are significantly regulated during the *T. cruzi* life-cycle. Among the differentially regulated genes were members of paralog clusters, nearly 10% of which showed divergent expression patterns between cluster members [81].

Results from several microarray studies in *Leishmania* showed that there is a surprisingly low level of differentially expressed genes, ranging from 0.2% to 9% of total genes, between the amastigote and promastigote life stages [82–87]. Thus, the *Leishmania* genome can be considered to be constitutively expressed with a limited number of genes showing stage-specific expression. Quantitative proteomic analysis of *Leishmania* relative protein expression showed that there is a weak correlation to gene expression [84]. Therefore, *Leishmania* protein expression is mainly regulated at the level of post-transcriptional mechanisms.

3.3. General Transcription. The three classical RNA polymerases, identified on the basis of their resistance to α -amanitin, have been detected in *T. brucei* [88]. The major

subunit of each of these enzymes has been cloned [89]. In most species of trypanosomes undergoing antigenic variant, with the exception *T. vivax*, two slightly different genes for RNA pol II subunit were found [90, 91].

In eukaryotes, RNA pol II is a protein complex of more than 500 kDa that contains 12 subunits, 5 of which (RPB5, RPB6, RPB8, RPB10 and RPB12) are shared with RNA pol I and III. The RPB1 is the largest subunit of the *T. brucei* enzyme and RPB1, RPB2, RPB3, and RPB11 are the functional and structural homologues of eubacterial core subunits of RNA polymerase [92]. RPB4 to RPB10 and RPB12 contribute to the ability of the enzyme to respond to activators to bind tightly with promoter regions, properly initiate RNA transcripts, and ensure efficient and accurate RNA synthesis [92]. Interestingly, the RPB5 homolog (TbRPB5) associated with RNA Pol II is different from the one previously found associated with RNA Pol I. Also two genes coding for different isoforms of TbRPB6 were identified [92], suggesting the existence of polymerase-specific isoforms for both TbRPB5 and TbRPB6.

A functional TBP was identified in *T. brucei*. This protein, called TRF4, was essential for cell viability and was recruited to the SL RNA gene promoter [93–95]. However, the TBP lacks two of the four important phenylalanines that are responsible for bending the DNA on either side of the TATA box. The significant divergence in the trypanosome TBP may indicate functional variation in its role in transcription [93, 94].

Tandem affinity purification (TAP) assays were used to characterize the subunits that form the RNA pol II and III in *L. major* [96]. Mass spectrometric analysis of the complex copurified with TAP-tagged LmRPB2 identified seven RNA pol II subunits: RPB1, RPB2, RPB5, RPB7, RPB10 and RPB11. With the exception of RPB10 and RPB11, and the addition of RPB8, these were also identified using a TAP-tagged construct of one of the two LmRPB6 orthologues [96]. The latter experiments also identified the RNA pol III subunits RPC1, RPC2, RPC3, RPC4, RPC5, RPC6, RPC9, RPAC1 and RPAC2 [96]. Significantly, the complex precipitated by TAP-tagged LmRPB6 did not contain any RNAPol I-specific subunits, suggesting that, unlike other eukaryotes, LmRPB6 is not shared by all three polymerases but is restricted to RNA pol II and III. In addition to these RNA pol subunits, several other proteins that copurified with RNA pol II and III complexes were identified, these include a potential transcription factor, several histones, the splicing factor PTSR-1, RNA binding proteins, and others, suggesting that they may be physically associated with the RNA pol II complex [96].

The Trypanosomatidae family possesses unusual mechanisms of gene expression such as polycistronic transcription [97, 98], trans-splicing processing of the pre-mRNA [99], RNA editing of the mitochondrial transcripts and transcription of protein-coding genes by RNA pol I [100]. In these organisms, the mature nuclear mRNAs are generated from primary transcripts by *trans*-splicing, a process that adds a capped 39-nucleotide minixon or spliced leader (SL) to the 5' termini of the mRNAs [101]. The steady-state levels of most of the mature mRNA appear to be

regulated posttranscriptionally by mechanisms that involve their 3' untranslated region sequences [102]. In *T. brucei*, the genes encoding the variant surface glycoprotein (VSG) and procyclic acidic repetitive protein (PARP) are transcribed by an RNA polymerase that is resistant to α -amanitin, indicating that trypanosomes can transcribe protein-coding genes by RNA pol I [103].

Promoters from RNA pol I have been extensively characterized in trypanosomatids [104–106], as have been some pol III promoters [107]. However, little is known about the sequences that drive the expression of protein-coding genes by RNA pol II. The apparent lack of regulation of pol II transcription and the observation that episomal molecules are transcribed in both strands have led to the hypothesis that in trypanosomatids, RNA pol II has very low specificity, and that transcription can initiate indiscriminately at several sites along the polycistronic units [108, 109]. Now it is generally accepted that the trypanosomatid genomes are organized in long polycistronic transcription units. The genes are usually separated by only a few hundred base pairs and, with a few exceptions, they do not contain introns.

3.4. Cis-regulatory Elements and Transcription Factors. Only a few promoters have been characterized in trypanosomes: those of the ribosomal (rRNA), the procyclic acidic repetitive protein (PARP) and variant surface glycoprotein (VSG) genes of *T. brucei* [110, 111] and those for some small RNA genes. In addition, a *cis*-acting regulatory element able to determine the strandedness of transcription has been found upstream of a multidrug resistance gene in *L. enriettii* [112]. There is not significant sequence homology among these promoters or with any known eukaryotic promoter.

In the case of the VSG promoter, the 70 bp region preceding the transcription start site is sufficient to ensure maximal activity in transcription [113]. In the case of the ribosomal and procyclin promoters, the -70 to $+1$ bp region constitutes a core element whose basal activity is stimulated by an upstream control element located around position -200 bp [114]. These promoters contain two stretches, centred approximately at -60 and -35 bp, that are essential for promoter activity and whose spacing appears to be critical. Interestingly, the binding of specific proteins requires that the target DNA be single-stranded [115]. These results suggest that these promoters should be partially denatured to be functional [116].

Many groups have compared and contrasted the promoter elements of the SL RNA gene in various Trypanosomatids [117–122]. In most cases, SL RNA gene promoter consists of three closely spaced short elements that are located upstream of and proximal to the transcription start site. Mutational analysis showed that nucleotides near the start site ($+1$), at position -10 to $+10$ bp in *L. amazonensis* serve to direct correct transcription initiation of the SL RNA gene and an upstream element in the promoter (-80 to -60 bp) is essential for efficient SL RNA expression in vivo [123]. The SL RNA genes in *L. tarentolae* are organized into two separate, head-to-tail tandem arrays, *MINA* and *MINB* [124]. The *MINA* array contains ~ 60 gene copies with 363 bp

repeat length of which 105 are transcribed, resulting in a 96 nt mature transcript. The *MINB* array has ~ 40 copies, with a periodicity of 296 nt. The transcribed regions in both arrays are identical. The non-transcribed regions contain a bipartite promoter at -67 to -58 bp (the -60 element) and -40 to -31 bp (-30 element) from the transcription start site in the *MINA* array [118, 122].

In *Leptomonas seymouri* a factor termed promoter-binding protein 1 (PBP-1) that specifically binds to SL RNA gene promoter in the region from -60 to -70 bp was identified [125]. PBP-1, the first sequence-specific, double stranded DNA-binding protein isolated in Trypanosomes is composed of 57, 46 and 36 kDa subunits [125]. The 46 kDa is a previously uncharacterized protein and may be unique in Trypanosomes. Its predicted tertiary structure suggests it binds DNA as part of a complex. The 57 kDa subunit is orthologous to the human Small Nuclear Activating Protein 50 (SNAP₅₀), which is an essential subunit of the SNAP complex [125]. In human cells, the SNAP complex binds to the proximal sequence element in both RNA polymerase II- and III-dependent small nuclear RNA gene promoters.

A protein complex that specifically binds to the -60 element of the SL RNA gene promoter of *L. tarentolae* was identified by EMSA [126]. The complex has an estimated mass of 159 kDa and it contains a homologue of TBP (LtTBP). Both LtTBP and LtSNAP₅₀ are found near the spliced leader RNA gene promoter and the promoters important for tRNA Ala and/or U2 snRNA gene transcription [126].

The use of CHIP-on-chip analysis to probe genome-wide transcription factor occupancy suggest that there are only 184 transcription-initiation sites for protein-coding genes in *L. major* [127]. This analysis also extend the understanding of the roles of TBP and SNAP₅₀ in *L. major* transcription, because these proteins appear to bind to all RNA polymerase II and III promoters and appear to have identical binding patterns genome-wide, laying open the interesting possibility that the SNAP complex may serve as a general transcription factor for protein coding transcription in these organisms [126].

The transcriptional analysis of chromosome 1 from *L. major* Friedlin, the reference strain of the *Leishmania* Genome Project, revealed the presence of 79 putative genes, the first 29 of which are in a cluster on the “bottom” DNA strand, while the remaining 50 are in a cluster on the “top” strand [128]. The DNA segment between both clusters is called the “strand switch region”. Importantly, nuclear run-on analysis of chromosome 1 showed that specific transcription, leading to the production of stable transcripts, initiated within the strand switch region and proceeds bidirectionally toward the telomeres [98]. Stable-transfection studies support the presence of a bidirectional promoter in this region of chromosome 1. It also appears that nonspecific transcription takes place over the entire chromosome 1, but at a level ~ 10 -fold lower than the specific transcription initiating in the strand-switch area. 5'-rapid amplification of cDNA ends (RACE) studies localized the initiation sites to a < 100 bp region [98]. Thus, while in most eukaryotes each gene possesses its own promoter, a single region seems to drive the expression of the entire

chromosome 1 in *L. major* Friedlin. Although these results showed that pol II drives transcription of chromosome 1, no typical pol II promoter elements are present in the 73 bp region that separates each transcription unit. Moreover, no sequence conservation is discernible between the strand-switch area on chromosome 1 and strand switch region on other chromosomes of *L. major* Friedlin [98].

The transcriptional analysis by nuclear run-on of chromosome 3 was also described [129]. This chromosome contains 97 putative protein-coding genes organized into two long convergent clusters, which are separated by a tRNA^{Lys} gene [129]. In addition, a single divergent gene is located at the “left” end of the chromosome. Data showed that pol II transcription on chromosome 3 initiates bidirectionally between the single subtelomeric gene and the adjacent 67-gene cluster and near the “right” telomere upstream of the 30-gene cluster. The tRNA^{Lys} gene is transcribed by pol III. Transcription on both strands terminates in the tRNA-gene region [129].

Promoters from RNA pol I have been characterized in different species of *Leishmania*. The analysis of the ribosomal RNA (rRNA) gene promoter of *L. donovani* showed that these genes are organized on chromosome 27 as tandem repeats of approximately 12.5 kb. Each repeat contains the subunit rRNAs and approximately 39 copies of a 64-bp species-specific sequence [106]. The transcription initiation site was mapped to 1020 bp upstream of the 18S rRNA gene. A 349-bp sequence located between the 64-bp repeats and the 18S rRNA gene appears to contain a promoter [106]. Three domains (−76 to −57, −46 to −27 and −6 to +4 bp, relative to the transcription initiation site) were found to mediate promoter activity, suggesting that the rRNA is not dissimilar to that of other eukaryotes [106]. Similar findings were reported in the rRNA promoters of *L. major* Friedlin and *L. amazonensis*, where the transcription initiation site of the rRNA units were localized to 1043 and 1048 bp upstream of the rRNA genes, respectively [130, 131]. The repetitive element (60 bp for *L. major* and 63 bp for *L. amazonensis*) was also identified in the intergenic spacers, and constructs containing the rRNA gene promoters were able to drive the expression of reporter genes [130, 131].

3.5. Chromatin Structure. Although gene expression in Trypanosomatids is predominantly regulated posttranscriptionally, several lines of evidence point to important roles for chromatin structure and modification in gene expression, cell cycle control and differentiation [132–134]. In Trypanosomes, all classes of histones are present and DNA is packed into nucleosomes [135].

Analysis of the genomic organization of the SL RNA genes of *L. tarentolae* showed that a single nucleosome is positioned on its intergenic region, leaving the promoter and the transcribed gene region free of nucleosomes [136]. The array periodicity of one nucleosome per 363 bp differed from the standard heterochromatin arrangement in this species of one nucleosome per 230 bp. The array is bent further by the interaction with transcription factors. Thus, nucleosome arrangement may be vital for efficient transcription initiation

of the SL RNA gene. On the other hand, ChIP-on-chip assays in *L. major* suggested that H3 histones at the origins of polycistronic transcription of protein-coding genes are acetylated [127]. Thus, global regulation of transcription initiation may be achieved by modifying the acetylation state of H3 histone on these origins [127].

Histone acetylation, methylation and phosphorylation have been described in *T. brucei* and *T. cruzi* [137–139]. In addition, a detailed analysis of histone modifications in *T. brucei* showed the lack of the initial methionine residue of H2A, H2B and H4 and that the N-terminal alanine of these proteins could be monomethylated [140]. These studies also found that the histone H4 N-terminus is heavily modified, while, in contrast to other organisms, the histone H2A and H2B N-termini have relatively few modifications [140]. *T. brucei* expressed three distinct MYST-family members, all of which have homologues in *T. cruzi* and *Leishmania* and was described nonredundant roles for each of these histone acetyltransferases in bloodstream-forms of *T. brucei* [141]. HAT1 modulates telomeric silencing and is required for growth, and possibly, for DNA replication; HAT2 is required for H4K10 acetylation and growth; and HAT3 is required for H4K4 acetylation and is dispensable for growth. The orthologues in *Leishmania* likely have similar roles. The nonredundant functions for *T. brucei* HAT1-3 appear to reflect unique substrates for each acetyltransferase and further support the idea of a simplified, nonredundant histone code in these divergent parasites [141].

3.6. Post-Transcriptional Regulation. The general organization of trypanosomatids genes in polycistronic units means that most of the genes are transcribed at an equivalent rate within large polycistronic clusters; consequently, there must be present a post-transcriptional mechanism in these organisms to control the gene expression. Constitutive synthesis of the transcriptome and selection of the right messages only at the maturation step probably enables the parasite to switch gene expression rapidly to survive and adapt to a new environment. Although this system requires the permanent degradation of an important fraction of the transcriptome, trypanosomatids have avoided the burden of encoding networks of specific transcription factors and target sequences.

Post-transcriptional regulation could be exerted through sequence elements in intergenic regions. Evidence of the primary role of untranslated regions (UTRs) to determine the relative stage-specific mRNA abundance has accumulated [142, 143]. The 3′-terminal region of VSG and procyclin transcripts regulates the expression of a reporter gene in an inverse manner, depending on the developmental form of the parasite [143]. In the case of VSG mRNA, the 97 nt sequence upstream from the polyadenylation site is responsible for these effects. The regulation occurs through a variation of mRNA abundance which is not due to a change in primary transcription. In the bloodstream form this effect is manifested by an increase in RNA stability, whereas in the procyclic form it seems to be related to a reduction in the efficiency of mRNA maturation [143]. The 3′-end

of VSG mRNA can obviate the 5- to 10-fold stimulation of transcription driven by the procyclin promoter during differentiation from the bloodstream to the procyclic form [143].

An analysis of synonymous codon bias in Trypanosomatids showed the enrichment of “favoured” codons in more highly expressed genes [144]. Consistent with translational selection, cognate tRNA genes for favoured codons are over-represented [144]. In addition, relative codon bias is conserved among orthologous genes from divergent Trypanosomatids (*T. cruzi*, *T. brucei*, *L. major*) even in genes thought to be expressed at low level [144]. Taken together, the results suggest that control of the level of translation is an important mechanism underlying differential protein expression in Trypanosomatids.

4. Regulation of Gene Expression in *Entamoeba histolytica*

Entamoeba histolytica, an enteric protozoan parasite, is the etiologic agent of human amoebiasis. It has been estimated that every year 50 million cases of invasive amoebiasis occur and approximately 110,000 deaths, but interestingly only 10% of the infected people present disease symptoms expressed as intestinal or extraintestinal amoebiasis [145]. The molecular mechanisms participating in the parasite invasiveness are not completely understood. Diverse populations of *E. histolytica*, including clones derived from a particular strain, display different virulence phenotypes [146–148]. In addition, long-time cultured trophozoites, which show poor capacity to produce liver abscesses in experimental animals, recover their virulence after incubation with cholesterol or with certain types of bacteria, or after their passage by Hamster livers [149–152]. This behavior could be due to changes in expression of certain genes. In addition, the life cycle of this parasite involving the reversible conversion of the infective forms (cysts) to the invasive cells (trophozoites) is expected to be due to differential expression of *E. histolytica* genes.

4.1. Genome. The genome of this parasite consists of 23,751,783 bp distributed among 888 scaffolds and contains approximately 9,938 genes [153]. It has been difficult to determine the number of *E. histolytica* chromosomes, because they do not condense [154]. However, the presence of 14 independent linkage groups has been reported [155]. In addition, the genome of this parasite contains a number of circular plasmid-like molecules [154]. The intergenic regions are from 400 bp to 2.3 kb, suggesting a tight packing of genes [154]. Approximately 25% of the *E. histolytica* genes contain introns, with 6% of genes containing multiple introns [153]. Intron sequences are relatively short (46–115 bp), and they contain the dinucleotides GU and AG at the donor and acceptor splice sites, respectively. Around 10% of the genome consist of tRNAs genes organized in tandem arrays that vary in unit length from 490 to 1775 bp and containing from 1 to 5 tRNA genes [156]. The rRNA genes are located exclusively on extrachromosomal circular DNA molecules

with an approximate size of 26 kb [157]. The genomes of *E. histolytica* and other species of *Entamoeba* have a high A/T content (77.6%), with the exception of *E. moshkovskii*, which has approximately 10% less A/T content [154].

4.2. Transcriptome. *E. histolytica* undergoes the reversible switch between the infective cysts and the invasive trophozoites. Identification of genes involved in the developmental pathway was examined by whole-genome transcriptional profiling [158]. Approximately, 15% of annotated genes are potentially developmentally regulated. Genes enriched in cysts (672 in total) included cysteine proteinases and transmembrane protein kinases. Genes enriched in trophozoites (767 in total) included genes involved in tissue invasion, putative regulators of differentiation, including possible G-protein coupled receptors, signal transduction proteins and transcription factors [158]. A number of *E. histolytica* stage-specific genes were also developmentally regulated in the reptilian parasite *E. invadens* [158], indicating that they likely have conserved functions in *Entamoeba* development.

The majority of human infections with *E. histolytica* remain asymptomatic. In some infections trophozoites invade the intestinal mucosa producing dysentery, and in a small fraction of infections, trophozoites disseminate to the liver, where they induce abscess formation. It is assumed that the ability of *E. histolytica* trophozoites to survive within the host and to destroy host tissues is accomplished by the specific regulation of a number of amoeba proteins.

A genome-wide transcriptional analysis of *E. histolytica* performed on trophozoites isolated from the colon of six infected mice and from in vitro culture revealed 523 transcripts (5.2% of all *E. histolytica* genes), whose expression was significantly changed in trophozoites isolated from the intestine [159]. The genes that modify their expression in trophozoites obtained from the mice encode proteins implicated in metabolism, oxygen defense, cell signaling, virulence, and antibacterial activity [159]. Control of the observed changes in the transcriptome might potentially rest with four related proteins with DNA binding domains that were down-regulated in the intestinal environment [159].

Comparison of RNA abundance between *E. histolytica* trophozoites isolated from liver abscesses of experimental animals and those obtained from in vitro culture found that at least seven *E. histolytica* genes were specifically up regulated and five were down regulated in trophozoites isolated from the livers [160]. The genes specifically up regulated encode proteins associated with heat shock, some ribosomal proteins, cyclophilin, ferredoxin 2, and the small GTPase RAB7D, whereas two of the genes down regulated encode members of a family of proteins containing repetitive stretches of sequences that are rich in lysine and glutamic acid residues [160]. All these results support the idea that host invasion requires the regulation and concerted action of a variety of amoeba proteins.

The varied outcome of infection by *E. histolytica* could be due also to differences in the virulence of the *E. histolytica* isolates. Comparison of the transcriptomes of strains HM1 : IMSS (highly virulent) and Rahman (low

virulent) showed 353 transcripts that exhibited at least a two-fold difference between those strains; 152 transcripts were expressed at higher levels in HM1 : IMSS and 201 transcripts were more expressed in Rahman strain [161]. The genes differentially expressed included cysteine proteinases (CPs), AIG family members, and lectin light chains [161]. The genes of the cysteine proteases EhCP4, EhCP6, and EhCP7 were expressed approximately three-fold in HM1 : IMSS than in Rahman strain [161]. In contrast, the expression of the genes of the cysteine proteases EhCP8, EhCP112 and EhCP3 was higher in Rahman strain than in HM1 : IMSS [161]. The most striking difference was seen in the expression of EhCP3, which was approximately 100-fold higher in Rahman than HM1 : IMSS [161]. Interestingly, in the non-pathogenic amoeba *E. dispar*, EhCP3 is expressed at higher levels compared to *E. histolytica* [162]. All these results validate the hypothesis that CPs have an important role in the *E. histolytica* virulence.

In addition, *E. histolytica* trophozoites require different proteins to survive when they are exposed to different environmental conditions. The gene expression pattern of *E. histolytica* trophozoites exposed to a heat shock stress showed a massive gene down regulation [163]. Of the 1,131 unique genes probed by the microarray, 471 (42%) were significantly repressed during the heat shock treatment. A small number of genes were up regulated by heat shock; including those that encode the heat shock proteins hsp90 and hsp70, some hypothetical proteins and regulatory factors such as BRE, and a putative reverse transcriptase [163]. Heat shock treatment also induced the transcription of some CP genes [163]. The EhCP6 gene was especially up regulated by heat shock, indicating the particular activity of this protease during stress due to its potential role in the degradation of damaged proteins. This study also found that some alleles of the genes encoding for heavy (Hgl) and light (Lgl) subunits of the Galactose/N-acetyl-D-galactosamine-inhibitable lectin (Gal/GalNac lectin) were involved in the heat shock response [163].

4.3. General Transcription. In *E. histolytica* very few sequences and, therefore, transcription factors have been identified, isolated, and characterized not only at structural level, but at the functional level, as well.

The transcription of protein-coding genes is monocistronic and mRNA is synthesized by an unusual RNA polymerase resistant to α -amanitin [164]. The core promoter of several protein-coding genes contains three conserved motifs [165, 166], and according to the study of different *E. histolytica* gene promoters, the size of the functional promoter region is between 200 to 900 bp [167–173]. TBP is the only member of the basal transcription machinery of this parasite characterized so far. EhTBP has 234 amino acid residues and its functional domain showed 55% sequence identity to TBP of *Homo sapiens* [174]. The recombinant EhTBP formed specific complexes with the consensus TATA-box sequence of *E. histolytica* and with other TATA-like motifs [175], indicating that this protein is more promiscuous than TBPs of human and yeast. This behavior of EhTBP

probably is due to the presence of modifications in some amino acid residues involved in the binding to DNA.

4.4. Cis-Regulatory Elements and Transcription Factors. At the structural level, the core promoters of *E. histolytica* contain three conserved elements: the TATA-box (~–35 to –25 bp) enriched in T and A bases (TAT/GT/G/AT/G/AA/GAAC/G)—the Initiator sequence AAAAATTCA (Inr) that is overlying the transcription initiation site, and the GAAC element (AA/TGAAC) [165, 176]. The GAAC element controls the rate and site of transcription initiation, mediates the transcriptional activation by some upstream regulatory regions, and functions in a context-dependent manner [176].

The development of DNA-mediated transfection for *E. histolytica* [177, 178] enabled the characterization of cis-acting promoter elements required for gene expression. However, until now few promoters upstream of protein coding genes have been analyzed at structural and functional level; among them are the promoters of genes encode the heavy subunit of the Gal/GalNac lectin (*hgl2* and *hgl5*), the multidrug resistance proteins *EhPgp1* and *EhPgp5*, the *EhADHCP* complex, and the small GTPase *EhRabB* [167–173].

The promoter of the *hgl2* gene includes two regulatory elements; a sequence located 100 bp upstream of the transcription start site similar to the CCAAT-box motif found in gene promoters of some higher eukaryotes, and a sequence of 15 bp situated at –520 bp [167]. This study also showed that mutations in the putative TATA-box or in the ATTCA element reduced the promoter activity from 20 to 56% with respect to that displayed by the wild type promoter [167].

The full transcriptional activity of the *hgl5* gene promoter was obtained through 272 bp upstream of the transcription initiation site [168]. Five upstream regulatory elements (UREs) were identified in this region; four of them act as positive regulatory elements: URE1 (–49 to –40 bp), URE2 (–69 to –60 bp), URE4 (–189 to –160) and URE5 (–219 to –200), whereas the URE3 motif (–89 to –80) performs a negative regulatory activity [168]. However, URE3 functions as a positive regulatory element in the ferredoxin (*fdx1*) promoter region [179].

URE4 is formed by two direct repeats of nine base pair and it functions as an enhancer in the *hgl5* gene [180]. Two polypeptides of 28- and 18-kDa, named EhEBP1 and EhEBP2, recognize the URE4 sequence [181]. These proteins contain two (EhEBP1) and one (EhEBP2) sequences homologous to the RNA recognition motif RRM. This domain has been found in a large number of RNA-binding proteins and in several sequence-specific DNA-binding proteins [182]. The over expression of EhEBP1 in trophozoites decreased the expression of a reporter gene under the control of the *hgl5* promoter [181], demonstrating the role of the protein EhEBP1 in the transcriptional control of *E. histolytica*.

Using a yeast one-hybrid screen, a 22.6 kDa protein that specifically binds to URE3 (URE3-BP) was identified [183]. This protein contains two EF-hand motifs, which is the most common calcium-binding motif found in proteins,

suggesting that the activity of URE3-BP may be regulated by calcium. Indeed, it was demonstrated that relatively high concentrations of calcium (100–500 mM) inhibited the DNA-binding activity of URE3-BP [183]. ChIP assays corroborated the calcium-dependent interaction of URE3-BP with both *hgl5* and *fdx1* promoters. Recently, was demonstrated that several genes of *E. histolytica* are regulated by URE3-BP [184]. The URE3 motif was found in the 59 promoter regions of the genes modulated by URE3-BP. These genes encode proteins involved in fatty acid metabolism and in potential membrane proteins, suggesting that URE3-BP could be engaged to remodel the surface of trophozoites in response to a calcium signal [184].

The analysis of the *EhPgp1* gene promoter revealed that it does not contain a TATA-box motif, but it has a putative Inr sequence and several transcription initiation sites [169]. Moreover, this promoter contains sequences similar to some *cis*-regulatory elements of higher eukaryotes, such as C/EBP, GATA-1, OCT, and HOX motifs. Several of these elements were able to compete the DNA-binding activity of nuclear extracts in EMSA [169]. Mutational analyses of some of these elements demonstrated the functional relevance of three regions of the *EhPgp1* core promoter; two of them correspond to C/EBP regulatory sites (–54 to –43 bp and –198 to –186 bp) [185]. Nuclear proteins from trophozoites specifically bind to these C/EBP sequences, and two polypeptides of 25 and 65 kDa were recognized by anti-C/EBP β antibodies [185]. These results suggest the presence of C/EBP like-proteins in *E. histolytica*. The other functional sequence identified in the *EhPgp1* gene promoter contains repeated sequences and GATA-1, Gal4, Nit-2, and C/EBP consensus sequences [186].

In the emetine-resistant clone C2, the *EhPgp5* gene displays an inducible expression pattern when trophozoites are exposed to the drug. The structural analysis of its promoter showed the presence of a TATA box at –31 bp and an Inr consensus sequence located only three nucleotides upstream from the start codon [170]. By primer extension assays, a single product mapping at the Inr sequence was detected in mRNA from clone C2 grown in the presence of 225 μ M of emetine (C2₂₂₅). However, this product was not detected in mRNA from trophozoites grown in the absence of the drug; instead, we found a minor primer extension product at 16 bases downstream the ATG, which has no ORF [170]. These results suggest that *EhPgp5* gene expression could be associated with the accurate selection of the transcription initiation site. Consensus sequences for the binding of AP-1, HOX, C/EBP, OCT-1, PIT-1, OCT-6, CF-1 and MYC were detected at the *EhPgp5* promoter [170]. Gel shift competition assays showed evidence that some nuclear proteins similar to those transcription factors could be specifically recognizing DNA binding sites. Functional promoter assays showed that the *EhPgp5* gene promoter was active in transfected trophozoites of clone C2 in the absence of emetine, but its activity increased when trophozoites were cultured in 40 μ M emetine, whereas the transcriptional activity was turned off in the drug-sensitive clone A [170]. These results suggest that emetine is an inductor of the *EhPgp5* over expression in trophozoites of clone C2. Deletion

analysis of the *EhPgp5* promoter region delimited a fragment of 59 bp (–170 to –110 bp) where the emetine response element could be situated [187].

The EhRabB protein is a Rab GTPase located in small vesicles that in wild-type trophozoites are translocated to plasma membrane and to phagocytic mouths during phagocytosis [188], whereas, in trophozoites deficient in phagocytosis most of these vesicles remain in the cytoplasm [189]. The *EhrabB* gene is located close to the *Ehpcp112* and *Ehadh112* genes, whose products form the EhCPADH complex, which is involved in the pathogenic mechanism of *E. histolytica* [190]. These three genes span a 4500 bp region named virulence locus (VI) [172], providing a good model to study gene transcription regulation of virulence-related genes. The *EhrabB* gene is situated 332 bp upstream of the *Ehpcp112* gene, but in the complementary strand [188]. *In silico* analyses of the 5'-flanking sequence of the *EhrabB* gene showed that it does not contain Inr elements or TATA-box consensus sequences, but it has a sequence similar to the GAAC element [173]. These analyses also showed the presence of sequences similar to C/EBP, GATA-1, and heat shock elements (HSE) of higher eukaryotes, and a sequence related to the URE1 motif of the *hgl5* gene promoter [173]. Functional assays of the *EhrabB* promoter showed that: (i) the C/EBP and GATA-1 sequences may not be relevant for *EhrabB* gene transcription, because their removal did not show significant effect in CAT activity; (ii) a DNA region located between positions –428 to –683 bp negatively controls the *EhrabB* transcription; and (iii) a DNA fragment located at –257 to –428 bp, where HSE and URE1 motifs were detected, activates the *EhrabB* transcription [173]. Deletion of the URE1 sequence showed a decrease in the expression of the CAT reporter gene, indicating that URE1 is a *cis*-activating element of the *EhrabB* transcription [173]. Finally, functional CAT assays with a construction that includes seven HSEs to transfect *E. histolytica* showed an increase in CAT enzymatic activity of approximately twice in heat shocked trophozoites with respect to the activity displayed by cells maintained at 37°C [173]. These results indicate that HSE motifs present into the *EhrabB* gene promoter could be functional under heat shock stress. All these results show that transcription of *EhrabB* is coordinated by different *cis*-elements that are specifically recognized by proteins under certain environmental conditions.

The heat shock transcription factors (HSTF), are proteins that under stress conditions rapidly activate and bind to the heat shock element (HSE) present in the *hsp* promoters. Then, this factor induces the expression of *hsp* genes, whose products ensure the survival of the cell during stressful conditions by providing defense against general protein damage [191]. Recently, Gomez-García et al. [192] described the *in silico* identification of three *hstf* genes (*Ehhstf1*, *Ehhstf2*, *Ehhstf3*) in *E. histolytica*. The proteins encoded by these genes possess a conserved DNA-binding domain with 24% of identity and 37% of similarity to the DNA-binding domain of different HSTFs from *Homo sapiens*, *Gallus gallus*, *Mus musculus* and *Arabidopsis thaliana* [192]. The phylogenetic tree constructed using the alignment of the EhHSTFs and HSTFs from other organisms showed a

closer relationship between EhHSTF2 and EhHSTF3, while EhHSTF1 exhibits high similarity with the HSTF1 from *A. thailiana* and appears to be deriving from the same root than HSTF1 from human, mouse, chicken, frog and fruit fly [192].

Another transcription factor that has been identified in *E. histolytica* is a protein similar to the tumor suppressor protein p53 [193]. The Ehp53 protein shows 30%–54% and 50%–57% homology with important domains of p53 from human and *Drosophila melanogaster*, respectively. This homology included the tetramerization domain, the nuclear export signal and a nuclear localization signal. Ehp53 also contains seven of the eight DNA-binding residues and two of the four Zn²⁺-binding sites described for p53 [193]. Heterologous monoclonal antibodies against p53 (Ab-1 and Ab-2) recognized a single 53 kDa spot in two-dimensional gels and they inhibited the formation of DNA-protein complexes produced by the interaction of nuclear extracts of *E. histolytica* with an oligonucleotide containing the consensus sequence for the binding of human p53. A recombinant Ehp53 polypeptide was recognized by Ab-2 antibodies and this protein also was detected in *E. moshkovskii* and *E. invadens* [193].

A member of the high-mobility group (HMGB) was identified in *E. histolytica* (HMGB1) [194]. Its amino acid sequence has significant homology with HMGB proteins from a diverse range of species, like *P. falciparum* (53% and 58% with PfHMGB1 and PfHMGB2, resp.), *Schistosoma mansoni* (40%), and *H. sapiens* (50%). Two residues have been predicted to be crucial to determine the structural DNA specificity [37], a serine at position 10 and a hydrophobic residue at position 32 according to residue numbering of *D. melanogaster* HMG-D. The corresponding conserved residues in EhHMGB1 are threonine at position 34 and phenylalanine at position 56 [194]. EhHMGB1 also has the acidic C-terminal tail seen in other eukaryotes. Thus, all these computational analyses supported the idea that EhHMGB1 is a *bona fide* HMGB protein. Moreover, recombinant EhHMGB1 shared the capacity of human HMGB1 to augment the binding of certain transcription factors to DNA, and it is localized at the nucleus [194]. Overexpression of EhHMGB1 in trophozoites led to modulation of 33 transcripts involved in a variety of cellular functions. Of these, 20 were also modulated in the mouse model of intestinal amoebiasis [159, 194]. Four genes known to be involved in virulence were modulated by the overexpression of EhHMGB1, including those coding for two of the five Gal/GalNac lectin light subunits, the cysteine proteinase EhCP-A7, and a potential enterotoxin peptide [194]. These results suggest a role of EhHMGB1 in parasite adaptation to, and destruction of, the host intestine.

The signal transducers and activators of transcription (STAT) factors are cytoplasmic proteins that after tyrosine-phosphorylation form homo- or heterodimers that are translocated to the nucleus, where they bind to DNA within a well defined consensus sequence called SIE [195]. *E. histolytica* contains transcription factors of the STAT family, where they could potentially function downstream of receptor kinases in processes related to pathogenesis [196]. The interaction of trophozoites with collagen type I and

calcium induces the expression and activation of proteins homologous to STAT1 and STAT3 [197]. Collagen induces a time dependent increase in tyrosine phosphorylation of both STAT1K and STAT1L. These proteins become tyrosine-phosphorylated as early as 15 minutes of stimulation with collagen, reaching a maximal stimulation after 120 minutes of collagen treatment [197]. When the phosphorylation status of STAT3 was explored, again, both isoforms (K and L) increase their phosphorylation content after exposure to collagen and calcium [197]. Then, there is an association between phospho-STAT1 and phospho-STAT3; these heterodimers are targeted to the nuclei and bind to SIE [197].

The Myb domain is a sequence-specific DNA binding domain that was originally identified in vertebrates. Myb is one of the largest transcription factor family in plants [198]. Myb proteins contain DNA-binding domains composed of one, two or three repeated motifs of approximately 50 amino acids surrounded by three conserved tryptophan residues [199]. A gene (*Ehmyb*) encoding for a 145 amino acid protein containing a Myb domain was identified in *E. histolytica* [200]. EhMyb protein belongs to the SHAKY family by the presence of a single Myb DNA-binding domain (80 to 130 aa) as well as the THAKQF motif [200]. Overexpression of the EhMyb protein resulted in a transcriptome that overlapped significantly with the expression profile of amoebic cysts [158, 200]. The analysis of several promoters of genes regulated by EhMyb identified a CCCCC motif to which nuclear proteins bind in a sequence-specific manner [200]. All results together strongly suggest that EhMyb is involved in the *E. histolytica* development.

4.5. Chromatin Structure. The chromatin of *E. histolytica* is packaged in nucleosome-like structures not unlike metazoan chromatin; however, linker regions between adjacent nucleosomes appear to be irregular in length as compared to the average 40 bp observed in metazoans [201]. *E. histolytica* genome encodes all the four proteins that comprise the core of histones [202–204]. The amino-terminal domains of the histones although divergent from metazoan sequences, are highly basic with several lysine residues that are potential targets for acetylation by histone acetyltransferases (HATs) [205]. Additionally, *E. histolytica* has some members of the HATs family such as GNAT and MYST with significant similarity to GCN5, MYST, TafII250, Hat1 y Elp3 [205]. The EhGCN5 protein has an acetyltransferase domain (GNAT motif) and conserved residues involved in the interaction with the cofactor CoA as well as with the histone H3 [205]. The EhMYST protein contains two domains, at the amino-terminal it has an Agenet domain of unknown function and at the carboxy-proximal a MOZSAS domain that is a common domain of the HATs [205]. One histone deacetylase (HDAC) called EhHDAC was identified in this parasite [205]. This protein is member of the Class I family of HDACs, which are subunits or co-repressors that function in association with known repressors in response to different events [205]. Although this parasite contains genes for acetylation and deacetylation of histones, until now we do not know the mechanisms of acetylation and deacetylation in *E. histolytica*.

However, we expect that these proteins perform a relevant activity implicated in the transcriptional control.

E. histolytica expresses a cytosine-5 DNA methyltransferase (Eh $meth$), and 5-methylcytosine (m5C) was found predominantly in repetitive elements. The 5' region of the gene encoding for the *E. histolytica* heat shock protein 100 (EHsp100) was isolated by affinity chromatography with 5-methylcytosine antibodies as ligand [206]. The expression of EHsp100 was induced by heat shock, 5-azacytidine (5-AzaC), an inhibitor of DNA methyltransferase and Trichostatin A (TSA), an inhibitor of histone deacetylase [206]. These data suggest that EHsp100 expression can be regulated, in addition to the initiation transcription level, by an epigenetic mechanism.

Lavi et al. [207] identified a 32 kDa nuclear protein (EhMLBP) that binds to the methylated form of a DNA segment encoding a reverse transcriptase of an autonomous non-long-terminal repeat retrotransposon (RT LINE). Deletion mapping analysis localized the DNA binding region at the C-terminal part of the protein. This region is sufficient to assure the binding to methylated RT LINE with high affinity [207]. By an affinity-based technique using the C-terminal of EhMLBP as ligand were isolated DNA sequences containing a 29-nucleotide consensus motif that includes a stretch of ten adenines [208]. Gel retardation analysis showed that EhMLBP binds to the consensus motif with a preference for its methylated form [208]. These results suggest that EhMLBP may serve as a sensor of methylated DNA.

Transcriptional silencing of the gene coding for the amoebapore a (*Ehap-a*) occurred following the transfection of trophozoites with a plasmid containing the 5' promoter region of *Ehap-a* as well as a truncated segment of a neighboring, upstream SINE1 element that is transcribed from the opposite strand [209]. Small amounts of short (approximately 140 nt) ssRNA molecules with homology to SINE1 were detected in the silenced amoeba but no siRNA. ChIP assays using an antibody against methylated K4 of histone H3 showed a demethylation of K4 at the domain of the *Ehap-a* gene, indicating transcriptional inactivation [209]. Transfections of *E. histolytica* trophozoites which already had a silenced *Ehap-a*, with a plasmid containing a second gene ligated to the 5' upstream region of *Ehap-a*, enabled the silencing, in-trans, of other genes of choice [210]. The nonvirulent phenotype of the gene-silenced amoeba was demonstrated in various assays and the results suggest that they may have a potential use for vaccination [211].

4.6. Post-Transcriptional Regulation. In *E. histolytica*, the 5' untranslated region (5' UTR) of the mRNAs are typically short in length (from 5 to 20 nucleotides) [165]. Only few mRNAs with extended 5' UTR have been reported: *Ehmc3* (126 bp), *Ehpk* (265 bp), *EhTBP* (420 bp), and *Ehpc112* (280 bp) [174, 190, 212].

Computational analysis of the 3' UTR of a large EST and genomic sequences collection from *E. histolytica* revealed the presence of conserved elements like an AU-rich domain

corresponding to the consensus UA(A/U)UU polyadenylation signal that could be involved in pre-mRNA polyadenylation [213]. Interestingly, the molecular organization of 3'-UTR *cis*-regulatory elements of the pre-mRNA appears to be roughly conserved through evolutionary scale, whereas the polyadenylation signal seems to be species-specific in protozoan parasites and a novel A-rich element is unique for the primitive eukaryote *E. histolytica* [213].

Measure of the mRNA half-life of *EhPgp5* in trophozoites of clone C2 grown at different concentrations of emetine showed that the stability of this mRNA is increased at high concentrations of the drug [214]. In trophozoites grown in the absence of the drug, the experimental half-life of the *EhPgp5* transcript was estimated in 2.1 hours, whereas in trophozoites grown in 90 (C₂₉₀) and 225 μ M (C₂₂₅) of emetine the half-life of the transcript was 3.1 hours, and 7.8 hours, respectively, confirming significant variations in the decay rates of the *EhPgp5* mRNA in the three conditions [214]. In addition, the *EhPgp5* mRNA contains a longer poly(A) tail in clone C₂₂₅ [214], and it is well known that large poly(A) tails give higher stability to mRNA and promote a more efficient translation. These results indicate that the higher levels of EhPGP5 protein in multidrug-resistant trophozoites (clone C2) could be influenced, in addition to transcriptional activation, by an increased mRNA stability.

5. Expression Regulation in *Trichomonas vaginalis*

Trichomonosis is a common but overlooked sexually transmitted human infection caused by *Trichomonas vaginalis*, a flagellated protist that resides in the urogenital tract of both sexes and can cause vaginitis in women and urethritis and prostatitis in men. The impact of this parasite in women is not only limited to vaginitis, but is also a major factor in promoting transmission of VIH, in causing low-weight and premature birth, and in predisposing women to atypical pelvis inflammatory disease, cervical cancer and infertility [215]. *T. vaginalis* causes an estimated 174 million sexually transmitted infections annually worldwide [216]. Among women, the prevalence of trichomonosis is estimated to have a range of 3%–48%. However, *T. vaginalis* infection is seldom diagnosed in men, primarily because of insensitive diagnostic test [217].

5.1. Genome. The *T. vaginalis* genome is estimated to be 160 Mb and about two thirds of the genome contains several repeats and transposable elements [218]. A core set of approximately 60,000 protein-coding genes was predicted, which are organized into six chromosomes [218, 219]. The analysis of the age distributions of gene families with five or fewer members indicate that the genome underwent a period of increased duplication, and possibly one or more large-scale genome duplication events [218].

Although *T. vaginalis* has an unusually large repertoire of genes, only ~65 of them appear to have an intron [218, 220]. The positions of these introns are often conserved in orthologous genes, indicating they were present in a

common ancestor of trichomonads, yeast, and metazoan. Introns that have been identified in *T. vaginalis* are uniformly short and characterized by a conserved 12-nt sequence (5'-ACTAACACACAG-3') at the 3' splice-site that includes the branch point (underlined) [220]. Recently were identified all five *T. vaginalis* spliceosomal snRNAs U1, U2, U4, U5 and U6 snRNAs [221]. Approximately 250 rDNA units were identified to one of the six *T. vaginalis* chromosomes [218].

5.2. Transcriptome. In order to identify the genes that are up-regulated during the interaction with target cells, a subtraction cDNA library enriched for differentially expressed genes from the parasites that were in contact with vaginal epithelial cells was obtained [222]. This strategy showed that genes encoding for the adhesins AP65 and AP33, α -actinin, enolase, a putative PDI gene, a phosphoglucomutase, and a conserved GTP-binding protein (GTP-BP) were up-regulated in parasites that were in contact with target cells [222]. Genes involved in transcription and protein translation in addition to six genes with unknown functions were also upregulated [222].

The phylogenetic analyses based on the rRNA and class II fumerase gene sequences have shown that *Trichomonas* species formed a closely related clade, including isolates of *T. gallinae*, *T. tenax*, and *T. vaginalis* [223]. To identify uniquely-expressed genes of *T. vaginalis* that may represent determinants that contribute to urogenital virulence and pathogenesis, genes differentially expressed in *T. vaginalis* with respect to *T. tenax*, usually regarded as a harmless commensal of the human oral cavity, were identified by: (i) the screening of three independent subtraction cDNA libraries enriched for *T. vaginalis* genes; and (ii) the screening of a *T. vaginalis* cDNA expression library with patient sera that were first pre-adsorbed with an extract of *T. tenax* antigens [224]. Noteworthy, clones identified by both procedures were found to be up-regulated in expression in *T. vaginalis* upon contact with vaginal epithelial cells [222], suggesting a role for these gene products in host colonization. Semi-quantitative RT-PCR analysis of select clones showed that the genes were not unique to *T. vaginalis* and that these genes were also present in *T. tenax*, albeit at very low levels of expression [224]. Of the transcripts whose relative abundance was found to vary significantly, the AP65, GAPDH, and hypothetical protein 2 are secreted or released during growth of *T. vaginalis* [225]. These results suggest that *T. vaginalis* and *T. tenax* have remarkable genetic identity and that *T. vaginalis* has higher levels of gene expression when compared to that of *T. tenax*. The data may suggest that *T. tenax* could be a variant of *T. vaginalis* [224].

5.3. General Transcription. Most of the studies regarding gene expression in *T. vaginalis* have been focused on the promoter region of protein-coding genes. The protein-coding genes in *T. vaginalis* are transcribed by a RNA polymerase II resistant to α -amanitin [226]. A metazoan-like TATA element appears to be absent in trichomonad promoters [227]. However, an initiator (Inr) sequence has been identified as the only known core promoter element

in this organism. This element is architecturally and functionally equivalent to its metazoan counterpart [227, 228]. In addition, the Inr promoter element was found in ~75% of 5' untranslated region (UTR) sequences of the protein-coding genes, supporting its central role in gene expression [218]. The finding of the Inr sequence in this early branched eukaryote strongly indicate that this promoter element evolved early in eukaryotic evolution, and it is likely that the trichomonad transcription machinery is highly optimized for Inr function.

5.4. Cis-Regulatory Elements and Transcription Factors. The Inr element is located within 20 nucleotides upstream of the start codon and may be as close as 6 nucleotides. This motif, with the consensus sequence TCA+1Py(T/A), surrounds the transcription start site of all genes studied [228]. An Inr-binding protein, a novel 39 kDa polypeptide (IBP39), from *T. vaginalis* was isolated by DNA affinity chromatography [229]. IBP39 shows no sequence similarity to any known protein and consists of two domains that are connected by a proteolytically sensitive linker; the N-terminal domain that is responsible for Inr binding (IBD) and the C-terminal domain, which binds the RNA pol II large subunit C-terminal domain (CTD) [230, 231]. The search of sequences similar to IBD revealed a family of at least 100 proteins in *T. vaginalis* containing an IBD motif with comparable architecture to IBP39, suggesting that IBD defines a lineage-specific DNA-binding domain that is utilized by specific transcription factors in this organism [196]. Sequence divergence in the recognition helix as well as the N-terminal positively charged loop across the IBD family suggest that different versions of the domain have potentially specialized to contact a range of target sites, other than Inr [196].

In *T. vaginalis*, iron is an essential nutrient for growth, metabolism and as determinant in modulating expression of multiple virulence phenotypes, such as cytoadherence, phenotypic variation and resistance to complement lysis [232–235]. However, the iron concentration in the human vagina is constantly changing throughout the menstrual cycle. Thus, *T. vaginalis* may respond to varying iron supply by means of differential gene expression mechanisms in order to survive, grow and colonize the vaginal hostile environment. Studies on the role of iron in the expression the *ap65-1* gene, which encodes a 65 kDa malic enzyme that is involved in cytoadherence, demonstrated that this element plays a crucial role in transcription. Transcription of *ap65-1* is critically regulated by the coordination of two similar but opposite oriented DNA regulatory regions, MRE-1/MRE-2r and MRE-2f, both of which are binding sites for multiple Myb-like proteins [236]. Myb1 protein exhibited variations in nuclear concentration with changes in the iron supply [237]. Overexpression of Myb1 in *T. vaginalis* resulted in repression or activation of *ap65-1* transcription in iron-depleted cells at an early and a late stage of cell growth, respectively, while iron-inducible *ap65-1* transcription was constitutively repressed. Myb1 protein was found to constantly occupy the chromosomal *ap65-1* promoter at a

proximal site, but it also selected two more distal sites only at the late growth stage [237]. Lou et al. [238] recently defined the minimal DNA-binding domain of Myb1, which consist in the sequence from Lys35 to Ser141 (tvMyb 35-141). Another Myb protein (Myb2) preferentially binds to MRE-2f than to MRE-2r [239]. The presence of iron caused the repression of the *myb2* gene, and the temporal activation/deactivation of Myb2 promoter entry, which was also activated by prolonged iron depletion [239]. The over expression of Myb2 in *T. vaginalis* during iron-depleted conditions facilitated basal and growth-related *ap65-1* transcription at similar level to that observed in iron-replete cells, whereas iron-inducible *ap65-1* transcription was abolished with knockdown of Myb2 [239]. In addition, another iron-inducible nuclear protein (Myb3) that binds only to the MRE-1 element was recently identified [240]. Changes in the iron supply resulted in temporal and alternate entries of Myb2 and Myb3 into the *ap65-1* promoter [240]. The over expression of Myb3 activates the basal and iron-inducible *ap65-1* transcription, and in agreement, the inhibition of Myb3 expression results in a decrease of *ap65-1* transcription [240]. In trophozoites overexpressing Myb3, an increased promoter entry of this protein was detected with concomitant decrease in Myb2 promoter entry under specific conditions, while Myb3 promoter entry was inhibited under all test conditions in cells overexpressing Myb2. In contrast, concomitant promoter entries by Myb2 and Myb3 diminished in cells overexpressing Myb1, except that Myb3 promoter entry was slightly affected under prolonged iron depletion [240]. All these results suggest that Myb2 and Myb3 may coactivate basal and iron-inducible *ap65-1* transcription against Myb1 through conditional and competitive promoter entries.

The existence of a large number of core histone genes in *T. vaginalis* genome was used to the identification of common nucleotide elements which may be involved in their transcription. The search of over represented nucleotide sequence elements in the 5' upstream sequence of *T. vaginalis* core histone genes revealed that these regions had three over represented motifs characterized with a string of conserved nucleotides: Motif I (TCAYWAKTT), Motif II (TTTTGGCGSS), and Motif III (TGHCAWWWWRRRYY) [241]. Four *T. vaginalis* core histone gene families (H2A, H2B, H3 and H4) have comparable motif architecture regardless of whether or not they are organized as gene pairs [241]. The 9 bp length Motif I is 3–7 bp upstream from the ATG initiation codon and a 5 bp length sub-motif (TCAYW) is highly similar to the Inr (TC₊₁AYW) [241]. Motif II predominately locates at the regions of –20 to –40 bp and is about 15 bp upstream from Motif I. Motif III is located at –40 to –80 upstream. Notably, the direction of Motif III related to gene transcription in H2A/H2B is opposite to that in H3/H4, indicating that Motif III may function in a direction-independent fashion [241]. These motifs are apparently enriched in the promoter region of several *T. vaginalis* genes and the positions of Motif II and Motif III related to translation start codon are similar with that in the promoter regions of the core histone genes, suggesting that the identified motifs are biologically meaningful transcriptional regulatory elements [241].

5.5. Chromatin Structure. *T. vaginalis* genome contains a large number of histone genes, and most of them organize as gene pairs in a head-to-head manner [241, 242]. It has a total of 74 functional core histones, including 11 H2A/H2B gene pairs, 6 solitary H2A, 3 solitary H2B, 19 H3/H4 gene pairs, 2 solitary H3 and 3 solitary H4 [241]. Comparison of the amino acid sequences of the *T. vaginalis* H3 and H4 histones with sequences from other organisms revealed a significant divergence not only from the sequences in multicellular organisms but also from the sequences in other protists [242].

T. vaginalis genome has also an expansion of genes encode both, HATs and HDAC deacetylases [196], but their role in gene expression in this parasite remain unknown.

5.6. Post-Transcriptional Regulation. The iron-responsive promoter elements that control the transcription initiation of *ap-65-1* have not been found until now in other genes coding iron-regulated proteins, suggesting that regulatory iron-mediated mechanisms at post-transcriptional level may exist in *T. vaginalis*.

In higher eukaryotes, the IRE/IRP system is a post-transcriptional mechanism of iron regulation based on the binding of cytoplasmic iron regulatory proteins (IRPs) to iron-responsive elements (IREs) situated into the untranslated regions of mRNAs of some iron-regulated proteins. In low-iron conditions IRPs bind to IREs blocking the translation of mRNAs containing IREs in their 5'-ends or increasing the stability of mRNAs that has IREs at their 3'-ends [243]. TvCP4 is a cysteine protease of *T. vaginalis*, which amount was found 3-fold increased in iron-rich than in iron-depleted parasites. However, the Tvcp4 transcript was expressed at similar level in both conditions, suggesting that the iron-regulated expression of TvCP4 is carried out at a posttranscriptional stage [244]. The search of IRE structures in the mRNA of *Tvcp4* revealed that the first 23-nt downstream of the start codon form an IRE-like stable stem-loop structure. The recombinant IRP-1 of human specifically binds to the IRE-like structure of the *Tvcp4* mRNA, and cytoplasmic extracts from *T. vaginalis* form RNA-protein complexes with the IRE sequence of the mRNA of human ferritin [244], suggesting that this parasite posses an IRE/IRP system that control the expression of some iron-regulated proteins.

6. Concluding Remarks

Development of an organism depends upon finely-tuned and accurate control of gene expression. Recent studies have identified various biological processes involved in the regulation of gene expression in protozoa parasites. These processes required several components, such as *cis*-regulatory elements, transcription factors, transcription cofactors, chromatin modification proteins, and proteins involved in post-transcriptional regulation. The list of these components will continue to grow, as future studies identify additional examples of direct communications between regulatory proteins, and reveal how gene networks are regulated

coordinately through these interactions. Further analyses of these regulatory mechanisms in the protozoa parasites should continue to provide broadly applicable information about their conserved functions in vivo, and new insights into the specific biological processes in which they are involved. In addition, assembled genome sequences are now available for different parasites, inclusively for some host and vectors that are part of their life cycles. These assemblies provide a powerful tool for the comparative analysis of gene regulation networks. As ever, new knowledge raises new questions. But these questions await their orderly resolution.

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Research Article

Improved Method for In Vitro Secondary Amastigogenesis of *Trypanosoma cruzi*: Morphometrical and Molecular Analysis of Intermediate Developmental Forms

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Trypanosoma cruzi undergoes a biphasic life cycle that consists of four alternate developmental stages. In vitro conditions to obtain a synchronic transformation and efficient rates of pure intermediate forms (IFs), which are indispensable for further biochemical, biological, and molecular studies, have not been reported. In the present study, we established an improved method to obtain IFs from secondary amastigogenesis. During the transformation kinetics, we observed progressive decreases in the size of the parasite body, undulating membrane and flagellum that were concomitant with nucleus remodeling and kinetoplast displacement. In addition, a gradual reduction in parasite movement and acquisition of the amastigote-specific Ssp4 antigen were observed. Therefore, our results showed that the in vitro conditions used obtained large quantities of highly synchronous and pure IFs that were clearly distinguished by morphometrical and molecular analyses. Obtaining these IFs represents the first step towards an understanding of the molecular mechanisms involved in amastigogenesis.

1. Introduction

Trypanosoma cruzi is an obligate intracellular parasite that is responsible for Chagas disease, which affects 16–18 million people in Latin America. This parasite has a complex biphasic life cycle in which four developmental forms alternate between the Reduviid beetle vector (epimastigotes and metacyclic trypomastigotes) and the mammalian host (amastigotes and bloodstream trypomastigotes). Transmission is initiated in the Reduviid beetle vector, which becomes infected by taking up circulating trypomastigotes during a blood meal. After trypomastigotes differentiate to epimastigotes in the insect gut lumen, the parasite divides by binary fission before migrating along the hindgut and rectum, where they transform to metacyclic trypomastigotes.

These trypomastigotes are released near the bite wound with the insect feces during the next blood meal. Following its introduction into mammalian blood, the trypomastigotes penetrate nonphagocytic and phagocytic cells through a parasitophorous vacuole to start the intracellular cycle. In this stage, they differentiate into amastigotes and replicate in the infected cell cytoplasm. Amastigotes develop into nondividing bloodstream trypomastigotes that can either initiate another round of infection to propagate to different organs or can be taken up by the insect vector to complete the life cycle.

Throughout its life cycle, *T. cruzi* survives under a wide range of environmental conditions that induce complex morphological changes among parasite stages. In addition to the four main developmental forms, it is possible to

observe intermediate forms (IFs) that seem to follow the same differentiation path, independent of whether they exist in a vertebrate or in an invertebrate host [1, 2]. Intermediate forms appear transiently during the differentiation of epimastigotes into metacyclic trypomastigotes (metacyclogenesis) in the triatomine, the differentiation of metacyclic trypomastigotes (primary amastigogenesis), and tissue-derived trypomastigotes (secondary amastigogenesis) into amastigotes and also into bloodstream trypomastigotes inside the mammalian host cell [2–5].

Adaptation of *T. cruzi* to diverse environments found in the different hosts undoubtedly induces a complex regulation of gene expression that apparently precedes the morphological changes observed during parasite transformation. Several researchers have studied some of the factors that represent physiological stress for the parasite and have demonstrated that temperature, nutritional conditions, and pH stimulate morphological differentiation during amastigogenesis [5–8].

The vast majority of the information regarding in vivo and in vitro amastigogenesis comes from data in which tissue-derived trypomastigotes were used because it was possible to obtain higher yields of these parasites [3, 5, 8–15]. Ultra-structural and molecular analyses during the trypomastigote to amastigote transformation have shown a complex and progressive morphological rearrangement of parasite shape and flagellum that has been associated with the differential expression of stage-specific antigens [3, 5, 8]. A comparative morphology study showed that even when metacyclic and bloodstream trypomastigotes share similar biological and morphological properties, primary and secondary amastigogenesis apparently display different developmental processes, which suggests that their intracellular mechanisms are different [5].

Although the basic features of the amastigogenesis transformation process are known, the molecular mechanisms involved are still unidentified. Analysis of the molecules implicated in the detonation and control of the transformation process will increase our knowledge about morphogenesis and gene expression programs that are involved not only in the differentiation between developmental forms but also during parasite transitions from the nonreplicative to the replicative stage. The usefulness of a differentiation system for molecular research in which a population of cells is involved depends first on the synchrony of the transition, second on the availability of easily analyzable markers for monitoring the process, and third on the system efficiency for obtaining large enough amounts of analyzable sample for further biochemical, biological, and molecular analyses. To date, in vitro conditions that allow for obtaining IFs that satisfy these needs have not been reported. Therefore, in vitro conditions that induce high rates of pure IFs during the transformation of culture-derived trypomastigotes into amastigotes are described in this work. Furthermore, morphological, cellular, and molecular characterizations of the different IFs obtained are presented.

2. Materials and Methods

2.1. Cells and Parasites. NIH 3T3 fibroblasts were grown in high glucose Dulbecco's minimal essential medium (hgDMEM) supplemented with 10% fetal bovine serum (FBS), 1% glutamine, and 5 $\mu\text{g}/\text{mL}$ penicillin-streptomycin at 37°C in a humidified atmosphere with 5% CO_2 in a 75 cm^2 Corning cell culture flask (catalog number 4306-41). Epimastigotes of *T. cruzi* CL-Brener strain were grown in liver infusion tryptose medium (LYT) containing 10% FBS and Hemin (25 $\mu\text{g}/\text{mL}$) at 28°C [16].

Fifty percent confluent fibroblasts were infected with 2×10^6 CL-Brener mid-log-phase epimastigotes per mL of hgDMEM (15 mL) supplemented with 2% FBS to get a parasite-host cell ratio of 10 : 1. The NIH 3T3 monolayers were washed every 2 days with hgDMEM medium until non-adherent parasites were removed, and then fresh hgDMEM plus 2% FBS was added. The parasites released from the early infections corresponding to the first 6 days after the first liberation were harvested every day at $2000 \times g$ for 5 minutes. The trypomastigotes were separated from amastigotes by immunoprecipitation using 500 μL of hybridoma supernatant of monoclonal antibody 2C2B6, which is specific for the Ssp4 surface antigen of amastigotes [3], per 3×10^8 parasites at 37°C for 1 hour. The trypomastigotes were separated from amastigotes at 800 rpm for 10 seconds in a microcentrifuge and were washed with 1X PBS as many times as necessary to obtain pure parasites. The amastigotes were found in the pellet, and the trypomastigotes were found in the supernatant.

2.2. In Vitro Amastigogenesis. Purified tissue culture-derived trypomastigotes (5×10^6) were harvested at $500 \times g$ for 10 minutes and were transferred to 100 \times 20 mm untreated Petri dishes containing 1 mL per experiment of hgDMEM at different pHs (4, 5, 6, 7, 8, and 9) without or with different FBS concentrations (0.5%, 1%, 1.5%, and 2%) incubated at 37°C in a 5% CO_2 atmosphere.

After 1, 2, 3, 4, 5, 6, 12, 24, 48, or 72 hours, the parasites were washed, fixed with 4% paraformaldehyde in 1X PBS, washed again, and dropped (3×10^4 parasites/ $10 \mu\text{L}$) over poly-L-lysine-treated slides (Silane-Prep slides SIGMA DIAGNOSTICS, catalog number S4651) inside circles delimited with a liquid-repellent slide marker pen (*Electron Microscopy Sciences* catalog number 71310). After the slides were air dried, the parasites were stained with Hema 3 according to the protocol supplied by the manufacturer (*Fisher Diagnostic* 840021), and the percentages of trypomastigotes, IFs, and amastigotes were determined for 100 cells analyzed randomly under an optic microscope.

2.3. Resistance to Complement-Mediated Lysis Assays. Trypomastigotes, amastigotes, epimastigotes, or IFs (2×10^6 parasites) were incubated with 500 μL of fresh human serum at 37°C for 30, 60, and 120 minutes. Parasite lysis was determined by adding one volume of trypan blue (0.4% in 1X PBS, Hyclon 23850) and counting the complement-resistant parasites in a hemocytometer

under a light microscope. Three independent experiments, repeated in triplicate each time, were carried out using serum from different donors.

2.4. Localization of *Ssp4* by Indirect Immunofluorescence. The parasites were harvested, washed, fixed, and dropped over poly-L-lysine-treated slides as described above. The parasites were permeabilized with 0.1% Triton-X100 for 5 minutes, rinsed extensively with 1X PBS, and treated with 2% BSA (*Albumin, bovine, Cohn fraction V* / 1X PBS pH 7) at 37°C for 30 minutes. These preparations were washed with PBS and incubated 1 hour at 37°C with hybridoma supernatant (1 : 50) of the amastigote-specific monoclonal antibody 2C2B6 [3]. After rinsing, a secondary antimouse antibody tagged with Alexa Fluor 488 green diluted 1 : 1000 was layered on the parasites and incubated for 1 hour at 37°C. The slides were rinsed, mounted with Vectashield Mounting Medium (Vector Laboratories Cat. H-1000), and observed under an Olympus fluorescence microscope (BX41) equipped with a 60X/1.25 Oil Iris Ph3 UPlanFL N objective. The images were captured using an Evolution VF Fast Cooled Color Media Cybernetics camera and were analyzed using the Image-Pro Plus V 6.0 Media Cybernetics program.

2.5. DAPI Staining. The parasites were processed and analyzed as per the indirect immunofluorescence protocol described above, with some specifications. After treatment with a polyclonal antibody against the epimastigotes (1 : 200) obtained from the mouse and the antimouse antibody tagged with Alexa Fluor 546 red (1 : 1000) as the first and second antibodies, the parasites were stained with 10 µg/mL DAPI (Molecular Probes). The slides were mounted and analyzed as described above.

3. Results

3.1. Serum-Free Culture at pH5 Stimulated the Highest Rates of Transformation from Tissue-Derived Trypomastigotes into Amastigotes. As mentioned before, conditions such as temperature, nutritional environment, and pH stimulate the in vitro differentiation of trypomastigotes into amastigotes [5–8]. To determine the best conditions for obtaining the highest rates of transformation, in vitro experiments were performed using tissue-derived trypomastigotes incubated at 37°C in hgDMEM medium at different pHs (4, 5, 6, 7, 8, and 9), without or with 0.5%, 1%, 1.5%, or 2% FBS for different lengths of time, as described in the materials and methods. The movement and morphology of the parasites were analyzed in fresh preparations under a light microscope, and the relative percentages of trypomastigotes, Ifs, and amastigotes were determined. In all of the conditions in which parasite differentiation was achieved, the trypomastigotes showed gradual morphological changes and size reduction; they transformed from the typical S- or C-shaped bloodstream morphology to diverse Ifs that finally reached the rounded or oval shape characteristic of amastigotes after 12 hours of incubation. A progressive reduction of parasite movement concomitant with the gradual shortening of the undulating membrane and flagellum was also observed.

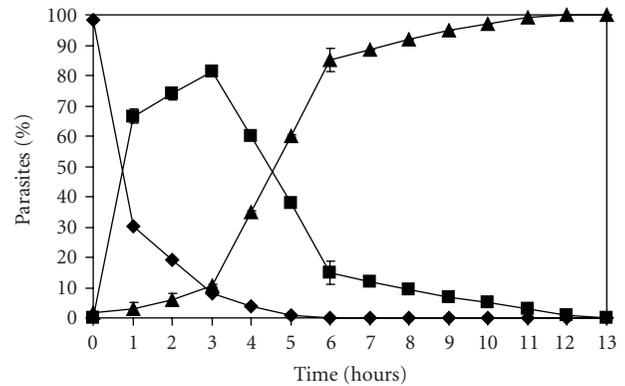


FIGURE 1: Transformation kinetics from tissue-derived trypomastigote to amastigote in hgDMEM at pH 5 without FBS at 37°C: relative percentage of trypomastigotes (—◆—), IFs (—■—), and amastigotes (—▲—) analyzed in fresh preparations under the light microscope. The results are the average of 3 independent experiments.

Although similar morphological patterns of differentiation could be seen in some of the different conditions tested, very different rates of transformation were obtained. Incubation at 37°C in hgDMEM at pH 5 without FBS induced the most efficient rates of differentiation. Under these conditions, the level of tissue-derived trypomastigotes decreased concomitantly with increasing amounts of IFs, which reached 85% within 3 hours of incubation (Figure 1). Subsequently, as the percentage of IFs decreased, the relative amount of amastigotes increased, and the transformation process was completed after 12 hours of incubation. A homogenous population (100%) of round-shaped forms, which displayed either no flagella or very short protruding flagella and a noticeable reduction of movement, was observed at this point. On the contrary, when tissue-derived trypomastigotes were incubated in hgDMEM at pH 4 without FBS, only 30% of the cells were transformed into amastigotes after 3 hours and 65% after 12 hours (data not shown). In addition, only 21% of the trypomastigotes differentiated into amastigotes after 24 hours of incubation at pH 6 without FBS (data not shown). Finally, at pHs 7, 8, and 9 in the absence of FBS as well as those same pHs with 0.5%, 1%, 1.5%, and 2% FBS did not induce parasite differentiation (data not shown).

These results indicate that the differentiation process during the in vitro secondary amastigogenesis involves several IFs as a result of the morphological change of the parasites. In addition, the optimal conditions to induce the highest rates of transformation from tissue-derived trypomastigotes into amastigotes were pH 5, serum-deprivation and 37°C.

3.2. Gradual Morphological Changes were Observed During In Vitro Secondary Amastigogenesis. The different developmental forms of *T. cruzi* can be identified by morphological and biological parasite features, such as the size and shape of the parasite, undulating membrane and flagellum, the position

of flagellum emergence, and the size, shape, and relative position of the nucleus and the kinetoplast. The amastigote has a spherical- to oval-shaped form that is 2 to 4 μm in diameter with no or very short free flagellum and a kinetoplast located in the middle of the parasite body close to the nucleus. The trypomastigote kinetoplast is located subterminal and posterior to the nucleus. The trypomastigotes have a flagellum and an undulating membrane that extends the entire length of the parasite (16–25 μm long and 2 μm diameter). Trypomastigotes found in the blood of infected mammals have a slender S- or C-shaped form, while the trypomastigotes found in infected insects usually have a straight form [17].

Once the best conditions to induce the transformation of the parasite were determined, a detailed morphological analysis was carried out on fixed and stained preparations, and the relative amounts of trypomastigotes, amastigotes, and IFs were determined at different points of the differentiation kinetics at 37°C using hgDMEM at pH 5 with serum-deprivation (Figure 2). This methodology allowed us to perform a morphometrical analysis and a more precise quantification of the transformation process compared to those obtained with fresh preparations. Before initiating the differentiation process, tissue-derived trypomastigotes showed the typical morphology previously described (Figure 2a). During the transformation process, the parasite progressively reduced its entire length by 22% at 1 hour, 23% at 2 hours, 39% at 3 hours, 48% at 4 hours, 50% at 5 hours, and 53% at 6 hours of transformation as shown in Figure 2 and Table 1. The quantitative analysis indicated that as little as 1 hour of transformation time was enough to induce high rates of IFs (80%) in which the parasites displayed a widening and shortening of their body, flagellum, and undulating membrane; however, this last one maintained the subterminal emergence site that is characteristic of trypomastigotes (Figure 2b and Table 1). After 2 hours of transformation, no trypomastigotes were detected, which indicates that the culture contained 100% IFs. These IFs showed size reduction, a light oval-shaped form, shortening of their flagellum, and an undulating membrane in which their emergence site was slightly displaced to the middle of the parasite body (Figure 2(c) and Table 1). After 3 hours, the morphology of the IFs was very close to the one observed at 2 hours of transformation, but their size continued to reduce and the undulating membrane was not appreciable (Figure 2(d) and Table 1). After 4 and 5 hours of transformation, the IFs showed similar size and morphology to the amastigotes and were only distinguished by the progressive shortening of their flagellum (Figures 2(e) and 2(f) and Table 1). Finally, after 6 hours of transformation, the parasites reached the spherical- to oval-shaped form with no or very short free flagellum (Figure 2(g)), and their morphology was indistinguishable from the one observed in the cell culture-derived amastigotes (Figure 2(h) and Table 1). To assess the reversibility of the differentiation process, the IFs were cultured again in hgDMEM at pH 7.2 complemented with 2% FBS after 1, 2, 3, 4, 5, 6, 12, 24, 48 or 72 hours of differentiation at 37°C using hgDMEM at pH 5 with serum deprivation. In all cases, the IFs continued their differentiation, thus indicating that

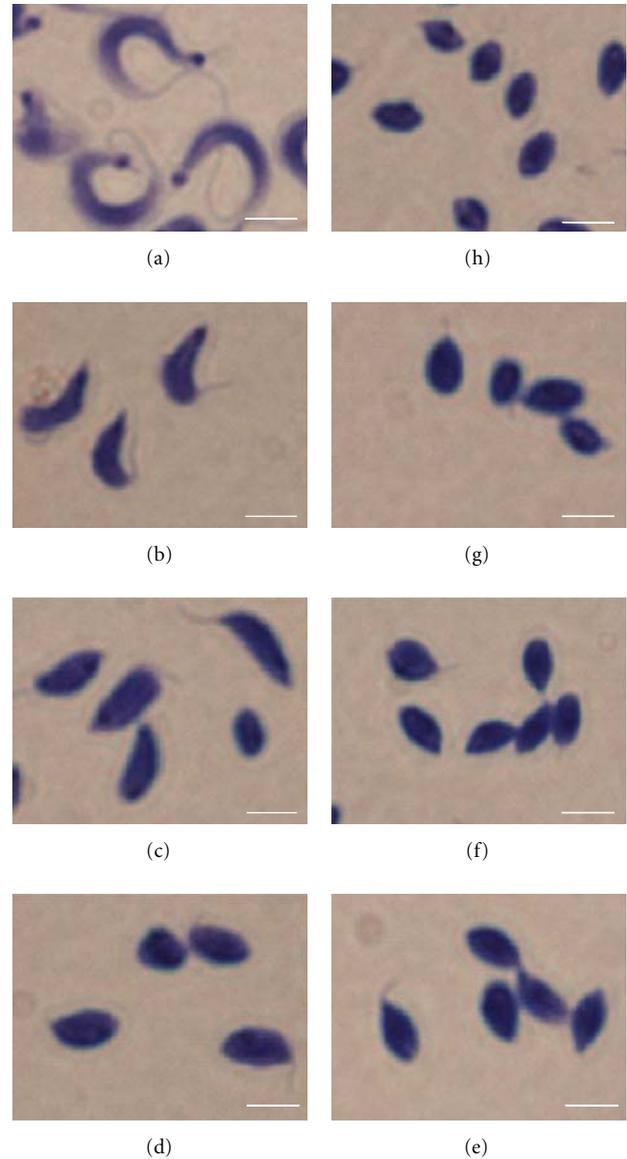


FIGURE 2: Morphological analysis of the kinetics of extracellular differentiation from tissue-derived trypomastigotes (a) to amastigotes (h), in hgDMEM at pH 5 without FBS at 37°C: intermediate forms at 1 hour (b), 2 hours (c), 3 hours (d), 4 hours (e), 5 hours (f), and 6 hours (g) of transformation stained with Hema 3. Bar = 25 μm .

once detonated, this process cannot be stopped and is not reversible (data not shown).

These results showed that, under the *in vitro* conditions used, the trypomastigotes differentiated very efficiently into highly homogeneous and pure IF populations after 2 hours of induction.

3.3. The Shape and Position of the Nucleus and Kinetoplast Determined the Different IFs. To continue with the morphological analysis of the IFs, the shape and position of the nucleus and the kinetoplast at each point of the transformation kinetics were determined. The parasites were then

stained with a polyclonal antibody against epimastigote total proteins, which had been previously shown to recognize all of the developmental forms and IFs of *T. cruzi*. The reactions were developed with an Alexa Fluor 546 red-conjugated antimouse antibody, and the kinetoplast and nucleus were counterstained with DAPI.

As shown in previous experiments, the same morphological transformation pattern of the parasite body was found with this technique (compare Figures 2 and 3). Nucleus remodeling and a progressive and continuous displacement of the kinetoplast from the posterior to the anterior position with respect to the parasite nucleus were also observed. As shown in Table 1, the distance between the nucleus and the kinetoplast became shorter during the transformation kinetics. At 1 hour of transformation (Figure 3(b)), the distance between both organelles was reduced by 28% (Table 1), even though the IFs showed a subterminal kinetoplast posterior to the nucleus, as commonly observed in trypomastigotes (Figure 3(a)). After 2 hours of transformation, the IFs showed a displacement of their kinetoplast to the middle of the parasite body and reduced the distance with respect to the nucleus (now remodeled from the lengthened-shaped form to an oval-shaped form) by 58% (Figure 3(c) and Table 1). After 3 hours of transformation, the kinetoplast remained located posterior to the nucleus, close to the middle part of the parasite body, with a 66% reduction in the distance between both organelles (Figure 3(d) and Table 1). At 4 hours of transformation, the kinetoplast was positioned next to and parallel to the nucleus in a transversal position in the middle of the parasite body and showed 85% reduction in the organelle distance (Figure 3(e) and Table 1). After 5 hours of transformation, the kinetoplast moved to an anterior position with respect to the nucleus, in the middle part of the parasite body and showed a 90% reduction in the distance between the organelles (Figure 3(f) and Table 1). Finally, at 6 hours of transformation (Figure 3(g) and Table 1), the parasite morphology and the position of the kinetoplast were very similar to those observed in the tissue-derived amastigotes (Figure 3(h) and Table 1).

These results showed that, concomitant with the morphological parasite shape transformation, nucleus remodeling and kinetoplast displacement are observed during the transformation kinetics under the *in vitro* conditions used.

3.4. Highly Synchronous Morphologic Changes were Obtained During *In Vitro* Transformation. The morphological analysis described above showed homogenous IF populations; nevertheless, the nucleus remodeling and kinetoplast displacement studies indicated that each point of the transformation kinetics is composed of 3 different IFs. The percentages of trypomastigotes, amastigotes and the different IFs were then determined for each time point evaluated in the previous experiment. As shown in Figure 4, 3 different IFs were observed at 1, 2, 3, 4 and 5 hours of *in vitro* differentiation (Figures 4(a) and 4(c)). At 1 hour of transformation The first IF, which was arbitrarily named IF1, showed similar morphology to the immediate previous parasite stage (trypomastigotes for the first hour and IF3 for 2, 3, 4, and 5 hours)

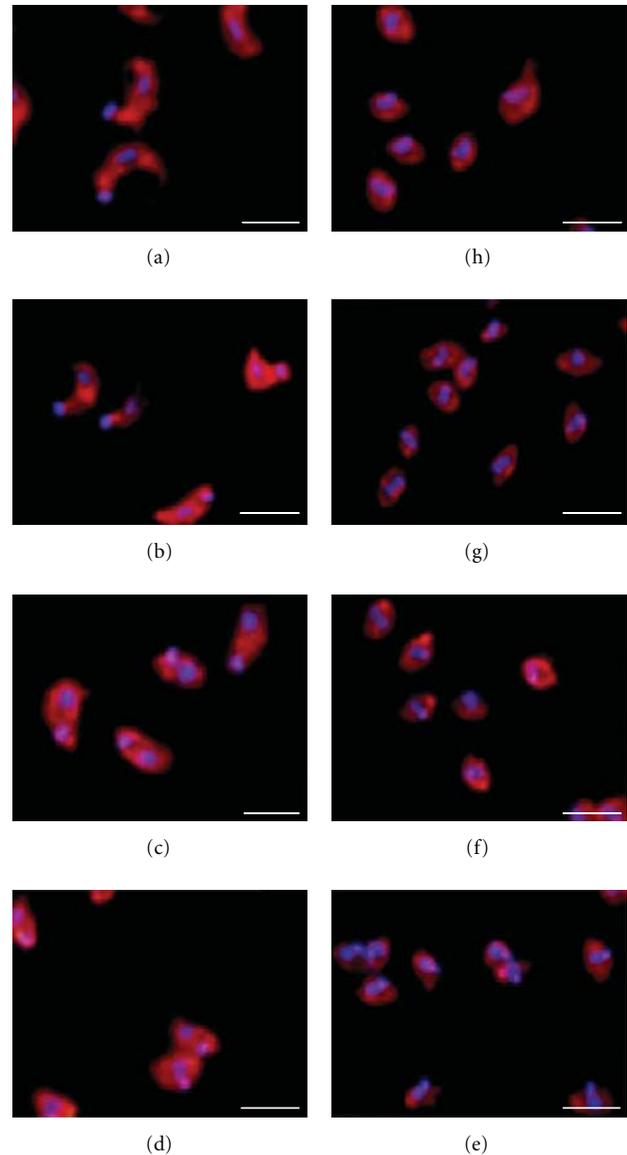


FIGURE 3: The shape and position of the nucleus and the kinetoplast identified the different IFs. The morphology and position of the nucleus and the kinetoplast were determined by indirect immunofluorescence and DAPI staining of trypomastigotes (a), IFs at 1 hour (b), 2 hours (c), 3 hours (d), 4 hours (e), 5 hours (f) and 6 hours (g) of transformation and amastigotes (h). Bar = 25 μ m.

and represented 2% to 3% of the total parasites. The second intermediary form, named IF2, showed a slightly, but clearly, more differentiated morphology than IF1 and represented the most abundant parasite population (85% to 90%). The third IF, still more differentiated than IF2 and named IF3 (2% to 15%), displayed characteristics similar to the IF1 that emerged from the next time of transformation. At 1 hour of transformation, trypomastigotes comprised only 2% of the culture, which confirms that the transformation process is very efficient under the established conditions (Figures 4(a), 4(b) and 4(c)). Moreover, from 1 to 5 hours of transformation were not observed any amastigotes (Panel A).

TABLE 1: Morphometrical analysis of *T. cruzi* developmental stages.

Parasite	Parasite length (μm)	Distance between nucleus and kinetoplast (μm)
Trypomastigote	24.17 ± 4.06	15.19 ± 3.02
If 1 hours	19.04 ± 2.62	10.89 ± 1.97
If 2 hours	18.52 ± 2.43	6.43 ± 1.60
If 3 hours	14.84 ± 2.47	5.12 ± 1.58
If 4 hours	12.52 ± 1.03	2.29 ± 0.72
If 5 hours	11.98 ± 1.44	1.47 ± 0.53
If 6 hours	11.26 ± 1.24	1.36 ± 0.38
Amastigote	11.45 ± 1.02	1.08 ± 0.22

The results are the average of 100 cells analyzed randomly.

Finally, at 6 hours of transformation, 100% of the parasites showed a very similar morphology to that of tissue-derived amastigotes (Figures 3(g) and 3(h), Table 1 and Figures 4(a), 4(b) and 4(c)).

The only forms detected after 2, 3, 4 and 5 hours of transformation were IFs with a gradually increasing degree of differentiation. Therefore, this experimental model could be useful for future analysis of the differential expression between the IFs and the completely differentiated forms of *T. cruzi*. In addition, even when the IFs at 1 hour of transformation showed morphometric characteristics that corresponded with an IF, the presence of contaminant trypomastigotes (2%) rendered this population not useful for differential expression studies (Figure 4).

3.5. Secondary Amastigogenesis was Accompanied by the Gradual Acquisition of the Amastigote-Specific Ssp4 Glycoprotein. The previous results demonstrated that different IFs, which can be identified by specific morphological characteristics, are generated during amastigogenesis. Since it was necessary to know more about their biological properties, a molecular marker was evaluated for the different IFs during the transformation kinetics. It has been reported that the morphological differentiation of the IFs implies the remodeling of parasite surface molecules [3, 8]. The expression of the amastigote-specific surface glycoprotein Ssp4 was evaluated by indirect immunofluorescence in the diverse IFs as well as in trypomastigotes and amastigotes used as controls.

The results presented in Figure 5 showed that, as expected, the trypomastigotes did not express the amastigote-specific surface glycoprotein (Figures 5(a) and 5(b)), while amastigotes displayed heterogeneous levels of Ssp4 (Figures 5(o) and 5(p)). Moreover, while the IFs generated at 1, 2, and, 3 hours of transformation did not express Ssp4 (from Figures 5(c) to 5(h)), approximately 8% and 36% of parasites at 4 (Figures 5(i) and 5(j)) and 5 hours of differentiation (Figures 5(k) and 5(l)), respectively, showed low expression levels. In addition, after 6 hours of transformation (Figures 5(m) and 5(n)), approximately 76% of the parasites showed variable and lower levels of Ssp4 compared to culture-derived amastigotes (Figures 5(o) and

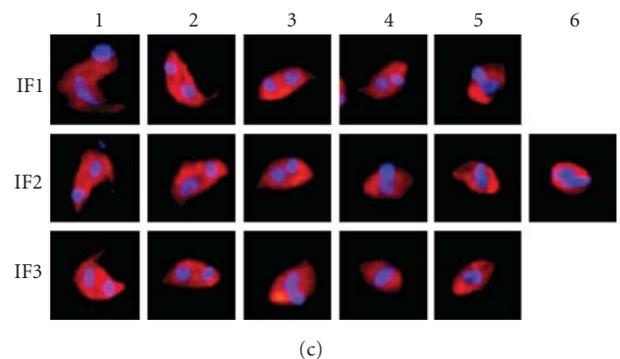
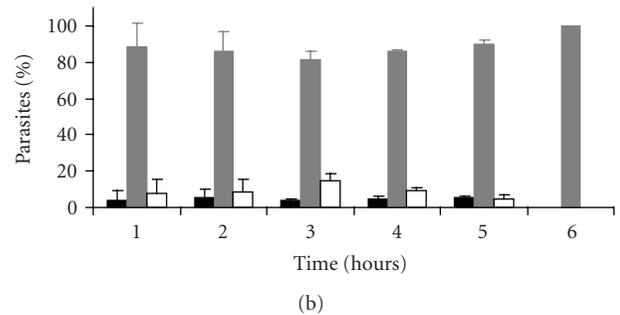
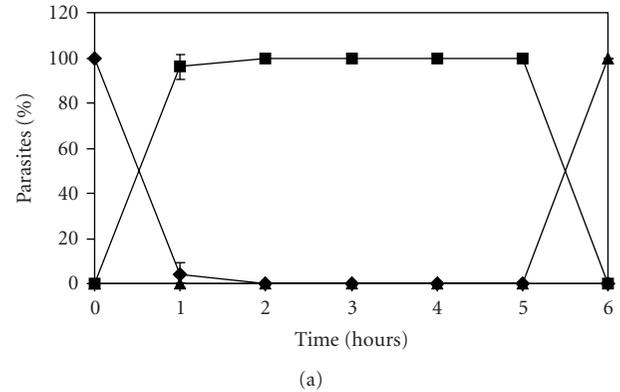


FIGURE 4: Highly synchronous morphologic changes are obtained during the in vitro transformation kinetics. Quantitative analysis of 2 independent experiments of extracellular differentiation from tissue-derived trypomastigotes into amastigotes stained by indirect immunofluorescence and DAPI after 1, 2, 3, 4, 5 and 6 hours of transformation. (a) Relative percentage of trypomastigotes (\blacklozenge), IFs (\blacksquare) and amastigotes (\blacktriangle). (b) Three different IFs arbitrarily designated as IF1 (black), IF2 (grey) and IF3 (white) were observed from 1 to 5 hours of transformation. (c) Representative parasite of the corresponding IFs of panel b.

5(p)). Finally, after 72 hours of transformation, all parasites showed equivalent expression of Ssp4 to that of culture-derived amastigotes (data not show).

These results indicated that the consecutive and gradual expression of Ssp4 was concomitant with the morphological transformation of trypomastigotes into amastigotes, in which the IFs with a similar morphology to amastigotes were the ones that started expressing the amastigote-specific glycoprotein. Moreover, even though the morphology of

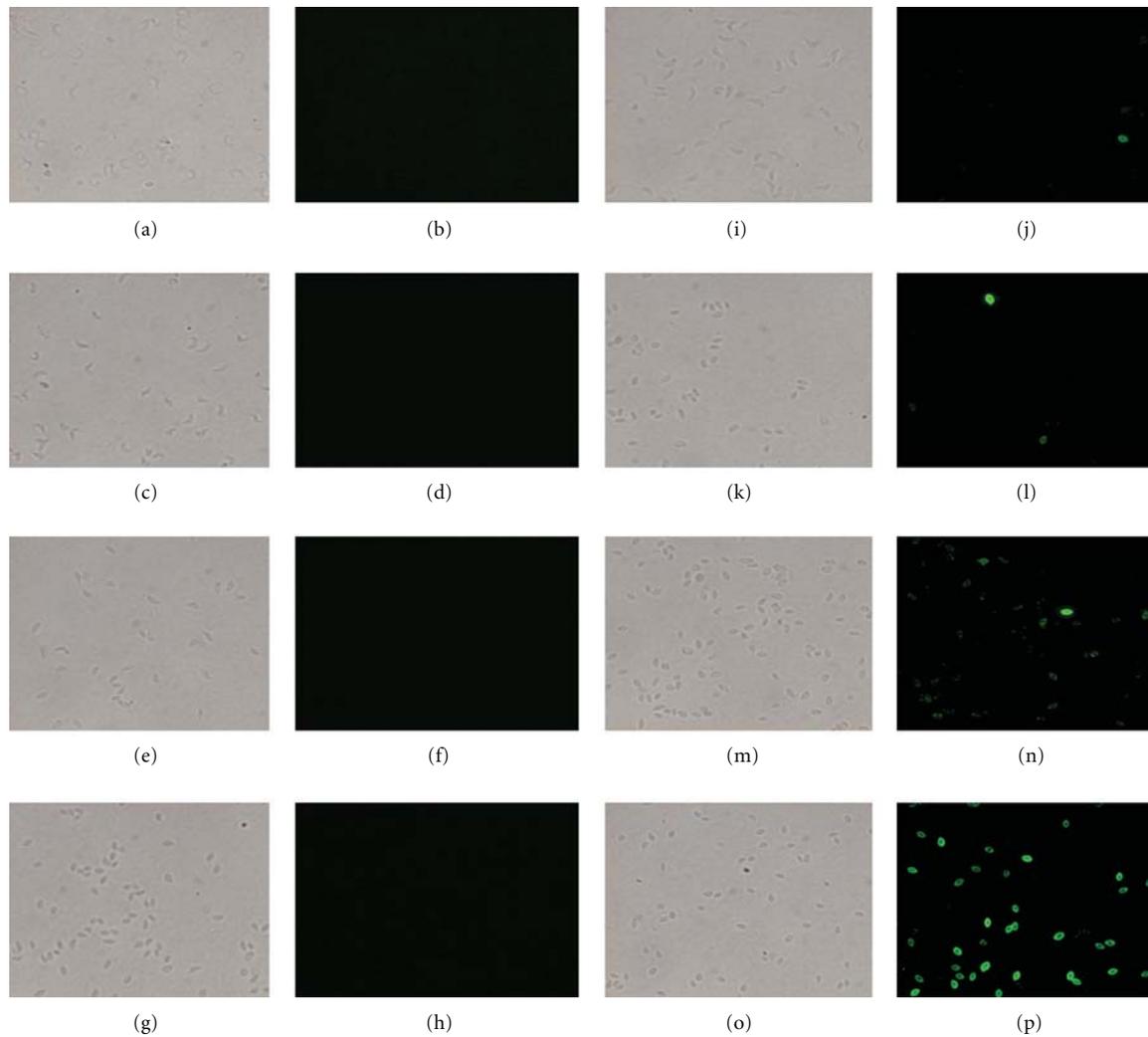


FIGURE 5: Secondary amastigogenesis is accompanied by the gradual acquisition of amastigote-specific Ssp4 surface glycoprotein. The localization of Ssp4 was determined by indirect immunofluorescence in trypomastigotes (a, b), IFs at 1 hour (c, d), 2 hours (e, f), 3 hours (g, h), 4 hours (i, j), 5 hours (k, l) and 6 hours (m, n) of transformation and in amastigotes (o, p). The right panels are the immunofluorescence image (b, d, f, h, j, l, n, p) of the corresponding light microscopy panels (a, c, e, g, i, k, m, o).

the parasites at 6 hours of transformation was similar to the tissue-derived amastigote morphology, the expression of this molecular marker indicated that these parasites were not completely transformed at the molecular level.

3.6. The IFs were Resistant to Complement-Mediated Lysis. Continuing with the biological analysis of the different IFs, their resistance to complement-mediated lysis was determined. As the IFs are obtained during trypomastigote differentiation into amastigotes and both developmental stages are resistant to complement-mediated lysis, it would not be surprising if the IFs are also resistant. However, it was important to evaluate this phenotype because (1) the IFs are usually intracellular and therefore not exposed to the blood and extracellular space in the vertebrate host and so they would not need to be resistant to complement-mediated lysis and (2) the IFs obtained during metacyclogenesis and

amastigogenesis show very similar morphological characteristics, and very little is known about biological properties that could be used to distinguish them. The IFs obtained by metacyclogenesis are sensitive to complement-mediated lysis [18]. However, this biological property has not been evaluated in the IFs obtained during amastigogenesis. Therefore, the complement-sensitivity of the IFs obtained was evaluated using fresh human serum as described in the materials and methods.

As shown in Figure 6, the IF at 3 hours of transformation, similar to trypomastigotes and amastigotes, was resistant to complement-mediated lysis. Moreover, all of the IFs obtained through the transformation kinetics were also resistant to complement-mediated lysis (data not shown). As expected, epimastigotes were sensitive to complement, thus reaching 100% lysed parasites in the first 30 minutes of treatment.

These results suggest that the IFs obtained during the *in vitro* amastigogenesis do not lose the ability to resist

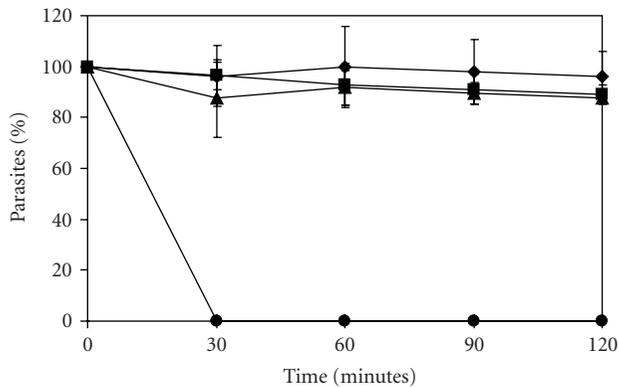


FIGURE 6: Analysis of resistance to complement-mediated lysis. The relative percentages of trypomastigotes (-◆-), amastigotes (-■-), IFs at 3 hours of transformation (-▲-) and epimastigotes (-●-) were determined after treatment with fresh human serum. The results are the average of 3 independent experiments.

complement-mediated lysis. Therefore, even if the IFs from metacyclogenesis and amastigogenesis are morphologically similar, they have different biological properties that can be used to distinguish them.

4. Discussion

The lack of effective *in vitro* conditions to obtain high rates of synchronic and pure IFs has delayed the understanding of the molecular mechanisms that mediate the cellular remodeling and the identification of molecular markers that are important for amastigogenesis. In the present work, we established an improved method to obtain high rates of pure IFs and correlated parasite differentiation with the expression of a specific developmental marker, complement-mediated lysis sensitivity and morphometrical analysis of the nucleus, kinetoplast and parasite body throughout the complete transformation process.

The efficient transformation of cell culture-derived trypomastigotes to amastigotes was induced using an acidic pH and serum deprivation at 37°C (Figure 1). The parasites showed dramatic cell restructuring with a gradual and progressive reduction of their size and the readsorption of their flagellum and undulating membrane until they reached the typical round shape of tissue-derived amastigotes (Figure 2). These results are consistent with previous reports that document the existence of several intermediate forms during the life cycle of *T. cruzi*, either under *in vivo* or *in vitro* conditions that showed the same gradual and progressive parasite morphometrical transformation, apparently as an obligatory step for the differentiation process [1, 3, 5, 8, 18–22]. The quantitative analysis demonstrated that parasite transformation was initiated immediately after the exposure to hgDMEM at pH5 and 37°C, thus reaching 100% IFs after only 2 hours of incubation (Figure 4); this indicates a faster and more efficient differentiation rate than previously reported [5, 8]. These differences could be the result of the absence of BSA in the differentiation medium used in this study in comparison with the 0.4% BSA contained in the previously reported medium [5, 8]. In addition, the condition

of the cell culture-derived trypomastigotes used to initiate parasite differentiation may play an important role in the process. The parasites used in all of the experiments in this study were early released trypomastigotes that were obtained from early infections corresponding to the first 6 days after the first trypomastigote liberation, because the parasites released later showed variable, not synchronous and inefficient transformation rates. Interestingly, Andrews et al [3], and Tomlinson et al [8], also used only trypomastigotes early released from infected cells for their transformation experiments. Even when these authors did not justify this specification, it was most probably the result of similar observations.

The morphological restructuring of *T. cruzi* during amastigogenesis has shown that the transition from the slender to the spherical or oval parasite shape comprises an extensive remodeling of cellular architecture, the flagellum and the undulating membrane, and a dramatic change in the overall parasite size, apparently as a result of the reorganization of their subpellicular microtubules[17]. This dramatic restructuring also affects the location and morphology of the parasite organelles during *T. cruzi* differentiation. The kinetoplast of the trypomastigotes is spherical; whereas in amastigotes, it is elongated [8, 21, 23, 24]. In contrast, the nuclei of the trypomastigotes are elongated, apparently following the parasite's shape; meanwhile, it is round in amastigotes. These morphometrical characteristics of the nucleus and the kinetoplast have been reported to allow the distinction of the *T. cruzi* developmental stages [17]. Therefore, this was the criteria used to initiate the characterization of the IFs obtained (Figure 3 and Table 1). The results showed that the morphometrical analysis of the nucleus and the kinetoplast allows the clear identification of the different IFs. The trypomastigotes showed a bigger distance between the nucleus and the kinetoplast than did the amastigotes. In addition, during amastigogenesis, together with the parasite shape change, we observed nucleus remodeling and a progressive and continuous displacement of the kinetoplast from the posterior to the anterior position with respect to the parasite nucleus. Similar observations were reported by Contreras and collaborators in 2002, during primary amastigogenesis. As in the quantitative analysis conducted during the morphometrical characterization of the IFs (Figure 2 and Table 1), the shape and position of the nucleus and the kinetoplast (Figure 3 and Table 1) allowed us to very precisely determine that one of these populations was highly predominant even when three different IF populations were present at each time of the transformation kinetics (from 85% to 90%), which confirmed a high efficiency of the homogeneity and synchronicity of the differentiation process in our *in vitro* conditions (Figure 4).

Differentiation of the diverse developmental stages of *T. cruzi* has been associated with changes in their biological properties and with the expression of stage-specific surface molecules [9]. Moreover, the morphological transformation during primary amastigogenesis has been associated with changes in the glycoprotein profile related to the expression of trans-sialidase and mucin previously described in *T. cruzi*. The same behavior was observed in this work during

the secondary amastigogenesis: contrary to the positive expression of Ssp4 observed in the amastigote surface, the trypomastigotes, epimastigotes and IFs at 1, 2 and 3 hours of transformation did not express this amastigote-specific glycoprotein (GP) (Figure 5). In the case of the IFs obtained at 4, 5 and 6 hours of transformation, low and heterogeneous levels of Ssp4 expression were observed. At these times, the total number of parasites expressing Ssp4 as well as the expression level of this GP on their surfaces, increased in parallel with the degree of transformation from trypomastigotes to amastigotes, which confirms that the progressive expression of Ssp4 can be useful as a molecular marker of differentiation [3, 8, 21].

To initiate and establish infection in the mammalian host, *T. cruzi* needs to develop several strategies to avoid the immune response. To survive and disseminate in the bloodstream and in the extracellular space, trypomastigotes and amastigotes have developed a stage-specific capacity to resist complement-mediated lysis [25–27], apparently using stage-specific regulators [28–30]. Conversely, epimastigotes found in the insect vector activate the complement cascade, but they are not able to inhibit its lytic action [27]. Complement-mediated lysis of the IFs obtained during in vitro secondary amastigogenesis was evaluated. As expected, trypomastigotes and amastigotes, but not epimastigotes, were resistant to the lytic effects of complement. Besides this, the IFs obtained during in vitro amastigogenesis were not sensitive to complement lysis (Figure 6). This result indicates that these IFs express the proteins that participate in the stage-specific inhibition of complement activation. Nevertheless, future studies will be necessary to elucidate which stage-specific proteins from trypomastigotes and/or amastigotes are expressed by the IFs in order to inhibit complement activation. It has been reported that the IFs generated during the transformation from epimastigote to trypomastigote are sensitive to complement lysis [18]. The IFs obtained during the metacyclogenesis and amastigogenesis are morphologically indistinguishable, which is remarkable, because this biological property might be used as a criterion to distinguish both types of IFs. Moreover, this result further supports the existence of the alternative parasite life subcycle that was previously proposed and the hypothesis that this could be the result of the premature lysis of the infected cells or the extracellular parasite differentiation [3].

In summary, we have shown that the experimental differentiation model reported in this work provides, for the first time, a highly synchronous system that could be useful for studying the molecular mechanism of the transformation process and the identification of the regulators involved in differentiation control, with the potential to identify routes to block the cycle of *T. cruzi* infection.

Acknowledgements

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Review Article

Immunobiology of African Trypanosomes: Need of Alternative Interventions

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Trypanosomiasis is one of the major parasitic diseases for which control is still far from reality. The vaccination approaches by using dominant surface proteins have not been successful, mainly due to antigenic variation of the parasite surface coat. On the other hand, the chemotherapeutic drugs in current use for the treatment of this disease are toxic and problems of resistance are increasing (see Kennedy (2004) and Legros et al. (2002)). Therefore, alternative approaches in both treatment and vaccination against trypanosomiasis are needed at this time. To be able to design and develop such alternatives, the biology of this parasite and the host response against the pathogen need to be studied. These two aspects of this disease with few examples of alternative approaches are discussed here.

1. Introduction

Trypanosomiasis is a fatal disease of both human and livestock. Besides death, it causes a heavy economic loss mainly in Africa. The etiological agent of the disease is a unicellular flagellated protozoan parasite of the genus *Trypanosoma*. Trypanosomes ($20\text{--}30\ \mu\text{m} \times 1.5\text{--}3.5\ \mu\text{m}$) are blood-borne unicellular protozoan parasites dwelling in various body and tissue fluids. These parasites are motile due to the undulatory motion of their flagellum. The parasite is known for more than a century, but still the control of the disease remains elusive. Trypanosomes are the causative agent of human African trypanosomiasis (HAT), also known as “sleeping sickness”. The term “sleeping sickness” describes the deregulation of the sleep-wake cycle and the intrasleep cycle which is observed in the late stage of the disease [1].

Sleeping sickness is caused by *Trypanosoma brucei rhodesiense* in Eastern and Southern Africa and by *Trypanosoma brucei gambiense* in Western and Central Africa. Both protozoan species are morphologically indistinguishable, but have drastically different epidemiological features. Both forms of the sleeping sickness affect the central nervous system. The typical East African form of trypanosomiasis is characterized by a rapid and acute development of the disease, and untreated patients can die within weeks or months of the

infection, whereas the West African form of this disease is more chronic that can last for several years. According to WHO reports, African sleeping sickness is the third most important contributor to the global burden of the parasitic diseases after malaria and schistosomiasis, if the Disability Adjusted Life Year (DALY) figures (i.e., loss of healthy life years by premature mortality and disability) are considered [2]. More than 60 million people are at risk of infection with human African trypanosomiasis, with about 45,000 new cases reported annually. It is estimated that at least 300,000–500,000 people are presently infected. However, less than 4 million people are under surveillance and as such, it is estimated that less than 10% of the new cases are diagnosed and treated [3].

Several species of hematophagous glossina, commonly known as tsetse flies (*Glossina spp.*) are the vectors of the African trypanosomes and are responsible for cyclical transmission of the parasitic protozoan between numerous vertebrate hosts. The vector is distributed over a wide range of habitats covering about 10 million square kilometer potential grazing lands in 37 countries which are rendered unsuitable for livestock breeding and farming in Africa [4].

Trypanosome infections in livestock are known as Nagana and Surra. Animal trypanosomiasis, caused by a wider number of trypanosome species and carried with

higher prevalence by a greater number of glossina species, is invariably the greater epidemic across the African continent with direct economic consequences. In general, trypanosome infections that threaten livestock have a 100 to 150-fold higher prevalence than the HAT [5]. Historically, the impact of animal trypanosomiasis has been so profound, that it has influenced the migration routes of cattle-owning tribes that were forced to avoid the *G. morsitans* “fly-belts” [6] (Figure 1), as well as the movements of early European and Arab settlers who depended on horses and oxen in Africa [7].

Although trypanosomiasis is often referred to as African trypanosomiasis, certain trypanosomes do cause infections outside this continent. *T. evansi*, the causative agent of “surra” occurs not only in Africa, but also in Central and South America, the Middle East, and Asia. It causes a disease in camels, horses, cattle, pigs, buffaloes, and dogs. In Southeast Asia, *T. evansi* infection is a disease of economic importance since it affects the health of buffalo, cattle, and swine [8]. The acute stage symptoms of this disease include abortion, central nervous system disorders, and even death, while in the chronic condition; working capacity and productivity of the animals are affected. Even though it is generally considered as a livestock disease, there are now recent reports of the human *T. evansi* trypanosomiasis in India [9, 10].

2. General Features of Trypanosomes

2.1. Classification of Trypanosome. The protozoal parasite trypanosomes are grouped in the order “kinetoplastida” because of the presence of a kinetoplast (discussed later). Based on the mode of transmission by their insect vector, the genus *Trypanosoma* is divided in two main groups: stercoraria and salivaria [11, 12] (Figure 2). The development of stercoraria parasites takes place in the intestinal track of the invertebrate vector and the infection to the vertebrate is via feces. *T. cruzi*, the causative agent of Chagas’ disease, is an example of the stercoraria group. On the other hand, salivarian parasites colonize the stomach of their invertebrate vector, but never pass to the intestinal track. Rather, they migrate towards the salivary gland of the vector where the infectious form for vertebrate host develops. Infection of the vertebrate occurs via saliva when the vector bites in order to take the blood meal. The African parasites, *T. brucei*, *T. congolense*, *T. evansi*, and *T. equiperdum* all belong to the salivarian group. *T. brucei* has three subgenera; while *T. b. brucei* is the causative agent of Nagana, a cattle disease in Africa, *T. b. rhodesiense* and *T. b. gambiense* are the causative agents of the sleeping sickness in human. The other two species *T. congolense* and *T. vivax* are the major causative agents of animal trypanosomiasis in Africa. *T. equiperdum* is the causative agent of an equine venereal disease that is called “dourine” where the parasites are transmitted during coitus. *T. evansi* causes a livestock disease called “surra”.

2.2. Life Cycle. Trypanosomes are the excellent examples of organisms that display an extreme adaptation to their environment, in many cases because they must evade

the immune response of the host. African trypanosomes are transmitted between mammalian hosts by tsetse flies. However, in each host, the parasites undergo many life cycle stages involving forms with discrete morphologies, patterns of gene expression, and proliferation status. In each case, these developmental changes are programmed precisely [13]. Infection in the mammalian host begins when the infective stage, known as the metacyclic stage, is injected intradermally by the tsetse fly (Figure 3). The organisms rapidly transform into the blood-stage trypomastigotes (long, slender forms), and divide by binary fission in the interstitial spaces at the site of the bite. The buildup of metabolic wastes and cell debris leads to the formation of a “chancre”. In the mammalian host, the metacyclic parasites rapidly undergo cell cycle reentry and morphological changes, and exchange the restricted repertoire for antigenic variation that is the characteristics of the metacyclic forms with a more elaborate system of the bloodstream forms [14]. Once established in the mammalian host, the bloodstream parasite is heterogeneous [15, 16] comprising the proliferative slender forms during the ascending phase of parasitemia and the nonproliferative stumpy forms at the peak of parasitemia [17]. The transition from the morphological extremes (i.e., the slender versus stumpy forms) involves a progression from proliferation to cell cycle arrest, accompanied by a series of biological and morphological transformations [18, 19]. Once stumpy forms develop during the course of parasitemia, the population is preadapted for transition to the procyclic forms, which occupy and proliferate in the midgut of the tsetse. The key features of the stumpy formation are the cell cycle arrest, the elaboration of some mitochondrial activities, and a relative resistance to lysis by antibodies [20, 21] or to the proteolytic environment that might be encountered in the midgut of tsetse [21, 22]. When the vector-fly (tsetse) bites an infected individual, it takes the parasites with the blood meal. The parasites undergo metabolic changes in the midgut of the fly. They lose their surface coat, which consists of about 10^7 molecules of the Variant Specific Surface Glycoprotein (VSG), and transform into the proliferative procyclic forms. In this form, they express their own surface proteins called the Procyclic Acidic Repetitive Proteins (PARPs, or procyclins). The defining events of the differentiation from the bloodstream forms to the procyclic forms are the loss of VSG and gain of the procyclins. VSG loss occurs very rapidly and involves the combined action of glycosyl-phosphatidyl-inositol-specific phospholipase C (GPI-PLC) and a proteolytic cleavage of the VSG via a zinc metalloprotease [23–26]. The transformation to procyclic form also changes the energy generation from being exclusively based on glycolysis in the bloodstream to a mitochondrion-based respiratory system, which requires a structural elaboration and the metabolic activation of organelle [27]. For successful transmission, the parasite undergoes two stages of differentiation in the fly: first, establishment in the midgut and then maturation in the mouthparts or the salivary gland. It is generally thought that during normal development in the fly, there are no intracellular stages and the parasites do not cross an epithelial barrier to enter the fly. After proliferation in the tsetse

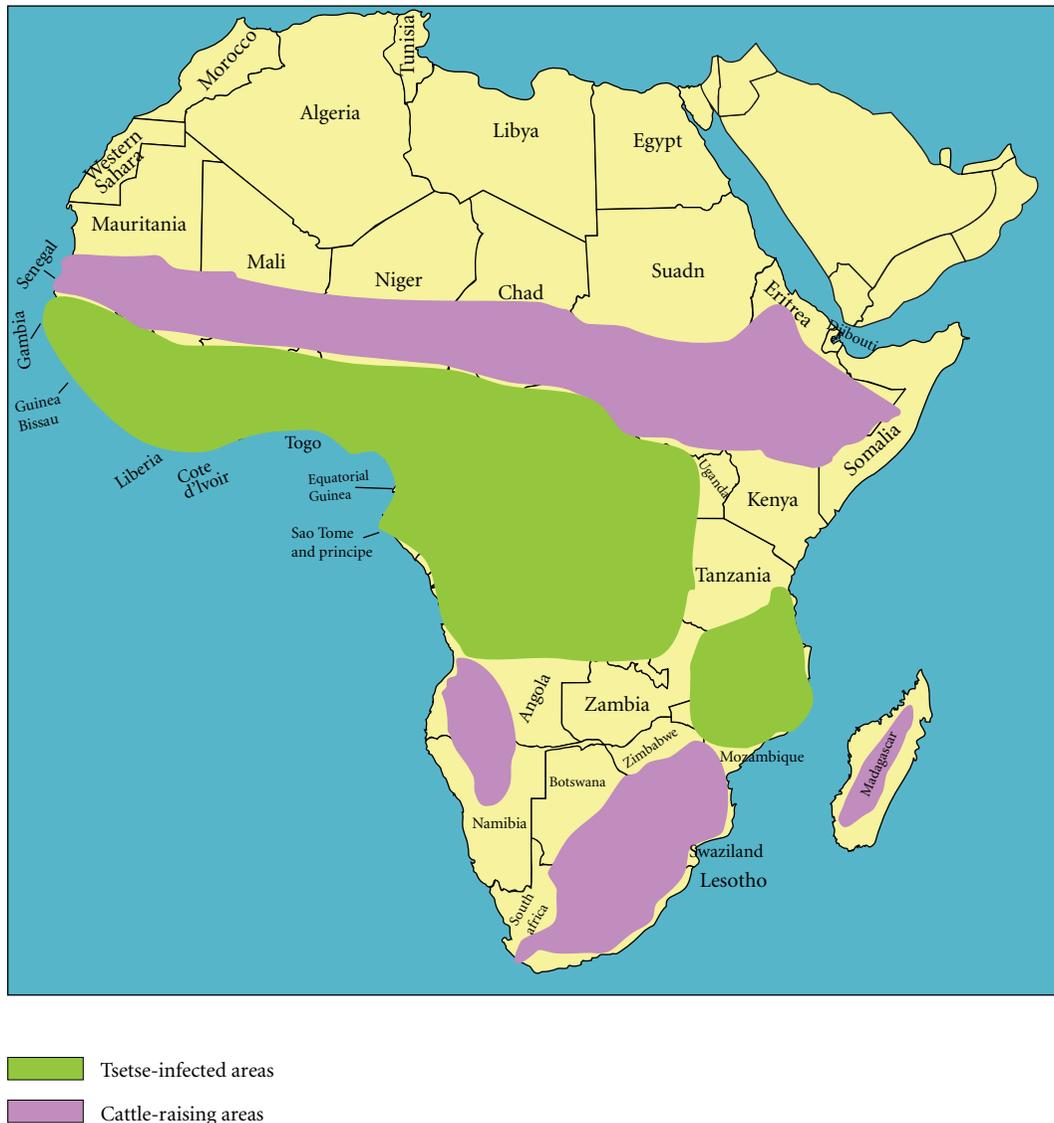


FIGURE 1: Distribution of tsetse and cattle raising area in Africa <http://pathmicro.med.sc.edu/lecture/trypanosomiasis.htm>.

midgut, the parasite migrates to the salivary gland. The epimastigote forms generated there attach to the gland through elaboration of the flagellar membrane. After further multiplication, the parasite undergoes division arrest, re-acquires a VSG coat, and is released into the salivary gland lumen, in preparation for an inoculation into a new mammalian host [27].

If the tsetse flies ingest more than one strain of trypanosome, there is the possibility of genetic exchange between the two strains, generating an increase in the genetic diversity in an organism that may not have a true sexual cycle. Indeed, it was shown by laboratory crosses that genetic exchange in the African trypanosome is possible [28–33]. Precisely at which stage of the life cycle this genetic exchange takes place is equivocal, suggesting at the midgut stage [34], in the salivary gland of the fly [35], and possibly at the proventriculus and foregut stage [36]. Though there are

conflicting results for the stage at which this exchange takes place, it is shown that this is not a compulsory process. The mechanism of genetic exchange in *T. brucei* is still unclear though it appears to be a true sexual process involving meiosis [35]. However, no haploid stage has been observed and the intermediates in the process are still a matter of conjecture. The frequency of sex in trypanosomes in nature is also a matter for speculation and controversy, with conflicting results arising from population genetics [37, 38].

In contrast to tsetse transmitted trypanosomes, *T. evansi* is transmitted mechanically by the blood sucking insects, in Asia especially by the horseflies (*Tabanus* spp.) and the stable flies (*Stomoxys* spp.), and in Africa the tsetse fly, like other biting flies, can act as mechanical vector. In South and Central America, in addition to blood sucking flies, *T. evansi* can also be transmitted by the vampire bats (*Dosmodus rotundus*). Besides mechanical transmission by insects and

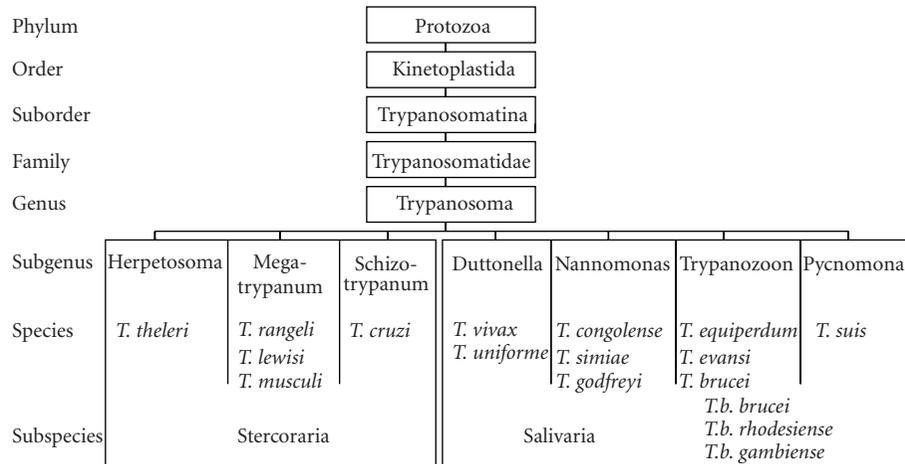


FIGURE 2: Classification of trypanosomes.

vampire bats, *T. evansi* can be transmitted through milk or during coitus [39]. Developmental stages were not observed in any of the vectors mentioned above. A procyclic or insect stage does not exist in *T. evansi* which is attributed to the lack of maxi circles in the kinetoplast DNA (discussed later) [40].

3. Special Features of Trypanosomes

Kinetoplasts are eukaryotes and hence exhibit conventional features such as the presence of a nucleus delimited by a nuclear membrane and organelles such as the endoplasmic reticulum, the Golgi apparatus, the endo/exocytosis system, the mitochondrion, and so forth [41]. However, many of these organelles exhibit specific and sometimes extreme features often found only in the kinetoplastids. As trypanosomes have a dual host life cycle, they have some specific adaptation characteristics at different levels, that is, DNA, RNA, and cellular organization. This includes the presence of a kinetoplast DNA, flagellum and flagellar pocket, unique gene regulation, RNA-editing, and the presence of glycosomes.

3.1. Kinetoplast DNA. Being a eukaryote, trypanosomes contain mitochondria, however, there is only a single mitochondrion per cell, which is extremely large and elongated, and its whole DNA content is condensed in a substructure called the kinetoplast [42]. The mitochondrial DNA makes up 10–20% of the total cellular DNA with an unusual network in the kinetoplast which is called kinetoplast DNA (kDNA) [43]. The kDNA is a planer network composed of several thousand topologically interlocked DNA circles. The network contains two types of circles of different sizes: minicircles of about 1 kb and maxicircles of 22 kb [44, 45]. Maxicircles number in dozen per cell with conserved sequences and code for ribosomal RNA and some mitochondrial proteins [46]. Many of the maxicircle transcripts are cryptic and require editing to form a functional mRNA. Editing specificity is controlled by the minicircle-encoded guide RNA (gRNA) that serve as a template [47]. The minicircles are in high

copy number (5000–10000 per cell) and their sequences vary between each other [48]. No transfer RNA (tRNA) genes are found neither in the maxi-circle nor the minicircles [49, 50]. So all the tRNAs necessary for protein translation of mitochondrial origin may be nuclear-encoded and imported from the cytosol into the mitochondrion [49, 51]. Unlike other trypanosomes, the kDNA of *T. evansi* does not contain maxicircle [40].

3.2. The Flagellum and Flagellar Pocket. Trypanosomatids possess a single flagellum that exits from a flagellar pocket, a specialized invagination of the plasma membrane where the entirety of endocytosis/exocytosis traffic takes place. In *Trypanosoma* spp., the flagellum is attached along the cell body for most of its length, with the exception of the distal tip [42]. The site of attachment defines a specialized region of the flagellum and of the cell body called the flagellum attachment zone (FAZ). The flagellum could accomplish several functions. Firstly, it is involved in the cell motility; trypanosomes are actively motile cells, swimming at average speeds of 10–30 μm per second in the culture medium [52]. The flagellar motility is required for the viability of the bloodstream trypanosomes [53]. A striking feature is the fact that trypanosomes swim with their flagellum leading, that is, the flagellum drags the cell behind it. This is related to the way wave forms are initiated: from tip to base, and not from base to tip as seen in the majority of flagellated organisms [42]. Secondly, the flagellum is involved in the attachment of the parasite to the host surfaces. Thirdly, the flagellum plays a role in the morphogenesis and the cell division [54, 55]. The flagellar pocket is a flask-shaped invagination of the plasma membrane where the flagellum emerges out from the plasma membrane [56]. This pocket constitutes a highly differentiated region that facilitates internalization of host macromolecules, while restricting host access to the exposed, endocytic receptors of the parasites [57]. The contribution of the flagellar pocket to protein trafficking, immune evasion, and other processes has been recently reviewed [58].

3.3. RNA Editing. Most trypanosomatid mitochondrial mRNAs undergo RNA editing by which the precursor mRNA (pre-mRNA) sequences are changed, often extensively, by the insertion and less frequently the deletion of uridine nucleotides (Us) [59]. The pre-mRNAs are encoded in the maxicircles, whereas the minicircles encode gRNA that specify the editing. The editing in *T. brucei* is catalyzed by compositionally and functionally distinct multiprotein complexes, called editosomes [60]. In this editing process up to half of the nucleotides can be added. This is a posttranscriptional process responsible for correcting the coding sequences of the mRNAs. Recently it had been shown that the structural conformation of gRNA and the thermodynamic stability of the gRNA-mRNA binary complex are very important for the editing to proceed [61]. It has been shown by comparing the rRNA synthesis in the bloodstream and the insect life-stages that the mitochondrial gene expression levels are controlled not at the transcriptional level, but rather by a mechanism which likely modulates the stability of the mature RNAs [62]. Thus it is speculated that editing may play a central role in controlling different mitochondrial functions during the development cycle of the trypanosomes.

3.4. The Nuclear Genome. The nuclear chromosomes of *T. brucei* can be grouped into three classes according to their sizes: 11 pairs of megabase chromosomes (1 to 6 Mb) that contain the house keeping genes and represent about 80% of the nuclear DNA content, a few intermediate-sized chromosomes (200 to 900 kb) and an undetermined number of minichromosomes (in the range of 100 that are 50 to 150 kb) which comprise about 10% of the nuclear DNA [63, 64]. The sequence of the 11 megabase-sized chromosomes of *Trypanosoma brucei* contains 9068 predicted genes, including approximately 900 pseudogenes and approximately 1700 *T. brucei*-specific genes. A large subtelomeric arrays contain an archive of 806 VSG genes used by the parasite to evade the mammalian immune system. Most VSG genes are pseudogenes, which may be used to generate expressed mosaic genes by ectopic recombination [65].

3.5. Glycosomes. The bloodstream form of trypanosomes has adapted its life in an abundance of glucose and relies entirely on glycolysis and substrate level phosphorylation for their energy production [66, 67]. The first seven to nine enzymes of the glycolytic pathway are present in the glycosomes, the peroxisome-related small globular organelles [68], found in all kinetoplastida. Expression of either phosphoglycerate kinase or triosephosphate isomerase in the cytosol inhibits parasite growth, suggesting that correct localization of the glycolytic enzymes is important [69, 70]. Various results, including the metabolic modeling, suggest that in bloodstream *T. brucei* the glycosome plays a vital role in the regulation of glycolysis [71–73]. The glycosomal membrane of *T. brucei* is impermeable to several metabolites [72], implying the presence of specific glycosomal metabolite receptors. Indeed, with the proteomic data it has been shown that there are certain receptors/carrier proteins in

the glycosomal membrane [74]. Because the glycosomes of the bloodstream *T. brucei* together make up-to about 4% of the total cellular volume, and the enzymes are present at relatively high concentrations within the organelle, it was postulated that their confinement to a small volume overcomes a diffusion limitation of the metabolites between the glycolytic enzymes [66]. This led to the more general notion that glycosomes would enable the trypanosomatids to maintain their high glycolytic flux. However, several arguments have been put forward that render such an explanation unlikely [71]. Even though the glycosomal protein content is dominated by the glycolytic enzymes, representing up to 90%, the glycosomes are not only involved in the glycolysis but are predicted to carry out also the gluconeogenesis, reactions of the hexose-monophosphate pathway, purine salvage and pyrimidine biosynthesis, β -oxidation of fatty acids, fatty acid elongation, and the biosynthesis of ether lipid [75].

4. Unique Features of Trypanosomes

4.1. Antigenic Variation and Immune Evasion. Trypanosomes growing in the bloodstream of mammalian host need mechanisms to circumvent the host immune response. Antigenic variation is one of the most spectacular adaptive mechanisms exhibited by the African trypanosomes. The bloodstream form of trypanosomes is entirely covered with a monolayer made of 10^7 copies of the VSG, which is a major antigen of the parasite whose antigenicity is in continuous changes. During the ascending phase of the parasitemia, the majority of parasites are of the same antigenic type (called homotype). The host immune system recognizes this homotype and makes antibodies against it. As the parasites of the major variable antigenic type (VAT) are eliminated the parasitemia goes in descending phase but at the same time, the parasites expressing the heterotype or the minor VATs are multiplying and one of them overgrows others. As a result this one becomes the new homotype, leading to a new wave of parasitemia and resulting in a long-lasting chronic infection. So expression of the VSG is central in the antigenic variation process and eventually for exhausting the host immune system in the benefit of the parasite. For the immune evasion, trypanosomes have also developed another method; macromolecular trafficking mechanism [76] whereby the VSG-complexed with antibody are sorted and endocytosed [77]. This mechanism most probably protects the parasites from the complement-mediated killing and as such the trypanosomes escape from the host immune system. For the purpose of escaping from the host immune system, trypanosomes have more mechanisms such as capping of the surface bound immune factors, restriction of the invariant receptors in the flagellar pocket, rendering them inaccessible to the host immune effectors [58].

4.1.1. VSG Expression. The trypanosome genome contains hundreds of VSG genes (VSG) of which very few (7%) are fully functional (encoding all recognizable features of known functional VSG), whereas the majority (66%) are

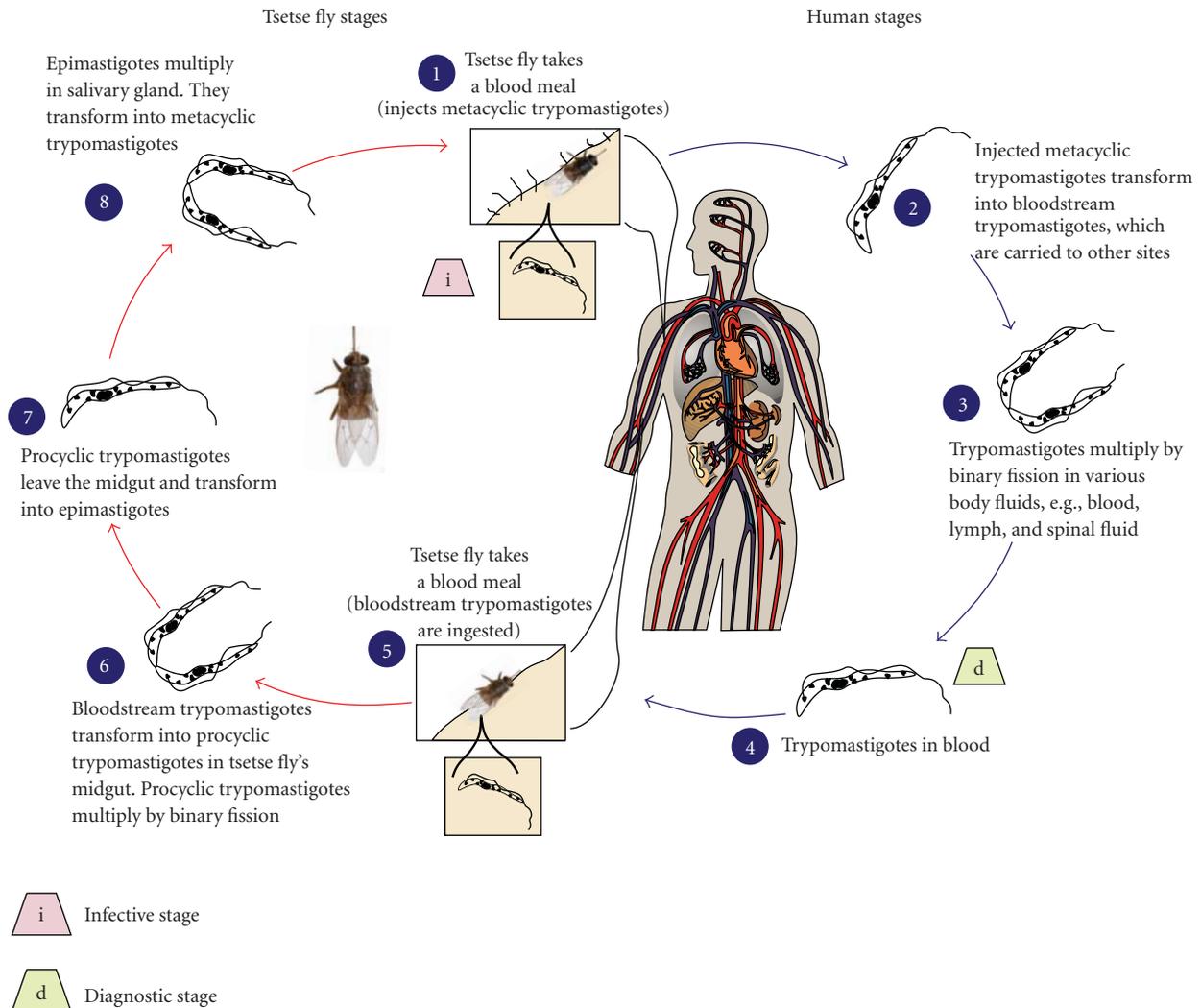


FIGURE 3: Life cycle of African trypanosomes, <http://www.dpd.cdc.gov/dpdx/HTML/TrypanosomiasisAfrican.htm>.

full-length pseudogenes (with frame shifts and/or in-frame stop codons) [65]. Most of these VSGs are clustered in the subtelomeric arrays. Transcription of the VSG occurs in one of the telomeres of the large chromosomes, which contain the VSG expression sites (VSG ESs) [78]. These expression sites are polycistronic transcription units having expression site associated genes (ESAGs) upstream of the VSG. These polycistronic mRNAs are matured by polyadenylation and addition of a spliced leader sequence by a process called transsplicing. Among the different expression sites only one is active at a given time. Thus only one of the VSG molecules is present within the trypanosome surface coat, resulting in the homogeneous display of an identical surface coat. Transcription starts simultaneously in all VSG ESs, but only in the "active" one there is complete transcription and all the others are aborted [79, 80]. In the rapidly dividing long slender form of trypanosomes, the active expression site was found to be present in a specialized region called expression site body (ESB) [81] and no similar structure was detected for the silent expression sites.

4.1.2. Mechanism of Antigenic Variation. There are several studies trying to unravel the different systems involved in the antigenic variation (reviewed by Pays et al. [80, 82, 83]. A first mechanism for the parasite to perform a VSG switch is to change the expression site. By switching off the active expression site [84] and activating a silent expression site, a VSG switch takes place. However, as the VSG genes are transcribed as polycistronic units, at the same time an ESAG switch will occur. This type of VSG switching is called *in situ* activation. This process could be one of the mechanisms which makes it possible for the trypanosomes to survive in various conditions and as such extending the host range [85]. A second system of the antigenic variation occurs possibly via the VSG gene rearrangements including a reciprocal recombination [86] and a gene conversion [87]. In this system, the active site is not changed (and the ESAGs remain the same) and only the VSG gene undergoes a modification. During the reciprocal recombination, the whole transcribed VSG gene in one telomere is replaced by another VSG gene present on a silent telomere [88]. This system occurs

by crossing over in the 70 bp repeat region flanking every VSG gene on the promoter site of the gene. For the gene conversion, an actively transcribed VSG gene or at least a part encoding for the surface epitopes is replaced by a copy of another VSG gene or a pseudogene, present on a silent telomere or another location in the genome. This event occurs more frequently than the reciprocal recombination [86].

4.2. Resistance to Normal Human Serum. Humans and some other primates [89, 90] are resistant to most of the trypanosomes because of the presence of a trypanolytic factor in their serum. The trypanolytic factor of the human serum is discussed below. Two subspecies of *T. brucei*, *T. b. gambiense* and *T. b. rhodesiense*, are resistant to the human serum trypanolytic factor and thus are able to infect human causing HAT or “sleeping sickness”.

The resistance to human serum, at least in case of *T. b. rhodesiense*, is linked to an antigenic variation. It had been shown that a gene called serum resistance-associated (SRA) gene is only expressed in the resistant clone of *T. b. rhodesiense* and not in the sensitive clone of the same parasite [91–93]. The SRA was necessary and enough for the resistance against normal human serum (NHS) [94]. It was found that the SRA is associated, as an ESAG, to a specific VSG ES, termed R-ES, which is selected in the human serum by antigenic variation [94]. The expression of SRA by *T. b. rhodesiense* is not a permanent phenomenon as it loses the resistance to NHS in the absence of the trypanolytic factor of the human serum, for example, after several passage in mice [95] or the *T. b. rhodesiense* which are found in nonprimate animals [96]. The SRA contains all the characteristics of a VSG except that the region coding for the surface-exposed epitope is missing because of an in-frame deletion [97–100]. So SRA is a kind of truncated VSG. As in the VSGs, the SRA contains the N-terminal α -helices [100], which in case of VSG are involved in a coil-coil interaction with the adjacent VSG to make the dimer [101], whereas this helix of the SRA is responsible for neutralizing the human serum trypanolytic factor by coil-coil interaction [102].

4.2.1. Trypanolytic Factor of the Normal Human Serum (NHS). It has been known already for a century, reported by Laveran and Mesnil between 1902 and 1912, (cited in [98, 103], that the sera from humans and other primates such as baboon and mangabey can kill trypanosomes, although there were differences in the killing activities within these sera. Recently it has been shown that gorilla serum also has trypanolytic activity but the serum from chimpanzees, which are evolutionarily most close to the human [104], does not have trypanolytic capacity [89]. Further characterization of trypanolytic factor (TLF) in the human serum showed that the factor is associated with high-density lipoprotein (HDL) [105, 106], and endocytosis of these HDL particles by the trypanosome is necessary for the lysis [107, 108]. The trypanosomal receptor for the HDL is yet to be defined, but it is most likely to be a lipoprotein scavenger receptor [109]. Two TLF complexes have been shown to

be present in the human serum; (i) TLF1, a 500 kDa high-density lipoprotein complex composed of apolipoprotein A-I (apoA-I), haptoglobin-related protein (Hpr), apolipoprotein L-I (APOL1), human cathelicidin antimicrobial peptide (hCAP18), GPI-specific phospholipase D (GPI-PLD), apolipoprotein A-II, and paraoxanase [106, 110], and (ii) TLF2, a 1000 kDa lipid poor immunocomplex composed of apoA-I, Hpr, APOL1, hCAP18, GPI-PLD, and IgM [111–113]. Among these components, Hpr has been thought for a long time to be the active trypanolytic component of TLF, because (i) it was considered to be the component recognized by parasite surface [114], (ii) anti-haptoglobin antibodies, which cross-react to the HPR, could inhibit the TLF mediated trypanolysis [110, 115], and (iii) it is not expressed in chimpanzees serum which lacks the trypanolytic capacity [90]. But now there are more and more confirmative evidences showing that APOL1 is the trypanolytic factor of normal human serum [89, 98, 102, 103, 116]. There are still ongoing discussions on which component of TLF is the most important for the trypanolytic activity. Some have suggested that there is a synergistic effect of Hpr and APOL1 for which HDL provides the platform [117]. Others have shown three human apolipoproteins (Hpr, APOA1, and APOL1) acting cooperatively for maximal killing capacity; however; the truncated APOL1 did not function in transgenic animal [118]. Furthermore, recently it has been shown that baboon contains orthologs of APOL1 and Hpr and when these two genes were expressed together with APOA-1; there was full protection against both animal and human infective *T. b. rhodesiense* infection [119]. In addition, TLF can also ameliorate infection by the intracellular parasite *Leishmania* [120, 121].

In humans, the *APOL* family has six members (*APOL1* to *APOL6*) which all are clustered in the chromosome 22 [122–124]. *APOL1* is only found in the sera from humans and gorilla which have the trypanolytic capacity but not in the chimpanzee serum which lacks the trypanolytic potential [89]. There are growing evidences that support *APOL1* to be the active trypanolytic component of TLF: (i) *APOL1* depleted NHS lost the trypanolytic capacity, (ii) this lost function could be rescued by adding a physiological concentration of both natural and importantly the recombinant *APOL1*, (iii) physiological concentrations of *APOL1* lysed trypanosomes in fetal calf serum (FCS), showing that the addition of only *APOL1* is enough to have trypanolytic activity in FCS, and (iv) *APOL1* was lytic only to the NHS sensitive strain and not to the NHS resistant strain, showing that killing by purified *APOL1* is not due to the toxicity acquired in the purification process [98, 102, 103]. *APOL1* contains a signal sequence (amino acid 1–27) and the secreted protein could be divided in three domains in relation with trypanolysis: a pore-forming domain, a membrane-addressing domain, and an SRA-interacting domain [116]. The pore-forming domain, which spans from Met60 to Trp235, has structural and functional similarities with the pore-forming domain of bacterial colicins. Next to the pore-forming domain there is the membrane-addressing domain, Ala238 to Pro304, which is predicted to bind to HDL particles only in neutral pH such as blood. At the C-terminal

of APOL1, there is an α -helix which interacts with the SRA of *T. b. rhodesiense* and this interaction leads to a loss of trypanolytic capability of the APOL1. The pore-forming and the membrane addressing domains are necessary for the trypanolytic capacity whereas the SRA-interacting domain is dispensable. In fact, removal of this C-terminal domain, by which the SRA can no longer interact, makes the APOL1 even lytic to NHS-resistant *T. b. rhodesiense* [98]. Therefore, this truncated molecule can be envisaged as a possible therapeutic molecule of human origin.

4.2.2. Mechanism of Trypanolysis by APOL1. The mechanism of trypanolysis by APOL1 has been proposed by Pays et al. [93] and Baral et al. [103]. The APOL1 which is associated with HDL particles are internalized by the bloodstream form of trypanosomes via HDL-receptor mediated endocytosis in the flagellar pocket. The endocytic pathway leads the APOL1-loaded HDL to the endosomes and then to the lysosomes. The acidic pH of the lysosome induces a conformational change in the pH sensitive membrane-addressing domain of the APOL1. This conformational change would cause a dissociation of the APOL1 from the HDL and leads to association with the lysosomal membrane. At this stage the pore-forming domain would be able to form ionic-pore in the lysosomal membrane causing an influx of chloride ions (Cl^-) from the cytoplasm to the lysosomal lumen. There would be a compensatory influx of Cl^- to the cytoplasm via ion channels in the plasma membrane. In the plasma membrane the anionic influx can be accompanied by some other cationic influx as well [112] as Hpr is suggested to permeabilize both anionic and zwitterionic membranes [125]. The ionic influx leads to the movement of water to the lysosome causing an osmotic swelling of this organelle. This uncontrolled swelling of the lysosome can cause an increased intracellular pressure which might cause the plasma membrane damage and ultimately kill the parasite. The SRA protein expressed by *T. b. rhodesiense* interacts with the C-terminal domain of APOL1 in the endosomes and/or lysosomes which prevents the APOL1 being able to form the pore, and as such makes the parasite resistant to the APOL1/NHS.

5. The Surface Proteins of the Trypanosomes

Trypanosomes are covered by their stage-specific surface proteins. In the bloodstream form they are covered by the VSG and in the procyclic insect form they are covered by another protein called procyclins. The procyclins are GPI-anchored glycoproteins with either five or six pentapeptide repeats (GPEET procyclin) or up to 30 glutamic acid-proline dipeptide repeats (EP procyclin) [126, 127].

5.1. Variant-Specific Surface Glycoprotein (VSG). The VSG is the most abundant surface protein in the bloodstream form of the trypanosomes. It forms a dense surface coat of 12–15 nm over the entire surface of the trypanosome [128] and accounts for about 10% of the total protein content of the bloodstream form of the parasite and more than 95% of

the externally disposed cell surface protein [128]. Nearly the entire cell surface of the parasite and the flagellum is covered by the VSG. The bloodstream form of the trypanosome is coated with a continuous layer of approximately five million densely packed identical homodimers of VSGs [128, 129] that provide the parasite a defense barrier against both innate and specific immune effectors of the host [130].

5.1.1. Structure of VSG. VSGs are antigenically distinct due to extensive differences in primary sequence. Despite the differences in the primary structure, it is believed that the VSGs have a conserved tertiary structure which could explain how arrangement of the VSGs with different primary sequences can perform the same apparent function of producing a monolayer barrier that prevents binding of the host complement components or other lytic components that are present in the serum of the host [131]. The main feature of the VSG tertiary structure is the formation of two long α helices per monomer that are perpendicular to the cell surface and define the elongated shape of the VSG [132]. Due to the elongated shape and densely packed composition, only a very limited number of amino acids are accessible to the extra cellular environment which might be hostile. The mature VSG polypeptide has 400–500 amino acid residues (most having between 420 and 460) consisting of two or three domains, namely, N- and C-terminal domains [133]. The majority of the sequence forms a single N-terminal domain of 350–400 residues and the remainder one or two smaller C-terminal domains of 40–80 residues each [133–135]. Other than cysteine residues, there is little conservation of primary sequence within the N-terminal domain whereas the C-terminal domain has a greater degree of primary sequence identity [135]. The C-terminal domain is attached to the membrane of the parasite by a glycosyl-phosphatidylinositol (GPI) anchor [136]. Both attachment of the GPI anchor and the N-glycosylation of the VSG occur in the endoplasmic reticulum during the transportation of the protein towards the surface. The attachment of the GPI is essential for further transport of the VSG [137]. An N-terminal part of the VSG, containing about two thirds of the mature polypeptide can be cleaved off by exogenous protease cleavage.

The three dimensional structure of this N-terminal domain of *T. brucei* VSG has been resolved at high resolution [138]. The dimer was found to be $\pm 100 \text{ \AA}$ long and having an asymmetrical cross section being $60 \text{ \AA} \times 40 \text{ \AA}$ at the bottom, $30 \text{ \AA} \times 20 \text{ \AA}$ in the middle and $45 \text{ \AA} \times 45 \text{ \AA}$ near the top in which the top of the molecule is normally exposed to the external environment. This top part represents the hypervariable part of the VSG molecule and is stabilized by two-conserved disulfide bridges [139]. The N-terminal domain of VSG ends with an α -helix which is followed by a single C-terminal domain which is attached to the parasite membrane with the GPI anchor.

5.1.2. The GPI Anchor. Each VSG and related molecules like the ESAG6 is attached to the bloodstream form *T. brucei* cell surface by a GPI anchor [136, 140, 141]. GPI anchors

influence the trafficking of the VSG in the early and late secretory pathway [142], and play an important role in the expression of the VSG on the surface of the parasite [143].

5.1.3. Structure of the GPI Anchor. The first GPI-anchor structure determination, as well as the first description of the mechanism of GPI biosynthesis were both established using trypanosome VSG [136, 144–146]. This GPI-anchor is preassembled as a GPI precursor with the following structure: $\text{NH}_2\text{CH}_2\text{CH}_2\text{-PO}_4\text{H-6Man}\alpha\text{1-2Man}\alpha\text{1-6Man}\alpha\text{1-4GlcN}\alpha\text{1-6myoinositol-1-PO}_4\text{H-3(sn-1,2-di-Myristoylglycerol}$, [147]. This core is attached to the mature C-terminal amino acid in exchange for a hydrophobic C-terminal GPI-addition signal peptide [140]. The VSG-linked GPI anchors are subsequently substituted with unique galactose side-chain residues, not present in other mammalian GPI anchored proteins [136]. Another unique feature of the trypanosome GPI anchor is that it contains exclusively myristate (a 14-carbon, saturated fatty acid) as lipid moiety [148]. As analogues of myristate are selectively toxic for the parasites, the GPI biosynthesis pathway can be a potential candidate for treatment of trypanosomiasis [149, 150].

Trypanosomes contain an endogenous phospholipase-C (PLC) known as the GPI-PLC that is capable of hydrolyzing the GPI-anchor of the membrane-bound form VSG (mfVSG) [151]. This PLC is located primarily in the membrane of the flagellar pocket and is possibly part of the VSG-membrane recycling system [152]. It is highly expressed in the bloodstream form parasites (30 000 copies per cell) and severely down-regulated in the procyclic form [153, 154]. The GPI-PLC expressed by one parasite only targets its own plasma membrane and as such is unable to release the VSG of another parasite [155]. As a consequence the death of one parasite would not harm others. The GPI-PLC under stress condition cleaves off the VSG leaving the dimyristoyl glycerol (DMG) in the membrane and the glycosyl inositol phosphate (GIP) fraction on the released soluble VSG (sVSG) [156–158]. This conversion can be detected immunologically as it results in the exposure of the cryptic cross-reacting determinant (CRD) [158–160]. A low rate of the sVSG release from trypanosomes has been observed in the cultures of the bloodstream forms and clearly demonstrated not to result from the lysis of a subset of the population [161]. In addition, release of the sVSG can be induced under stress conditions that do not lyse the parasites [162]. Both of these observations suggest that GPI-PLC acts on the mfVSG in living trypanosomes and not just on cell lysis. The VSG and the GPI-PLC show the same developmentally regulated expression, being found in the blood-stream form and not in the procyclic form [154]. The ability of the GPI-PLC to catalyze the shedding of the VSG coat *in vitro*, and the contemporaneous expression of the two proteins, has led to the models that suggest an important role for the enzyme in the developmental changes that involve alterations in the expression of cell surface proteins [163]. However, by analyzing a GPI-PLC null mutant trypanosome it was shown that the GPI-PLC is not essential and is not necessary for antigenic variation, though it influences parasitemia in mice [164]. Moreover, beside the GPI-PLC

activity, there is a zinc metalloproteases (MSP-B) activity which also causes shedding of pre-existed VSG and which even could be playing a quantitatively major role than the GPI-PLC [25, 26].

5.2. Other Surface Proteins. Even though trypanosomes are covered mainly by their stage specific major surface proteins, some other surface proteins are also present which might be located either beneath the surface coat of the VSG/procyclicin or within the flagellar pocket. Some of these are invariant surface proteins, others are receptors and transporters [165].

5.2.1. Invariant Surface Glycoproteins (ISGs). Due to the low abundance of ISGs, it is not very easy to identify minor surface proteins in the trypanosome. However, by use of different techniques some invariant surface glycoproteins (ISGs) have been identified, such as ISG 60, ISG 65 and ISG 75 [130, 166], ISG 64, and ISG 70 [167]. The ISGs 65 and 75 both are predicted to be composed of large extracellular domains, a single transmembrane domain, a small C-terminal intracellular domain and are not accessible for antibodies on live parasites. Using fixed parasites, it was shown that both of them were distributed over the entire cell surface. The ISG65 and not the ISG75 has been shown to elicit an antibody response in the chronically infected mice [130]. Another invariant protein with a single copy gene, ISG 100, has been described. It is present in the flagellar pocket and is associated with the endo/exocytosis compartments. This suggests that it might play a role in the pathway for the endocytosis or it may have a structural role in the compartments involved in intracellular trafficking of *T. brucei* [168].

5.2.2. Surface Receptors. For the uptake of different molecules from the host, trypanosomes use different receptors. Not many receptors have been characterized in the trypanosomes, so far. One of the best characterized receptors in the trypanosomes is the transferrin receptor. African trypanosomes grow in the bloodstream of different mammals where they take up their nutrients required for growth. Iron is one of the crucial molecules for the trypanosome survival [169]. Iron requirement of the trypanosomes is fulfilled by a receptor-mediated uptake of the host transferrin (Tf) [170–172]. Unlike the mammalian transferrin receptor (Tf-R) which is a homo-dimer transmembrane protein distributed over the surface of the cell and binding two Tf molecules [173], the trypanosomal Tf-R, located in the flagellar pocket membrane, is a hetero-dimer constructed from two very similar VSG-like N-terminal domains [174] and binds only one molecule of Tf [170]. The trypanosomal Tf-R differs in primary structure, subunit organization and mode of anchorage from its human counterpart [175]. Another important receptor might be the lipoprotein scavenger receptor which is involved in uptake of the LDL, HDL as well as the human trypanolytic factor (TLF) [109]. In addition to the scavenger receptor another LDL receptor has been described to be present in the flagellar pocket [176, 177]. There are reports

that small molecules are taken up by the trypanosomes via transporters like the glucose transporters [178] to transport glucose, the nucleoside transporters with diverse substrate specificities and distinct patterns of expression during the trypanosome life cycle [179].

6. Immune Responses during Infection with African Trypanosomes

The immune response of vertebrates consists of two arms: the innate immune response which has a low specificity and the adaptive immune response which is antigen specific. The immunology of infections by the African trypanosomes is a complex process and has been recently reviewed [180, 181]. Being an extracellular parasite, the African trypanosome encounters both the innate as well as the adaptive immune response from the host.

6.1. Innate Immune Response against African Trypanosomes. Once in the bloodstream of the mammalian host, the trypanosomes encounter the innate host immune system as the first barrier. As already mentioned, human and some other primates have trypanolytic factors in their serum that aid the primary defense mechanism.

In a cellular innate immune response, different host cells are activated by different trypanosomal factors, initiating an acute inflammatory response [182, 183] (Figure 4). Among many molecules, the trypanosomal DNA that might be released from the dead trypanosomes has been shown to activate macrophages in a process called classical activation, to secrete proinflammatory molecules like TNF, IL-12 and NO [184, 185]. In this regard, the involvement of toll like receptors (TLR) and in particular the TLR9, in parasitemia control [186], would suggest that the DNA from trypanosomes plays a role in disease progression. The GPI anchor of the VSG also interacts with the macrophages (via a putative receptor which is still elusive) and induces secretion of pro-inflammatory cytokines [187–189]. So, the first response of the host immune system consists of classically activated macrophages (caM ϕ) secreting pro-inflammatory molecules such as TNF, IL-1, IL-6, NO (219–221). The caM ϕ s can phagocytose antibody-opsonised parasites [190] as well as secrete trypanotoxic molecules such as TNF and NO [191–194] that are involved in the control of the first peak of parasitemia.

6.2. Adaptive Immune Response. The initial inflammatory response is beneficial to the host at the early stage of the infection, but a sustained inflammation can cause pathology. Hence, it is essential for the host to reduce the inflammation which is obtained by down regulating the caM ϕ and their pro-inflammatory cytokines. Production of type II cytokines such as IL-4, IL-10 and IL-13 which can modulate the macrophages to become more anti-inflammatory type alternatively activated macrophages (aaM ϕ) are involved in a longer survival of the host (Figure 4). So a type I inflammatory response at the beginning of the infection and a shift to the type II immune response in the late

stage of the infection are correlated with the capacity of the host to control the parasite and the pathology respectively. In a murine model, it has been shown that the VSG-specific cytokine responses associated with the resistance to the murine African trypanosomiasis are infection-stage dependent, with the type-I cytokine responses being critical during the early stage of infection while the type-II cytokine responses to be more important during the late and chronic phases of the disease [195]. Several studies suggest that the cytokine responses influence the outcome of African trypanosomiasis [196–199]. However, the precise role of the individual cytokines is still equivocal and may be dependent on the parasite strain, the mouse model or both. In this context the role of IFN- γ [196] for resistance against *T. b. rhodesiense* and the role of IFN- γ and NO together with the antibody response have been shown to be crucial in the control of *T. congolense* infection [200]. However, in the *T. evansi* model even though TNF, IFN and NO levels are elevated in the early stage of infection, none of these molecules seem to be important for the parasitemia control as well as the survival of the host [201]. In another trypanosome model, *T. borelli*, a blood parasite of carp, NO hinders antibody clearance from the surface of the parasite and increases susceptibility to the complement lysis [202]. Moreover, Magez et al. [203, 204] demonstrated that TNF plays a key role in both parasitemia controls as well in the development of pathology in *T. brucei* infections. Concerning the role of type II cytokines, some have shown that CD4+ T cell regulated IL-4 production was crucial for controlling *T. b. gambiense* infections in mice [198] and a role for IL-4 in resistance to bovine trypanosomiasis was also proposed [199] while others [196] reported that IL-4 knockout mice do not show any alteration in the parasite control. Namangala et al. [205] showed that during the chronic stage of infection a Th2 cytokines production as well as a IgG1 antibody response to the trypanosome antigens are linked to the longer survival of the host in *T. brucei* infection model. Moreover, the levels of IL-10 and IL-6 in the brain have been shown to be associated with the protection from neuroinflammatory pathology of HAT [206, 207].

6.2.1. Humoral Responses. During the trypanosome infection a dominant humoral response of the host is expected, since the location of the parasite is extra-cellular. Both the murine and bovine trypanosomiasis is characterized by a polyclonal B cell activation as evidenced by an increased number of B cells and a significant elevation in plasma Igs [209–211]. Because of the polyclonal B cell activation, a significant component of the resultant antibody is either polyspecific or auto reactive [212–214]. Although the VSG molecules are highly immunogenic for all mouse strains upon immunization, dramatic differences in the ability of animals to mount the VSG-specific B cell response occur after infection [215]. It is shown in different independent studies that specific antibodies directed against the trypanosome VSG mediate the destruction and clearance of parasites in successive parasitemic waves and hence contribute to antibody-mediated trypanotolerance

[197, 213, 216, 217]. Animals immunized with the irradiated trypanosomes or the VSG are successfully protected against a challenge with the homologous parasites [218, 219]. The antibodies directed against the specific surface-exposed epitopes of the VSG coat opsonize the parasites and the immune complexes are efficiently phagocytosed and destroyed, mainly in the liver, by the macrophages (Kupffer cells) [190, 220, 221].

During African trypanosomiasis, the VSG-specific B cell responses can occur in a T-cell independent manner [222]. However, the T-cells improve the B-cell responses, mainly by secreting cytokines mediating antibody class switching. In this context an increased IL-4 mRNA level and a concomitant increase in the IgG1 antibodies against the VSG was observed in the trypanotolerant N'dama cattle infected with *T. congolense* but not in the trypanosusceptible Boran cattle [199]. In animal trypanosomiasis, trypanotolerance is a combination of the humoral response needed for parasite control as well as the ability to control the immunopathology (described below) which is the cause for loss of productivity. Schofield et al. [223] described a rapid major histocompatibility complex (MHC)-unrestricted antibody response to the diverse pathogens including the trypanosomes. These authors demonstrated the CD1d-restricted IgG formation in response to *Plasmodium* and *Trypanosoma* GPI anchored antigens mediated by IL-4 producing CD4+, NK1.1+ helper T-cells (NKT cells) and proposed that this may represent a general mechanism for a rapid response to the GPI-anchored surface antigens and the parasite control.

Although the trypanosome-specific antibodies are produced in the early stage of infection and may be protective as they mediate parasite clearance [224–228], remove immune complexes [229], and possibly neutralize the parasite products, yet a significant proportion of the antibodies is either polyspecific or auto-reactive [209, 213, 214, 230, 231]. Moreover, later in the infection, the B-cells become suppressed or exhausted, resulting in a total absence of IgG responses and a strongly reduced IgM response [232]. Using B-cell (μ MT) and IgM-deficient mice, it has been shown that in the murine experimental *T. brucei* trypanosomiasis, B-cells were crucial for periodic peak parasitemia clearance, whereas the IgM antibody played a limited role [233]. However, in the *T. evansi* infection model, the IgM has been shown to play an important role in the control of the disease [201] suggesting the role of different antibodies can vary with different trypanosome strains.

6.3. Immunosuppression. One of the striking features of the trypanosome infections is the dramatic suppression of the immune responses, which might result in a high susceptibility to opportunistic infections. The generalized immune suppression has been reported to affect a large variety of both the humoral (B cell) and the cellular (T-cell and macrophage) immune functions [234], consequently leading to occurrence of the trypanosome-induced immunopathology [235–237]. Although the existence of the immunosuppression has been known for long time, the unresolved question was whether the immunosuppression was mediated by the macrophages

or the T cells. There are suggestions that both cells might be involved [238]

Suppressive macrophages elicited by the *T. brucei* infection play a central role in the immunosuppression observed in this infection [239–241]. The immunosuppression is characterized by an inhibition of the T cell proliferation due to down regulation of both IL-2 production and expression of IL-2 receptor [235, 240]. Prostaglandins and nitric oxide (NO) impair mitogen-induced T-cell proliferation in the spleen, peritoneal cavity, and lymph nodes of *T. brucei* infected mice but only during the early stage of infection [241–243]. At the early stage of the infection, the involvement of TNF and IFN- γ in the inhibition of T-cell proliferation seems to be involved in an up-regulation of prostaglandins and NO synthesis [204, 244]. In addition, TNF promotes the development of suppressive cells by inducing IFN- γ production in the lymph nodes of *T. brucei* infected mice [244]. Moreover, *T. brucei* infection also impairs the MHC class II antigen presenting capacity of the classically activated macrophages [245] resulting in a reduced T-cell activation. But at the late stage of infection, inhibition of T-cell proliferation in the lymph nodes occurs through NO/prostaglandin independent pathway, whereby IFN- γ released by CD8+ T-cell plays a crucial role [242, 246]. There are reports showing at the late stage of infection, macrophages displaying an anti-inflammatory cytokine production, which might modulate in several aspect of the immune system as the infection progresses [247, 248]. Factors like IL-10 secreted by the macrophages of the infected animals are shown to inhibit antigen presentation [249] and contributing to the impairment of T-cell activation. However, the mechanisms of suppression by the alternatively activated macrophages elicited at later stage of the African trypanosome infections are not fully understood. Regulatory T cells (Tregs) have also been shown to limit the production of IFN- γ by CD4+ and CD8+ T cells and also down regulate the activation of macrophages [250, 251]. Furthermore, these Tregs are suggested to suppress the NKT cell [238]. Presentation of glycolipids to the NKT cells in the context of CD1d have been suggested in the trypanosome infection [238] or a GPI treatment [252].

6.4. Immunopathology. As mentioned above, uncontrolled type I immune reaction of the host leads to a pathological condition. The major pathological complication associated with the human trypanosomiasis is the neurological disorder which is finally manifested as 'sleeping sickness'. However the pathological symptoms observed in experimental trypanosomiasis are mainly loss of body weight, fever, reduced locomotory activity, splenomegaly, and liver damages. One of the common pathological features observed in human, bovine as well as the experimental murine trypanosomiasis is the loss of red blood cell count, that is, anemia. Here, the degree of anemia might be considered as an indicator of the disease severity [253]. At least in case of the bovine trypanosomiasis, one aspect of the trypanotolerance is the measurement of the ability to control the infection-associated anemia and subsequently the loss of productivity

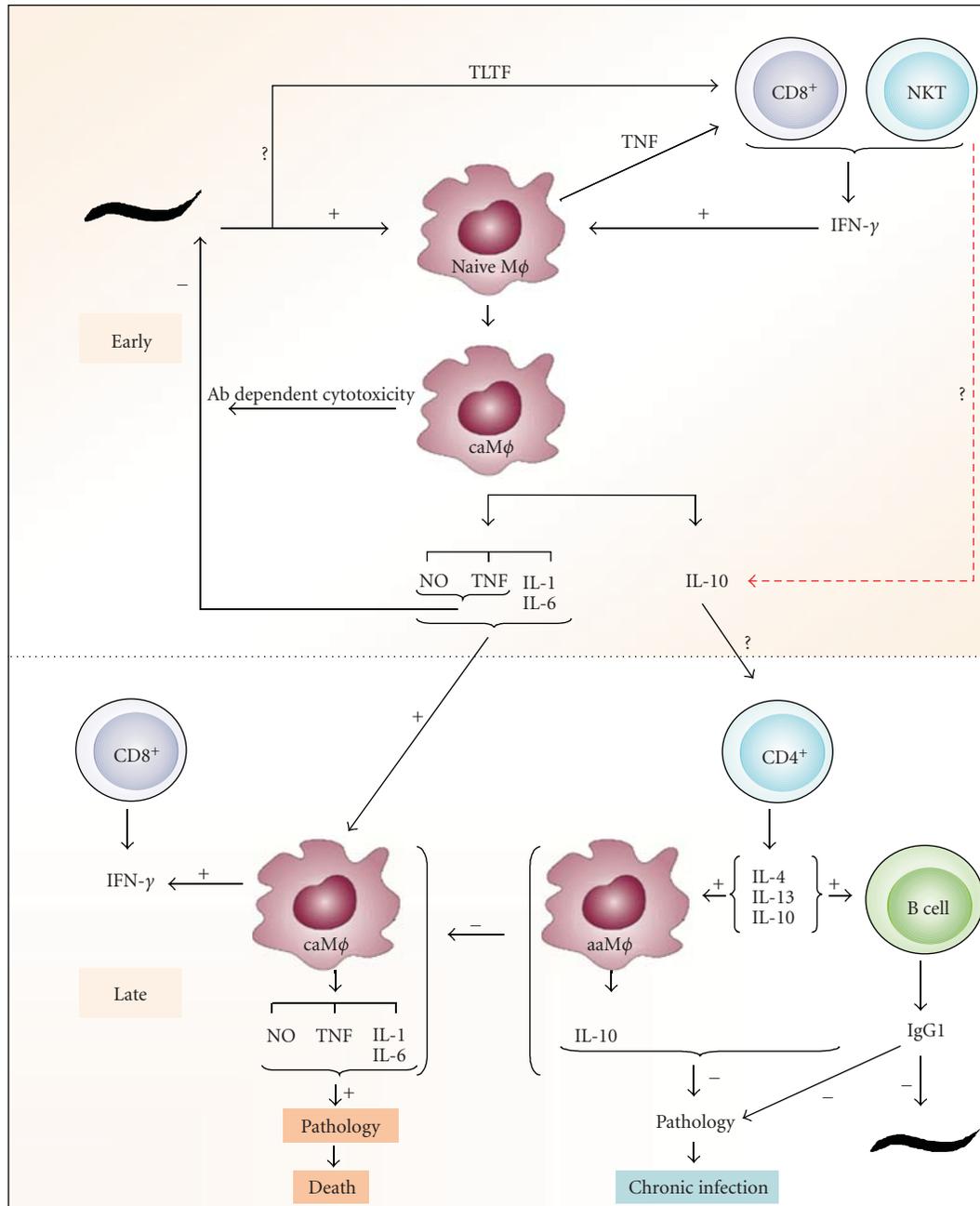


FIGURE 4: Trypanosome-host interaction (from [208]). Model for induction classical (caM ϕ) and alternative activation (caM ϕ) of macrophages during trypanosome infection; TLTf: trypanosome-derived lymphocyte-triggering factor.

of the host [254]. Anemia during trypanosomiasis might be due to either loss of RBC, for example, the cytokine-activated macrophages (M1 cells) are suggested to be responsible for the enhanced phagocytosis of parasites as well as the RBCs [255] or due to inability to mount a vigorous compensatory erythropoietic response [255, 256].

During trypanosome infections, TNF is involved both in parasitemia control and infection associated pathology [204] such as anemia, neurological disorders, fever and cachexia during both human and animal trypanosomiasis [234, 257]. In view of this dual role, lots of works reveal

how trypanosomal components induce TNF. In this regard, VSG was identified as major TNF inducing component in trypanosome-soluble extract. Both sVSG and mfVSG were shown to manifest similar TNF inducing capacities but by a detailed analysis it was indicated that they are working in a different way. The GIP moiety of the VSG via the GIP associated galactose side chain is responsible for direct induction of TNF. Yet, the mfVSG, but not the sVSG, stimulates macrophages toward IL-1 secretion and acquisition of the LPS-responsiveness and is, as such, involved in indirect TNF production. Thus, the VSG has two distinct

macrophage activating components [258]. TNF can signal for cellular activities through 2 different receptors; TNF-R1 (CD120a) and TNF-R2 (CD120b). It is suggested, that TNF-R2 signaling in trypanosomiasis mediates infection-associated pathology, whereas TNF-R1 signaling has no impact on infection [259].

It was also shown that serum TNF levels correlate with the severity of neuropathological symptoms in the human sleeping sickness [260]; however, some studies found no correlation between the TNF serum level and pathology of the HAT [261, 262]. In the same line of research, there are reports that demonstrate the enhanced expression of TNF mRNA in the brain of *T. brucei*-infected mice [263, 264] and the correlation in trypanosome-infected cattle between TNF production by monocytes and the severity of diseases-associated anemia [265]. Hence, the accumulated knowledge about trypanosome-elicited production of TNF indicates that while this cytokine might be beneficial during the early stage of infection through its role in parasite clearance, the overall pathology-inducing aspect overrules in a negative way. As outlined before, due to highly sophisticated antigenic variation, an antiparasitic vaccination seems to be very difficult. However, the knowledge of the immunopathology gives a basis for a design of an antidisease vaccination.

7. Current and Alternative Control Strategies for Trypanosomiasis

The control of trypanosomiasis can be approached from three different aspects: control the vector, vaccinate the host as a preventive measure, or treat the infected host. All three approaches have their own benefits but at the same time suffer from some difficulties.

There are different vector-control methods currently available; use of insecticide (through the sequential aerosol spraying technique, insecticide-treated targets or insecticide treated animals), use of traps, and the sterile insect technique (reviewed [266]). Technical improvements to make these methods cheaper, more efficient and less time consuming are still needed [267]. In an attempt to develop a cost effective control method for *Glossina fuscipes*, a prospect for developing the odour baits based on the kairomones present in lizard odour and pig odour have been suggested [268]. In all these and other studies it has been agreed that more precise and possibly new method of vector control are still in demand. One of the problems associated with the use of chemical insecticides as a vector control is the evolution of resistance against that chemical. As an alternative approach, one may think about an “evolution-proof” insecticide as has been discussed for the malaria control [269].

The current treatment of HAT is based on four main drugs, namely suramin, pentamidine, melarsoprol, and eflornithine (difluoromethylornithine or DFMO), with nifurtimox undergoing evaluation [270, 271]. Most of these drugs were developed in the first half of the twentieth century and there has been no new registered drug since 1981. The candidate drug called DB 289, a dimidine derivative has finished clinical trial II [270–272]. Early-stage disease

is treated with an intravenous injection of suramin in *rhodesiense* disease and with an intramuscular pentamidine in *gambiense* disease. The arsenical melarsoprol is the only effective drug for the late-stage disease in both forms of the HAT, as the drug crosses the blood brain barrier [272]. Nifurtimox taken orally for 1 to 2 months and DFMO with an administration scheme spread over five weeks including 14 days of intravenous injection can be other alternatives. However, the DFMO is not commonly available and is considered too expensive for routine use. Moreover, the DMFO is known to be active only against *T. b. gambiense* [273].

The major problems of therapy are the frequent and often serious adverse events due to drug toxicity, relapses, and the long duration of treatment. The worst adverse events are encephalopathic syndromes, which occur in 5–10% of patients, and result in the death in 10–50% of those in whom the encephalopathy develops. Other severe adverse reactions reported are polyneuropathies (up to 10%), exfoliative dermatitis, fever, headache, diarrhea, maculopapular eruptions, pruritus, and abdominal and chest pain [274, 275]. There are reports for a shorter treatment regime of melarsoprol [276, 277] or a combination of melarsoprol with suramin [278]. Drug resistance in trypanosomes appears to be increasing in the field and is now hindering efforts to control the HAT. Failures of the melarsoprol treatment, the preferred treatment during the encephalitic stage of *T. b. rhodesiense* infections, have reached alarming levels. The unacceptable toxicity of the currently available drugs for HAT underpins the urgency of developing more effective and safer drugs [270].

Control of African bovine trypanosomiasis mainly relies in endemic areas on chemotherapy and chemoprophylaxis using three trypanocidal compounds; isometamidium, homidium and diminazine. Suramin and quinapyramine are also in practice since long time in animal trypanosomiasis treatment. All of these drugs have been in widespread use for about 40 years and resistance has been reported in many parts [279–282]. Resistance to the Berenil (a drug commonly used for treatment of livestock trypanosomiasis [283]) has also been reported in several foci [284].

The resistance to drugs for the African trypanosomes has been shown to be associated with reduced drug uptake by the parasites stressing the importance of drug transporter (reviewed by [285]). Reduction of net drug uptake can be caused by either a decreased drug import or an increased drug export. Either mechanism implies mutations in the transporters since most trypanocides do not freely diffuse through the plasma membrane. In this context, the adenine nucleoside transporter (P2-transporter) has been found to be the transporter for arsenical as well as pentamidine [286, 287]. That provides an explanation for the frequent occurrence of cross-resistance between arsenicals and dimidines in *T. brucei* spp. [288]. A trypanosome gene (*TbAT1*) product exhibits P2-like transport activity but this is not the only transporter for the trypanocidal drugs, indicating that drug resistance involves the loss of more than one transporter [289]. Besides loss of import to the parasite, increased export of the drug also can be responsible for the drug resistance.

In this aspect, over expression of one exporter, *TbMRPA* (*T. brucei* multidrug-resistance associated protein) caused 10-fold resistance to the melarsoprol in vitro [290, 291] but such over expression in patients with treatment failure with melarsoprol has not been reported yet. Similarly, over expression of a gene *TeDR40* has been shown to correlate with Berenil resistance observed in *T. evansi* [292]. Besides, alterations in drug import and export, there can be other alternative mechanisms like failure to undergo apoptosis may also contribute to the drug resistance. Different resistance mechanisms are not mutually exclusive. On the contrary, drug resistance is often multifactorial.

Due to the facts that (i) there are very few drugs available for the treatment of trypanosomiasis, (ii) the drugs being used cause severe toxic effects, and (iii) there are increasing field cases of drug resistance and there is a clear demand for alternative treatment schemes. Furthermore, due to antigenic variation and immunosuppression, the conventional vaccinations strategies are not able to give promising results. So, also in this regard alternative approaches are necessary.

Even a century after its first description, the trypanosomiasis is still one of the major parasitic disease for which control is far from reality. Most antitrypanosome drugs are taken up by the parasite via specific transporters (e.g., P2 transporter). As a result, mutation on the gene encoding that transporter can lead to multiple drug resistance [286, 287]. Therefore when new drug strategies for trypanosomiasis are designed, they should rely on novel molecular and biochemical pathways. The human trypanolytic factor, APOL1, has been exploited as an alternative approach for the treatment of HAT. APOL1 lyses trypanosomes except the ones which are causing HAT [102]. In case of *T. rhodesiense* the resistance to the trypanolytic capacity of APOL-1 is due to the expression of serum resistance-associated (SRA) protein [94] which neutralizes APOL1 through the interaction with the C-terminal of this lipoprotein. Deletion of the SRA interacting domain of APOL1 results in the generation of a new molecule, that is, Tr-APOL1 that is lytic to both NHS-sensitive as well as resistant *T. rhodesiense*. Hence, Tr-APOL1 represents a possible drug against all *T. rhodesiense* parasites. However, to avoid the possible competition with the native APOL1 in the serum, specific trypanosomal targeting of Tr-APOL1 is essential. One such approach where the Tr-APOL1 has been conjugated with a single domain camel VHH that targets the oligomannose moiety of VSG has been reported [293]. The results in that study showed that treatment with this conjugated Tr-APOL1 cured mice infected with either NHS-resistant *T. rhodesiense* or NHS-sensitive *T. brucei*. The treatment also had beneficial effect when used in the chronic stage of the trypanosomiasis, although in this case a complete elimination of the parasite was not obtained [103]. The results thus may suggest that, Tr-APOL1 could be developed as an alternative drug for treatment of the early stage HAT. Due to the fact that APOL1 is a human self-antigen, and since VHH is highly homologous to human VH, the authors of this paper suggest very minimal, if any, immune response against the conjugated protein, when administered to HAT patients. Furthermore, it has been shown that the full length baboon APOL1 (which does not interact with

SRA) is protective against both the animal-infective and human-infective *T. rhodesiense* in an experimental mouse model, and, as such, it has been suggested to create transgenic livestock that would be resistant to animal and human-infective trypanosomes, which is envisaged to result in the reduction of the livestock trypanosomiasis and zoonotic transmission of human infective trypanosomes [119].

Beside treatment, effective vaccination strategy is a second approach for the control of any infectious diseases. In the case of trypanosomiasis, all conventional anti-parasitic vaccination efforts undertaken so far, that used dominant surface protein, have failed due to the antigenic variation of the trypanosomes surface coat. Therefore, an alternative strategy of the vaccination is demanding. As alternative vaccination approaches, different molecules such as trypanosomal cystein protease (congopain) [294], trypanosomal tubulin [295, 296] or trypanosomal GPI have been attempted. The GPI-anchor of the VSG as one of the major parasitic components causing the inflammatory response associated to the infection has been identified [258]. In one of the studies, this information has been used to evaluate GPI-based vaccination as an alternative strategy with antidisease potential [252]. Using liposomes as slow delivery system, the GPI administered prior to the infection had been shown to result in a better control of the parasitemia and a longer lifespan of the infected mice. The treated animals were better protected from various pathological conditions including anemia which is considered as one of the major pathological parameters of the trypanosomiasis [254]. These results are related to the fact that the treatment oriented the classically activated inflammatory macrophages, to more counter-inflammatory alternatively activated macrophages [208], subsequently resulting in reduced TNF production and reduced pathology. With the GPI-based treatment, though there were positive effects on the infection-induced pathology as well as survival, but the animals were not cleared from their parasites. Therefore, this strategy is more an anti-disease approach rather than an anti-parasitic strategy. While this solution would not be advisable for the HAT where an antiparasitic treatment would be needed, the persistence of a low level infection that would prevent the severe disease might be preferred in the livestock field conditions, where the host has controlled geographical movement and is under continuous threat of re-infection by the infected tsetse flies. The needs, possibilities and requirements of further knowledge for the way to develop an anti-disease control strategy for trypanosomiasis has been recently highlighted [297].

8. Conclusion

Both the prophylactic as well as the therapeutic aspects of trypanosomiasis need alternative approaches as currently there is no vaccine and the drugs that are in use have several short comings. Some such alternative approaches have been initiated but still more detail and comprehensive strategies should be envisaged. These approaches should be based on the strong background of understanding the biology of the

parasite as well as the host-pathogen interaction needs to be further studied. One final point of consideration for such alternative approach would be its economical viability since the disease affects the poorest of the poor of the world.

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Research Article

Mexican *Trypanosoma cruzi* TCI Strains with Different Degrees of Virulence Induce Diverse Humoral and Cellular Immune Responses in a Murine Experimental Infection Model

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It has been shown that the majority of *T. cruzi* strains isolated from Mexico belong to the *T. cruzi* I (TCI). The immune response produced in response to Mexican *T. cruzi* I strains has not been well characterized. In this study, two Mexican *T. cruzi* I strains were used to infect Balb/c mice. The Queretaro (TBAR/MX/0000/Queretaro)(Qro) strain resulted in 100% mortality. In contrast, no mortality was observed in mice infected with the Ninoa (MHOM/MX/1994/Ninoa) strain. Both strains produced extended lymphocyte infiltrates in cardiac tissue. Ninoa infection induced a diverse humoral response with a higher variety of immunoglobulin isotypes than were found in Qro-infected mice. Also, a stronger inflammatory TH1 response, represented by IL-12p40, IFN γ , RANTES, MIG, MIP-1 β , and MCP-1 production was observed in Qro-infected mice when compared with Ninoa-infected mice. We propose that an exacerbated TH1 immune response is a likely cause of pathological damage observed in cardiac tissue and the primary cause of death in Qro-infected mice.

1. Introduction

Chagas' disease is a major endemic disease caused by the protozoan *Trypanosoma cruzi*. This parasitic disease is widely distributed throughout Latin America, affecting 18 million people [1]. In the past, it was believed that Chagas' disease was very rare in the northern part of America, including Mexico, as few human disease cases were reported. More recently, however, it has been estimated that there are as many as two million infected individuals and more than 72 000 cases in Mexico and Central America [2]. Even though these figures are only estimates, other field studies support the notion that the prevalence of *T. cruzi* infection might be higher in some regions of the country than previously thought [3–5]. *T. cruzi* has also been repeatedly found infecting insects and mammals in Mexico and the United States of America [6–8].

T. cruzi strains have been divided into six discrete typing units (DTUs) according to their genetic background. These

groups are designed the *T. cruzi* I to VI [9]. The geographical distribution of these groups indicate that *T. cruzi* II to VI are the main causal agent of Chagas' disease in the southern parts of South America, with *T. cruzi* I only present in the sylvatic cycle [9–11]. In contrast, *T. cruzi* I has been reported as the primary parasite present in human cases in Colombia, Venezuela, and Central America [12–14]. In Mexico, most of the *T. cruzi* strains that have been genetically analyzed to date belong to the *T. cruzi* I group [15–17]. We have reported that this Mexican *T. cruzi* I strain possesses different biological characteristics such as growth rates, metaciclogenesis, and infectivity in vitro [15]. However, the pathology and immune response that these strains can induce has largely gone unstudied.

Knowledge of the pathology and immune response to *T. cruzi* infection has been benefited by data obtained from murine models. These models have shown that the innate and adaptive immune responses play an important role in parasite control, depending on the combined action

of various cellular types including NK, CD4+ and CD8+ as well as on the production of antibodies by B cells [18, 19]. Resistance to *T. cruzi* infection has been associated with the production of the pro-inflammatory cytokines IL-12 and IFN- γ and with the local production of RANTES, MIP-1 α , MIP-1 β and MCP-1. These cytokines activate the production of nitric oxide by macrophages, which is responsible for elimination of the parasite [20–23]. TNF- α has also been associated with macrophage activation as a secondary signal for nitric oxide production [24]. In contrast, the Th2 cytokines IL-10, IL-4 and TGF- β are associated with parasite susceptibility [25, 26].

Since the majority of published data has been obtained from studying *T. cruzi* II-VI-infected mice and the genetic differences between *T. cruzi* II-VI and *T. cruzi* I strains are large, the pathology and immune response to Mexican *T. cruzi* I Qro and Ninoa strains were evaluated in a murine model. Even though these two strains were genetically indistinguishable using the genetic markers available until recently [16, unpublished data], differences in pathology and immune responses were found in mice infected with both strains.

2. Materials and Methods

Parasites. Mexican *T. cruzi* I Qro (TBAR/MX/0000/Queretaro) and Ninoa (MHOM/MX/1994/Ninoa) strains were used in this study [15, 16]. The Qro strain was isolated from the *Triatoma barberi* vector from the Queretaro State in Central Mexico. The Ninoa strain was obtained from a human case in Oaxaca State in the southern Pacific coast of Mexico. Both strains were maintained by serial passage in Balb/c mice.

Mice. Six–eight-week-old female Balb/c mice were obtained from the Animal House of the Instituto de Investigaciones Biomedicas (UNAM, Mexico City, Mexico) and maintained under standard conditions. Groups of 9 to 12 mice were injected intraperitoneally with 1×10^4 or 1×10^5 blood-form trypomastigotes (BT). Parasitemia was determined every third day by blood microscopy observation. Groups of 28 mice were used for daily monitored of survival. All animal research followed the Instituto de Investigaciones Biomedicas ethical committee's guidelines.

Histopathological Evaluation. Groups of four Ninoa- or Qro-infected mice were sacrificed under anesthesia on day 21 post infection (pi). Groups of three age-matched control mice were sacrificed at the same time point. The myocardium was fixed in neutral 4% paraformaldehyde and embedded in paraffin. Serial 5- μ m sections were prepared and stained with hematoxylin and eosin (H-E) and examined using light microscopy on an Optiphot-2 microscope (Nikon).

The inflammatory infiltrates were subdivided into focal and diffuse infiltrates, depending on how closely the inflammatory cells were associated [27].

Characterization of Specific Antibodies and Antigens. Specific *T. cruzi* antibodies were detected by an indirect ELISA using an epimastigote extract as previously described [28]. The antibody isotypes and antigens recognized by the

specific antibodies were determined at days 0, 4, 8, 12, 16, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, and 150 post-infection by western blot. Briefly, proteins from the total extract from both strains were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. After overnight saturation in PBS containing 10% skim milk at 4°C with constant shaking, the nitrocellulose membrane was cut into strips that were individually incubated (2 hrs at 37°C) with 1 ml of mouse serum diluted 1 : 500 in PBS/10% skim milk. Each strip was washed three times with PBS-0.1% Tween 20 and incubated with goat antimouse IgG1, IgG2a, IgG2b, IgG3, IgA and IgM (diluted 1 : 2,000) for 2 hrs at room temperature. Then the strips were washed as above and a peroxidase-conjugated anti-goat antibody was added. After washing, the reaction was developed with 0.5 μ g/mL of diaminobenzidine in PBS containing 0.02% H₂O₂. The reaction was stopped with water. Positive (chronically infected mice) and negative control (non infected mice) sera were included in each experiment.

T Cell Proliferation Assay. Splens were removed aseptically from anesthetized Balb/c mice at 15, 21 and 98 days post-infection and spleen cell suspensions were prepared. Cultures of spleen cell suspensions (1×10^5 in RPMI medium containing 10% heat inactivated serum, 100 U/mL penicillin, 100 μ g/mL streptomycin and, 100 U/mL gentamicin) were set up in triplicate in microculture plates. Then cells were stimulated with 1 μ g/mL of concanavalin A (Con A) or 20 μ g/mL of epimastigote total extract. After culturing for 48 hours at 37°C with 5% CO₂, 1.0 μ Ci of (³H) thymidine per well was added. Twenty hours later, cells were harvested onto filters, dried and placed in scintillation fluid and counted in a scintillation counter. The stimulation index (SI) was obtained by dividing the infected cells' counts per minute between the control group's counts [29].

CD4+ and CD8+ Cells. FACS analysis was used to quantify CD4+ and CD8+ lymphocytes. Briefly, spleen cell suspensions (1×10^6) were obtained from infected and control mice and were stained with 1 μ g of FITC-labeled antibodies against CD4 or PE-labeled antibodies against CD8. Cells were analyzed by FACS (Becton and Dickinson) [29].

Quantification of Murine Cytokines. Levels of IFN- γ , IL-12p40, IL-4 and IL-10 cytokines in sera (diluted 1 : 10) were assayed in a two-site ELISA following the manufacturer's instructions (ENDOGEN, Cambridge). Cytokine levels were calculated by reference to a standard curve constructed with recombinant cytokines included in the commercial kit.

RT-PCR Assay for Measuring Expression of Chemokine mRNA. RNA was isolated from cardiac muscle of infected mice and controls by TRIZOL (Invitrogen). Before RT-PCR, RNA was incubated with amplification grade DNase I (Invitrogen). Levels of MIG (CXCL9), MCP-1(CCL2), MIP-1 β (CCL4), RANTES (CCL5), and β -actin (as control) mRNA were determined by SuperScript One-step RT-PCR with Taq Platinum (Invitrogen). The primer (sense and antisense) sequences, the number of cycles, and expected product sizes have been published previously [21, 30]. For all primer pairs, a negative control (without template) was

run in parallel. Reactions were performed using a PTC-100 thermal cycler (MJ Research, INC). PCR products and molecular weight marker were run on 2% agarose gels and stained with Ethidium bromide.

PCR products were quantified with a densitometer (Fluor-S MultiImager, Bio-Rad) using the Quantity One version 4.4.1 (Bio-Rad) program. Densitometry values were corrected using the mouse β -actin value for the same sample.

Statistical analysis. Data regarding parasitemia, percentage of survival, SI, percentage of CD4+ and CD8+ cells and cytokine/chemokine quantification represent the values derived from three independent experiments containing at least 3 mice per group. Arithmetic means and standard deviations of the means were calculated. Student's *t*-test was used to analyze the statistical significance of the differences observed in RT-PCR analysis. Differences were considered statistically significant when $P \leq .05$. To analyze data regarding parasitemia, cytokine levels, SI values, and percentage of CD4+/CD8+ cells, ANOVA and X^2 Test were used, with significance assigned for values of $P \leq .05$ [31].

3. Results

Parasite infectivity was determined by blood microscopy observation. When 1×10^4 *T. cruzi* BT were used, the parasites were observed at day 3 post-infection for Qro-infected mice and at day 7 post-infection for Ninoa infected mice. A rapid and continuous increase in parasitemia for both strains was observed, with blood stream parasites reaching a peak at day 28–31 post-infection (Figure 1(a)). Mice infected with Qro tripomastigotes showed significantly higher levels of blood parasitemia (2.9 ± 0.327 million blood tripomastigotes/mL) than the mice infected with the Ninoa strain (1.6 ± 0.306 million blood tripomastigotes/mL) at the peak of blood parasitemia. Nevertheless, no significant differences were found throughout the rest of the blood parasitemia curve. Based on this result, the acute phase was defined as ranging from the initial infection until three months post-infection, with the chronic phase following immediately after. Similar results were found when 1×10^5 parasites were used (data not shown).

Importantly, significant differences in mortality percentages were observed when mice infected with different strains were compared. In the majority of the experiments, all mice infected with 1×10^4 Qro BT were killed by day 57 postinfection. In other experiments, the mortality rate was 60% (data not shown). In contrast, no mortality was observed in Ninoa-infected mice (Figure 1(b)). Macroscopic enlarged spleen was observed in infected mice, with a significant difference in weight and size when comparing Qro and Ninoa infected mice (Figure 2).

Both strains were cardiotropic, as a number of amastigote nests were observed in this organ. Lymphocyte infiltrates were observed in H&E stained cardiac tissue. At day 21 post-infection, visible lymphocyte infiltrates were observed in the cardiac tissue of mice infected with both strains. Some regions also displayed visible edema. Both diffuse and focal lymphocyte infiltrates were observed (Figure 3).

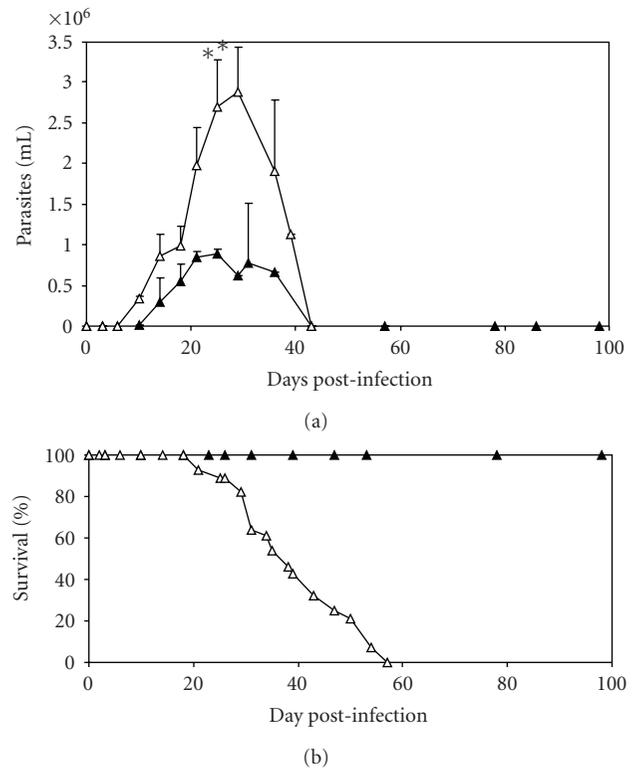


FIGURE 1: Parasitemia and Survival curve in mice infected with *T. cruzi* BT. Mice were inoculated ip with 1×10^4 BT Ninoa (\blacktriangle) or Qro (\triangle) strains. (a) Parasite number in blood was determined by counting in a Neubauer chamber using an optic microscopy. Statistical differences in blood parasite number are indicated ($*P < .05$) at 25 y 29 days post infection. (b) Survival of mice infected with *T. cruzi* strains was evaluated using groups of 28 mice infected with *T. cruzi* strains. Daily direct observation was done and dead mice were recorded.

Pooled sera from 3 to 6 Ninoa- or Qro-infected mice were tested by ELISA. Infected mice showed an increase in specific IgG isotypes during the acute phase of infection, reaching similar OD values by day 29 post-infection. In Ninoa-infected mice, antibodies were detected until the early chronic phase of the infection (80 days post-infection) (Figure 4).

Interestingly, when antibody isotypes were determined, a clear difference was observed. Ninoa-infected mice produced IgM and IgG2a during the early phase of infection. Later in the course of infection, (day 35–40 post-infection) IgG1, IgG2b, IgG3, and IgA were produced. In contrast, Qro-infected mice displayed a notable reduction in specific isotype production. IgM (25 days post-infection) and IgG2a (40 days post-infection) were the only isotypes detected in the sera of these mice (Table 1). In western blots of sera from Ninoa-infected mice, three main antigens (70, 45–50, and 30 kDa) were recognized by the majority of isotypes being produced by day 20 post-infection. A fourth major antigen (100 kDa) was recognized later in the course of the infection by IgG1 and Ig2a isotypes. Sera from Qro-infected mice recognized antigens of 70, 40, and 35 kDa (Table 1).

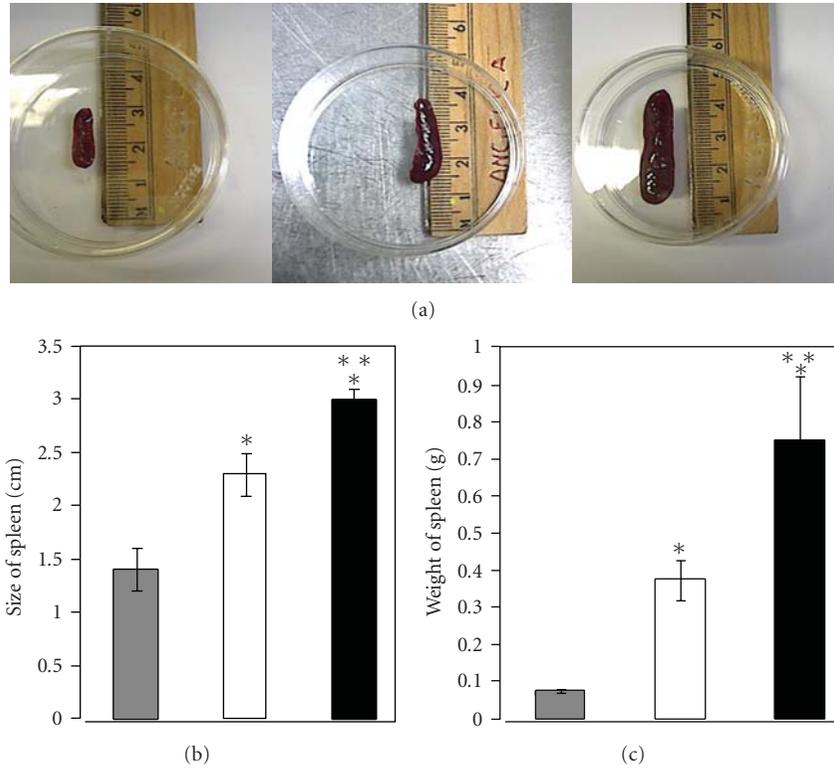


FIGURE 2: Spleen changes of mice infected with *T. cruzi* strains. Groups of three mice were inoculated with PBS (grey bar) or $1 \times 10^5 T. cruzi$ BT of Ninoa (white bar) or Qro (black bar) strains and spleens were obtained at acute phase of infection. (a) Growth of spleen from mice infected with Ninoa (center) and Qro (right) strains compared with control non infected mice (left). Size (b) and Weight (c) of spleen from mice infected. Media value and standard deviation are shown. Statistical differences ($P < .001$) were determined between control and infected mice (*) and between Ninoa and Qro strains (**) by ANOVA Bonferroni post-test.

TABLE 1: Immunoglobulin isotype and kinetics of antigen recognized in mice infected with *T. cruzi* I strains.

Strain	Isotype	Antigens recognized (kDa)	Days post-infection												
			20	25	30	35	40	50	60	70	80	90	100	150	
Ninoa	IgG1	45-50				X	X	X	X	X	X	X	X	X	
		70				X	X	X	X	X	X	X	X	X	
		100									X	X	X	X	
	IgG2a	45-50		X	X	X	X	X	X	X	X	X	X	X	
		70			X	X	X	X	X	X	X	X	X	X	
		100								X	X	X	X	X	
	IgG2b	45-50				X	X	X	X	X	X	X	X	X	
		70					X	X	X	X	X	X	X	X	
		100											X	X	
	IgG3	45-50					X	X	X	X	X	X	X	X	
		70					X	X	X	X	X	X	X	X	
	IgM	30	X	X	X	X	X	X	X	X	X	X	X	X	
		45-50		X	X	X	X	X	X	X	X	X	X	X	
		70		X	X	X	X	X	X	X	X	X	X	X	
	IgA	45-50					X	X	X	X	X	X	X	X	
		70						X	X	X	X	X	X	X	
Qro	IgG2a	70					X	X							
		40		X	X	X	X	X							
		35		X	X	X	X	X							

Isotype of IgG and molecular weight of antigens recognized during infection course (X) are showed in days post-infection.

TABLE 2: Percentage of CD4+ and CD8+ lymphocytes in mice infected with *T. cruzi* I strains.

Strain (infection phase)	Parasites	CD4+	CD8+
Ninoa (acute)	PBS	28.8 ± 1	11.8 ± 0.1
	1 × 10 ⁴	15.1 ± 2.6*	11.8 ± 2.6
	1 × 10 ⁵	18 ± 2.5*	16.3 ± 1.9
Ninoa (chronic)	PBS	25.8 ± 8	9.9 ± 4.1
	1 × 10 ⁴	30.3 ± 11.6	23.2 ± 7.8*
	1 × 10 ⁵	25.3 ± 7.4	18 ± 8.7*
Qro (acute)	PBS	24 ± 3.3	10.5 ± 4.6
	1 × 10 ⁴	17 ± 3.3*	11.7 ± 4.3
	1 × 10 ⁵	14 ± 2.5*	10 ± 6

Mean ± standard deviation of three independent assays. Statistical differences between parasitized and control mice are indicated (* $P < .05$). Control mice were inoculated with phosphate buffer saline (PBS).

When spleen cells from acute phase mice were obtained and stimulated with parasite antigens (20 µg/mL), a significant increase in proliferative response to these antigens was observed in both groups of infected mice. No significant differences were found between the SI of spleen cells from Ninoa- or Qro-infected mice (2.9 ± 0.05 versus 2.2 ± 1, resp.). During the chronic phase of infection, which was only reached by Ninoa-infected mice, no differences were observed compared to noninfected mice. When Con A was used for stimulation, no differences were found between infected and control mice (data not shown).

Percentages of CD4+ T lymphocytes were significantly lower in infected mice than in noninfected controls in the acute phase, regardless of strain. Additionally, no differences were found when CD8+ cells were analyzed in the acute phase (Table 2). In the chronic phase of Ninoa infection, the levels of CD4+ cells were reestablished, reaching levels similar to noninfected controls. Furthermore, a significant percentage of CD8+ T cells were observed in Ninoa-infected mice during the chronic phase. These phenomena were observed independently of the initial parasite inoculum used (Table 2).

The concentration of TH1 IL-12p40 was higher in the blood of Qro-infected mice than in Ninoa-infected mice. Significant differences were observed even in the first days of infection. The expression of IL-12 correlated with the expression of IFN γ , which increased significantly within 10 days post-infection in Qro-infected mice. In Ninoa-infected mice, IFN γ production was dampened, reaching a maximum concentration eight days after Qro-infected mice (Figure 5). With respect to the TH2 cytokines, the concentration of IL4 increased slowly, reaching a maximum concentration by day 29 post-infection in Ninoa-infected mice. A similar pattern was observed in Qro-infected mice. With respect to IL10, we observed a slight increase in the first day of infection followed by a decline in overall levels, with no significant differences observed between strains.

Because the heart is the primary site of parasite infection, it was important to measure the concentration of the major chemokines associated with *T. cruzi* infection. MCP-1, MIP-1 β , RANTES, and MIG mRNA expression in cardiac

tissue were measured during the acute phase (21 days post-infection). In all cases, a significant increase in chemokine mRNA expression was observed in mice infected with either strain. A significant increase in the expression of MCP-1, MIP-1 β , and MIG mRNA was found in the cardiac tissue of mice infected with Qro compared with Ninoa-infected mice. In contrast, no differences in RANTES expression were observed in mice infected with either strain (Figure 6).

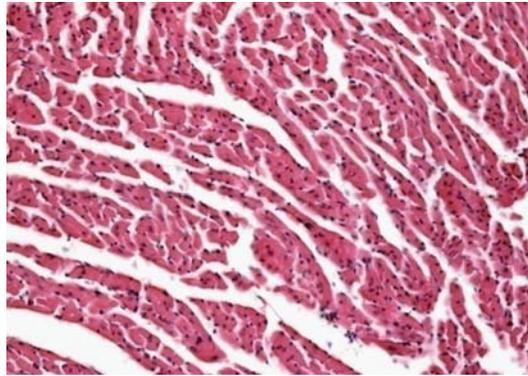
4. Discussion

The human parasite *T. cruzi* contains many strains and clones that show a great deal of genetic heterogeneity [32]. It is now accepted that *T. cruzi* strains can be divided into six DTUs, *T. cruzi* I to VI [9]. As mentioned earlier, the geographic distribution of these genetic types is different and has important epidemiological implications [32, 33]. Our group has demonstrated the existence of *T. cruzi* I parasites in the domestic cycle as well as a few TCII strains in the sylvatic cycle in Mexico [15, 16]. Furthermore, differences in growth, metaciclogenesis and in vitro infectivity have been demonstrated for these strains [15].

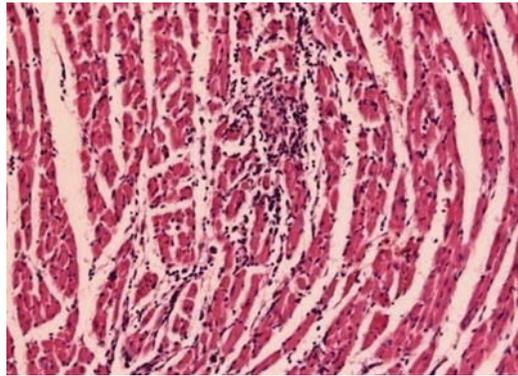
It has also been found that different *T. cruzi* populations correlate with differences in pathology observed in patients [34]. Also, the fact that genetically similar parasite strains can be obtained from patients with distinct clinical forms of the disease suggests that the host immune response may represent an important factor in determining the outcome of infection [34, 35].

In the present study, we have shown that two genetically similar *T. cruzi* I strains can possess notable differences in their in vivo infectivity and pathogenesis as well as in the immune response induced in a susceptible murine model.

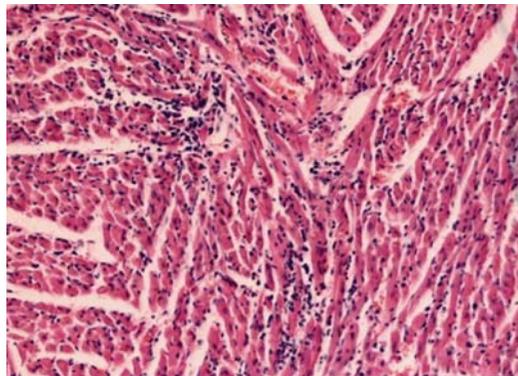
We demonstrated that the two *T. cruzi* I strains analyzed here result in different levels of blood parasitemia, with Qro-infected mice having double the number of parasites observed in Ninoa-infected mice at the peak of blood parasitemia. However, no difference in blood parasitemia was found during other stages of infection. This phenomenon was observed with two different initial inoculum. Both strains were cardiotrophic, showing intense lymphocyte



(a)



(b)



(c)

FIGURE 3: Cardiac tissue histology of mice infected with *T. cruzi* strains. Mice infected with 1×10^4 parasites were sacrificed at day 21 post infection and heart was recovered. Tissue was fixed in 4% paraformaldehyde and embedded in paraffin. Five μm sections were Hemotoxilin-Eosin stained and observed at $20\times$ in optical microscope. (a) Control noninfected mice, (b) Mice infected with Ninoa strain, and (c) Mice infected with Qro strain.

infiltration in the cardiac tissue along with diffuse infiltration over large areas in the heart. It is worth mentioning that the physical aspects of Qro-infected mice were different from Ninoa; the former presented bristly hair and paralysis of the posterior legs. Also a significant increase of the weight and size of the infected mice spleen was observed.

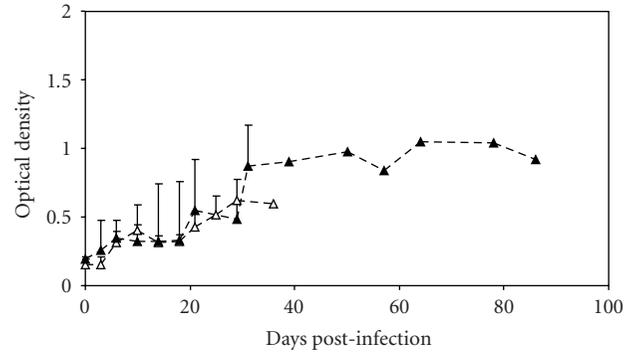


FIGURE 4: Immunoglobulin profile in serum of mice infected with *T. cruzi* I strains. Mice were inoculated ip with 1×10^4 *T. cruzi* BT of Ninoa (▲) or Qro (△) strains. IgG antibodies were determined by ELISA as described in materials and methods and are showed as media value \pm SD.

More important were the differences in mortality rates that were induced by the two strains. Qro infection resulted in 60% to 100% mortality (depending on the initial inoculum). In contrast, Ninoa infection did not produce significant mortality, regardless of initial inoculum. These results indicate an enormous difference in virulence between these two strains. To find a possible explanation for this observation, the host immune response was analyzed.

In the acute phase, SI data indicated an increase in the number of spleen cells activated by parasite antigens. Surprisingly, in our model, no increase in CD4+ T cell activation was observed. On the contrary, a significant decrease was found following infection with either strain, conflicting with other previously published murine models using *T. cruzi* I and *T. cruzi* II-VI strains [36, 37]. It is possible that macrophages, NK, and CD8 cells are responsible for the increased levels of TH1 cytokines observed in this study, which would agree with other murine models [21, 30, 38, 39]. In the chronic phase of Ninoa infection, recovery of CD4+ T cells was observed, reaching similar levels to uninfected controls. CD8+ T cell levels remained unchanged in the acute phase and showed a significant increase in the chronic phase of Ninoa infection, in agreement with their timing for expansion and contraction phases in *T. cruzi* infection [40]. Furthermore, as has been recently postulated *T. cruzi*-specific CD8+ T cells develop in the absence of CD4+ T cells and display similar effector functions but fail to control parasite load [41, 42].

The TH1 response was characterized by high levels of IL-12p40 and IFN γ . Interestingly, higher concentration and early appearance of these cytokines was only observed in Qro-infected mice. Levels of the regulatory cytokine IL-10 were low and levels of IL-4 peaked later, around 30 days post-infection. Also, chemokine profiles in the acute phase were different, with high levels of RANTES found in mice infected either both strains, but with higher levels of cardiac MIG, MCP-1, and MIP-1 β being observed only in Qro-infected mice. These chemokines are known to induce production of nitric oxide in infected macrophages

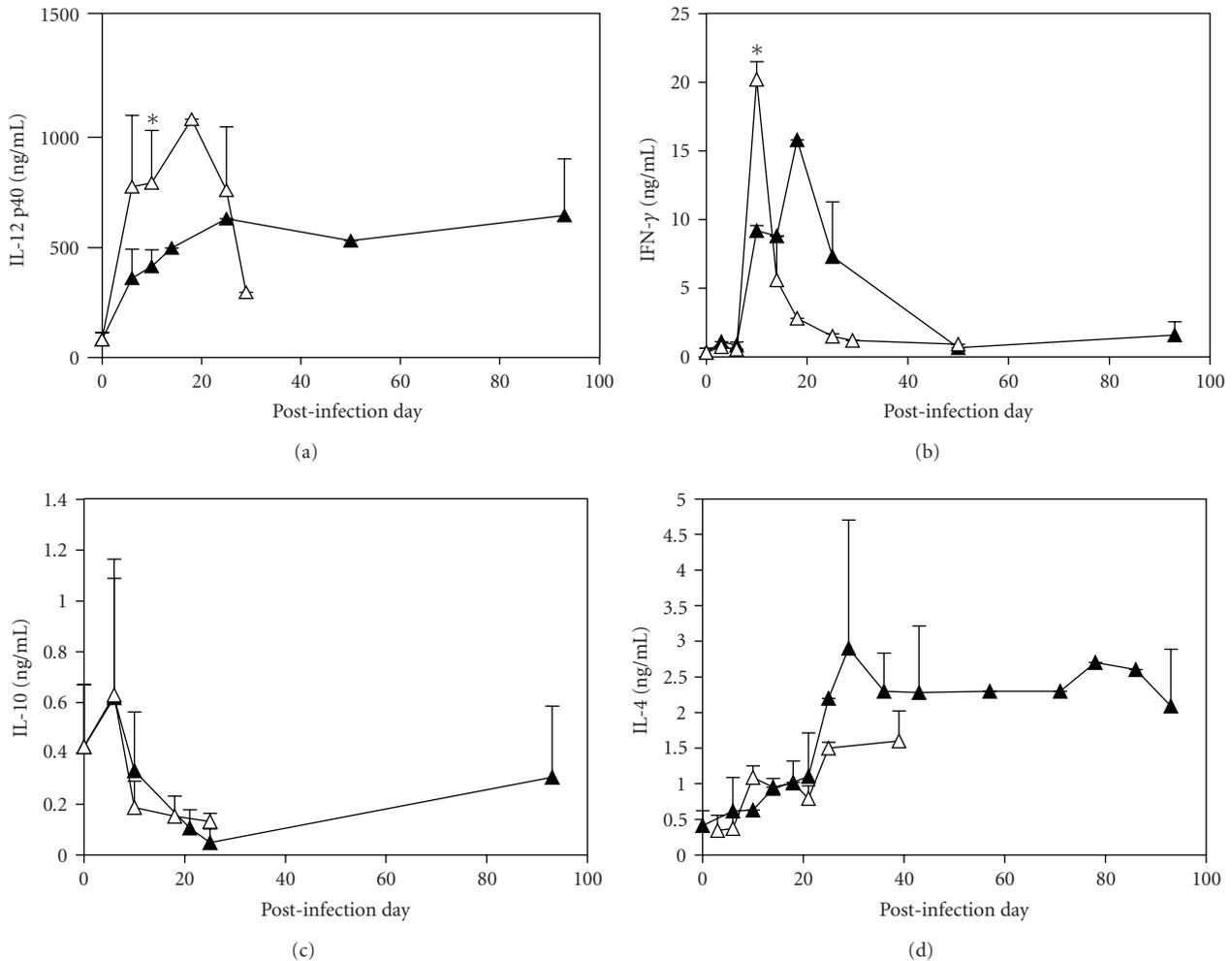


FIGURE 5: Serum cytokine concentration in mice infected with *T. cruzi* I strains. Groups of nine mice were inoculated with 1×10^4 BT Qro (Δ) or Ninoa BT (\blacktriangle). Serum was obtained from ocular plexus each 3rd day and pooled. Cytokines were measured by ELISA and presented as average of two experiments with standard deviation. (a) IL-12 p40, (b) IFN γ , (c) IL-10, and (d) IL-4. Statistical differences between mice infected with either strain are indicated (* $P < .05$).

[21, 43, 44]. They are also known to induce attraction of immune cells to cardiac tissue, as has been demonstrated with the Colombiana strain [45].

Interestingly, an enormous difference was observed when antibody isotypes were analyzed. The Ninoa strain induced IgM and IgG2a in the early acute phase and IgG1, IgG2b, IgG3, and IgA after 30 days post-infection. In contrast, the Qro strain induced only IgM and IgG2a in the early acute phase, with a notable difference in recognized antigens. The presence of a higher number of isotypes in Ninoa-infected mice could be one of the factors responsible for improved control of parasitemia, as IgG antibodies have been related to parasite resistance in murine models [46, 47]. Interestingly, it has been reported that genetic diversity between *T. cruzi* I and *T. cruzi* II-VI strains influences the immunoglobulin profile elicited during murine infection, but no differences between *T. cruzi* I strains were found [48].

In the *T. cruzi* I infection model presented here, a vigorous humoral response accompanied by a significant but moderate TH1 cellular response resulted in control of parasitemia and limited pathology, allowing the recovery and survival of Ninoa-infected mice. In contrast, a weak humoral response with a potent TH1 proinflammatory profile produced an uncontrolled inflammatory reaction. The decrease of CD4+ cells together with high in vitro secretion of TNF α by Qro-infected J774 cells (unpublish data) suggest that this *T. cruzi* strain could induce a phenomenon similar to the toxemic state observed in severe infections, between other pathological mechanisms that could explain the death of Qro-infected mice [49, 50]. Studies looking for apoptotic death of T lymphocytes and TNF and Fas ligand measurements must carry on with this model in order to clarify this point.

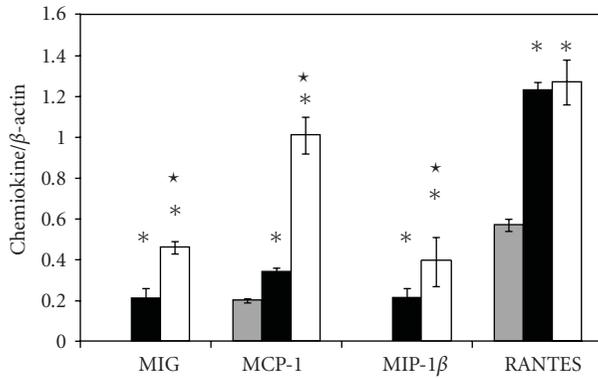


FIGURE 6: mRNA expression of MIG (CXCL9), MCP-1 (CCL2), MIP-1 β (CCL4), and Rantes (CCL5) in cardiac tissue of Balb/c mice infected with 1×10^5 BT of *T. cruzi*. Total RNA was obtained at day 21 post infection and chemokines expression was determined by RT-PCR as described in material and methods. PCR products were analyzed in 2% agarose gels stained with ethidium bromide. Semicuantitative analysis was done. Values are average of 4 mice from 3 independent assays. Grey bar control, black bar Ninoa strain and White bar Qro strain. In the case of MIG and MIP-1 β no detectable levels of chemokines were found in the control tissues. Significant differences ($*P < .05$) in chemokine expression were found in cardiac tissue of control versus infected mice. Differences between *T. cruzi* strains were also founded ($*P < .05$).

As in our model, a TH1 response has been reported in active Chiclero's ulcer produced by *Leishmania mexicana* [51], indicating that the TH1/TH2 paradigm is not always present in these complex host-parasite relationships.

In conclusion, intense cardiomyopathy in the acute phase with vigorous humoral immune response, followed by the reestablishment of CD4+ and CD8+ cells and associated animal survival observed in Ninoa infection represents a useful model for the study of immune mechanisms that allow the host to overcome parasite infection. On the other hand, the highly virulent *T. cruzi* I Qro strain provides a useful model to study virulence factors that result in the death of infected mice. Additional studies should be conducted on the *T. cruzi* I strains from the northern part of the American Continent, as they are responsible for most cases of Chagas' disease in this area.

Acknowledgment

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Research Article

Cysteine-Free Proteins in the Immunobiology of Arthropod-Borne Diseases

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One approach to identify epitopes that could be used in the design of vaccines to control several arthropod-borne diseases simultaneously is to look for common structural features in the secretome of the pathogens that cause them. Using a novel bioinformatics technique, cysteine-abundance and distribution analysis, we found that many different proteins secreted by several arthropod-borne pathogens, including *Plasmodium falciparum*, *Borrelia burgdorferi*, and eight species of Proteobacteria, are devoid of cysteine residues. The identification of three cysteine-abundance and distribution patterns in several families of proteins secreted by pathogenic and nonpathogenic Proteobacteria, and not found when the amino acid analyzed was tryptophan, provides evidence of forces restricting the content of cysteine residues in microbial proteins during evolution. We discuss these findings in the context of protein structure and function, antigenicity and immunogenicity, and host-parasite relationships.

1. Introduction

Microbial pathogens transmitted by hematophagous arthropods are responsible for some of the most devastating human diseases and the design of vaccines to control them represents one of the main collective efforts in global health. These arthropod-borne diseases (ABDs) are caused by hundreds of species of viruses, bacteria, protozoa, and metazoan parasites and are transmitted by hematophagous mosquitoes, flies, fleas, lice, biting midges, kissing bugs, ticks, and mites [1]. Some ABDs are emerging, reemerging, or out-of-control as a result of high mobility of vertebrate and invertebrate hosts, environmental degradation, and global warming [2]. Further complicating the bleak scenario that ABDs represent, several of the pathogens that cause them could potentially be used as weapons [3–5]. Vaccination remains one of the most cost-effective interventions in public health but only a handful of effective vaccines are available for the control of ABDs [6, 7]. Central to attempts to control infectious diseases through use of vaccines is the idea that species-specific immunity is the best way to induce safe and effective protection. In light of the monumental task of the development of vaccines

directed to each species of arthropod-borne pathogen and/or their vectors, it is necessary to revisit the idea of cross-reactive immunity as a potential venue to protect humans and animals against several ABDs simultaneously. Thanks to advances in synthetic peptide libraries and phage expression libraries, evidence is accumulating that supports the concept of functional poly-specificity of antibodies [8, 9]. Such poly-specificity could be expected to be more apparent when antibodies interact with protein antigens that, due to the high degree of structural disorder and flexibility defined in its amino acid sequence, display a high tendency to engage in promiscuous interactions with other proteins [10, 11]. Some of these features can be identified in proteins using a variety of predictive algorithms that help in the identification of structural disorder [12, 13] and B-cell epitopes [14]. A complementary approach that could be used to identify such sequences is to scan proteins looking for the absence of structural features known to limit protein flexibility and that induce, through an effect on protein folding, the formation of conformational B-cell epitopes. One such structure, the disulfide bridge, is formed when a protein that has at least 2 cysteine residues is allowed to

fold under the oxidative conditions prevalent in extracellular compartments of tissues. It follows that secreted proteins incapable of forming disulfide bridges because of a lack of cysteine residues represent potential targets of cross-reactive vaccines directed to linear, nonconformational, and flexible epitopes. In order to understand both the biological significance that the absence of cysteine residues in pathogen proteins might have in the immunobiology of ABDs and the potential of cysteine-free proteins (CFPs) as vaccine targets, we conducted a cysteine-abundance and distribution analysis on secreted proteins from ten species of arthropod-borne pathogens that cause serious human pathologies, including malaria, Lyme disease, plague, tularemia, Q fever, rocky mountain-spotted fever, human granulocytic anaplasmosis, human monocytic ehrlichiosis, scrub typhus, and Carrion's disease. This analysis allowed for the identification of three major patterns of cysteine expression in secreted proteins of arthropod-borne pathogens. For comparison purposes, a similar abundance and distribution analysis was conducted for another amino acid residue of low abundance in proteins: tryptophan. The significance of the patterns described is discussed in the context of microbial evolution, host-parasite relationships, and prospects for the development of vaccines directed to control several ABDs simultaneously.

2. Materials and Methods

2.1. Genomes Analyzed. Ten arthropod-borne pathogens were selected for cysteine- and tryptophan-distribution analysis, including five species of Alphaproteobacteria (*Anaplasma phagocytophilum*, *Bartonella bacilliformis*, *Ehrlichia chaffeensis*, *Orientia tsutsugamushi*, and *Rickettsia rickettsii*), three Gammaproteobacteria (*Coxiella burnetii*, *Francisella tularensis*, and *Yersinia pestis*), one Spirochaetes (*Borrelia burgdorferi*), and one protozoan parasite (*Plasmodium falciparum*). In order to reveal potential links between the expression of secreted cysteine-free proteins (sCFPs) and/or secreted tryptophan-free proteins (sWFPs) with virulence and pathogenicity, the genomes of two nonpathogenic bacteria harbored by arthropods (*Wigglesworthia glossinidia* endosymbiont of *Glossina brevipalpis* and *Wolbachia* endosymbiont of *Drosophila melanogaster*) were included in the analysis.

2.2. Bioinformatics Tools and Procedures Used. FTP files of the genomes of selected species of arthropod-borne microorganisms were downloaded from NCBI Reference Sequences (RefSeq) and transferred to word processing files for analysis of cysteine- and tryptophan-abundance and distribution patterns. We limited the analysis to proteins of at least 200 amino acid residues in length because cysteine and tryptophan are low-abundance amino acids (average content of 1.38% and 1.09%, resp., in UniProtKB/Swiss-prot protein knowledgebase, release 57.2), and thus the significance of their absence is highly dependent on sequence length. Incomplete sequences or those with sequence ambiguities were not included and care was taken to minimize the degree of redundancy of the list of proteins analyzed by

manually removing exact copies. The selected sequences were then analyzed using the Simple Modular Architecture Research Tool (SMART) [15] to detect the presence of domains and motifs that can be used to assign functionality and to track evolutionary trends via analysis of orthologs. SMART has a built-in SignalP 3.0 predictor that was used to identify proteins secreted through a classical signal-peptide-dependent pathway [16], which allowed for the identification of a subset of proteins with cysteine or tryptophan residues confined to the predicted signal peptide segment.

3. Results

3.1. Frequency of CFPs and WFPs in the Genomes of Arthropod-Borne Microorganisms. While it is possible to retrieve the sequence of CFPs from the UniProtKB/Swiss-Prot database with the ExpASY ScanProsite tool using the $\langle\{C\}^*\rangle$ syntax for pattern recognition, a very important subset of proteins is overlooked: those that have cysteine residues confined to an amino-terminal segment predicted to be excised upon secretion via the signal-peptide-dependent pathway. The precursor of those proteins would not be cysteine free but the mature-secreted protein would be (Figure 1). For that reason, all proteins of at least 200 residues with cysteine residues confined to a 70-amino acid NH₂-terminal segment were analyzed for the presence of potential signal peptides. Similar attention was taken while screening for the presence of WFPs. The frequency of CFPs found in the genome of the 12 species of arthropod-borne microorganisms studied was found to be as low as 0.94% in *A. phagocytophilum* and as high as 16.8% in *B. burgdorferi*. For WFPs, the lowest percentage (3.86%) was found in *C. burnetii* and the highest (16.21%) in *B. burgdorferi* (Table 1). When only those proteins predicted to be secreted through the signal-dependent pathway were considered (sCFPs and sWFPs), a clear difference was detected with preferential secretion of WFPs by four species of Alphaproteobacteria (*A. phagocytophilum*, *E. chaffeensis*, *O. tsutsugamushi*, and *R. rickettsii*) and the only eukaryote included in the study, *P. falciparum*. In contrast, the other arthropod-borne pathogenic bacteria (*B. bacilliformis*, *C. burnetii*, *F. tularensis*, *Y. pestis*, and *B. burgdorferi*) preferentially secrete CFPs. Of interest, the two arthropod endosymbionts included in the study (*W. glossinidia* and *W. endosymbiont* of *D. melanogaster*) do not have preference for the secretion for either CFPs or WFPs (Table 1). When the ratio of sCFPs to sWFPs was plotted against the percentage of sCFPs, a clear separation of pathogenic and nonpathogenic microorganisms became evident in a logarithmic plot (Figure 2). *P. falciparum* and four species of pathogenic Alphaproteobacteria segregated into the lower left corner of the plot while *B. bacilliformis*, *B. burgdorferi*, and the three species of pathogenic Gammaproteobacteria segregated to the upper right corner of the plot. The two species of arthropod endosymbionts remained at the center of the plot.

3.2. CFPs and WFPs in Functionally Defined Proteins Secreted by Proteobacteria. In order to define the biological

>gi|110671084|ref|YP_667641.1| lipoprotein [*F. tularensis*]

*MYFFANLNMRIILMKLNKSTLLLSLLGVTLSS***CS***SLK*ATKDNRDPFESYNRKYAFNDKAYETLTPAANSYEVKVPDPTLKSGLFNI FQNLAEPARVANDM
FQGEWDYAGDDGFRFLTNTTLGLAGYFDVADSWFNKPMRYHQSFVAVTLHKWGVYKDNKASPYVWVPLIGPGLTLEDITTTGVDALFNPLTYIFFFAPVGSAS
WGVSVGTTGAYVNVQGVSYLPSYSNLKEVSDPYIAMRNAYLQNYDYGMAKVLKQQLSKDDATIQTDQAVLGVVLGLSDNVNAQIATTGGTKVKSSEPPVI
FKSNI STVNKASQVEEAFDQDYSNDSTALDLANVDNDDTTQSEASKLKEIRDAKADLPGDADSPASAVQTLAEQG

>gi|108811011|ref|YP_646778.1| adhesin system component [*Y. pestis*]

*MNSKLYKLI***FCRR***LGCLIA*VGEFTRSYGRAFS SKGGQAGNNQRRVAVGILSRLAMMTGLALGIFPLLVLAHPVLPVNGHVVIQGGMLDQSSSTLTVTQQTDK
LA INWDSFDIAHGHSVIYAQPQSQSIALNQVQGSASQIYGRLLQANGQVFLNPRGILFGKEAQNVVGGVLA STKYMSNPEFLSGDYRLIGGESEGNIIINQ
ANLR SAPGGYIALVGNRIDNQRSGSITTPQGNVLA VGHVSVTLNLDHGNL LGVQIQGETVAALIQNGGLIQADGGVIQLTAKGKMDLMDTVIDNTGILQAK
GLSAKNGAIYLDGGGEGVVSQMGITDNNQQRGGRVVEGKRIYLNKNSNIKAKGTAGGGTVLVGGGAGKAKIIRSEMPRLW

>gi|121602140|ref|YP_989591.1| surface antigen/outer membrane protein, OMP85 family [*B. bacilliformis*]

*MI***QAKVLLQSGILRTA***LGL***LVFSFPQPLAA***FE*IFGIHLFGKESNAPSDGIQGAQKSYKVDVVA PPGAPLEGVKIVKTVS LVADQDKSVSSSAGLLA
KARSNYREILSALYADGRYGGVVISIKINGLEVADVSPMTELPDKSTIVITVDAGPQYVFNVRINKASPLAKYKTGGMISVEDLGKYGALAKSETILNAE
RWAIEGWRQRGYAKADVSRDIVADHARRIDAQIVVDPKQKAYYGSINVRDVNKHPHIDPMYVWMTGLKPGQYDS EAVAKANKRLARLDVFRSIVDRE
ADTINSDGHLPLTVVVEERKLRRLLKMGGSYSTLDGSGEYIYTHSNLFGHAERFKIETKINSIGRRKEQSYHPKNFNLYLLEVTFKPGIITPDDDLGAEK
VQRDVLDSEYITTAIKGKLSLTHVINDNLSGRVAMVITRGLHLDNHLGNRFVTVIGLQSDLIYDSRNNKLNATKGLYSEVILNPFYEMNLS S FMTKMTVEGR
SYWVLDTKNRFVATRAKLGITIGNNKILLPPDVLFFSGGGSVRGYAYHNIGIKTENGII GGRSLIEGSAELRFLNDDIGLVS FVDGGRVEKANFGFL
QKIKWGMIGGRYMTGLGPVRFVAVWPKREKGDPRIGIYVGIQAF

>gi|124805602|ref|XP_001350486.1| blood stage antigen 41-3 precursor [*P. falciparum*]

*MLLRHNS***F***IC***II***LVLC***SVQR***LS***D**EQNINDWPIDFEYNSKSLPSIEVKLSPENPLPQVSAEIKILESARLKEEGMMQKLEDEYNSLSMAKLIKDTV
ENSLSIFNDPNILSVISNSVKILKKKNLRKIKETTDEEKTSDNVSQMYERKGGPLPPELRKHTLFLQNYLNKTI PSVKISLTEISEPSVLIKEKIEE
IEQYRTDEEVTMFETAISELDILTDTIMLELEKQMLQLNPLVDKQIVHRSLEKELKEMGKAEQRENVNKSQTQS S FLEQEEENTGNILNVKISQTDY
SYPTIDELVMQMQRKRDITEKLERQKILELQMKLLKAQSEMIKDALHFSISKVIAQYSPIVETLKLQTLKNF

FIGURE 1: Amino acid sequence of proteins secreted by four arthropod-borne pathogens (*F. tularensis*, *Y. pestis*, *B. bacilliformis*, and *P. falciparum*) with cysteine residues (shaded in gray) confined to the predicted signal peptide segment (boxed italics) of their precursors. Signal peptide excision sites were identified using the SMART online tool. The number of cysteine residues present in the signal peptide segments shown varies from one in *F. tularensis* lipoprotein to four in *P. falciparum* blood stage antigen. The mature proteins produced following the removal of signal peptide segments during the secretion process are free of cysteine residues.

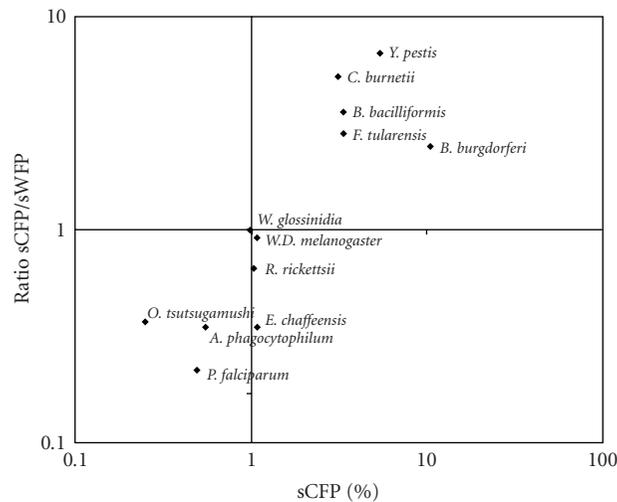
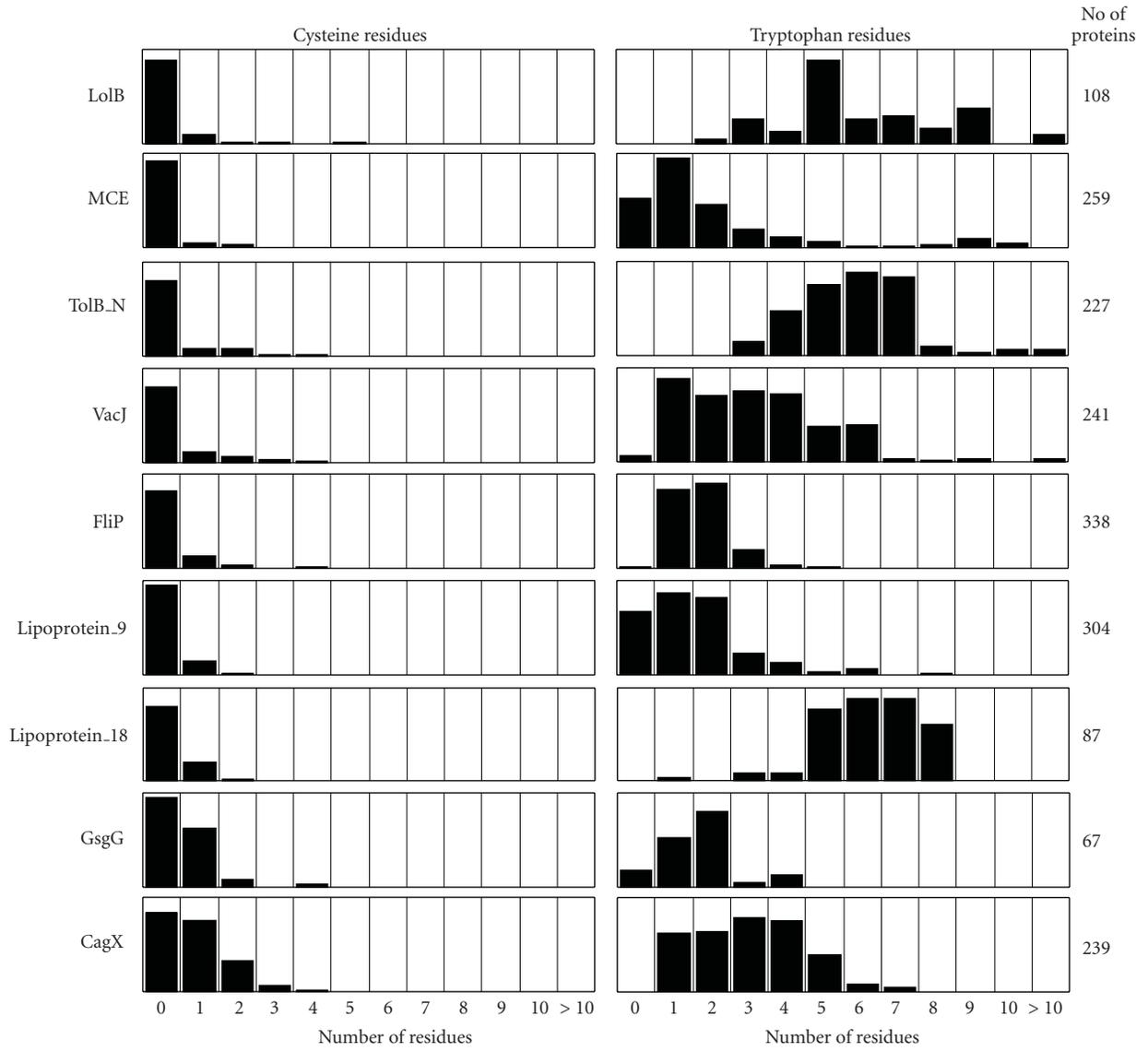


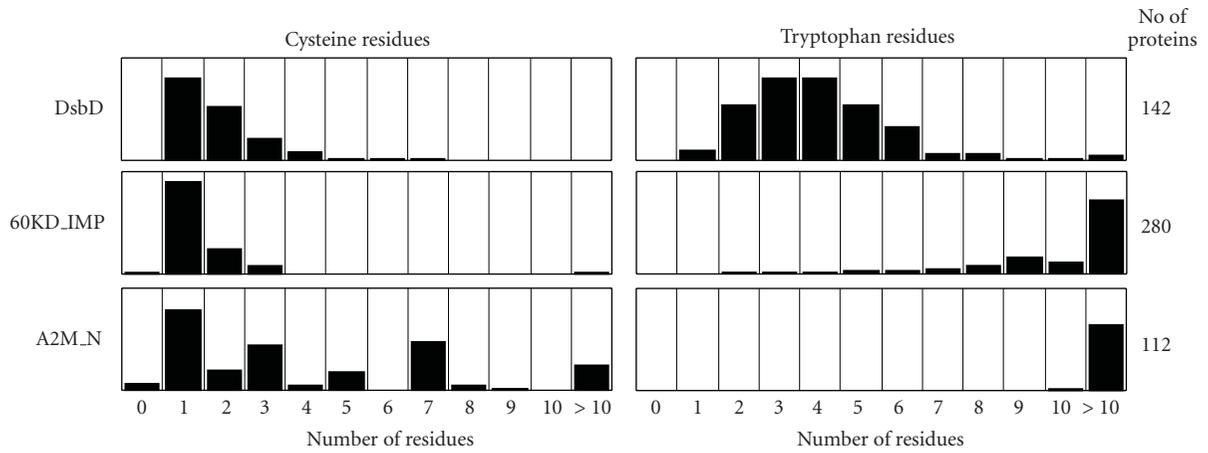
FIGURE 2: Frequency of sCFPs (percentage) plotted against a ratio of sCFP/sWFP in 12 arthropod-borne microorganisms. A logarithmic scale of the x and y axes was used to improve visual separation of microorganisms with low sCFP/sWFP ratios.

significance of the absence of cysteine or tryptophan residues in proteins secreted by arthropod-borne pathogens, it is necessary to determine the abundance of such residues in the corresponding orthologs secreted by other pathogenic and nonpathogenic microorganisms that are not transmitted by arthropods. We selected to conduct this analysis in Proteobacteria because it is a large taxonomic group

with 466 complete genomes sequenced, including many species of pathogenic bacteria with well-characterized virulence and pathogenicity determinants. Furthermore, the majority of the arthropod-borne microorganisms included in this study (10 out of 12) belong to the Proteobacteria phylum. We selected 28 different families of proteins secreted by Proteobacteria and belonging to



(a)



(b)

FIGURE 3: Continued.

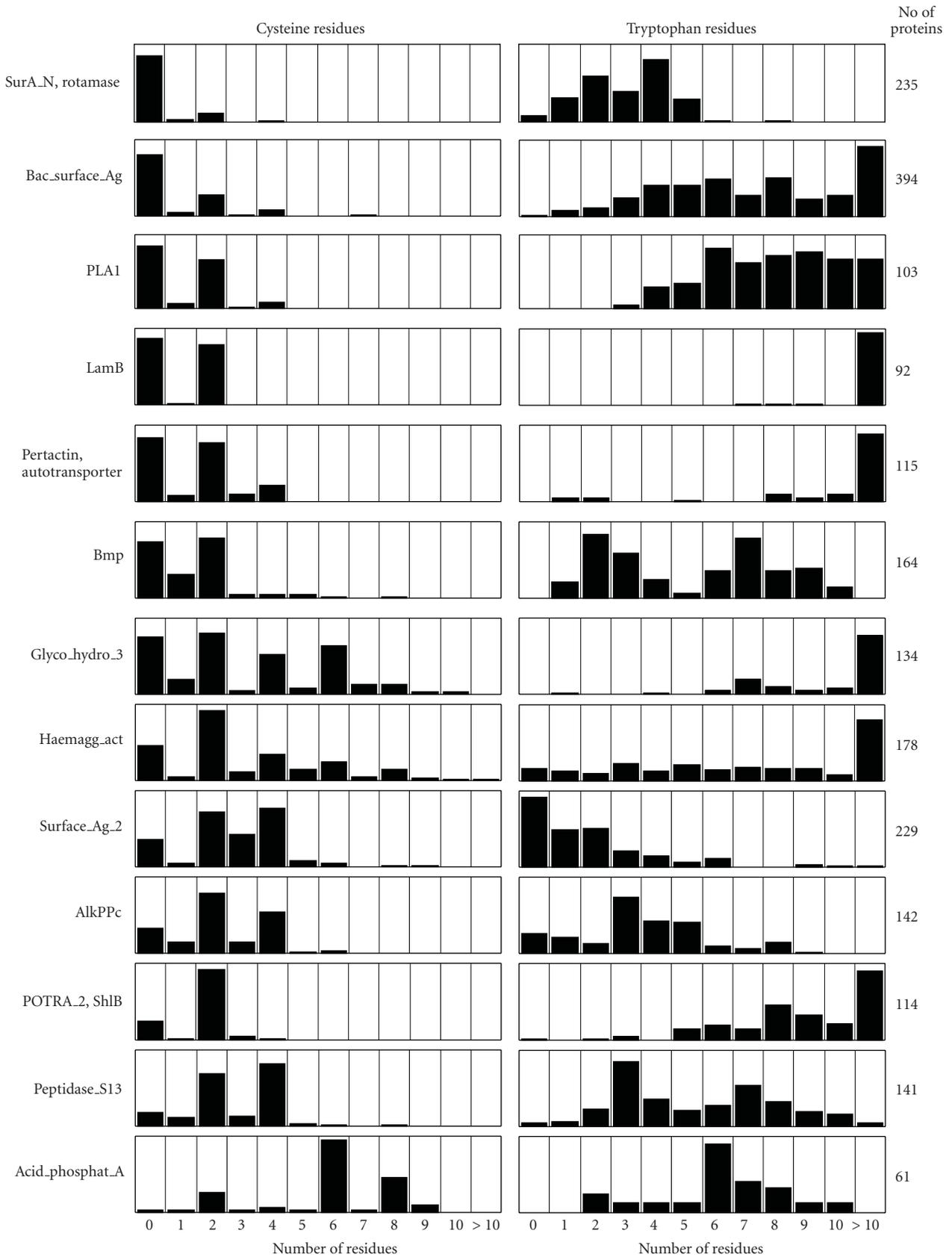


FIGURE 3: Continued.

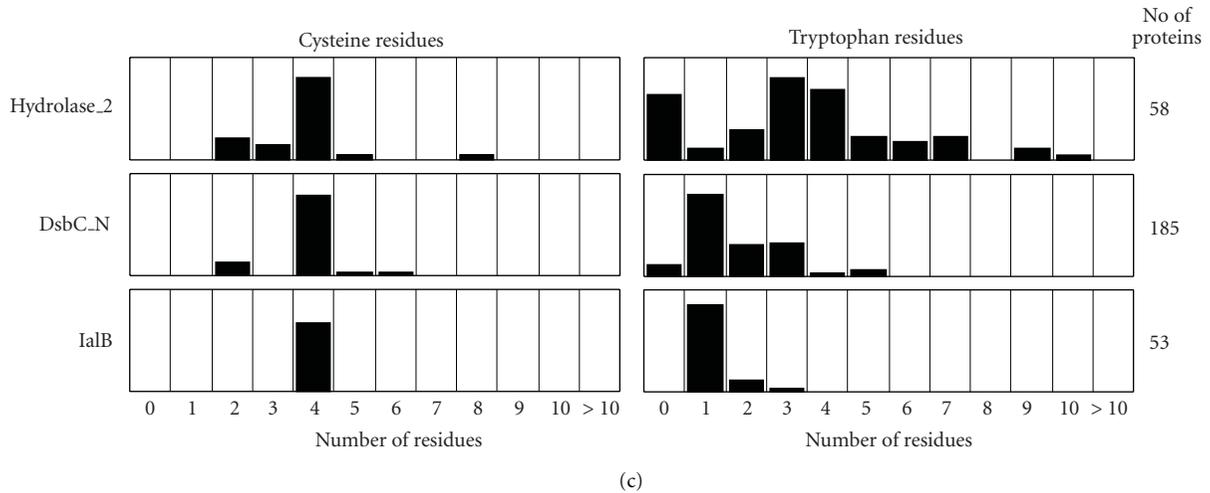


FIGURE 3: Abundance and distribution analysis of cysteine (left panel) and tryptophan residues (right panel) in secreted proteins belonging to 28 different families and secreted by many species of pathogenic and non/pathogenic Proteobacteria. The protein families were arranged by cysteine abundance and distribution to optimize the visualization of patterns.

TABLE 1: Abundance of proteins devoid of cysteine residues (CFP) or tryptophan residues (WFP), included those predicted as secreted (sCFP and sWFP), in 12 species of arthropod-borne microorganisms.

Species	Proteins*	CFP (%)	WFP (%)	sCFP	sWFP	sCFP/sWFP
Alphaproteobacteria						
<i>A. phagocytophilum</i> HZ	1264	12 (0.94)	77 (6.09)	7	20	0.35
<i>O. tsutsugamushi</i> str. Boryong	1182	15 (1.26)	71 (6.00)	3	8	0.37
<i>E. chaffeensis</i> str. Arkansas	1105	20 (1.80)	133 (12.03)	12	34	0.35
<i>R. rickettsii</i> str. "Sheila Smith"	1345	40 (2.97)	99 (7.36)	14	21	0.66
<i>W. endosymbiont of D. melanogaster</i>	1195	36 (3.01)	90 (7.53)	13	14	0.92
<i>B. bacilliformis</i> KC583	1282	86 (6.70)	74 (5.76)	43	12	3.58
Gammaproteobacteria						
<i>W. glossinidia</i> endosymbiont of <i>G. brevipalpis</i>	611	26 (4.25)	71 (11.62)	6	6	1.00
<i>F. tularensis</i> subsp. <i>tularensis</i> FSC198	1605	78 (4.85)	133 (8.28)	54	19	2.84
<i>C. burnetii</i> RSA 493	2016	114 (5.65)	78 (3.86)	63	12	5.25
<i>Y. pestis</i> Nepal 516	3981	339 (8.51)	157 (3.94)	216	32	6.75
Spirochaetes						
<i>B. burgdorferi</i> B31	851	140 (16.45)	138 (16.21)	89	36	2.47
Apicomplexa						
<i>P. falciparum</i> 3D7	5267	64 (1.21)	567 (10.76)	26	115	0.22

*Chromosomal proteins only.

different functional categories to conduct the cysteine- and tryptophan-abundance and distribution analysis. The domains identified in the proteins selected for this analysis allowed for access (using a SMART window to proteins of similar composition) to the sequence of orthologs present in other species of Proteobacteria. The number of cysteine and tryptophan residues present in the mature proteins selected was recorded and a figure indicating their relative abundance was visually inspected for emergence of patterns (Figure 3). When considering the abundance and distribution of cysteine residues in these

protein families, three different patterns were detected. Pattern-I, which was found in proteins carrying 9 different domains (LolB, MCE, TolB N, VacJ, Flip, Lipoprotein_9, Lipoprotein_18, CsgG, and GagX), is characterized by a predominance of CFPs over proteins expressing one or more cysteine residues. Pattern-II, which was found in proteins carrying the DsdB, 60KD_IMP, or A2M_N domains, shows a predominance of proteins expressing one cysteine residue and rarely expressing CFPs. Finally, Pattern-III was found in proteins expressing 16 different sets of domains (Sur_A_N/Rotamase, Bac_surface_Ag, PLA1,

TABLE 2: Domain and functional groups associated with each cysteine-abundance and distribution-pattern detected in pathogenic and nonpathogenic Proteobacteria.

Patterns	Functional category	Domains
Pattern-I	Lipoproteins	CsgG, Lipoprotein_9, Lipoprotein_18, LolB, VacJ
	Part of protein-secretion systems	CagX, FliP, MCE, TolB_N
Pattern-II	Endopeptidase inhibitory activity	A2M_N
	Generation of reduced environment in periplasm	DsdB
	Protein insertion into membranes	60KD_IMP
Pattern-III	Adhesin	Haemagg_act, Pertactin, Autotransporter
	Invasin	IalB
	Hemolysin	POTRA_2, ShlB
	Enzyme	PLA1, Acid_phosphatase_A, AlkPPc, Glyco_hydro_3, Hydrolase_2, Peptidase_S13
	Surface antigen	Bac_surface_Ag, BMP, Surface_Ag_2
	Membrane transporter	LamB

LamB, Pertactin/Autotransporter, Bmp, Glyco_hydro_3, Haemagg_act, Surface_Ag_2, AlkPPc, POTRA_2/ShlB, Peptidase_S13, Acid_phosphatases_A, Hydrolase_2, DsbC_N, and IalB) and was characterized by a wider variety of cysteine abundance, but with a clear preference for even numbers of residues. It is possible that this segregation into 3 patterns is a reflection of a functional spectrum defined by structural requirements of reduced sulfhydryl groups (as in proteins of Pattern-II), correct folding and rigidity conferred by a defined number of disulfide bonds (as in proteins of Pattern-III), and high flexibility and promiscuous interactivity conferred by the absence of cysteine residues (as in proteins of Pattern-I). In support of this interpretation, a correlation was found between functionality and cysteine abundance and distribution patterns for proteins secreted by Proteobacteria (Table 2). In contrast to the clear definition of cysteine-expression patterns in the proteins analyzed, no clear patterns emerged when analyzing the abundance and distribution of tryptophan residues. With the exception of proteins carrying the Surface_Ag_2 domain (which have a dominant WFPs profile) and those carrying the DsbC_N and IalB domains (which preferentially express a single tryptophan residue), no particular pattern of tryptophan residues was observed, with most proteins expressing a variable number of tryptophan residues, frequently more than 10 per protein.

3.3. Secreted CFPs in Pathogenic Arthropod-Borne Proteobacteria. Many sCFPs were found in pathogenic arthropod-borne Proteobacteria, including 79 in Alphaproteobacteria and 333 in Gammaproteobacteria (Table 1). In order to identify those that could represent virulence and pathogenicity determinants or potential vaccine targets, we eliminated from consideration those with domains detected in sCFPs of any of the arthropod endosymbionts included in the study. Still, a large list of sCFP-associated domains remained with 42 present in Alphaproteobacteria (Table 3) and 137 in Gammaproteobacteria (Table 4). In addition to these proteins, a group of sCFPs, characterized by the absence of any of the domains

TABLE 3: Domains associated with secreted CFPs in four species of arthropod-borne Alphaproteobacteria.

Species	Domains
<i>A. phagocytophilum</i>	OEP, Peptidase_M16, Peptidase_M17, Peptidase_S49, TPR_2
<i>B. bacilliformis</i>	ABC_sub_bind, Ami_3, Autotransporter , Bac_surface_Ag , BPD_transp_1, DPBB_1, DUF165, DUF374, DUF541, FlgH, FlgI, FliP , HAMP, HATPase_C, HisKA, HlyD, Lipoprotein_9 , LolA, MotA_ExbB, OEP, Oxidored_q3, PDZ, Peptidase_M23, Peripla_BP_2, Pertactin , Porin_2, SBP_bac_9, Surf_Ag_VNR , TolB_N , PD40 , Trypsin, TSPc
<i>E. chaffeensis</i>	Bac_surface_Ag , BON, OEP, PDZ, Peptidase_S49, Surf_Ag_VNR , TerC, Trypsin
<i>R. rickettsii</i>	AAA, Bac_surface_Ag , DUF6, FTR1, OmpW, PBPb, PDZ, Peptidase_M23, Surf_Ag_VNR , Trypsin, TSPc

Bold font domains selected for definition of cysteine-abundance and distribution patterns (Figure 3).

registered in the SMART and Pfam databases and conformed by segments of low complexity, intrinsic disorder, coiled-coil structure, transmembrane regions, and/or internal repeats, was found among the CFPs secreted by these pathogens. These sequences were found to be particularly abundant in Gammaproteobacteria, with 14 present in *C. burnetii*, 22 in *F. tularensis*, and 28 in *Y. pestis*. Only one was found in both *A. phagocytophilum* and *O. tsutsugamushi*, three in *R. rickettsii*, four in *E. chaffeensis*, and seven in *B. bacilliformis*. One strategy to identify proteins of interest in such a large list of candidates is to focus on those with the longest sequence of amino acid residues. After all, the significance of absence of a particular residue in a protein is highly dependent on protein length. When only the precursors of at least 500 amino acid residues were considered, few domains were identified (Table 5). Of these, the Pertactin and Autotransporter domains are of interest to understand

TABLE 4: Domains associated with secreted CFPs in arthropod-borne Gammaproteobacteria.

Species	Domains
<i>C. burnetii</i>	AAL_decarboxy, Acid_phosphat_B, Bac_surface_Ag , Bac_Ubq_Cox, BPD_transp_1, Cation_efflux, DUF2066, DUF502, FKBP_N, FtsQ, FtsX, HlyD, Lipoprotein_9 , LolB , Lys M, MCE , MotA_ExbB, MreC, MVIN, OmpA_membrane, OpuAC, Oxidored_q1, PDZ, Peptidase_M16, Peptidase_M22, Peptidase_M23, Peptidase_M50, Peptidase_S24, PNTB, Polysacc_deac_1, POTRA_1, Sec_GG, SecD_SecF, SecY, SLT, SPOR, Surf_Ag_VNR , TolB_N , PD40 , Toluene_X, Trypsin, TSPc
<i>F. tularensis</i>	Bac_surface_Ag , DUF1239, DUF165, G_glu_transsept, GDPD, HlyD, LolB , Lys_M, MCE , Neur_chan_LBD, OEP, PD40 , Peptidase_M50, Peroxidase, PQQ, RmuC, SBP_bac_9, Sec_GG, SecD_SecF, Secretin, SOUL, Sugar_tr, SurA_N , Surf_Ag_VNR , TolB_N , VacJ , VWA, WZZ
<i>Y. pestis</i>	Abhydrolase_BP_2, ADC_lyase, Ami_2, ArsB, AsmA, Autotransporter , Bac_surface_Ag , BON, BPD_transp_1, Cache_1, CsgG , DPBB_1, DUF1284, DUF1454, DUF1471, DUF187, DUF218, DUF481, DUF541, DUF6, DUF799, DUF945, Ell_Sor, FKBP_N, FlgI, FlIP , FMN_bind, GDPD, Glyco_hydro_3 , GntP_permease, Haemagg_act , HAMP, HATPase_C, HIM, HisKA, HlyD, KdgM, KdpC, Kelch_2, Lactamase_B, LamB, Lipase_GDSL, Lipoprotein_18 , Lipoprotein_9 , LolB , Lyase_8, Lyase_catalyt, Lyase_N, Lys M, MA, MalM, MgtC, MotA_ExbB, MS_channel, OEP, Omptin, OmpW, Oxidored_q1, PAS, Patatin, PbH1, PBP5_C, PBPb, PD40 , PDZ, Pectate_lyase_2, Peptidase_M16, Peptidase_M23, Peptidase_M48, Peptidase_S11, Peripla_BP_1, Peripla_BP_2, Pertactin , PG_binding_1, Pili_assembly_N, PLA1 , Plug, Polysacc_deac_1, Porin_1, PQQ, Rotamase , RpiB, SBP_bac_1, SBP_bac_5, SBP_bac_7, SBP_bac_9, Secretin, Slp, SLT, SPOR, STAS, Sulfatase, SurA_N , Surf_Ag_VNR , TM_helix, Tol_tol_Ttg2, TolB_N , Toluene_X, TonB, TonB_dep_Rec, TPR_2, Transgly, Trypsin, TSPc, UPF0118, VacJ , VWA, YscJ_FliF, ZipA_C

Bold font domains selected for definition of cysteine-abundance and distribution patterns (Figure 3).

the role of sCFPs in the immunobiology of ABDs because they have been identified as virulence and pathogenicity determinants [17, 18] and because both have been found to be coexpressed in very large sCFPs, including a *Y. pestis* protein of 3710 amino acids (GenBank accession number gi: 45443160). Another domain of interest, Bac_surface_Ag, represents a potential target for multipathogen vaccines directed to sCFPs because it is the only domain found associated with sCFPs of six species of arthropod-borne Proteobacteria (Table 5) and in a 821-amino acid residue

outer membrane CFP secreted by *B. burgdorferi* Spirochetes (GenBank accession number gi: 15595140).

4. Discussion

Several technical hurdles have complicated the development of effective vaccines to control the transmission of ABDs, including (1) identification of protective epitopes expressed on the pathogen and/or its arthropod vector, (2) induction of safe and long-lasting immunity that overcomes immune evasion mechanisms used by the pathogens and amplified by immuno-modulators present in arthropod saliva [19, 20], and (3) induction of an immunity expected to operate effectively in human populations of tremendous diversity. Some of these problems derive from the assumption that the induction of species-specific immunity is the most effective approach to develop vaccines to prevent infectious diseases. Research into an alternative approach, the induction of immunity of broad specificity, can benefit from the great diversity of pathogens and vectors involved in the etiopathogenesis of ABDs in order to identify the cross-reactive epitopes required for the induction of cross-protective immunity. Just as adaptation of arthropods to hematophagy is an example of convergent evolution [21], the adaptation of microbial pathogens to the complex biological processes that guarantees their survival and multiplication in two very different kinds of hosts, vertebrate and invertebrate, can be seen as an example of convergent evolution as well. Under such consideration, the identification of common structural determinants in molecules secreted by pathogens into the host-parasite interface might shed light into vulnerabilities of the host defense systems, providing opportunities for the design of alternatives to control ABDs. The availability of complete genomes in many of the etiologic agents of ABDs offers the possibility to identify, via the comparative analysis of their secretomes described herein, such structural determinants.

4.1. Cysteine Residues in Protein Function and Antigenicity/Immunogenicity. One of the amino acid residues with a prominent effect on the structure of proteins, cysteine, can be expected to play critical roles in protein function, antigenicity and immunogenicity. The role played by cysteine residues in protein function has been demonstrated in numerous experiments using chemical modification techniques and cysteine scanning mutagenesis [22, 23], but its role in antigenicity and immunogenicity of proteins is only beginning to be characterized using the same techniques [24]. The flexibility that characterizes a group of proteins with a clear cysteine use bias, Intrinsically Unstructured Proteins [10–13], can play a prominent role in host-parasite relationships by favoring protein-protein interactions of low affinity and specificity. From an interactomics perspective, this kind of protein-protein interactivity might favor the interaction of pathogen-secreted proteins with nondefense proteins present in host tissues, thus interfering with pathogen recognition by defense proteins [25]. As a result, immunization with these cysteine-depleted proteins

TABLE 5: Domains associated with sCFPs of at least 500 residues in length and present in more than one species of the indicated species of arthropod-borne Proteobacteria: *A. phagocytophilum* (A.p), *B. bacilliformis* (B.b), *E. chaffeensis* (E.c), *R. rickettsii* (R.r), *C. burnetii* (C.b), *F. tularensis* (F.t), and *Y. pestis* (Y.p).

Domains	A.p	B.b	E.c	R.r	C.b	F.t	Y.p
PBP_dimer/Transpeptidase	—	—	—	—	1	1	—
Peptidase_M16	1	—	—	—	—	—	1
Pertactin/Autotransporter	—	1	—	—	—	—	3
Sec_GG/SecD_SecF	—	—	—	—	1	1	—
Secretin	—	—	—	—	—	1	1
Bac_surface_Ag	—	2	1	1	1	1	2

might shift the balance toward protective immunity against pathogens that secreted them [26].

4.2. Evolutionary Considerations on the Secretion of CFPs by Proteobacteria. Judged by the contrast between cysteine- and tryptophan-abundance and distribution expression patterns identified in 28 families of proteins secreted by Proteobacteria, a restrictive evolutionary process must be in operation to keep some of these proteins in a cysteine-free state (Figure 3). This is particularly evident in all proteins classified in Pattern-I which includes nine families of surface lipoproteins and components of protein-transport systems. This expression pattern is better explained by the idea that these families of proteins were derived from ancestors that were themselves free of cysteine residues. During the radiation of species a variable number of mutations might have occurred that introduced additional tryptophan residues in the proteins without restriction. While a similar process might have led to the diversification in the number of cysteine residues, a restrictive force must have kept it to a minimum. Given that this occurred in all species of Proteobacteria, including those known to be pathogenic and nonpathogenic to vertebrates and arthropods, it is unlikely that the immune systems of these hosts were responsible for the restriction. Most likely, the function of the proteins belonging to this group of proteins is seriously compromised by the introduction of cysteine residues. A somewhat similar mechanism might have operated during the evolution of the three families of proteins classified in Pattern-II. An ancestor with a single cysteine residue may have evolved under conditions that allowed the acquisition of cysteine residues but restricted its loss. The case of proteins carrying the A2M_N domain is peculiar because it is the only family of proteins where a variable, and odd number, of cysteine residues predominates. This suggests that while the accumulation of cysteine residues through the evolution of this family was fully permitted, those individuals with an odd number of residues had a survival advantage, probably because the function of the A2M_N domain, just as it is known to occur in vertebrate proteins carrying it, depends on the availability of a free sulfhydryl group [27]. One scenario that could explain the more complex cysteine-abundance and distribution analysis of the families of proteins classified in Pattern-III is that the ancestor of these protein families,

starting with an even number of cysteine residues, evolved under conditions that allowed either the acquisition or loss of cysteine residues. Once again the survival advantage of having, in this case, an even number of cysteine residues explains the overall pattern. Under this hypothetical scenario it becomes necessary to explain a peculiar jump in protein evolution. In the case of protein families carrying the Bmp, Glyco_hydro_3, Haemagg_act, Surface_Ag_2, AlkPPc, POTRA_2/ShlB, and Peptidase_S13 domains (which would be predicted to derive from ancestors with two cysteine residues and thus the potential to have a disulfide bond in the protein structure, Figure 3) could be transformed into a protein where the formation of disulfide bonds is no longer possible because of the loss of two cysteine residues. How much protein functionality is affected by the disappearance of the only disulfide bond present in the structure of these proteins? Similarly one could ask: How much is the function of a protein devoid of cysteine residues affected when accumulation of two cysteine residues allows the emergence of disulfide bonds in the protein structure? This complementary scenario could occur with proteins carrying the Sur_A/Rotamase, Bac_surface_Ag, PLA1, LamB, and Pertactin/Autotransporter domains.

5. Conclusions

It is apparent that cysteine residues play a dramatic role in protein function and that their removal, or introduction, into protein sequences represents a critical event of great significance in pathogen evolution and host-parasite relationships. The use of the a simple and novel bioinformatics tool, amino acid-abundance and distribution analysis, can prove useful in the clarification on some of these ideas and address others that were not included in the discussion, such as the role of sWFPs in host-parasite relationship, in particular in the context of two arthropod-borne diseases that are caused by pathogens that secrete them abundantly, *B. burgdorferi* and *P. falciparum*. The use of cysteine- and/or tryptophan-directed mutagenesis will prove instrumental in clarifying the role that these amino acid depleted proteins may have in the immunobiology of ABDs. It will also open to experimentation the usefulness of vaccines based on a new kind of epitope, one defined not by what it contains in its sequence, but rather by what it lacks.

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Research Article

Differential Midgut Attachment of *Leishmania* (*Viannia*) *braziliensis* in the Sand Flies *Lutzomyia* (*Nyssomyia*) *whitmani* and *Lutzomyia* (*Nyssomyia*) *intermedia*

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The interaction between *Leishmania* and sand flies has been demonstrated in many Old and New World species. Besides the morphological differentiation from procyclic to infective metacyclic promastigotes, the parasite undergoes biochemical transformations in its major surface lipophosphoglycan (LPG). An upregulation of β -glucose residues was previously shown in the LPG repeat units from procyclic to metacyclic phase in *Leishmania* (*Viannia*) *braziliensis*, which has not been reported in any *Leishmania* species. LPG has been implicated as an adhesion molecule that mediates the interaction with the midgut epithelium of the sand fly in the Subgenus *Leishmania*. These adaptations were explored for the first time in a species from the Subgenus *Viannia*, *L. (V.) braziliensis* with its natural vectors *Lutzomyia* (*Nyssomyia*) *intermedia* and *Lutzomyia* (*Nyssomyia*) *whitmani*. Using two in vitro binding techniques, phosphoglycans (PGs) derived from procyclic and metacyclic parasites were able to bind to the insect midgut and inhibit *L. braziliensis* attachment. Interestingly, *L. braziliensis* procyclic parasite attachment was ~ 11 -fold greater in the midgut of *L. whitmani* than in *L. intermedia*. The epidemiological relevance of *L. whitmani* as a vector of American Cutaneous Leishmaniasis (ACL) in Brazil is discussed.

1. Introduction

Leishmania (*Viannia*) *braziliensis*, the etiological agent of American Cutaneous Leishmaniasis (ACL), has the widest geographic distribution in the Americas and can be transmitted by the sand flies *Lutzomyia* (*Psychodopygus*) *wellcomei*, *Lutzomyia* (*P.*) *complexa*, *Lutzomyia* *migonei*, *Lutzomyia* (*Nyssomyia*) *whitmani*, *Lutzomyia* (*N.*) *intermedia* [1, 2], and *Lutzomyia* (*N.*) *neivai* [3]. From those, *L. (N.) whitmani* is considered of the highest epidemiological importance [4].

Leishmania parasites in their sand fly vectors spend their life cycle as flagellated promastigotes within the gut, where

they must confront several challenges to survive including the activity of digestive enzymes, the need to escape from the peritrophic matrix, elimination of unattached parasites from the sand fly gut with the digested blood products, and the need to develop infective forms which can be transmitted to the vertebrate host [5–8].

Many studies have demonstrated that the promastigotes' dominant surface lipophosphoglycan (LPG) protects the parasites against those adverse conditions preventing loss of the parasite in the gut for many species of the subgenus *Leishmania*. LPG has been biochemically characterized and implicated in the *Leishmania* specificity to

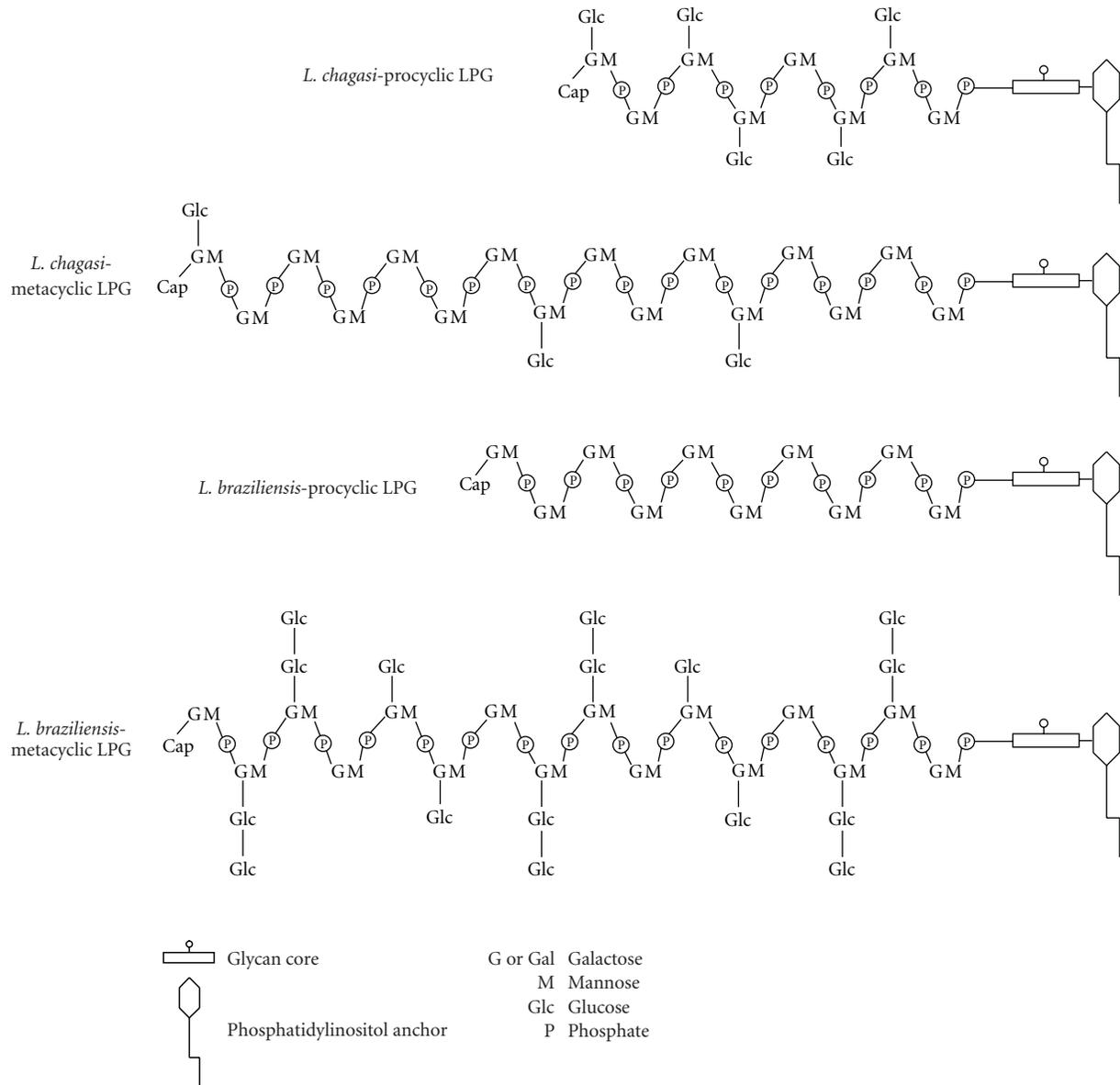


FIGURE 1: Opposite mechanisms in the glucose regulation in the LPGs from procyclic and metacyclic *L. chagasi* (syn. *L. infantum*) and *L. braziliensis* [9, 10]. The structure of the glycan core is Gal(α 1,6)Gal(α 1,3)Gal_f(α 1,3)[Glc(α 1-PO₄)-6]-Man(α 1,3)Man(α 1,4)GlcN(α 1,6) linked to 1-O-alkyl-2-lyso-phosphatidylinositol anchor. The repeat units are 6-Gal(β 1,4)Man(α 1)-PO₄.

different vectors [9, 11–17]. All LPGs have a conserved glycan core region of Gal(α 1,6)Gal(α 1,3)Gal_f(β 1,3)[Glc(α 1)-PO₄]Man(α 1,3)Man(α 1,4)GlcN(α 1) linked to a 1-O-alkyl-2-lyso-phosphatidylinositol anchor. LPG intra- and interspecific polymorphisms are in the size and variability of side-chains attached to the repeat unit Gal(β 1,4)Man(α 1)-PO₄ backbone and in the cap [18]. In *L. braziliensis* LPG, a novel mechanism in the carbohydrate regulation in the LPG side-chains was observed. The LPG from the procyclic form is devoid of side chains, while in the metacyclic phase it contains one or two β (1,3)glucose residues as side chains [10]. In other species such as the Indian strain of *L. donovani* and *L. infantum*, both belonging to the subgenus *Leishmania*, the opposite occurs, resulting in downregulation of

β (1,3)glucose residues during metacyclogenesis (Figure 1). The consequence is a loss of attachment by metacyclic LPG and midgut detachment of these *Leishmania* species [9, 15].

The developmental patterns of *Leishmania* within the sand fly gut do not depend on the vector but rather on the inherent behavior of the parasite species involved. Those behavior patterns were used to determine the currently accepted classification of *Leishmania* into the subgenera *Viannia* and *Leishmania* for species that show peripylarian and suprapylarian patterns, respectively [19]. Development of *Leishmania* (*Leishmania*) spp is restricted to anterior regions of the pylorus, beginning in the thoracic and abdominal midgut [20]. However, *Leishmania* (*Viannia*) spp involves an obligatory phase in the posterior gut (mainly in

the pylorus) prior to anterior migration and establishment of the parasites in the abdominal and thoracic midgut with following colonization of the foregut and mouth parts [21]. In this manuscript, we report for the first time the interaction of LPG with *L. whitmani* and *L. intermedia* midguts, vectors of ACL in Brazil.

2. Materials and Methods

2.1. Parasites. *Leishmania braziliensis* World Health Organization reference strain (MHOM/BR/75/M2903) was used. Starter cultures of promastigotes were grown in Medium 199 supplemented with 10% heat-inactivated FBS, penicillin (100 units/mL), streptomycin (50 µg/mL), 12.5 mM glutamine, 0.1 M adenine, 0.0005% hemin, and 40 mM Hepes, pH 7.4 at 25°C. Cells were grown at 26°C to a density of $1\text{--}1.2 \times 10^7$ cells/mL [10].

2.2. Purification of Metacyclic Cells. Parasites from stationary phase were harvested and resuspended in Medium 199 containing peanut agglutinin (PNA) from *Arachis hypogaea* (35 µg/mL). After 30 minutes incubation at room temperature, procyclic parasites that were agglutinated by the lectin (PNA+) were removed by low-speed centrifugation (150g, 5 minutes, 4°C). Non-agglutinated metacyclic cells remaining in the supernatant (PNA-) were washed 2 times by centrifugation with phosphate-buffered saline (PBS) (2100g, 15 minutes, 4°C) [10]. The yield of PNA+ and PNA- parasites was approximately 1.0×10^{11} and 5.0×10^9 cells, respectively.

2.3. Extraction and Purification of LPG from *L. braziliensis*. LPGs from procyclics (PNA+) and metacyclics (PNA-) parasites were extracted in solvent E (H₂O/ethanol/diethyl ether/pyridine/NH₄OH; 15:15:5:1:0.017), dried by N₂ evaporation, and resuspended in 0.1 N acetic acid/0.1 M NaCl. Then, they were applied to a column of phenyl-Sepharose (2 mL) equilibrated in the same buffer and LPG was eluted using solvent E. For binding studies, purified LPG was treated with PI-specific phospholipase C from *Bacillus cereus* (16 hours, 37°C). The dilapidated PG was separated from the cleaved lipid anchor by passage through a column of phenyl-Sepharose (2 mL) [22].

2.4. Midgut Binding Studies. *Lutzomyia whitmani* and *L. intermedia* sand flies were captured in Corte de Pedra, Bahia state, Brazil (13°26'23''S, 39°39'3''W). Taxonomical identification was performed prior to dissecting the midguts using the taxonomic key of Young and Duncan [23]. Binding of promastigotes was quantified by an in vitro technique [12]. Blood-unfed females maintained on 30% sucrose were dissected in PBS. Midguts (6–13 per group) were opened along the length of the abdominal segment with a fine needle, placed in concave wells of a microscope chamber slide, and incubated for 30 minutes with procyclic and metacyclic promastigotes (2×10^7 cells/mL, 50 µl). Then, guts were washed in successive drops of PBS and the number of attached parasites is determined with a Neubauer-counting chamber.

In a second experiment, the midguts were incubated for 20 minutes with PGs (10 µg/mL) derived from procyclic and metacyclic promastigotes, washed with PBS, and then incubated with procyclic promastigotes (2.0×10^7 cells/mL) for 20 minutes at room temperature. Controls were dissected guts incubated only with procyclic promastigotes. The guts were then individually washed and counted as described above.

For binding of purified PG to midguts in vitro, opened, dissected midguts were fixed with 2% formaldehyde in PBS (4°C, 20 minutes). After several washes in PBS, the guts were incubated for 20 minutes with PG (10 µg/mL) from procyclic or metacyclic parasites. After several washes, the guts were incubated in a 1:400 dilution of ascites containing the anti-LPG antibody CA7AE followed by incubation with fluorescein antimouse IgG (FITC) (1:1000). Stained guts were examined with a fluorescence microscope [9].

2.5. Data Analysis. The D'Agostino-Pearson omnibus test was made to test the null hypothesis—that the data are sampled from a Gaussian distribution—*P* value (*P* < .01) shows that the data deviate from Gaussian distribution. For this reason, nonparametric Kruskal-Wallis was performed to test equality of population medians among groups and independent samples. Data were analyzed by GraphPad Prism 4.0 software and *P* < .05 was considered significant.

3. Results and Discussion

Leishmania parasites have to face adverse conditions to accomplish their life cycle either in the invertebrate or in the vertebrate hosts [24]. For that purpose, the parasites developed a range of molecules represented by the secreted acid phosphatases (sAPs), glycoinositolphospholipids (GIPLs), filamentous proteophosphoglycans (fPPGs), lipophosphoglycans (LPGs), and lectins [25–27]. From these molecules, LPG is the most studied molecule and considered a multivirulent factor in *Leishmania* [28]. During the life cycle of *L. braziliensis* and other species of the subgenus *Viannia*, a crucial step for parasite survival is attachment to different parts of midgut. In this article, we focused on the interaction of this species with the midgut epithelium, where the parasites have to attach prior to arrival in the foregut and mouth parts. In the vector midgut, the ingested amastigotes need to transform into promastigotes. After metacyclogenesis, procyclic promastigotes differentiate in metacyclics, the infective forms to be passed to a new vertebrate host [20]. The relationship between stage differentiation and midgut adhesion has been previously reported for *L. major*, *L. infantum*, and *L. donovani* (India and Sudan). In those species, procyclic promastigotes attach to the midgut using the LPG, while metacyclic forms detach. In the case of *L. major*, the side chains comprising the LPG repeat units from procyclic parasites are often terminated with β(1,3)galactose, while metacyclic LPG side chains are capped with α(1,2)arabinose. In *L. donovani* from Sudan, procyclic LPG is devoid of side-chains, but metacyclic LPG increases in size, resulting in masking of the cap. The procyclic LPG of

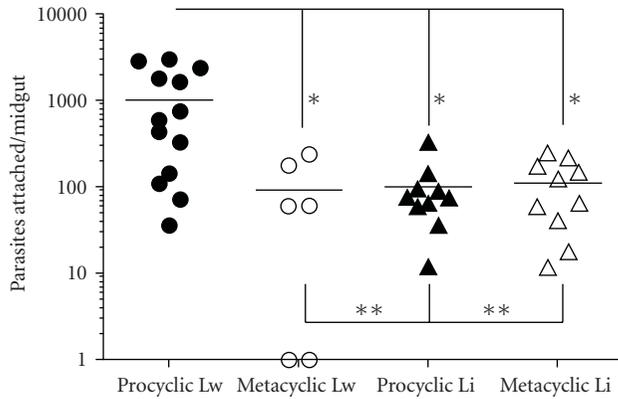


FIGURE 2: Differential attachment of *L. braziliensis* (procyclic and metacyclic) to *L. whitmani* (Lw) and *L. intermedia* (Li) midguts in vitro. * $P < .01$; ** $P > .05$. Data are the representation of two experiments.

L. infantum and *L. donovani* (India) has β -glucose residues that are downregulated in the metacyclic phase [9, 12, 14, 15].

Using a similar quantitative in vitro assay for the attachment of living *L. braziliensis* promastigotes, dissected midguts from *L. intermedia* and *L. whitmani* were analyzed. A differential pattern of attachment was observed in procyclic and metacyclic *L. braziliensis*. In *L. whitmani*, the average number of attached procyclics was ~ 11 -fold compared to the metacyclics (1027.71 ± 299.30 versus 90.00 ± 40.25 , $P = .0014$). For *L. intermedia*, the attachment of procyclics (101.40 ± 16.10) and metacyclics (112.80 ± 27.10) was very low and not statistically different ($P = .70$) (Figure 2). While comparing procyclic attachment between the two sand fly species, in *L. whitmani* the number of parasites that attached was ~ 10 -fold higher than in *L. intermedia* (1027.71 ± 299.30 versus 101.40 ± 16.10 , $P = .001$). Those data suggest that procyclic *L. braziliensis* were able to attach to the midgut with a mechanism different from hemidesmosomes seen in the pyloric and anterior regions [21], since they were able to easily detach and being counted in our in vitro system.

The number of metacyclic promastigotes that attach was very low in both *L. whitmani* and in *L. intermedia* ($P = .71$) (Figure 2) and therefore, only procyclics were used for the subsequent inhibition experiments. To test if the parasite attachment could be intermediate by LPG molecules, a competitive binding experiment was developed, where midguts were previously incubated with PGs derived from procyclic and metacyclic forms. Both PGs strongly blocked (~ 10 -fold) the attachment of procyclic *L. braziliensis* in *L. whitmani* and *L. intermedia* midguts (Figure 3) ($P < .0001$). Interesting observation arose regarding the unexpected binding of metacyclic PG (Figure 4) to the midguts and its inhibition of parasite attachment (Figure 3). The possible explanation is that the attachment of *L. braziliensis* metacyclics to the midguts of *L. whitmani* and *L. intermedia* was very low in numbers. Furthermore, *L. braziliensis* has 10–20 less LPG molecules expressed on its cell surface than

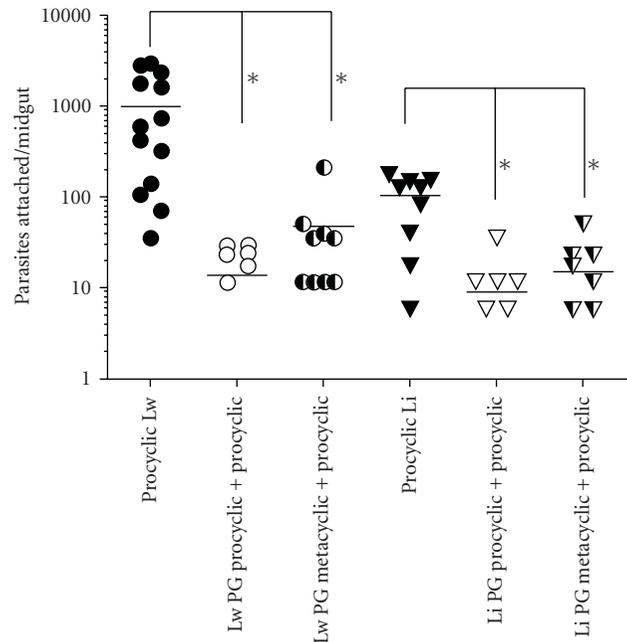


FIGURE 3: Inhibition of attachment of procyclic *L. braziliensis* to the midgut of *L. whitmani* (Lw) and *L. intermedia* (Li) in the presence of phosphoglycans (PGs) ($10 \mu\text{g/mL}$). Midguts were incubated with PGs from procyclic and metacyclic *L. braziliensis* and further incubated with procyclic promastigotes. Control midguts were incubated only with procyclic promastigotes. PG procyclic: PG derived from procyclics; PG metacyclic: PG derived from metacyclics. * $P < .0001$. Data are the representation of two experiments.

species from the subgenus *Leishmania* [10] and thus much less ligands for midgut attachment. These data also suggest the existence of an LPG-ligand in the sand fly midguts, which is necessary for *L. braziliensis* to establish and maintain the infection with further passage of the parasites towards the mouth parts. A galectin receptor for *L. major* LPG (subgenus *Leishmania*) in *P. papatasi* midgut was recently reported [29].

To confirm if PGs from procyclic and metacyclic promastigotes were recognizing any ligand in the midguts, the molecules were incubated with the dissected organs and revealed by immunocytochemical fluorescent staining with a specific antibody CA7AE, which recognizes PGs from procyclic and metacyclic *L. braziliensis* [10] (Figure 4). Consistent with our observations using live parasites, a similar pattern of stage-specific bindings was observed, where procyclic and metacyclic PGs were able to attach to opened *L. whitmani* and *L. intermedia* midguts (Figure 4(a) and 4(b)). The attachment of both types of PGs in this model may be attributed to the presence of $\beta(1,3)$ glucose residues in the procyclic promastigote cap and in the repeat units of metacyclics [10]. The role of $\beta(1,3)$ glucose residues in attachment was also demonstrated in *L. donovani* (India) [15] and *L. infantum* (Brazil) [9]. Those data suggest that upregulation of glucoses is necessary for *L. braziliensis* development. By adding glucoses in its LPG, perypilariam metacyclics might have to traverse midgut following their

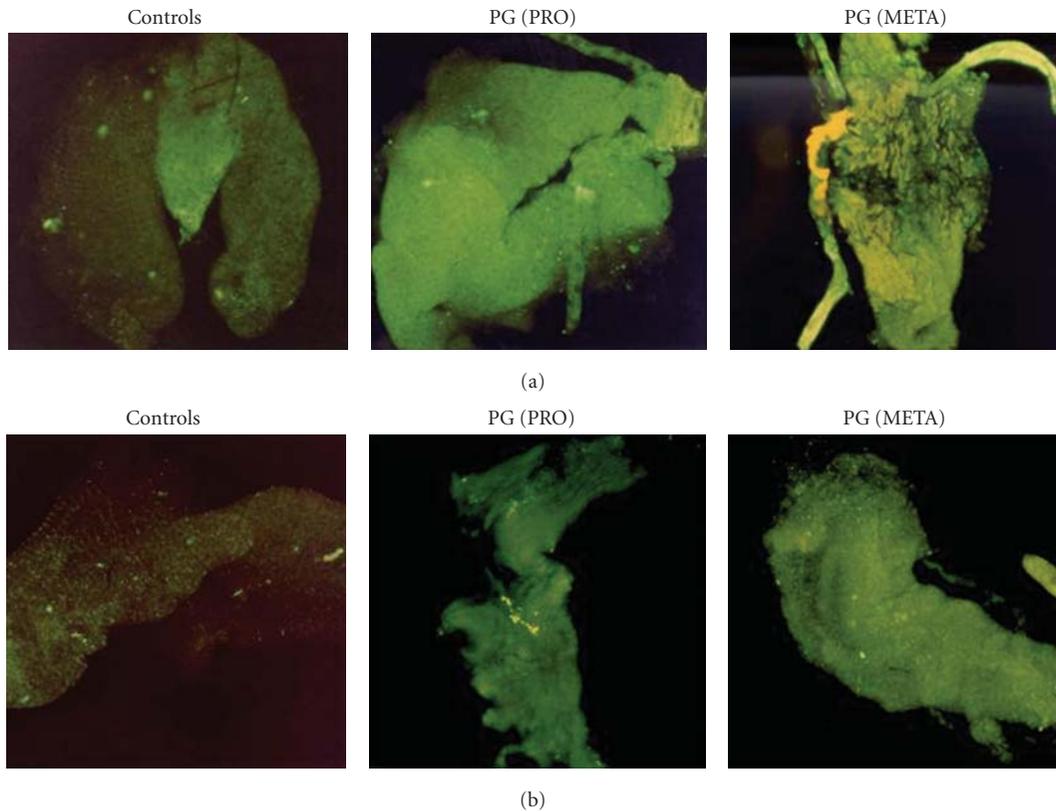


FIGURE 4: Fluorescent staining of *L. intermedia* (a) and *L. whitmani* (b) midguts incubated with PGs from procyclic (PRO) and metacyclic (META) *L. braziliensis*, probed with CA7AE antibody (1:400) and developed with FITC (1:1000). Control midguts were incubated with primary and secondary antibodies only.

early development in the hindgut. Given that the term metacyclic normally refers to the detached infective stage available for transmission in the subgenus *Leishmania*, in the subgenus *Viannia*, this term should perhaps be renamed as suggested elsewhere [26]. However, how those parasites would detach from the midgut is still a missing step, which cannot be demonstrated in our in vitro system. A possibility is that a “second metacyclic-like” stage, if any, could detach from midgut prior to migration to foregut.

Although both studied sand flies are known to be successful vectors, the adhesion of the parasites in *L. whitmani* was more pronounced (~11-fold) than in *L. intermedia*. Another interesting result is that procyclics were able to attach more than the metacyclics in *L. whitmani*, while in *L. intermedia* this attachment was low for both forms (Figure 2). This finding has a major epidemiological relevance, once *L. whitmani* is known to be the most important and widely distributed ACL vector [2, 4, 30–32] mainly in the Northeast of Brazil. On the other hand, *L. intermedia* is more concentrated in Southeast, especially in the State of Rio de Janeiro where is the main vector of *L. braziliensis* [2, 33–36]. However, in Corte de Pedra, State of Bahia, the border state between Northeast and Southeast, where the insects were captured for this study, both species occur sympatrically being able to transmit ACL all over the year [37].

Recently, it was shown that *L. longipalpis* was more efficient vector of *L. infantum* than *Lutzomyia evansi* [38]. Infection success was dependent on the establishment of the parasite in the midgut, which was very irregular in *L. evansi*. Consequently, those results explain the irregularity in the Visceral Leishmaniasis transmission where *L. evansi* occurs. To be considered a good vector, many conditions have to be followed: the distribution of the sand fly vector has to be coincident with the human disease; the insect must be found infected in the peridomestic or domestic areas and it has to feed avidly on man and many hosts [39]. However, if differences in *Leishmania* attachment have an impact on the efficacy of disease transmission by *L. whitmani* and *L. intermedia* still awaits further investigation.

4. Conclusions

In the Subgenus *Leishmania*, there are strong biochemical and genetic evidences that LPG is a critical molecule for the attachment process to sand fly midguts. However, for *L. braziliensis*, which makes less LPG [10], the role of this molecule seems to be necessary at least during the transient parasite passage through the midgut. The term metacyclics refers to the vertebrate infective parasite stage able to detach from the midgut as it was observed in the species from the subgenus *Leishmania*. Nonetheless, in

the subgenus *Viannia*, we found two patterns of metacyclic attachment: (1) similar to the Subgenus *Leishmania* as observed for *L. whitmani*, a very competent vector and (2) very low as observed in *L. intermedia*, where LPG seems to have a less important role. We thus conclude that the unusual pattern of attachment of *L. braziliensis* in the midgut may be a result of its perypylarian behavior related with the vector susceptibility/specificity. Yet to be elucidated is how other glycoconjugates may be critical on the anterior migration of the parasite to the mouth part in the sand fly digestive tract. In the species of the Subgenus *Viannia*, the pattern of membrane glycoconjugates is different from those of the Subgenus *Leishmania*, which results in higher expression of GPIs in *L. braziliensis* and *L. panamensis* [40]. However, the current study reinforces our understanding that LPG may still play a key role in the interaction with those vectors.

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Letter to the Editor

The Vectorial Potential of *Lutzomyia (Nyssomyia) intermedia* and *Lutzomyia (N.) whitmani* in the Transmission of *Leishmania (V.) braziliensis* Can Also Be Related to Proteins Attaching

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We read with great interest the report by Soares et al. [1] on the potential of *Leishmania (Viannia) braziliensis* to attach to the midgut of the sand flies *Lutzomyia (N.) whitmani* and *Lutzomyia (N.) intermedia*. This manuscript assesses relevant information concerning the biomolecular phenomena between *Leishmania* promastigotes and the midgut of *Lutzomyia* species that act as vectors of American Cutaneous Leishmaniasis (ACL). However, it is necessary to comment that the basis of these physiological processes is not directly driven by glycolipid lipophosphoglycan (LPG) only. Other *L. (V.) braziliensis* promastigotes surface components, as proteins, can also be implicated in many steps of the midgut attachment.

Since 2007 we have been investigating the potential of heparin binding proteins (HPBs) from *L. (V.) braziliensis* promastigotes in the attachment of parasites to gut proteins from *L. (N.) intermedia* and *L. (N.) whitmani* [2]. We have indicated the existence of physicochemical conditions for the binding between the gut proteins from *Lutzomyia* spp. and the HPBs—a new macromolecule class involved in the recognition of the sand fly gut epithelium by *L. (V.) braziliensis*. We proposed that the five HPB ligands (67.0, 62.1, 59.5, 56.0, and 47.5 kDa) observed in both *L. (N.) intermedia* and *L. (N.) whitmani* are involved with the promastigote attachments to sand fly gut epithelium. Also, we suggested that the physicochemical conditions for the interaction between HBP and their ligands are more favourable in the midgut of *L. (N.) whitmani* than in

L. (N.) intermedia. Furthermore, heparin similar molecules, synthesized by cells of midgut epithelium seem to act as anchoring sites for *L. (V.) braziliensis* promastigotes.

The ability of promastigotes to adhere to epithelial microvillii of the *Phlebotominae* digestorium tube is an essential stage for the maintenance of the parasite life cycle, being a factor of distinction between infective and noninfective stains. Similarly to LPG, the HBPs are related to the infective forms of the parasite [3, 4]. In such a way, its presence can be an essential factor for the setting of promastigotes in the digestorium tube and for the continuity of the life cycle, since parasites unable to adhere to the intestinal epithelium would be rejected together with the “feces” of the insect vector [5].

In addition, *L. (N.) intermedia* and *L. (N.) whitmani* are related to *L. (V.) braziliensis* transmission in the same endemic area [6]. The detection of ligands with similar molecular weights in the digestorium tube of both insect species is a biochemical indicative of vectorial homogeneity of these species in the transmission of ACL. The mapping of the interactions between molecules from both parasite and vector molecules can help in the understanding of adhesion to epithelial cells through the parasite surface.

Thus, our results considered together with the recent findings by Soares et al. [1] present biochemical indicatives of the epidemiological relevance of *L. (N.) whitmani* as a primary vector of ACL in Brazil.

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Review Article

***Echinococcus multilocularis* and Its Intermediate Host: A Model of Parasite-Host Interplay**

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Host-parasite interactions in the *E. multilocularis*-intermediate host model depend on a subtle balance between cellular immunity, which is responsible for host's resistance towards the metacestode, the larval stage of the parasite, and tolerance induction and maintenance. The pathological features of alveolar echinococcosis, the disease caused by *E. multilocularis*, are related both to parasitic growth and to host's immune response, leading to fibrosis and necrosis. The disease spectrum is clearly dependent on the genetic background of the host as well as on acquired disturbances of Th1-related immunity. The laminated layer of the metacestode, and especially its carbohydrate components, plays a major role in tolerance induction. Th2-type and anti-inflammatory cytokines, IL-10 and TGF- β , as well as nitric oxide, are involved in the maintenance of tolerance and partial inhibition of cytotoxic mechanisms. Results of studies in the experimental mouse model and in patients suggest that immune modulation with cytokines, such as interferon- α , or with specific antigens could be used in the future to treat patients with alveolar echinococcosis and/or to prevent this very severe parasitic disease.

1. Introduction

The infection of the intermediate hosts by the metacestode stage ("larval stage") of *Echinococcus multilocularis*, a cestode of the genus *Echinococcus*, is a good example to demonstrate the pivotal role the host immune response plays, favoring on one hand a partial control of infection, but yielding also a partial impairment of its own responsiveness [1–3]. Both tolerance and immunopathology may be observed in Alveolar Echinococcosis (AE), the disease due to *E. multilocularis* [4], and most of the mechanisms occurring seem to be triggered by a sophisticated modulation born out of a stage-specific parasite metabolism and respective immunomodulating strategy.

Tolerance is essential to ensure growth and development of the larval stage of the parasite in its host. In fact, parasite survival relies upon a mutual tolerance of the parasite and the host, and like all parasites, *E. multilocularis* modulates and even "uses" the host's immune system to ensure its

survival. Early killing of the parasite by the host would, of course, not allow the larva to grow and proliferate to reach the fertile stage (i.e., the production of protoscoleces) which allows its passage to the definitive host and thus its "adult" stage. Conversely, early killing of the host by the *Echinococcus* sp. larvae would lead to premature death of the host, and thus would also not allow the parasite to develop until the production of the fertile stage of the larva. *E. multilocularis* has evolved suitable strategies, at the recognition and effector stages of the immune response, to achieve its goal: to avoid both its premature death and the death of its host.

The immune response which develops against the larval stages of *E. multilocularis* accounts for a controlled parasite tissue development, but also for immunopathological events. In AE, hypersensitivity and immunopathology encompass several mechanisms: (1) mostly cell-related immune reactions leading locally, in the liver and other involved organs, to necrosis and fibrosis, (2) a rather Th2-dominated hypersensitivity reaction, although IgE-dependent clinical

manifestation are rare despite a constant IgE production by the host. Combination of epidemiological data on AE in endemic areas with immunological findings in humans and in experimentally infected murine intermediate hosts, has allowed us to design a rather comprehensive picture of the way how parasite and host survive, an understanding that may contribute to develop new treatment strategies comprising immune modulatory and stimulatory tools.

2. Susceptibility and Resistance of Animal Intermediate Hosts to *E. multilocularis*

E. multilocularis exhibits different growth rates and maturation characteristics in various species of hosts, that is, species of rodents or lagomorphs for *E. multilocularis*, but also of multiple other animal species such as swine and primates. These differences were first assigned to differences in strains/subspecies of the parasites; however, there is very little variation in *E. multilocularis* species [5, 6]. As rodents are the natural intermediate hosts of *E. multilocularis* in the conventional parasitic cycle, differences in host immune responses have been extensively studied in *E. multilocularis* experimental infection, and actually, differences in susceptibility/resistance, putatively related to respective immune responses, do occur in different murine models [7–11]. Impairment of cellular immunity (immune suppression) is followed by an increase in susceptibility to *E. multilocularis* in experimental animals. This was shown more than 30 years ago in immunosuppressed mice by Baron and Tanner [12] and was further demonstrated later on using SCID mice, which were shown to be highly susceptible compared to the wild strain and to reconstituted mice [13], and in nude mice [14]. A similar increase of susceptibility of experimental mice, associated with a decrease of delayed type hypersensitivity, was also observed in mice infected with *E. multilocularis* and treated with an immunosuppressive drug, cyclosporine, which interferes with IL-2 production in T-cells [15].

Conversely, cellular immune response against parasitic antigens is stronger in infected resistant mice, tested either using specific delayed-type hypersensitivity reactions *in vivo* [8] or specific proliferation of lymphocytes *in vitro* [11]. Resistance is increased by stimulation of the cellular immune response, as was shown with Bacille Calmette Guérin administration [16]. It was also shown that the antiparasitic effect of Isoprinosine treatment in mice infected by *E. multilocularis* [17] was at least partially due to immune stimulation by this immunomodulating agent [1].

3. Susceptibility and Resistance of Human Hosts to *E. multilocularis*

Liver transplantation has been performed in patients with very severe cases of AE since 1986. Observations in transplanted patients, who received immunosuppressive agents to prevent liver rejection, confirmed the increased susceptibility to *E. multilocularis* growth in humans upon impaired immune responsiveness. Increased susceptibility

was evidenced by a rapid increase in size of lung metastases, the development of brain metastases, late re-invasion of the transplanted liver by parasitic cell remnants, and even early re-invasion of the transplanted liver from a spleen metastasis [18, 19]. Similarly, a case of co-infection by *E. multilocularis* and Human Immunodeficiency Virus (HIV), leading to AIDS, has been reported, with a rapid and irreversible growth of *E. multilocularis* larvae in a young patient [20], leading to fatality. Associated to AIDS, restoration of immunity by appropriate antiretroviral therapy has led to reinstallation of the control of metacestode development [21]. Other cases have been observed in several European countries since then, as well as cases of AE associated with administration of other immunosuppressive drugs for autoimmune diseases (see [22], and personal communications through the EurEchinoReg network). AE thus appears to be another example of “opportunistic infection”.

In humans, a variety of clinical presentations of AE may be seen; however, pathological features and the frequent absence of protoscoleces suggest that, generally speaking, humans are relatively resistant to *E. multilocularis*. In fact, the implementation of mass screenings in endemic areas has revealed that the number of established infections in humans was far lower than the number of exposures to parasitic eggs [23, 24]. It may be assumed that a minority of individuals among humans (estimated to 1 out of 10 subjects) allows the development of the *E. multilocularis* metacestode after a contact with *E. multilocularis* oncospheres, the infectious component produced by the adult worm in the intestine of carnivores, which are definitive hosts [25].

The conceptual consequences of these findings in humans, added to the observations made in experimental rodents, cover two complementary, albeit non-mutually exclusive, assessments: (1) natural (immunological) mechanisms of defence (innate or acquired) are at work in the majority of human hosts, which are able to stop the larval growth at the very first stages or after the beginning of its development in the liver, (2) strategies are operating at the parasite’s level, which may counteract the immune system of the host and even take advantage of it for its own growth and survival. Studies performed in experimental animals as well as in humans with AE currently offer a rather comprehensive picture of the main events which operate at the very beginning of infection, when *E. multilocularis* settles within the host’s liver, and at the effector stage of the immune response, when *E. multilocularis* develops and progressively invades the liver and other organs of the hosts. These two stages will be presented successively although they are actually linked and interdependent.

4. Role of Parasite-Derived Molecules in the Modulation of the Host Response in View to Induce Immune Tolerance to the Parasite in Its Intermediate Host

In the host-parasite interplay, metacestode surface molecules as well as excretory/secretory (E/S) metabolic products are considered to function as important key players (reviewed

in [3]). The intraperitoneal murine infection model of AE offers the opportunity to study the direct effect of metabolic metacestode molecules on periparasitic peritoneal cells, including especially DCs, but also other immunologically relevant populations such as macrophages ($M\phi$), lymphocytes and other (inflammatory) cells that play a significant role in the putative control of (or respective failure to control) metacestode proliferation, and thus triggering of disease development. The *E. multilocularis* metacestode actively secretes or expresses molecules that putatively have potent effects on the immune system of the murine host. The production of these molecules and their chemical compositions might depend on the stage of the parasite (oncosphere, early vesicle or fully mature metacestode). However, little is known about biological effector molecules arising metabolically or somatically from the intrahepatic stages of the metacestode, although various *E. multilocularis* antigens, their epitopes and respective genes have been characterized. Every parasite molecule reaching the host environment has to pass the metacestode laminated layer. This laminated layer is an acellular carbohydrate-rich surface structure that protects the parasite from immunological and physiological reactions on part of the host. Among the main antigens described, a major carbohydrate named Em2 (G11) localizes on the surface of the laminated layer of the metacestode [26]. Em2 is a T-cell independent antigen, and the corresponding antibody response lacks antibody maturation [27]. Another polysaccharide-containing antigen, antigen C has been isolated and characterized from crude metacestode extract [28]. Similar investigations have yielded the finding of EmP2 [29, 30] and Em492 [31]. Em492 antigen is continuously released into the exterior medium and is also abundantly present in the parasite vesicle fluid. A suppressive effect on Con-A- or crude parasite extract-induced splenocyte proliferation in infected experimental mice was observed upon addition of Em492 antigen [31]. Protease treatment of Em492 antigen does not change its splenocyte proliferation inhibitory potential, while periodate treatment severely alters the functional properties of Em492 antigen, indicating that the effects of Em492 antigen is mediated through the carbohydrate part. Em492 antigen stimulates peritoneal macrophages from *E. multilocularis*-infected and -uninfected mice to produce increased levels of nitric oxide, leading to inhibition of splenocyte proliferation. On the other hand, Em492 antigen increases the levels of anti-CD3-induced apoptosis, which may contribute to a decrease of immune effector mechanisms. Another neutral glycosphingolipid has been identified as suppressor of human PBMCs proliferation following stimulation by phytohemagglutinin [32]. Hülsmeier and co-workers [33] have then isolated novel mucin-type glycoforms from the metacestode of *E. multilocularis*, and these glycoforms contained mucin-type core-I type and core-II type structures that were further diversified by addition of GlcNAc or Gal residues. Koizumi et al. [34] reported on the synthesis of the glycan portions of a glycoprotein antigen of *E. multilocularis* in order to elucidate the interactions between oligosaccharides and sera of *E. multilocularis*-infected hosts. Stereocontrolled synthesis of branched tri-, tetra-, and pentasaccharides displaying

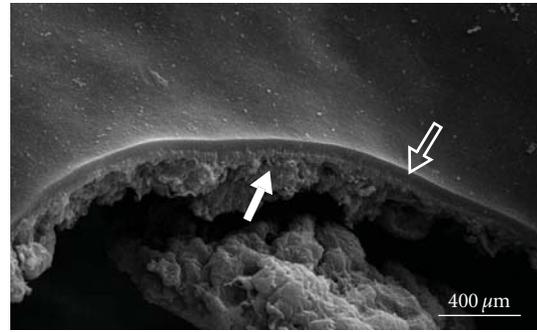


FIGURE 1: The laminated layer of *Echinococcus multilocularis*. Scanning electron microscopic visualization of an *Echinococcus multilocularis* metacestode vesicle trans-section: hollow arrow indicates outer laminated layer, white arrow indicates the inner germinal layer (Photo: A. Hemphill, IPB, Bern, Switzerland).

a Gal β 1- \rightarrow 3GalNAc core in the glycan portion of the glycoprotein antigen was achieved, which may become an interesting tool for further studies on their putative biological function. With regard to metabolized proteins, an *E. multilocularis*-protoscolex associated antigen of 62 kDa [35], two 70 and 90 kDa proteins [36], and several recombinant *E. multilocularis*-proteins (such as antigen II/3 [37] and its subfragments II/3-10 [38] and Em18 [39], or EM10 [40]), have all been published and discussed in view of a potential biological role. However, these antigens were mainly used to investigate respective immune responses with emphasis on immunodiagnosis of AE, and their biological functions have not been appropriately studied. EmAP (alkaline phosphatase) [41], an antigen which was shown to induce the production of antibodies associated with disease severity and resistance to treatment in AE patients [42], was also shown to induce only Th2-type cytokine secretion [43]. Aumüller et al. [44] used extracts from metacestodes of *E. multilocularis* to induce basophil degranulation, as well as the secretion of histamine, IL-4 and IL-13, in a dose-dependent manner. They concluded that *E. multilocularis* induces a Th2 response upon IL-4 release from basophils. Siles-Lucas and coworkers [45] identified and cloned a 14-3-3-gene of *E. multilocularis*, which appeared to play a key role in basic cellular events related to cellular proliferation, including signal transduction, cell-cycle control, cell differentiation, and cell survival [45, 46]. In a similar context, Kouguchi et al. [47] identified a cDNA clone, designated EMY162, that encoded a putatively secreted protein. EMY162 shares structural features with the EM95 antigen, for example, 31% amino acid sequence identity to EM95.

5. The Cross-Talk between *E. multilocularis* Larvae and Their Host through the Laminated Layer

As outlined above, the laminated layer is considered as a barrier between the parasite and the host (Figure 1). And, in fact, it may protect the growing larva from a direct

contact with the immune cells of the host, and especially the macrophages known as “epithelioid cells” that line the border of the germinal layer at the very early stage of parasitic development, and then the border of the laminated layer (Figure 2(a)); in addition, it may also protect the parasite against the attack by cytotoxic compounds such as activated complement proteins and NO. However, as described above, its main role in the protection of the parasite against the immune attack from the host seems to be mostly related to its immunomodulatory properties which inhibit immune cell activation directly or through the induction of immunoregulatory cytokines. Electron microscopic examination of the laminated layer suggests that, rather than a barrier, the laminated layer could well rather function as a “gate” between the parasitic germinal layer and vesicle and its host. The tegument of the germinal layer builds up a syncytium with numerous microtriches that protrude into the laminated layer; in addition, the release of membranous and vesiculated structures into the matrix of the laminated layer may be observed [3]. This “gate” could prove to be essential to ensure a regulated traffic of various substances between *Echinococcus* sp. and their hosts.

In fact, evidence of a “cross-talk” between the parasitic larva and its host is provided by a number of observations, the first one being the presence of high molecular weight host proteins within the “hydatid fluid” which was demonstrated at least 30 years ago both for *E. granulosus* [48, 49] and *E. multilocularis* [50]. Host immunoglobulins and albumin [48, 49, 51, 52], activators and inhibitors of the complement cascade [50, 53] and, recently, host-derived active matrix metalloproteinase 9, which was assumed to play a role in the periparasitic granulomatous reactions [54], were found in *Echinococcus* sp. hydatid fluid or bound to the cyst wall.

In addition, several lines of evidence now suggest that the larval development of *E. multilocularis* is triggered by cell signaling originating from the intermediate host [55, 56]. The phosphorylation of EmMPK1, a parasitic orthologue of the Extracellular signal Regulated Kinase (ERK) MAPK, is specifically induced in in vitro-cultured *E. multilocularis* metacestode vesicles, in response to exogenous host serum, hepatic cells and/or human epidermal growth factor (EGF). The *E. multilocularis* metacestode is thus able to “sense” host factors which results in an activation of the parasite MAPK cascade [57]. Cross-functioning between parasite-derived molecules and host liver was also described for parasite-derived enzymes: for instance, *E. multilocularis*-derived transglutaminase was shown to efficiently catalyze human liver-derived osteonectin cross-linking [58]. The fact that tissue-dwelling *E. multilocularis* expresses signaling systems with significant homologies to those of the host raises the highly interesting question whether cross-communication between cytokines and corresponding receptors of host and parasite can occur during an infection, that is, whether the parasite may also influence signaling mechanisms of host cells through the secretion of various molecules which might bind to host cell surface receptors. Such interactions could contribute to immunomodulatory activities of *E. multilocularis* or be involved in mechanisms of organotropism

and/or in host tissue destruction or regeneration during parasitic development. In a recent preliminary study, a significant influence of *E. multilocularis* metacestode on the activation of MAPKs signalling pathways was found in the liver cells both in vivo in infected patients and in vitro in cultured rat hepatocytes [59]. Significant changes in JNK phosphorylation was observed in hepatic cells in vivo, using hydatid fluid which contains a significant amount of host proteins. They were also observed using supernatant from axenic cultures of *E. multilocularis*, which is totally free of host components. This observation suggests that parasitic components, and not only factors from host origin, were actually acting on hepatocyte metabolic pathways.

It is possible that vesicle fluid from *E. multilocularis*, in addition to the already recognized proteins listed above, may also contain cytokines and growth factors from host origin and serve as storage for continuous release of factors both to the parasite and to the host through the laminated layer which appears critical at the host-parasite interface [3]. Dual interactions could thus ensure growth and survival of the parasite while interfering with host liver cells. As some of the cytokines which were proven to be important immunoregulatory factors leading to sustained tolerance, such as TGF- β , are quite important in the balance between cell proliferation and death/apoptosis, it may be suggested that the laminated layer could play a role similar to that of the placenta at the materno-foetal interface [60]: ensuring parasite growth and hepatic cell homeostasis while ensuring proper immune tolerance. Indeed, the *E. multilocularis* metacestode is sensitive to TGF- β signaling [61, 62] and the metacestode ERK-like kinase, EmMPK1, phosphorylates EmSmadD, a metacestode analogue of the Co-Smads of the TGF- β signaling pathway [63]. This is a novel and important field of research to understand better the subtle *Echinococcus*/host cross-talk and the complex triggering of the tolerance process.

6. Cells and Mechanisms Involved at the Early Stage of *E. multilocularis* Infection

At the time of initial encounter with its murine host, the metacestode might modulate the immune response. The changes that it induces are dynamic and depend on the stage of development, for example, ranging from oncosphere, to early stage vesicles up to a fully matured and fertile metacestode. Dendritic cells (DCs) and macrophages (M ϕ s) are among the first cells encountered by the parasite, which, by secreting and expressing certain molecules, has evolved mechanisms to suppress the major inflammatory and thus immunopathological pathway. Interaction of parasite metabolites with Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) that are expressed largely, but not exclusively, on DCs and M ϕ s is assumed to result in phenotypic changes and modification of the cytokine profiles produced by these cell types, but this has not yet been experimentally shown at the early post-oncospherical stage of infection of murine AE. Several experiments have suggested that *E. multilocularis*, either by cell-cell contact or

by its products, could inhibit and/or modulate the immune response at the very early stage of antigen presentation to T-cells. *E. multilocularis* was shown to modify the accessory cell function and interfere with antigen presentation through a parasite-derived macrophage modifying factor [64]. It was also shown that immature dendritic cells of human origin did not mature and had a reduced capacity to take up dextran via mannose receptors in the presence of crude non-fractionated *E. multilocularis* antigen; however, further maturation could be induced by proinflammatory cytokines; these mature dendritic cells, pulsed with *E. multilocularis* antigen, were slightly better inducers of T-cell proliferation than non-pulsed dendritic cells [65].

E. multilocularis-infected mouse macrophages as antigen-presenting cells exhibit a reduced ability to present a conventional antigen to specific responder lymph node T-cells when compared to normal macrophages [66]. Co-stimulatory signals are crucial for T-cell activation and it is well known that failure in the expression of one of the components of the receptor-ligand pairs may severely impair T-cell activation and induce tolerance by a mechanism generally called “anergy”. In the above mentioned experiment, using macrophages from *E. multilocularis*-infected mice, B7-1 (CD80) and B7-2 (CD86) remained unchanged, whereas CD40 was down-regulated and CD54 (=ICAM-1) slightly up-regulated. FACS analysis of peritoneal cells revealed a decrease in the percentage of CD4+ and CD8+ T-cells in *E. multilocularis*-infected mice. Taken together, the obstructed presenting activity of *E. multilocularis*-infected host macrophages appear to trigger an unresponsiveness of T-cells leading to the suppression of their clonal expansion during the chronic phase of AE infection [66].

By inducing functional changes in DCs and M ϕ s, the metacestode can achieve important shifts in T-cell subsets. An initial acute inflammatory Th1 response is subverted gradually to a Th2 response during the chronic phase of AE. Cytokines, such as IL-4, IL-5, IL-9 and IL-13, secreted largely by immune-cell types in response to parasite antigens, not only down-modulate the Th1 response but can also promote parasite expulsion and tissue renewal and repair [67]. The metacestode most likely achieves the Th2 expansion through the induction of regulatory cytokines, such as IL-10 and TGF- β [68]. As mentioned above, in murine AE, the cell-mediated immune response of the host plays an important role in controlling metacestode proliferation.

7. Cytokines Involved in the T-Cell Activation Stage of Immunity after *E. multilocularis* Infection

7.1. Th2 Cytokines. Cytokine profiles, due to the secretion of characteristic cytokines by (mostly but not only) T “helper” (Th) cells give an insight into immune mechanisms involved in host-infectious organism relationship and in the types of immune responses that are developed after the early stage of antigen and “pattern” recognition. Th1-cytokines [Interleukin- (IL)-2, Interferon- (IFN- γ)] are induced by IL-12 and mostly involved in T-cell-mediated cytotoxicity;

Th2-cytokines (IL-4, IL-5, IL-13) are induced by IL-4, and mostly involved in antibody-mediated non-cytotoxic immune responses; TH17-cytokine (IL-17) is induced by IL-23, modulated by IL-21 and IL-22 and TGF- β , and mostly involved in T-cell-mediated activation of innate immunity/inflammation, but also in tolerance [69].

High levels of Th2 cytokines are observed, including IL-4, IL-13 and IL-5, in addition to a relatively low level of secretion of IFN- γ , in patients with AE [70, 71], and in *E. multilocularis* infected mice [72]. Production of IL-5 by PBMCs, and among those very specifically by CD4 T lymphocytes, is induced by *Echinococcus* antigens not only in patients with AE but also in normal subjects [71]. Secretion of IgE and IgG4 antibodies is associated with the Th2 profile. Total IgE and specific IgE and IgG4 against *E. multilocularis* antigens are highly elevated in those patients with the most aggressive disease [73]. Disappearance of IgE and decline of IgG4 specific antibodies are significantly associated with regression of the lesions in patients treated with antiparasitic drugs, and both Th2-related antibody isotypes are the first to disappear after surgical cure [74, 75]; this represents indirect evidence of the prominent stimulation of Th2 cytokines by the viable parasite at a chronic stage.

In the experimental mouse model, a Th2 profile is also, globally, the hallmark of *Echinococcus* sp. infections at the chronic stage. In mice experimentally infected with *E. multilocularis*, three stages of cytokine secretion can be identified: (1) a first stage of Th1 cytokine secretion including IL-2 and IFN- γ , associated with a slow parasite growth; (2) a second stage characterized by a mixed secretion of Th1 and Th2 cytokine secretion, especially IL-5 and IL-10, associated with rapid parasite growth; and finally (3) a last stage of immune suppression with a nearly complete inhibition of lymphocyte proliferation and of cytokine secretion following specific or non-specific stimulation [76].

7.2. Cytokines Leading to Tolerance. The main cytokines involved in immune tolerance are IL-10 and TGF- β . Most of the studies in AE as well as in the experimental models have first focussed onto IL-10. The anti-inflammatory properties of IL-10 are well known, especially through the inhibition of macrophage activation and cytotoxic functions [77].

Spontaneous secretion of IL-10 by the PBMCs is the immunological hallmark of patients with progressing lesions of AE [71]. Conversely, IL-10 is significantly lower in patients with abortive lesions [78]. IL-10 is measurable in the serum of the patients with AE at higher concentrations than in control subjects [79]. A variety of cell types are involved in the secretion of IL-10 by resting and stimulated PBMC in patients with AE, especially CD4 and CD8 T-cells, but also non-T non-B cells [71]. “Suppressor” CD8 T-cells, induced by parasite products, were reported to be involved in tolerance to *E. multilocularis* [80, 81]. However, the relationship between the capacity of these cells to secrete IL-10 and their “suppressor” activity is unknown. A preliminary report has confirmed that locally, in the periparasitic granuloma, T-cells secreted IL-10 and the data suggest that IL-10 production is highest closer to the parasitic vesicles [82]. After experimental infection with *E. multilocularis*, IL-10 secretion by spleen

cells is slightly delayed and is part of the cytokine profile observed in the second phase of *E. multilocularis* growth [76]. Similar changes were also observed when measuring IL-10 levels in the serum of infected mice: they remained low before 80 days post-infection and then increased sharply at 100 days post-infection when they reached a peak [83].

The presence of TGF- β secreting cells in the periparasitic granuloma surrounding *E. multilocularis* vesicles in the liver of patients with AE has been recognised only very recently [68] and exploring TGF- β in its multiple functions in *E. multilocularis* infection is still an open field of research. To our knowledge, no information is available on the production of TGF- β by PBMCs in AE. Evidence of TGF- β production in experimental *E. multilocularis* infection is given by a recent study on the effect of combined pentoxifylline and albendazole on parasite growth: with this combination the inhibition rate of cyst growth was 88% and was associated with a marked decrease of IL-10 and TGF- β which were elevated in control mice [84].

7.3. Pro-Inflammatory Cytokines and Chemokines. All studies on the cytokine profile in AE, in humans and in the experimental models, have stressed that it was never a “pure” Th2 profile, but always a rather mixed profile, including the so-called “Th0” profile, even in late stages of the disease. Significant amounts of Th1 cytokines such as IL-2, IL-12, IFN- γ , as well as pro-inflammatory cytokines (IL-1, TNF- α , and IL-6) have been found to be secreted by peripheral blood mononuclear cells in most studies in humans infected by *E. multilocularis*. However, the relative ratios of these cytokines as well as of the chemokines produced when PBMCs from patients are cultivated with parasitic antigens give a rather complex picture. A recent study showed that production of the proinflammatory cytokines IL-1 α and IL-18 by *E. multilocularis* vesicle antigen-stimulated PBMC was reduced in AE patients, regulatory IL-10 was similar, but parasite vesicle-induced IL-8 was dominant and clearly elevated in patients [85]. Such selective and opposite dynamics of inflammatory cytokines and chemokine release may prevent pathogenic inflammation, and constitute an appropriate response for attraction of effector cells into the periparasitic tissues with the capacity to limit *E. multilocularis* metacyst proliferation and dissemination.

In the experimental model of secondary infection in mice, the levels of Th1 cytokines as well as pro-inflammatory cytokines was initially elevated, and then progressively decreased while Th2 cytokines and IL-10 increased [76]. Unfortunately, nothing is known on the involvement of Th17, IL-17 secreting T-cells, and of IL-21, -22 and -23 in the development of immune cell infiltration around the parasitic vesicles and their relationship with immunoregulatory cells in echinococcosis. This newly recognised subset of T helper cells appears to be essential for the development of T-cell-derived inflammatory reactions (delayed-type hypersensitivity reactions and granuloma-type pathological pictures) [69]. No doubt that this discovery opens a new and exciting field of research to better understand the tolerance/cytotoxicity balance and time-course in AE. The recruitment and presence of all potential actors of Th17-

driven immune reaction in the lesions highly suggests that the IL-23/IL-21/IL-22/IL-17 pathway is actually operating in echinococcosis; however, it has to be formally demonstrated and its modulation/inhibition by the regulatory cytokines should be carefully studied.

Attempts at enhancing Th1-related immune responses have resulted in increased resistance to *E. multilocularis* infection in experimental mice. Treatment with IFN- γ either before or after experimental infection has been shown to be only partially effective in reducing larval growth, although it was able to moderately increase the periparasitic fibrotic process [86]. Conversely, pretreatment of mice with IL-12 is extremely efficient in preventing the development of lesions and leads to abortive parasitic vesicles surrounded by fully efficient periparasitic immune cell infiltration and fibrosis [87]. IFN- α is also able to prevent larval growth in experimental mice [72]. Associated with this preventive effect of IFN- α , a significant modulation of cytokine secretion, with a significant decrease in IL-13 and increase in IFN- γ by peritoneal macrophages and spleen cells was observed in the treated mice [72]. Isolated attempts of treatment with IFN- γ in patients at a late stage of AE were no more successful than those performed in experimental mice and they could not modify host's cytokine profile significantly [88]. Conversely, IFN- α was shown to favour the regression of AE lesions while reversing the abnormal Th2-skewed cytokine profile in a patient with AE [89].

8. Effector and Regulatory Cells and Mechanisms in *E. multilocularis* Infection

Anti-inflammatory cytokines do interfere in T helper/regulatory/cytotoxic lymphocyte differentiation. In patients with AE, long after the initiation of the disease, the generation of memory Th1 CD4+ T-cells was shown to be impaired [90]. Furthermore, in such patients a biased CD4/CD8 T-cell ratio is observed: a marked increase of the CD4/CD8 ratio, mainly due to a decreased number of CD8 T-cells among peripheral T lymphocytes, and the predominance of CD8 T lymphocytes within the periparasitic granuloma characterize “susceptible” patients with a severe and progressing disease [91] (Figure 2(b)). In patients with progressing/severe AE, CD8-T-cells have been shown to produce Th2 cytokines as well as IL-10 and TGF- β [68, 71, 92]. In infected mice, the first two stages of cytokine production are also characterized by differential infiltration of the periparasitic liver with CD4, and then CD8 T lymphocytes, following different kinetics in resistant versus susceptible mice [9].

In addition, cytokines and other mechanisms may play a role in specifically inhibiting effector immune mechanisms, and particularly cytotoxicity mediated by cells of the innate immune response (NK cells, macrophages) and by cells of the adaptive immune response (cytotoxic T-cells). The crucial role of macrophages in the effector phase of the immune response towards *E. multilocularis* has been reported, and their stimulation results in enhanced parasite killing and protection of the host [25, 93]. On the other hand, an impairment of chemotactic and phagocytic properties of cells of

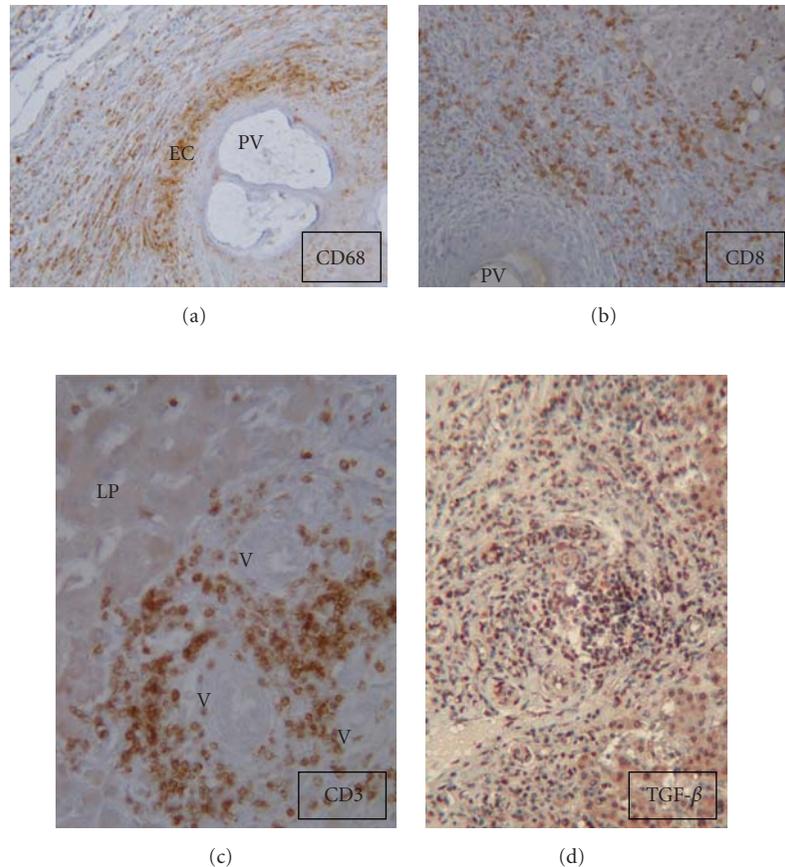


FIGURE 2: Cells of the periparasitic granuloma in *E. multilocularis* infection in humans; immunostaining of liver sections of patients with alveolar echinococcosis. (a) the “epithelioid cells” (EC) are CD68+ macrophages with an epithelium-like arrangement, located at close contact to the laminated layer of the parasitic vesicle (PV); (b) CD8+ T-cells represent the major cell population of the periparasitic infiltrate; (c) infiltration by CD3+ T-cells is especially prominent around the new vessels (V) developed at the periphery of the granuloma, at the border of the invaded liver parenchyma (LP); (d) most of the infiltrating T-cells express TGF- β . (Photo: B. Kantelip and DA Vuitton, WHO-Collaborating Centre; Université de Franche-Comté, Besançon, France).

the phagocytic system has been shown in *E. multilocularis* hosts [72, 94, 95]. This suggests that anti-inflammatory effects, mediated by cytokines, are actually operative at the systemic level. A crucial- and ambiguous- role of macrophages regarding NO secretion has been disclosed in murine *E. multilocularis* infection. In fact, NO may be both cytotoxic against parasites and immuno-modulatory by inhibiting cell activation. Both were found to be operating and it was especially demonstrated that the high periparasitic Nitric Oxide (NO) production by peritoneal exudate cells contributed to periparasitic immunosuppression [96, 97]; this explains why, paradoxically, iNOS deficient mice exhibit a significantly lower susceptibility towards experimental infection [98].

Contact inhibition of effector macrophages could be induced either by anti-inflammatory cytokine secretion or, more directly, by germinal layer-derived molecules through the laminated layer. Analysis of the cell surface markers of the epithelioid macrophages lining the parasitic vesicles in humans (Figure 2(a)) has shown a very unusual expression of these markers, particularly a high expression of CD 25,

the inducible chain of the IL-2 receptor, a chain usually only expressed by activated T-cells and especially CD4 T-regulatory cells [99]. Such an abnormal expression of CD 25 on macrophages has been shown in other granulomatous diseases and could be related to a particular functional state of the macrophages, either involved in tolerance induction or inhibited in its cytotoxic properties, or both.

NK- and/or T-cell-dependent cytotoxic mechanisms may be impaired by the cytokines secreted abundantly in the periparasitic immune cell infiltrate and/or through cell-cell interaction mechanisms. The quasi-absence of NK cells and the inhibition of the expression of the co-stimulatory receptor NKG2D at the surface of CD8-T-cells in the periparasitic granuloma has been shown in patients with AE. Despite the presence of its ligand, MICA/B, at the surface of hepatic cells, epithelioid cells and of the parasite germinal layer itself, cytotoxicity of CD8 T-cells might thus be severely impaired [68]. The lack of expression of NKG2D on CD8 T-cells was not related to the presence of the soluble form of MICA/B, since this soluble form could not be detected in these patients. A role for TGF- β is highly likely since it

was massively expressed by the lymphocytes surrounding the parasitic vesicles (Figure 2(d)).

Characterisation of regulatory cells is just at its beginning in *E. multilocularis* infection. An important role of CD4+ CD25+ T-regulatory cells has been suggested by a recent study in patients with AE [85]. Such a phenotype is quite characteristic of T-regulatory cells and they are likely responsible, at least partially, for the secretion of the anti-inflammatory/tolerogenic cytokines, such as IL-10 which is constantly elevated in AE patients. In this study, an increased number of CD4+ CD25+ T-cells was observed in the patients, compared to healthy controls. In the same study, after 48 h of co-culture, *E. multilocularis* metacystode culture supernatant and *E. multilocularis* vesicles depressed the release of the pro-inflammatory cytokine interleukin (IL)-12 by PBMC. This effect was dose-dependent and a suppression of tumour necrosis factor (TNF)- α and IL-12 was observed even when PBMC were activated with lipopolysaccharide (LPS). Comparing proinflammatory cytokine release by AE patients and controls showed that the release of IL-12 and TNF- α was reduced in AE patients, and was accompanied by a reduced release of the Th2-type chemokine CCL17 (*thymus and activation regulated chemokine*, TARC), suggesting an anti-inflammatory response to *E. multilocularis* metacystode in AE patients.

9. Relationship between the “Delayed Type Hypersensitivity” Responses and Local Pathological Events/Clinical Presentation, Signs and Symptoms in AE

Despite the tolerance that is exerted to protect the parasite against host's immune defences, effector mechanisms are nevertheless present. They are responsible for the attraction of various types of immune cells to the site of larval growth, and especially to the infected liver. “Delayed-type hypersensitivity reactions”, the immune effector mechanisms also known as “type IV” hypersensitivity, are characterised by immune cell recruitment, tissue infiltration, and neo-angiogenesis, followed by necrosis and fibrosis. Involvement of such reactions is particularly striking in AE, where chronically established larval development is characterized by a sustained infiltration of the host's immune cells which becomes organised as a “granuloma” and associated with necrosis and fibrosis [9, 91]. Granulomatous reactions are typical of delayed-type hypersensitivity reactions that are not fully efficient. Their association with histopathological characteristics such as “giant cells” (macrophages with multiple nuclei) and calcifications (which are the ultimate outcome of macrophage activation) is unique. Such granulomatous reactions contribute to the immunopathological events responsible for some of the complications of the underlying diseases, as observed in tuberculosis, leprosy, leishmaniasis or schistosomiasis.

Necrosis of the lesions is one of the hallmarks of AE in humans. In the liver, expression of IL-1, IL-6 and TNF- α mRNAs were observed in macrophages at the periphery of the granuloma in all studies patients, and close to the para-

sitic vesicles in patients with severe diseases and pathological pictures of lesion necrosis [99]. TNF- α could thus contribute to the necrotic process which gives the typical ultrasound and CT-scan pictures of AE, and leads to several complications in patients with AE. However, TNFs represent also a major factor to prevent metacystode growth, as is exemplified by the exacerbation of *E. multilocularis* metacystode growth in transgenic mice deficient for Lymphotoxin and TNF- α [100].

Fibrosis is also a hallmark of AE, leading to a complete disappearance of the liver parenchyma and to the death of the metacystode, with vesicles embedded in an acellular tissue composed nearly entirely of cross-linked collagens [101]. The diffusion of the fibrotic process even far from the parasitic lesions strongly suggests a major role for cytokines in collagen synthesis. They may also be involved in cross-linking the collagen bundles in humans [58, 101–104], as well as in the experimental models [10]. TGF- β , present in the cell infiltrate surrounding the parasitic lesions, in addition to its role in maintaining tolerance, is likely involved in the development of fibrosis in AE; however, this has never been studied until now. The parasite itself could also be involved in the collagen cross-linking process, since a transglutaminase of parasitic origin has been shown to be strongly expressed in and at the border of the parasitic vesicles and is able to efficiently cross-link collagens of human origin in vitro [58]. Development of fibrosis has been shown to be either quantitatively or qualitatively correlated to protection in experimental animals [10, 86], and is thus, usually, considered to be beneficial to the host. Fibrosis, in addition to the laminated layer, could be responsible for the protection of the parasite against any contact with both cytotoxic and antibody-secreting cells of the host and vice-versa. It may explain the low rate of anaphylactic symptoms in patients with AE [104, 105]: the extremely fibrotic lesions of AE cannot rupture, and the echinococcal fluid may well be never in contact with mast cell-bound IgE, despite their constant presence, which could be demonstrated in vitro [104]. In fact allergic symptoms rarely occur in patients with AE, only while parasitic cells are migrating to other organs than the liver and are eventually leading to metastases, especially through pulmonary embolism [105]. However, fibrosis is also the main cause for bile duct and vessel obstructions and thus, the pathophysiological background of chronic cholestasis, angiocholitis, portal hypertension, Budd Chiari syndrome and/or vena cava obstruction [105]. It may also be one of the reasons for the poor transport of antiparasitic drugs to the lesion.

One of the striking features observed in experimental murine AE (and also in naturally acquired AE of humans [104, 105]) is the absence of hypereosinophilia, a common feature of helminth-related diseases. The mobilization of eosinophils is known to be a crucial immunological event that plays an important role in the host defense against helminths. Eotaxin, a CC-proinflammatory chemokine, is one of several described chemoattractants for eosinophils. In addition, also IL-5 may mobilize these cells [106] but its role remains controversial. Eosinophils possess granules containing a variety of toxic molecules [major basic protein

(MBP), peroxidase, neurotoxin, histaminase and others] which are active against many multicellular parasites, in particular helminths [107]. In many examples of nematode infections, eosinophilia is a marked characteristic, and eosinophils directly cause profound damage to the tegument of the worms, in which a marked reduction of fertility and longevity is observed [108]. On the other hand, IL-5 and eosinophils have no detectable effects on the infection with selected cestodes or trematodes [109]. An extravasation of eosinophils causing eosinophilia in the peritoneal cavity has been demonstrated to be beneficial for the host by causing damage to the immigrant immature worm of *Fasciola hepatica*, resulting in the erosion of the tegumental syncytium [110]. Recently it could be shown that metacystode antigens (VF and E/S) exhibit proteolytic activity on eotaxin in vitro [111]. Inhibition of eotaxin activity may suppress the mobilization of eosinophils in *E. multilocularis*-infected hosts. Eotaxin is considered one of the main activators and chemo-attractants of resident eosinophils [112]. In experimental murine AE, the detected eotaxin inactivation by VF and E/S products may contribute to explain the absence of eosinophils within the peritoneal cavity of AE-secondary infected mice. Absent eosinophils thus may be a part of a series of events that maintain a low level of inflammation in *E. multilocularis*-infected hosts.

Finally, angiogenesis is one of the aspects of the periparasitic cell-mediated immune reactions which have been most neglected in the immuno-pathological studies of echinococcosis. Immunostaining of extracellular matrix proteins in the periparasitic areas of *E. multilocularis* infections have shown impressive pictures of neo-angiogenesis in the periparasitic granuloma [10, 101]. Angiogenesis is likely involved in the traffic of immune cells to and from the lesions; T-cells are especially numerous around the neo-vessels at the periphery of the periparasitic granuloma (Figure 2(c)). It may also explain some aspects of AE imaging, with a delayed reinforcement of the periphery of the lesions at CT-scan after injection [113] and an uptake of fluoro-deoxyglucose on PET-scan images [114]. Last but not least, as observed in malignant tumours, it may also be involved in the metastatic process observed in AE, a major component of the severity of this disease. Preliminary unpublished observations suggest that angiogenesis could be promoted by cytokine-like substances secreted by the metacystode itself.

10. Role of Host Immunogenetics in the Balance between Tolerance and Effector/Hypersensitivity Mechanisms

The role of some specific parasite-derived substances/antigens for the final orientation of the immune response and the relative importance of cell-mediated effector immune response *versus* tolerance in *Echinococcus* sp. infection is ambiguous. Until the beginning of the 1980s, every exposure to *E. multilocularis* oncospheres was believed to be followed by larval development and occurrence of the disease. A more careful evaluation of epidemiological data

in humans and animals in the same endemic areas first questioned this assumption [115, 116]. Then the implementation of mass screening in endemic areas revealed that the number of serologically “positive” cases were far higher than that of patent cases, disclosed by liver ultrasonography [23, 24, 117]. Positive serological results in individuals living in endemic areas (i.e., subjects with specific antibodies against *E. multilocularis* and who share the same risk factors) may account for at least 4 different situations: (1) “patent”, overt disease with symptoms, (2) “latent”, non-apparent disease, (3) calcified dead lesions in the liver and (4) no apparent lesions [2]. The clustering of subjects with specific antibodies in those areas where the number of “patent” cases is highest strongly suggests that these subjects actually had contacts with the parasite in the previous years. Some genetic factors have been found to associate with these various outcomes in humans. There is a significant association between HLA DR 11 and protection, HLA DPB1*0401 and susceptibility, and HLA DR3 and DQ2 and severe clinical evolution of the disease [118]. The HLA B8, DR3, DQ2 haplotype is observed with an unusual frequency in patients with autoimmune diseases characterised by an increased and inappropriate humoral immunity and a relatively inefficient cellular immune response. In fact, studies on cytokine secretion by PBMC from HLA DR3+, DQ2+ patients with AE, compared with patients without this HLA haplotype, have shown that the spontaneous secretion of IL-10 was much higher in HLA DR3+, DQ2+ patients [43]. A limited number of family cases have been observed in endemic areas that suggest an inherited pattern in populations which share the same risk factors [119]. Preliminary data indicate that relatives with progressive forms of *E. multilocularis* infection shared both HLA haplotypes and the associated clinical presentation of the disease [120]. Other genes within the MHC that are known to be related to the initiation and/or the effector phase of the immune response could also be involved. No significant correlation was observed between occurrence and/or severity of the disease and polymorphism in TNF promoter gene. However, 63% of 94 patients with AE from the same European endemic area as mentioned above had the homozygote Thr-Thr form of the TAP2 665 codon site, versus 45% of controls [121].

11. Conclusion

Results of observations in humans and experimental studies in animals suggest that, in the absence of fully effective anti-parasitic chemotherapy for AE, modulation of the host's immune response could be envisaged to fight against the parasite and to prevent the disease and/or its complications. As shown above, recombinant IFN- α 2a would be the best “immunological drug” candidate. Other approaches are based on the use of specific antigenic components of *Echinococcus* sp. as preventive or therapeutic vaccines [122–124]. The Eg95 vaccine has already reached field validation for its use in *E. granulosus* infection of sheep [125], and similar or novel antigens could be adapted to *E. multilocularis* infection of intermediate hosts. In murine AE, preliminary experimental reports suggest that several

antigenic compounds may provide good protection against primary infection, for example, Em14-3-3 [122], Em95 [123], EMY162 [126] and EmTetraspanin [124]. Dependent of the economical and ethical feasibility of such an approach, a preventive vaccine for *E. multilocularis* infection in humans may theoretically be envisaged, while a therapeutic approach still deserves further detailed investigation.

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Research Article

Release of Glycoprotein (GP1) from the Tegumental Surface of *Taenia solium* by Phospholipase C from *Clostridium perfringens* Suggests a Novel Protein-Anchor to Membranes

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In order to explore how molecules are linked to the membrane surface in larval *Taenia solium*, whole cysticerci were incubated in the presence of phospholipase C from *Clostridium perfringens* (PLC). Released material was collected and analyzed in polyacrylamide gels with sodium dodecyl sulfate. Two major bands with apparent molecular weights of 180 and 43 kDa were observed. Western blot of released material and localization assays in cysticerci tissue sections using antibodies against five known surface glycoproteins of *T. solium* cysticerci indicated that only one, previously called GP1, was released. Similar localization studies using the lectins wheat-germ-agglutinin and Concanavalin A showed that N-acetyl-D-glucosamine, N-acetylneuraminic, sialic acid, α-methyl-D-mannoside, D-mannose/glucose, and N-acetyl-D-glucosamine residues are abundantly present on the surface. On the other hand, we find that treatment with PLC releases molecules from the surface; they do not reveal Cross Reacting Determinant (CRD), suggesting a novel anchor to the membrane for the glycoprotein GP1.

1. Introduction

The tegumental surface of larval cestodes is in direct contact with the host tissues and plays a crucial role in the survival of the parasite. Glycoproteins and complex carbohydrates have been detected on the surface of the larvae in several species of cestodes through the use of histochemical techniques [1–4].

The tegumental membrane of *Taenia solium* cysticerci exhibits a dense glycocalyx composed of abundant carbohydrates and glycoproteins such as GP1, GP2-3, GP6, and GP7 [5–7]. However, little is known about the anchorage of the glycoproteins and glycolipids to the membrane. In other platyhelminths, several studies have also shown that alkaline phosphatase, acetyl-cholinesterase, and several surface proteins of 18, 22, 28, 32, 38, and 200 kDa are anchored to the tegumental membrane via glycosyl-phosphatidyl-inositol (GPI) in adult and somules of *Schistosoma mansoni* [8–10]. Moreover, the apical gut surface protein (p46^{Gal}) of *Haemonchus contortus* is also anchored through GPI [11].

Likewise, Sm25, a major schistosome tegumental glycoprotein, is attached by palmitic acid to the membrane [12].

Cestodes, trematodes, and other platyhelminths have triglycerides and cholesterol as the major neutral lipids and phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine as the major phospholipids [13, 14]. Glycolipids, galactosylceramides, and glycosphingolipids have been identified in tegumental membranes of *Spirometra mansonioides*, *Echinococcus multilocularis*, and *Hymenolepis diminuta* [15–17]. A novel glycosphingolipid named AGL containing inositol phosphate as acidic group has been found in the nematode *Ascaris sum* [18].

The purpose of this study was to determine the components that are released from the surface of *T. solium* cysticerci by phospholipase C from *Clostridium perfringens* (PLCs). The GP1 molecule was removed by PLC. Finally, complex carbohydrates that are ligands for wheat germ agglutinin (WGA) and concanavalin A (ConA) were also released from the tegumental surface. These results suggest that a different kind of glycoprotein anchor might be present in taeniids.

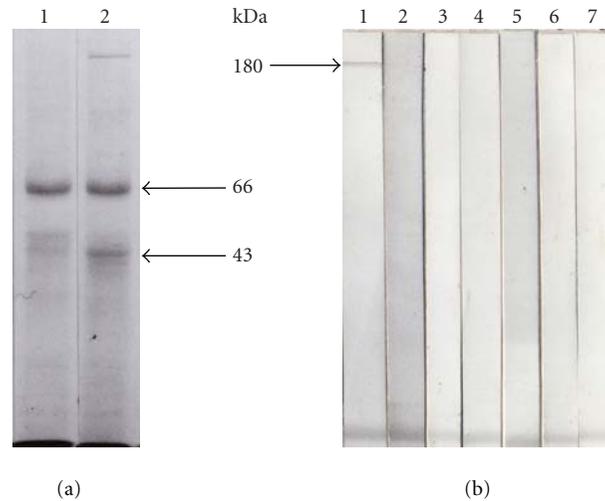


FIGURE 1: Materials released by PLC from the surface of *T. solium* cysticerci. (a) SDS-PAGE showing the material released after 1 hour incubation in Lane 1: PBS and Lane 2: PBS-PLC. (b) Western Blot of the material released with PLC probed with IgG fraction of Lane 1: anti-GP1, Lane 2: anti-GP2-3, Lane 3: anti-GP6, Lane 4: anti-GP7, Lane 5: anti-pig IgG, Lane 6: Preimmune rabbit serum, and Lane 7: anti-cross reacting determinant (CRD).

2. Materials and Methods

2.1. Biological Material. *Taenia solium* cysticerci were dissected from skeletal muscle of naturally infected pigs obtained in local abattoirs. Cysticerci were immediately washed three times with sterile phosphate buffered saline, pH 7.2 (PBS), containing 100 $\mu\text{g}/\text{mL}$ of penicillin and 100 U/mL of streptomycin. Crude extract of *T. solium* was obtained as described before [19].

2.2. Phospholipase C Treatment of Intact Cysticerci. Two groups of 20 cysticerci each were incubated for 1 hour at room temperature with PBS or PBS containing 10 U/mL of phospholipase C (PLC), type XIV, from *C. perfringens* (Sigma Chemical Co.) in the presence of proteinase inhibitors (0.1 mg/mL aprotinin, 5 mM PMSF, and 2.5 mM TLCK). Supernatants were collected and the amount of protein released was determined in each fraction with the Bio-Rad Protein Assay (Bio-Rad Laboratories, California, USA).

2.3. Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot Analysis. Samples of 10 μg of the released material were mixed with sample buffer (3% SDS, 62.5 mM Tris-HCl and 5% 2-mercaptoethanol, pH 6.8) and boiled for 2 minutes. Samples were run on 10% polyacrylamide gels and stained with Coomassie Blue R-250. Protein bands were transferred onto nitrocellulose membranes (Millipore, Illinois, USA) and incubated for 1 hour at 37°C with the appropriate dilution of the IgG fraction of one of the following antisera: αGP1 , $\alpha\text{GP2-3}$, αGP6 , αGP7 , $\alpha\text{pig IgG}$, and anti-CRD of GPI. The IgG fraction of preimmune rabbit serum was used as negative control. Peroxidase-conjugated goat anti-rabbit IgG (Zymed) was used as secondary antibody. Bound antibodies were developed incubating the blots with

0.5 mg 3-3'-diaminobenzidine and 20 μL of 1% H_2O_2 in PBS for 15 minutes at room temperature [20].

2.4. Histochemical Studies. Cysticerci that were treated with and without PLC were washed three times with PBS and fixed as described before [21]. Fragments of the bladder wall of cysticerci treated with PBS or PBS-PLC were incubated for 1 hour at room temperature with the appropriate dilution of the IgG fraction of one of the following antisera: αGP1 , $\alpha\text{GP2-3}$, αGP6 , αGP7 , and $\alpha\text{pig IgG}$. The IgG fraction of a preimmune rabbit serum was used as negative control. Peroxidase-conjugated goat anti-rabbit IgG (Zymed) was used as secondary antibody. The bound antibodies were developed incubating the fragments with 0.5 mg 3-3'-diaminobenzidine and 20 μL of 1% H_2O_2 in PBS, for 15 minutes at room temperature. Ruthenium red (RR) was also used in the fixation of intact cysticerci [22], before and after PLC treatment following the procedure described before. Similar localization studies were carried out using horseradish peroxidase (HRP) conjugated to ConA or WGA. Peroxidase reaction was stopped in cold PBS and all cyst fragments were postfixated in osmium tetroxide and processed for light and electron microscopy as described before [19]. Semithin (2 μm) and thin (80 nm) sections with or without staining were photographed in a Nikon Optiphot microscope and a JEOL-1200 EXII electron microscope.

3. Results

Treatment of the cysticerci with PBS and PBS-PLC resulted in the release of several proteins (Figure 1(a)). The supernatant from the cysticerci treated with PBS alone (lane 1) shows one intense band with an apparent molecular weight of 66 kDa and several weak bands around 90, 50, 45, 30, and 15 kDa. In contrast, treatment with PBS-PLC resulted in a similar

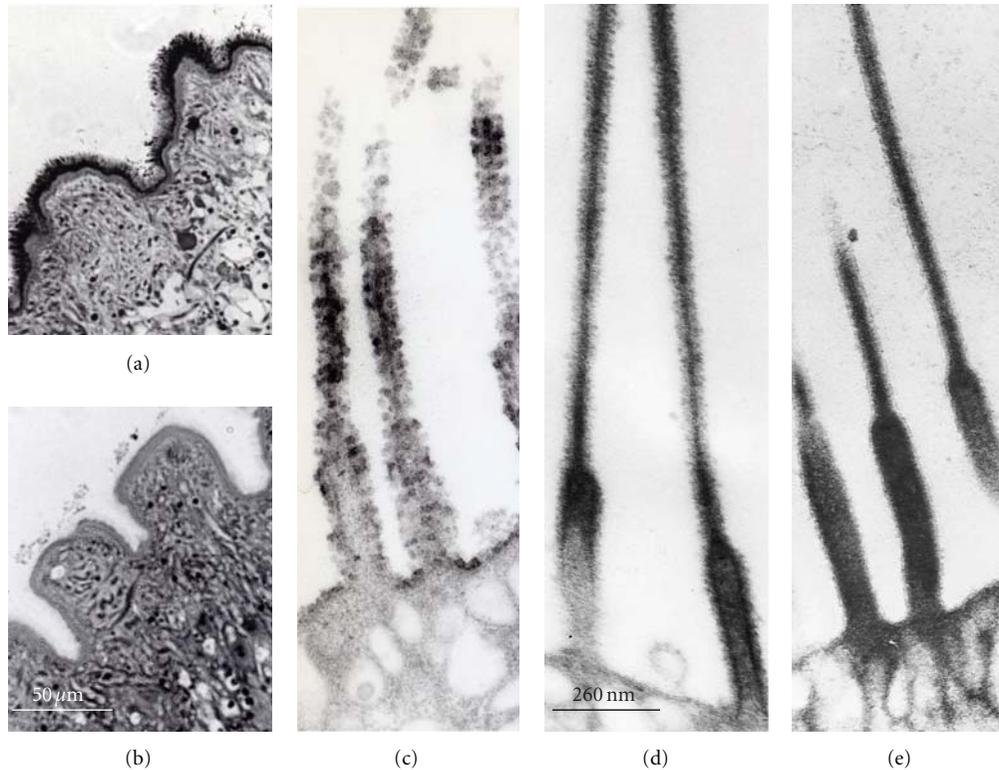


FIGURE 2: Micrographs of the tegumentary surface of *Taenia solium* cysticerci: light and electron micrographs of sections of the bladder wall of cysticerci incubated for 1 hour with PBS ((a) and (c)) and PLC ((b) and (d)) probed with anti-GP1. (e) IgG from preimmune serum rabbit was used as control. HRP-conjugated goat anti-rabbit IgG was used as secondary antibody.

band pattern as well as three major bands of 180 kDa, 66, and 43 kDa and a light smear under 45 kDa (Lane 2). Western blot assays using different antibodies against previously described tegumental glycoproteins α GP1, α GP2-3, α GP6, and α GP7, as well as against α pig IgG, showed that the only protein released by the PBS-PLC treatment was recognized by α GP1 antibodies, with an apparent molecular weight of 180 kDa (Figure 1(b)). The molecules contained in the PBS-PLC fraction were negative to the anti-CRD antibodies. Exposure of CRD requires cleavage by phosphatidylinositol-specific phospholipase C (PIPLC) of GPI at the junction between the phosphate and the hydrophobic diacylglycerol moiety [23, 24].

Localization studies with light and electron microscopy were carried out to determine if changes on the surface glycoprotein pattern could be observed after PBS-PLC treatment. As shown in the light and electron micrographs in Figures 2(a) and 2(c), a positive peroxidase reaction is present on the tegumentary and microtriche surface with anti-GP1 antibodies, but the same antibodies did not recognize it on cysticerci after PLC treatment (Figures 2(b) and 2(d)), illustrating that GP1 is almost completely removed from the tegumental surface. In contrast, the molecules GP2-3, GP6, GP7, and pig IgG were still bound to the microtriche surface after treatment with PLC (Figures 3(a)–3(d)). For these experiments preimmune rabbit IgG was used as negative control (Figure 2(e)).

To ascertain that the PLC treatment induced changes in the general pattern of surface carbohydrates on cysticerci, RR and lectin staining were carried out. The PBS-PLC treatment of cysts did not induce a noticeable change on regular distribution of RR stained particles on the surface of microtriches (Figures 4(a) and 4(b)). Images obtained after exposure to ConA and WGA normally appear as electron dense particles on the outer surface of microtriches (Figures 4(c), 5(a), and 5(c)). Particles that bind ConA were less abundant after PBS-PLC treatment (Figure 4(d)). In contrast, PBS-PLC treatment abolished almost completely the binding of WGA as illustrated by the negative peroxidase reaction in Figures 5(b) and 5(d). Interestingly, the patches recognized by WGA were destroyed by the PBS-PLC treatment whereas the patches for Con A were preserved.

4. Discussion

We have examined the effect of PLC treatment on plasma membrane lipids in *T. solium* cysticerci. PLC preferentially hydrolyses phosphatidylcholine but has a broad specificity for other lipids such as sphingomyelin, phosphatidylserine, and lysophosphatidylcholine, but not for glycosylphosphatidylinositol [25]. We have already demonstrated that glycoproteins GP1, GP2-3, GP6, and GP7 and pig IgG are abundant molecules seen as electron dense particles on the external surface of the tegument of *T. solium* cysticerci. GP1

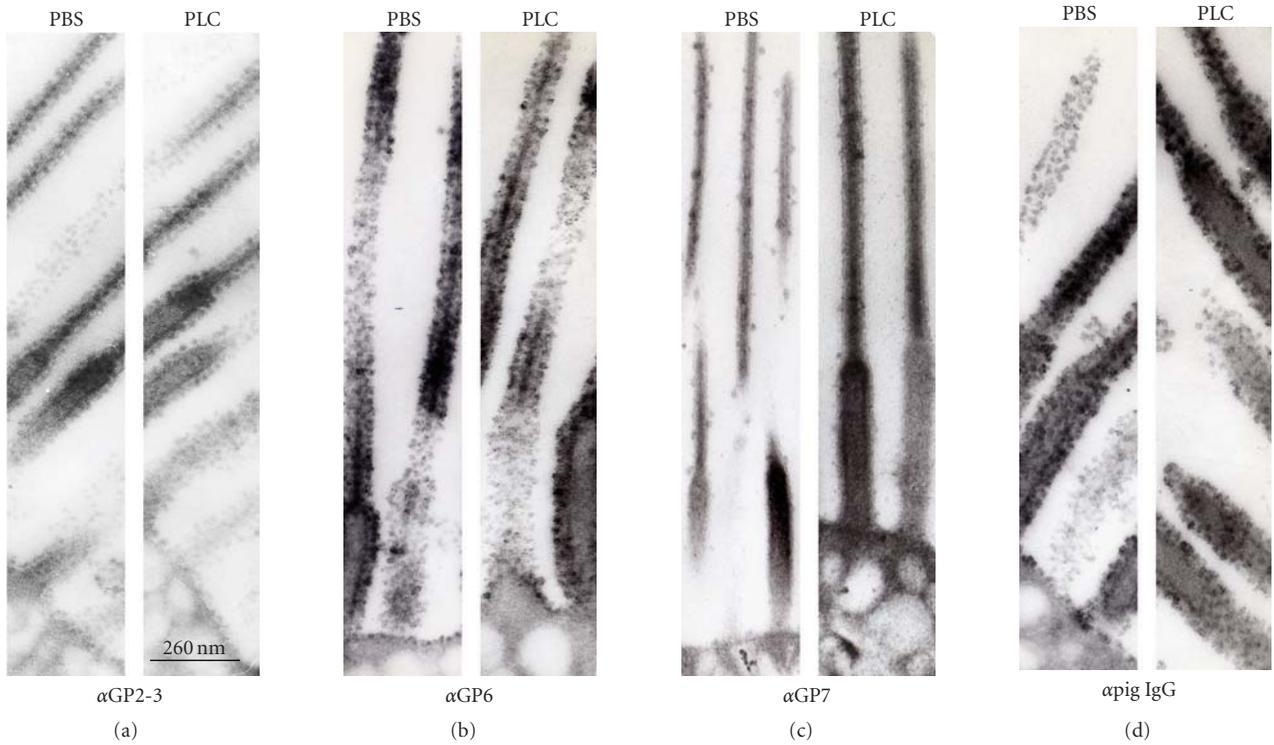


FIGURE 3: Tegument surface of *Taenia solium* cysticerci, after 1 hour incubation with PBS or PLC. Sections of the bladder wall of cysticerci were incubated with the IgG fraction of (a) anti-GP2-3, (b) anti-GP6, (c) anti-GP7, and (d) anti-pig IgG.

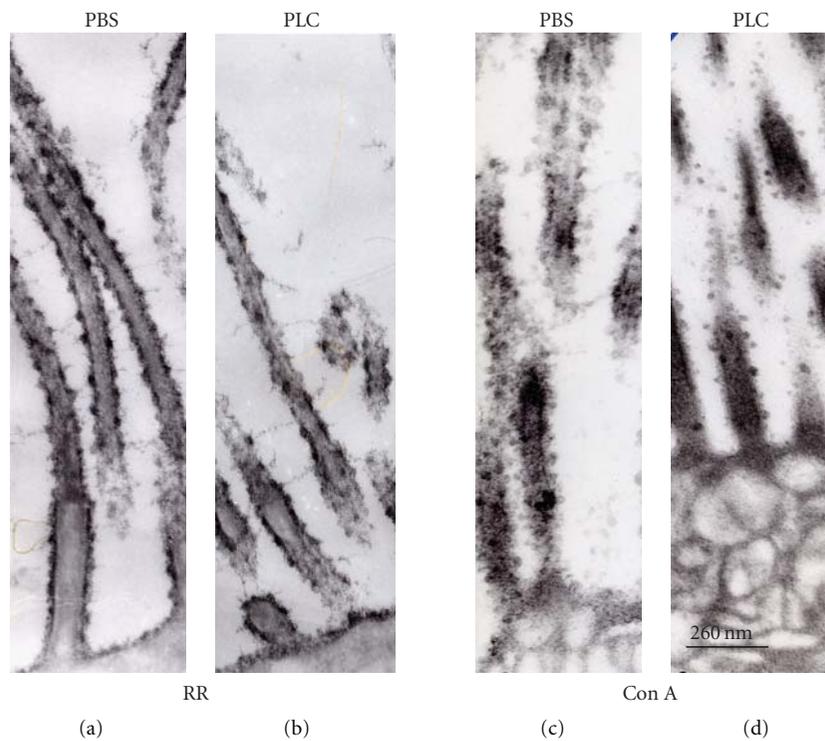


FIGURE 4: Electron micrographs of the tegumentary microtriches of *Taenia solium* cysticerci, after 1 hour incubation with PBS and PLC. Sections of the bladder wall were (a) and (b) stained with ruthenium red (RR) and (c) and (d) incubated with concanavalin A (ConA)-peroxidase.

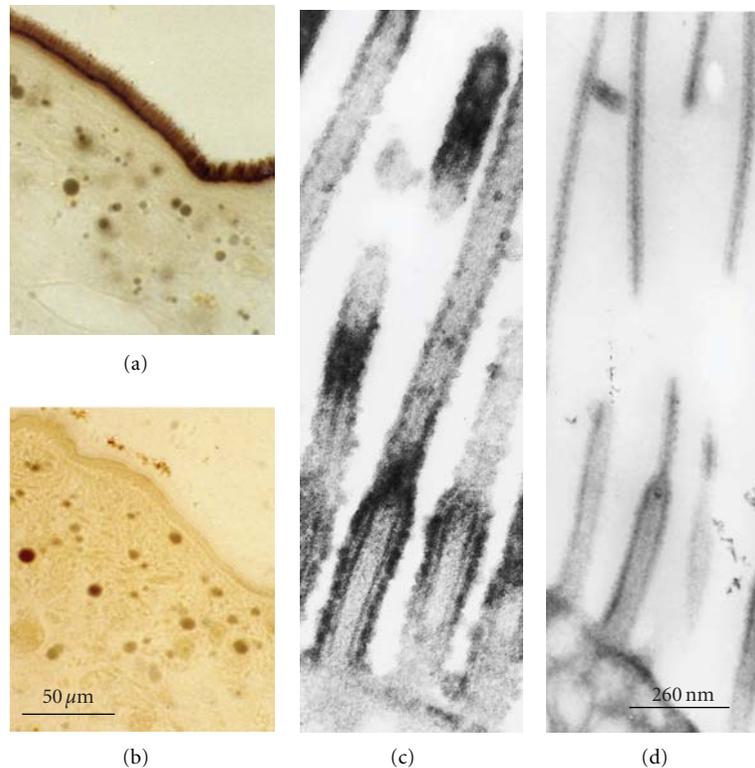


FIGURE 5: Light and electron micrographs of sections of the bladder wall of cysticerci incubated for 1 hour with PBS ((a) and (c)) and PLC ((b) and (d)) and probed with WGA-peroxidase.

is also present on the surface of *T. solium* and *T. saginata* adult worms as well as in *T. saginata*, *T. pisiformis*, and *T. crassiceps* cysticerci [7].

Western blot results showed that GP1 was present in the PLC released material, whereas CRD was absent (Figure 1). Moreover, light and electron microscopy images showed the loss of GP1 from the surface of cysticerci after treatment with PLC (Figure 2). All assays to determine the lipolytic activity of PLC were carried out in the presence of proteinase inhibitors that inhibit the effect of proteases in our preparation but do not affect the activity of PLC. In addition, data showed that the receptors to IgG and the other glycoproteins also present on the surface of cysticerci were not affected by PLC. This suggested that GP1 association with the tegumental membrane depends on an interaction with the phospholipids mentioned before. It is worth mentioning that PLC has an MW of 43 kDa and it is therefore possible that the band of around 43 kDa found in the PLC-released material could correspond to this enzyme used in the assay.

On the other hand, analysis of the general changes in the surface carbohydrates of *T. solium* cysts using ruthenium red and the lectins WGA and Con A suggested that the action of PLC is specific for a few surface components. The basic pattern of particles in the glycocalyx is clearly maintained after PLC treatment, as observed in the assays using RR and Con A. The removal of most of the binding sites for WGA suggested that N-acetyl-D-glucosamine, N-

acetylneuraminic, and sialic acid are important components of the released materials including the glycoprotein GP1. The ConA lectin principally binds to α -methyl-D-mannoside, D-mannose/glucose, and N-acetyl-D-glucosamine. The almost complete removal of binding sites to WGA and slight decrease in the binding sites for ConA after PLC treatment suggests that sugars recognized by both lectins are present on the surface. These findings are in agreement with results reported in other cestodes [1, 26]. It seems clear that the particles observed on the surface are complex structures composed of different molecules, among them, the sugars and glycoproteins.

It is known that products of the phospholipases such as phosphatidylinositol and diacylglycerol are recognized as important reservoirs of second messenger precursors and as anchors for membrane enzymes.

Our report is the first demonstration of a novel type of membrane anchor; however the biological role of these structures in *Taenia* genus remains to be explored.

Acknowledgments

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Research Article

Progesterone Induces Scolex Evagination of the Human Parasite *Taenia solium*: Evolutionary Implications to the Host-Parasite Relationship

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Taenia solium cysticercosis is a health problem in underdeveloped and developed countries. Sex hormones are involved in cysticercosis prevalence in female and male pigs. Here, we evaluated the effects of progesterone and its antagonist RU486 on scolex evagination, which is the initial step in the development of the adult worm. Interestingly, progesterone increased *T. solium* scolex evagination and worm growth, in a concentration-independent pattern. Progesterone effects could be mediated by a novel *T. solium* progesterone receptor (TsPR), since RU486 inhibits both scolex evagination and worm development induced by progesterone. Using RT-PCR and western blot, sequences related to progesterone receptor were detected in the parasite. A phylogenetic analysis reveals that TsPR is highly related to fish and amphibian progesterone receptors, whereas it has a distant relation with birds and mammals. Conclusively, progesterone directly acts upon *T. solium* cysticerci, possibly through its binding to a progesterone receptor synthesized by the parasite.

1. Introduction

Cysticercosis, caused by the metacestode stage of *Taenia solium*, is a serious health and veterinary problem in many developing countries [1–3]. In humans, *T. solium* cysticerci cause neurocysticercosis, which affects ~ 50 million people worldwide, and it has been recently considered as an emergent disease in the USA [4]. *T. solium* also infects pigs, its intermediate host, leading to major economic losses [5, 6].

When humans ingest undercooked contaminated pork meat, the adult worm develops in the small intestine. After two months of asymptomatic infection, this tapeworm starts producing thousands of eggs that, once released with the stools, can contaminate the environment, infecting pigs (rapidly differentiating into cysticerci mainly in the muscle)

and humans (where most severe symptoms are observed due to the presence of cysticerci in the brain) [1, 7].

Thus, maintenance of the parasite's life cycle depends on the adult tapeworm development [8]. In fact, even in communities which do not rear or consume pigs, human neurocysticercosis can be found, because of the presence of a tapeworm carrier [9, 10]. Furthermore, tapeworm development in turn depends on scolex evagination, the initial step through which a single cysticercus becomes an adult parasite with capability of producing infective eggs [11].

Recent information reveals that sex hormones can affect the course of a parasite infection [12–16], as in the case of taeniasis/cysticercosis [17–19]. Moreover, frequency of *T. solium* pig cysticercosis is increased during pregnancy, when there is a significant increase in progesterone levels [19, 20].

It has also been demonstrated that castration in naturally infected male boars, induces an increase in the prevalence of cysticercosis, which highlights the possible role of host androgens to restrict parasite establishment and estrogens to facilitate it [19].

Furthermore, *Taenia crassiceps* (a close relative of *T. solium*) has shown to be affected by in vivo and in vitro sex steroid treatment. Specifically, 17β -estradiol increases the reproduction of *T. crassiceps* cysticerci in vitro, while testosterone or dihydrotestosterone decreases it [21]. When castrated mice are treated with 17β -estradiol, the number of parasites as well as their infective capacity increases up to 200% [22, 23] meanwhile progesterone has the opposite effect in castrated mice of both sexes: a decrease in the parasite loads of almost 100% [24].

Since an mRNA sequence similar to that of estrogen receptor has been found in *T. crassiceps* [21], it is possible that the direct effect of estradiol on *T. crassiceps* reproduction could be due to its binding to this receptor [21]. In fact, numerous sex steroid actions in vertebrates are mediated by the binding to their nuclear receptors, which in this form regulate gene expression, as in the case of estrogens [25], androgens [26], and progesterone [27]. The latter interacts with two main progesterone receptor (PR) isoforms [28], whereas other hormones such as androgens only have one specific receptor [26]. Interestingly, a similar mechanism could occur also in parasites [21, 29, 30].

Steroid hormone effects are not restricted only to cestode parasites but also to nematodes such as *Ancylostoma dudodenale*, whose number of larval and adult stages is increased by sex steroid hormones in several organs of mice [31]. Moreover, adult and muscle larvae of *Trichinella spiralis* are increased in ovariectomized female rats [14], suggesting that estrogens are restrictive factors for parasite establishment, while androgens should play a permissive role to the infection. Concomitantly, trematode parasites are also affected by sex steroids. For instance, in vitro, testosterone has an antifecundity effect upon male and female *Schistosoma mansoni* adult worms [29], as well as dehydroepiandrosterone, which in vitro reduces the viability and oviposition of *Schistosoma mansoni* [32].

As it can be seen, direct effects of sex steroids upon helminth parasites (cestodes, nematodes, and trematodes) are not unusual. In fact, previous results suggest that these pathogens are not only directly affected by hormones, but they have also developed several strategies to exploit the host's endocrine microenvironment [33, 34], which include degradation of host proteins as an alternative source of aminoacids [35], development of parasitic-sex steroid receptors [29, 30], and cross-activation of signal transduction pathways [36, 37].

Taking into consideration this information, the aim of the present study was to explore the role of progesterone on *T. solium* cysticerci development, evaluating its in vitro effects on scolex evagination and adult worm growth, key processes in the maintenance of the infectious cycle in pigs and humans. The in vitro effect of progesterone on *T. solium* was studied through pharmacological (the use of RU486, a progesterone antagonist which binds to intracellular PR) and

molecular (RT-PCR, western blot, phylogenetic analyses) approaches, in order to figure out the mechanism of progesterone actions in the parasite.

2. Materials and Methods

2.1. Obtention of Parasites. *T. solium* cysticerci were dissected from the muscle of infected pigs, which were euthanized at the Veterinary School of the Universidad Nacional Autónoma de México. The method was previously evaluated by the University Animal Care and Use Committee to ensure compliance with international regulations and guidelines. The fibrous capsule that surrounds each parasite was carefully separated with the use of a dissection microscope. Once dissected, cysticerci were placed in tubes containing sterile PBS (1X) supplemented with 100 U/mL of antibiotics-fungizone (Gibco, Grand Island, NY, USA) [38]. Samples were centrifuged for 10 minutes, at 800 g at 4°C and the supernatant was discarded. Pellets containing cysticerci were incubated in Dulbecco's Modified Medium (DMEM) without fetal serum supplementation (Gibco, BRL, Rockville, Md, USA). They were then washed by centrifugation 3 times for 10 minutes at 800 g with DMEM. After the final wash, viable parasites (complete and translucent cystic structures) were counted using a binocular microscope.

2.2. Progesterone and RU486 Dose-Response-Time Curves. Culture grade progesterone and RU486 were obtained from Sigma (Sigma-Aldrich, USA). For in vitro tests, progesterone-water soluble (powder cell culture tested, Sigma-Aldrich, USA) was dissolved in DMEM free-serum culture medium, while RU486 was dissolved in pure ethanol (Sigma) to the desired stock concentration, and sterilized by passage through a 0.2 mm millipore filter. For concentration-response curves, the experimental design was as follows, using four wells per treatment: (a) progesterone vehicle (only DMEM); (b) RU486 vehicle (ethanol at the final concentration of 0.06% per well); (c) progesterone at 0.06, 0.25, 2.5, 3.175, and 63.5 μ M; (d) RU486 (at the same concentrations of progesterone); and (e) a combination of progesterone and RU486 in all concentrations described before. For time-response curves, cysticerci from all treatments were cultured during 20 days, with daily inspections of scolex evagination and worm length. Culture wells contained 5 mL of DMEM-medium and were incubated at 37°C and 5% CO₂. Progesterone and RU486 were prepared in a final volume of 100 μ L and added to 5 mL of medium in each well. From concentration-response curves of each steroid, we selected an optimal concentration for progesterone (0.25 μ M) and RU486 (2.5 μ M), to be used in the time-response curves. Culture media, as well as hormone treatments, were completely replaced every 24 hours during 20 days of culture. Scolex evagination and worm length were daily determined in all cultured cysticerci using an inverted microscope (Olympus, MO21, Tokyo) at 10X and 20X magnification. Worm length was considered as the millimetric addition of scolex, neck, and strobila.

2.3. RNA Extraction of Cultured *Cysticerci* in Presence of Progesterone and RU486. Total RNA was isolated from *T. solium* cysticerci of each in vitro treatment as well as from uterus of mouse (positive expression control) using Trizol reagent (Invitrogen, Carlsbad, Calif, USA). In brief, cysticerci were disrupted in Trizol reagent (1mL/0.1 g tissue) and 0.2 mL of chloroform was added per mL of Trizol. The aqueous phase was recovered after 10 minute of centrifugation at 14 000 g. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol, and redissolved in RNase-free water. RNA concentration was determined by absorbance at 260 nm and its purity was verified after electrophoresis on 1.0% denaturing agarose gel in presence of 2.2 M formaldehyde.

2.4. *T. solium* Progesterone Receptor (TsPR) Gene Amplification. Total RNA from all treated cysticerci was reverse-transcribed followed by specific PCR amplification of the putative TsPR by using one specific pair of primers designed to amplify corresponding fragments of the DNA-binding domain (one of the most conserved regions of all PR sequenced genes reported in the NIH Gene Data Bank). β -actin was used as a control gene of constitutive expression, as we have previously described [21, 39]. Primer sequence of PR was: sense 5'-GGAGGCAGAAATTCAGACC and antisense 5'-GACAACAACCCTTTGGTAGC; for β -actin, primer sequence was sense 5'-GGGTCAGAAGGATTCCTATG and antisense 5'-GGTCTCAAACATGATCTGGG. PCR products were visualized in 2% agarose gel stained with ethidium bromide. In all cases, a single-band corresponding to the expected base pair size of the amplified gene fragment was detected. Uterus from intact mouse was used as positive expression control for PR expression. TsPR expression is presented as the ratio of the optical density (OD) of the studied gene relative to the expression in the same preparation of the β -actin gene.

2.5. PCR Product Sequencing. TsPR was directly purified from the gel using a commercial kit (DNeasy Tissue Kit, QIAGEN) and sequenced. DNA sequences were determined by using a Thermo Sequenase cycle sequencing kit (Biorad) and an automatic sequencer (Model LIC-4200, Aloka Co.). Sequence data were analyzed by using DNASIS Software (Hitachi Software Engineering, Tokyo, Japan). In addition, nucleotide sequences were translated to their corresponding protein sequences by means of ExpASy Molecular Server.

2.6. TsPR Detection by Western Blot. Protein was obtained from cultured *T. solium* cysticerci. Untreated cysticerci and those treated with progesterone and/or RU486 were disrupted in Tris-HCl (1mL/0.1 g tissue), proteinase K (100 units/mL), and proteases inhibitor cocktail (Calbiochem). The supernatant was recovered after 15 minutes of centrifugation at 8000 g. Protein concentration was obtained by absorbance at 320 nm using the Bradford-Lowry method. Total protein of *T. solium* (50 μ g per well) was boiled in reducing Laemmli sample buffer, separated by SDS-PAGE (10% acrylamide) and electro-blotted onto nitrocellulose membranes.

Membranes were blocked for 2 h with PBS 1X buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.47 mM KH₂PO₄) containing 0.25% of BSA. For protein immunodetection, membranes were subjected overnight to immunoblotting with 1 μ g/mL of anti-PR polyclonal antibody (C-20, Santa Cruz Biotech.), diluted 1 : 1500, followed by HRP-conjugated anti-rat IgG (Santa Cruz Biotechnology; diluted 1 : 5000) for 1 hour, at room temperature. Next, membranes were washed five times in 1X PBS and bands were visualized using the Enhanced ChemoLuminiscence system, according to manufacturer's instructions (Super Signal ECL, Pierce). Chemiluminiscent signals were captured on Kodak Bio-Max film, and bands were quantitatively analyzed from digitized images captured from the films with the Gel-Doc system (BioRad, Richmond, Calif, USA), using the Bio-Rad Quantity One software. Rat uterus was used as positive expression control for this experiment. The content of the protein band corresponding to the TsPR is presented as the ratio of the optical density of the studied protein relative to the content of α -tubulin in the same preparation, used as a constitutively loading control protein.

2.7. Phylogenetic Analysis of the TsPR. The TsPR protein sequence was aligned to the PR protein sequences of other species (including mammals, birds, fish, one reptilian, and one amphibian) obtained from protein data sets in GenBank. Sequence alignment was done using Clustal W software [40]. Alignment of TsPR contained 88 amino acids from 12 different taxa. Phylogenetic relationships were inferred using the Neighbor joining (NJ) method. Robustness of the NJ tree was evaluated using bootstrap of 10 000 replicates. The tree was drawn using RETREE and DRAWGRAM from PHYLIP [41]. The genetic differentiation between taxa was estimated using the mean character difference with the help of PAUP* 4.0b10 software [42]. It is important to point out that the number of species used for the analysis was selected based on the PR sequence found in the gene data bank (for some species there is only one sequence).

2.8. Experimental Design and Statistical Analysis. Hormone dose-response-time curves were estimated in 4 independent experiments. The response variable used in statistical analyses was the total number of evaginated scolices that showed worm growth and motility in all wells of each hormone concentration and time of exposure, for every experiment. Hormones, their concentrations and times of exposure, were the independent variables. The data for the 4 replicates of each treatment were expressed as an average. Data were analyzed using one way variance analysis (ANOVA). If ANOVA showed significant differences among treatments, a Tukey Test was applied for test significance. Differences were considered significant when $P < .05$.

3. Results

When *T. solium* cysticerci were in vitro exposed to progesterone, an increase in the scolex evagination was observed in all treated parasites compared to control groups, where

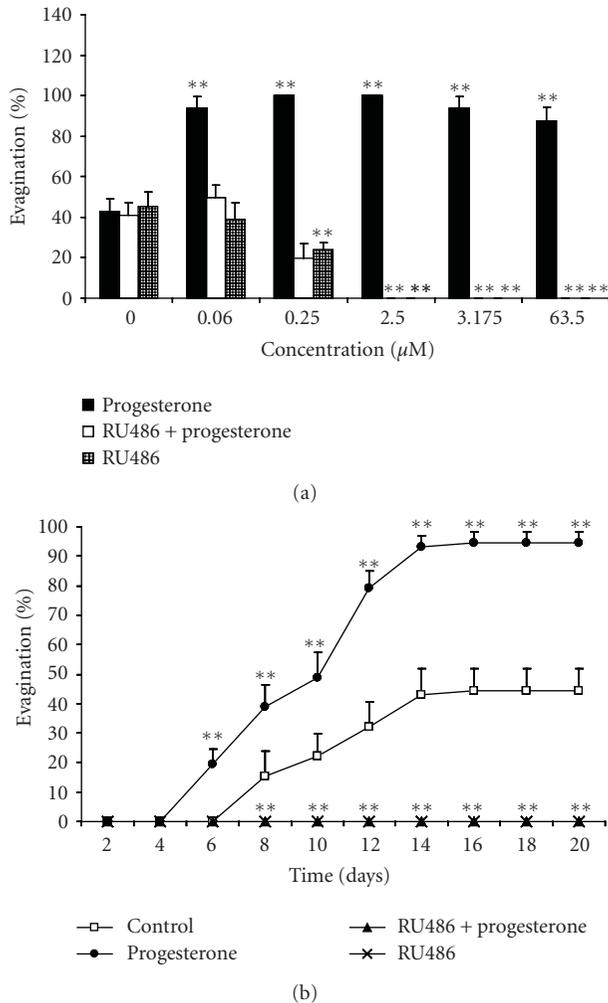


FIGURE 1: Progesterone induced scolex evagination of *Taenia solium* in a concentration-independent pattern (a) maintained along the time (b). On the contrary, RU486 exerted a potent concentration-dependent evagination-inhibitory effect (a), even in presence of progesterone and during the total 20 days of in vitro culture (b). In concentration-response curves (panel a), cysticerci treated with vehicle are referred as concentration zero. Data are represented as mean \pm SD; $**P < .05$.

only 40% spontaneously evaginated (Figure 1). However, this evagination-promoting effect mediated by progesterone was independent of the tested concentrations (Figure 1(a)). RU486 showed a strong anti-parasite effect, since progressively inhibited scolex evagination, reaching its maximum effect at $2.5 \mu\text{M}$, even in the presence of progesterone (Figure 1). Interestingly, in the case of RU486, a concentration-dependent pattern was evident, and no significant differences were observed between RU486 plus progesterone and RU486 alone-treated groups (Figure 1(a)).

Concomitantly, the evagination-promoting effect of progesterone ($0.25 \mu\text{M}$) was maintained through all 20 days of in vitro culture, reaching its highest response on day 14 in culture, in relation to untreated parasites (Figure 1(b)). Consistently, when cysticerci were exposed to $2.5 \mu\text{M}$ of RU486, scolex evagination was observed neither during

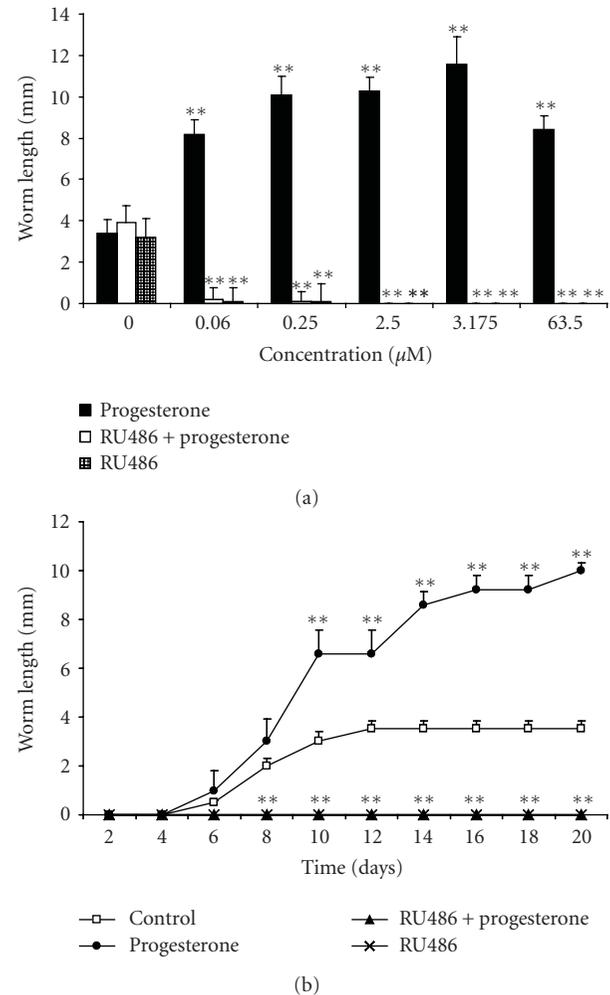


FIGURE 2: Progesterone stimulates worm growth in a concentration-independent pattern (a), reaching its maximum effect at 20 day of in vitro culture (b). In contrast, RU486 inhibits worm development with the lowest concentration ($0.06 \mu\text{M}$) (a) and during the whole culture time (b). Progesterone-treated parasites were motile and undamaged on the culture plate, typically distinct to those treated with RU486. Worm length was considered as the addition (mm) of scolex, neck, and strobila. In the concentration-response curves (panel a), cysticerci treated with vehicle are referred as concentration zero. Data are represented as mean \pm SD; $**P < .05$.

the first days of culture nor at the end of the process (Figure 1(b)).

It is important to mention that viability of evaginated cysticerci was verified daily by means of worm motility in the culture plate, which was constant through all days of in vitro culture. Injured parasites were recognized by a progressive internal disorganization: development of opaque areas in the tegument and loss of translucence of the vesicle (data not shown).

Progesterone also affected in vitro worm growth. From the lowest concentration ($0.06 \mu\text{M}$) progesterone duplicated worm length on day 10th (measured as the addition of scolex, neck, and strobila of the developing parasite) with respect to the control group, and reached a plateau (Figure 2(a)).

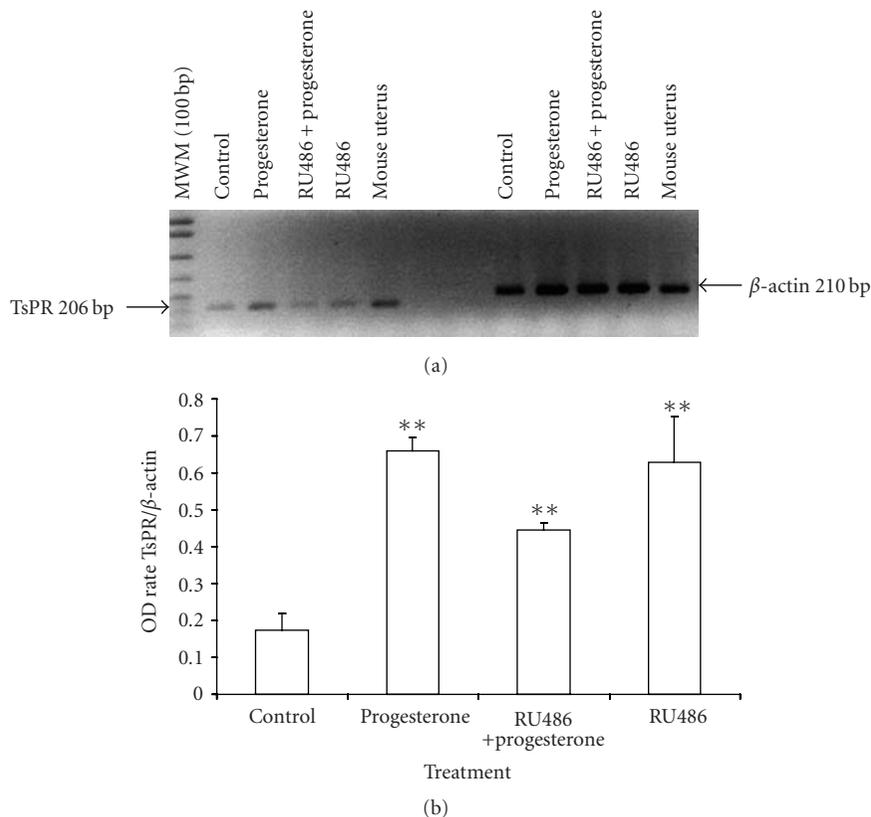


FIGURE 3: TsPR gene expression in *T. solium*. A single band of 206 bp, corresponding to the *Taenia solium* Progesterone Receptor (TsPR) was detected in *T. solium* and mouse uterus (a). Progesterone as well as RU486 increased TsPR expression in *T. solium* cysticerci. Densitometric analysis is shown in (b). β -actin was used as constitutive expression gene. Data are represented as mean \pm SD; ** $P < .05$.

The opposed effect was observed with RU486 treatment: when *T. solium* cysticerci were exposed to $2.5 \mu\text{M}$ and higher concentrations of RU486, a total inhibition of worm development was seen, even in the presence of the highest concentration of progesterone (Figure 2(a)). Dissimilar to the results of evagination percentage, the effect of RU486 on worm length was independent of the tested concentration. In addition, the *T. solium* worm gradually grew up in response to $0.25 \mu\text{M}$ of progesterone (Figure 2(b)). Differentiated worms in absence of hormones or anti-hormonal stimulus had a spontaneous development, reaching their maximum length (3.5 mm) at 12 day in culture (Figure 2(b)). Once again, in the presence or absence of progesterone, no worm differentiation was observed with $2.5 \mu\text{M}$ of RU486 along all the time of in vitro culture (Figure 2(b)).

A single band corresponding to the expected molecular weight of the amplified fragment of PR (approximately 206 bp) was detected from TsPR and mouse uterus (Figure 3(a)). Moreover, progesterone and RU486, separately and/or combined, increased TsPR mRNA content related to the control group (Figure 3(b)).

TsPR protein was detected by western blot as a main band of approximately 116 KDa (Figure 4(a)). This TsPR matches to PR-B isoform that has been previously reported for rodents and human cell lines [43–46]. Nevertheless, no bands corresponding to PR-A isoform were identified in

any of the tested treatments in the parasite (Figure 4(a)). On the contrary, by using the same antibody, a couple of bands of 87 and 116 KDa were well recognized in the control tissue used (rat uterus), corresponding to PR-A and PR-B, respectively (Figure 4(a)). TsPR content was increased in response to RU486 but not when parasites were exposed to both progesterone and RU486 (Figure 4(b)).

A preliminary sequence of TsPR was obtained by PCR product sequencing and then translated to protein sequence (ExpASY Proteomics Server). A posterior analysis of this TsPR showed homology of around 60% to the protein sequences previously reported for mouse, rat, rabbit, and human PR in the GenDataBank. It is important to mention that the analyzed conserved motif was situated in a region of approximately 50 aa, located in the DNA-binding domain of the C-terminal motif (from aa position number 110 to 160 of the mammal sequences described earlier) (data not shown). A more precise analysis of the TsPR sequence involved a Neighbor Joint Tree (NJT) for studying phylogenetic relationships (Figure 5). This NJT was inferred from the PR dataset, producing a single tree composed by 5 groups. The first contained sequences of 6 species of mammals (including pig and human, both natural hosts of the parasite), the second group consisted of one reptilian and one bird. The third group included only one amphibian. The fourth group was composed of 3 fish species, and the fifth group was

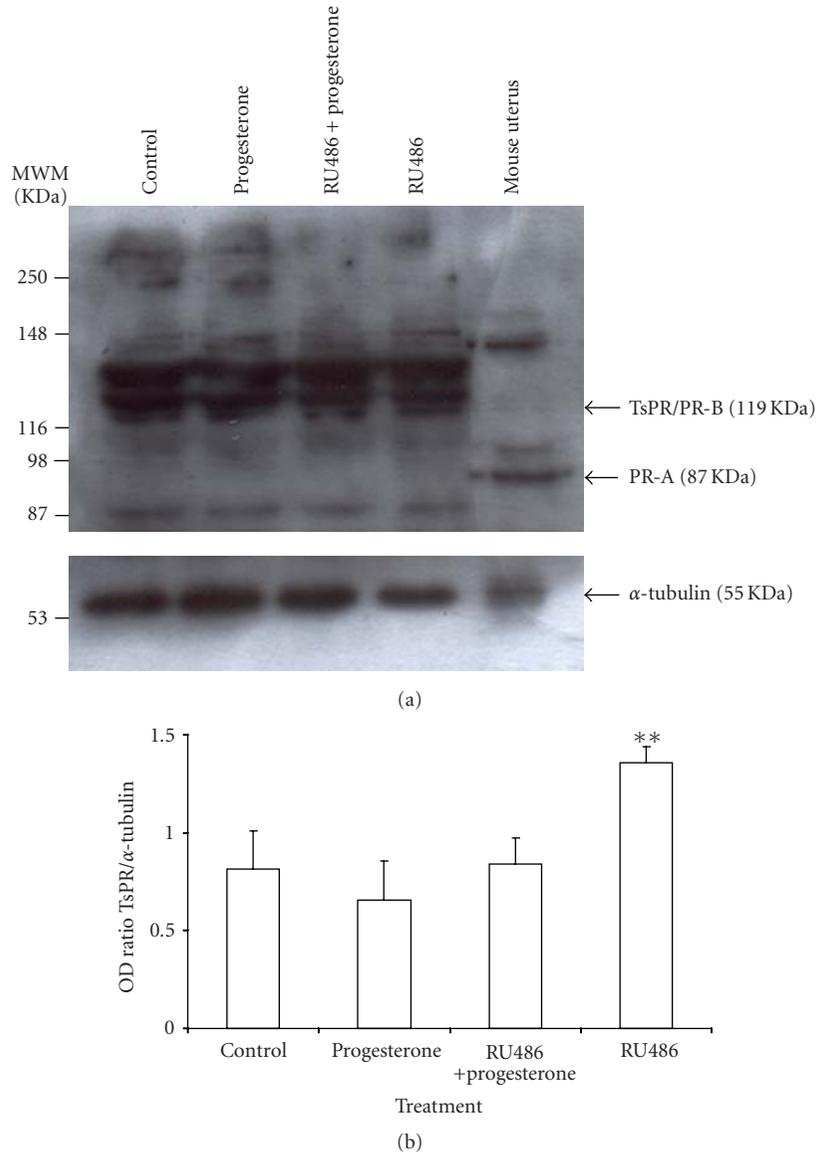


FIGURE 4: TsPR protein detection in *T. solium*. A main band of approximately 116 KDa, equivalent to TsPR was detected in *T. solium* (a). This TsPR matches to the molecular weight of PR-B isoform from rat uterus, but not to PR-A (~ 87 KDa), where no protein bands in the parasite were observed (a). TsPR protein was significantly up-regulated by RU486 alone, but not by progesterone and the combination between progesterone and RU486 (b). α -tubulin was used as control constitutive protein. Data are represented as mean \pm SD; ** $P < .05$.

only composed by *T. solium*. The phylogenetic relationships among the 5 groups received good bootstrap support ranging from 66 to 100% (see Figure 5). Additionally, TsPR is related to the PR family of vertebrates, more closely associated to reptilian and amphibian (Figure 5). This finding suggests that TsPR is definitively not a product of host cell contamination, specifically not of pig nor human cells, because of the big distance between *T. solium* and mammals in the NJT.

4. Discussion

Here we describe the effects of progesterone and its antagonist RU486 upon scolex evagination and adult worm

growth. First of all, it was clear that progesterone has a direct stimulatory effect on *T. solium* in vitro. In fact, progesterone exerts a marked evagination-promoting effect in a concentration-independent pattern, maintained entire time in culture, making that all parasites differentiate at 14 days of in vitro culture.

T. solium cysticerci not only showed evagination but they also presented a constant motility in the culture plate, which suggests that progesterone did not affect parasite viability and, therefore, they were alive during the culture process. Moreover, progesterone also induced the growth of the worm in the evaginated parasites by 2 folds, with respect to untreated cysticerci.

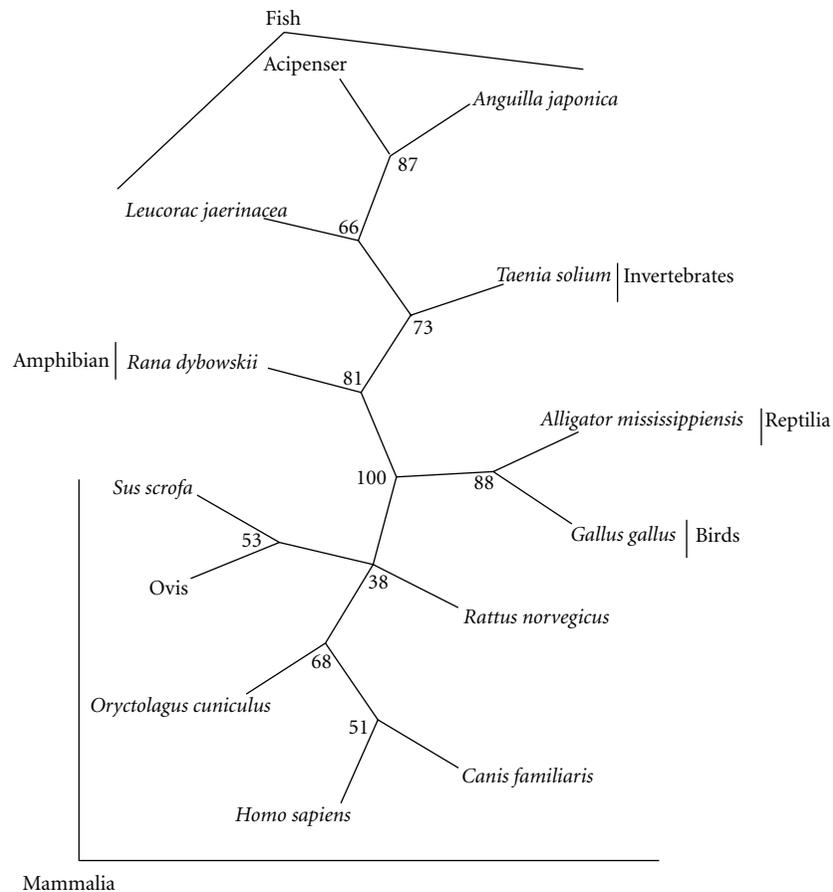


FIGURE 5: Neighbor Join Tree (NJT) for phylogenetic relation analysis. PRs from several species of fish, amphibian, reptilian, bird, and mammals were analyzed through a NJT for searching probable relationship to the *T. solium* PR identified and sequenced. TsPR showed close relation to PRs from fish and amphibian, but distant to their counterparts in mammals. Numbers on the NJT means bootstrap support ranging among analyzed species.

Interestingly, neither evagination nor worm size depend on progesterone concentrations, which are different from those typically found in mammals [47–50]. This finding highlights that sex steroids could conserve several effects on invertebrate organisms (such as helminth parasites), but their action mechanisms may differ from those reported in rodent models and human cells.

We also provide elements for a possible action mechanism through which progesterone exerts its effects upon *T. solium* differentiation. In fact, this helminth seems to have developed a molecule able to recognize progesterone and mediate its effects, a protein we named TsPR (*Taenia solium* Progesterone Receptor). This putative steroid receptor is expressed in *T. solium* and was upregulated by progesterone and RU486.

It is important to highlight that TsPR has a counterpart sequence, recently identified in the sequencing of the *T. solium* genome, performed by The Consortium of the *Taenia solium* Genome Project of the Universidad Nacional Autónoma de México (data not shown) [51]. These additional data confirm the existence of a PR in *T. solium*.

Besides, TsPR was detected both at mRNA and protein levels. Interestingly, this TsPR matches only to PR-B isoform, suggesting that *T. solium* presents only one form of PR-like, as it has been also described in other organisms such as rabbits [52, 53]. This result indicates that a single form of TsPR is actively expressed and translated to protein, which probably has repercussions on *T. solium* physiology and its relationship to the host.

Furthermore, this TsPR showed high degree of relation to their PR counterparts in fish and amphibian, but it is distant to mammalian sequences. This finding has two important connotations: firstly, it suggests that TsPR is a close relative of the steroid nuclear receptors that bind to progesterone. Secondly, this PR in *T. solium* definitively is not a contamination product from pig or human cells because it has a far relation to PRs sequenced in these organisms.

RU486 (2.5 μ M) totally inhibited scolex evagination and consequently worm development. This remarkable antiparasite effect was maintained even in the presence of the highest concentration of progesterone (63.5 μ M). Moreover, RU486-treated cysticerci showed internal disorganization;

development of opaque areas in the tegument, loss of translucence in the vesicule, and no differentiation along all the in vitro culture time. This result suggests a possible toxic effect of RU486 upon *T. solium* cysticerci, independent of the TsPR that has not yet been reported for this progesterone-antagonist.

RU486 induced TsPR expression as well as the concomitant protein production, clearly suggesting that this anti-hormone regulates PR expression in *T. solium*, as it has been reported in rodent models and human cell lines [54, 55]. Then, although RU486 had upregulatory effects on TsPR, the same toxic response in *T. solium* was observed, indicating that RU486's antiparasite mechanism does not involve TsPR nor progesterone-dependent pathways inhibition, but a potent cysticide effect on the parasite, worthy of further investigation.

5. Conclusions

Here, we describe two different effects of progesterone and RU486 that probably have different action mechanisms directly upon *T. solium* development. Progesterone effects could explain, at least partially, the higher prevalence of cysticercosis in pregnant than in nonpregnant swine [19]. Then, *T. solium* could respond to progesterone through a TsPR with capacity to bind the hormone, and in this way regulate parasite viability and survival inside an immunocompetent host. To the opposite, the RU486 antiparasite effect was lethal for *T. solium*. In fact, RU486 permanently blocked the evagination of *T. solium* cysticerci, which has negative consequences to the adult tapeworm development and production of infective eggs. Thus, RU486 could be considered as a potential new agent in the interruption of the *T. solium*'s life cycle, once we already know optimal doses of this antihormone as well as adverse and secondary effects in humans and other animal models.

To our knowledge, this is the first report where anticysticercus effects are described for RU486 and open a promissory field in the design of new strategies that include the antihormone therapy in the control of taeniasis/cysticercosis caused by *Taenia solium*.

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Research Article

Budding of *Taenia crassiceps* Cysticerci In Vitro Is Promoted by Crowding in Addition to Hormonal, Stress, and Energy-Related Signals

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Taenia crassiceps cysticerci (cysts) reproduce by budding. The cysts' production of buds was measured in vitro to explore parasite and environmental-related factors involved in the extreme individual variation in parasite loads of inbred mice. Cysts were placed in in vitro culture for 10 days at initial parasite densities of 1, 5, 10 cysts/well in 1 ml of RPMI Medium 1640 without serum. Results showed that there is considerable intrinsic initial variation among inoculated cysts in their production of buds and that increasing parasite density (crowding) stimulates the overall production of buds and recruit into budding most of the cysts. Identical cultures were then subjected to various treatments such as heating and exposure to peroxide to induce stress, or to 17 β -estradiol, insulin, glucose, or insulin+glucose to supplement putatively limiting hormonal and energy resources. All treatments increased budding but the parasites' strong budding response to crowding alone overshadows the other treatments.

1. Introduction

Taenia crassiceps is a cestode that, when adult, lives in the intestinal lumen of some carnivore species (i.e., fox) and in the subcutaneous connective tissue and pleural and peritoneal cavities of rodents (i.e., mice) in its metacestode (cysticercus) stage [1]. For experimental conditions, the infection with *T. crassiceps* is simply attained by an ip injection of a few cysticerci in laboratory mice. Once in the peritoneal cavity, the cysticerci reproduce asexually by budding [2], until reaching massive parasite loads in a matter of 3–6 months that weigh as much as the host [3]. Measuring parasite intensity in such conditions is an easy task requiring no more than a magnifying glass to count the number of parasites installed in the host at the time they are harvested by way of thoroughly washing the infected peritoneal cavity. Thus, Experimental murine intraperitoneal (ip) cysticercosis by *T. crassiceps* ORF strain (ExpMurIPTcrasCistiOrf) has been extensively used for

genetical, immunological, endocrinological, and behavioral studies of host-parasite relationships [3–7].

Notwithstanding its usefulness, ExpMurIPTcrasCistiOrf is plagued by unexplained great individual mouse variability in parasite loads and in IgG antibody responses, even within the same genetic strain and sex of the murine host and time of infection [8]. A number of factors from the individual host, the parasite, and the environment have been invoked as being involved in such variability [3]. The possible role of inherent variation in the putatively identical parasites composing the infecting inoculums has received less attention. There are two major sources of possible parasite variation between inoculums, one technical and the other biological. The technical sources are the number of infecting cysticerci in the inoculums, the time of infection studied, and the degree of injury suffered by the cysticerci upon their passage through the syringes' very tight caliber needles when squirted into the peritoneal cavities of the infected mice. The biological sources of unexplained variation are also plenty

and involve both host and parasite genetic and epigenetic physiological factors at the individual level [3].

To test the hypothesis of inherent budding variability among infecting cysticerci, without the participation of the host's responses, we counted the number of buds they produced in *in vitro* cultures in 1, 5, or 10 mL of RPMI Medium 1640 without fetal serum or added supplements, in isolated conditions (1 cyst/culture well), and in crowded conditions (5 and 10 cysts/well) or with various supplements during 10 days in the different culture conditions.

2. Material and Methods

2.1. Parasite Collection. The cysticerci employed came from two different BalbC/AnN female mice that had been infected ip 2 months before to develop a massive parasite load [3]. Harvesting the cysticerci implies killing the donor mice by etherization (in accordance with our institute's ethical procedure in dealing with experimental animals (at http://www.biomedicas.unam.mx/CodEtico_archivos/Reglamento_Bioterio.pdf) and immediately afterwards slitting its peritoneal cavity to release hundreds of cysticerci into a Petri dish containing phosphate-buffered saline (PBS) and 100 $\mu\text{g}/\text{mL}$ antibiotic (penicillin/streptomycin) at room temperature. Typically, the collected cysticerci are presented in three phases: initial (no buds and transparent vesicle), larval (filled with buds and transparent vesicle) and final (no buds and opaque vesicle), [8]. A significant fraction of the harvested cysticerci (~10%–20%) is the subpopulation of tiny (0.1–0.3 mm) nonbudded motile and transparent cysticerci, from which 10 cysticerci are selected to constitute each of the inoculums with which to infect experimental mice. Such selection of cysticerci expected would reduce variability in the resulting parasite loads between infected mice; and it does so to some extent, but significant individual variation in parasite loads usually subsists and not rarely, depending on strain and sex of infected recipient mice, some of the challenged mice are totally spared from infection [4–9]. It is from this subpopulation of tiny nonbudded cysticerci that the cysticerci employed in this *in vitro* study of their budding process were selected.

2.2. Parasite Culture. Microscopically nonbudded cysts were employed in two experiments. The independent variables were the initial density of cysts cultured in each well (density = 1, 5, 10 cysts/well with 1 mL of medium/well; in a dish with 6 wells), the nature of supplements to the culture medium, and the days of culture (0 to 10) at 37 or 42°C with 5% CO₂. The dependent variables were the number of buds found under light microscopy attached to each cyst (buds/cyst) in each well and the sum of all buds in each well (\sum buds). The culture medium employed was RPMI Medium 1640 without serum. The cysticerci came from two different donor mice and were cultured in the three density conditions without supplements in Experiment number 1 (from donor number 1) or were subjected to various treatments in Experiment number 2 (from donor number 2), such as heating (42°C) and peroxide (30 mM) to induce

TABLE 1: The final budding efficiency (final $\sum\text{buds}/\sum\text{cysticerci}$) in Experiments 1 and 2 at each density.

Culture condition	number of buds/number of cysts		
	$d = 1$	$d = 5$	$d = 10$
Control 1	0.17	3	10.3
Control 2	3	3.7	5.9
Heat	4	2.6	3.9
β -estradiol	6	6.1	4.5
Insulin	10	6.8	6.7
Glucose	6	5.8	5.9
Ins+Glu	6	5.4	7.9
Peroxide	5.5	5.1	5.1

stress in the cultured parasites, or supplemented with 17 β -estradiol (30 nM), insulin (1.5 U/mL), glucose (56 mM), or insulin+glucose (same concentrations as when by themselves only) to provide with energy resources and restore putatively energy limiting conditions. The culture medium in the wells was changed by fresh medium every 24 hours for the first two days and every 36 hours thereafter.

2.3. Statistics. Statistical analysis was performed with SPSS a Student's *t*-test to study the significance of contrasts between the different densities. Statistical significance levels were set at $P < .05$.

3. Results and Discussion

Figure 1 shows that in Experiment number 1 the sum of buds produced in each well (budding) progressively increases with increasing parasite densities in a wave-like fashion more clearly visible at density = 10. It took 8 days for density = 1 to initiate budding and by only 1 of the 6 cysts, while it took 3 and 2 days for densities = 5 and 10, respectively, for most or all cysts to bud and 7 days for the higher densities to start a second wave of budding. The first line of Table 1 shows that in Experiment number 1 the final budding efficiency (final $\sum\text{buds}/\sum\text{cysticerci}$) at each density increased from 0.17 to 3.00 to 10.3 for densities 1, 5, and 10, respectively. From these results it is clear that there is considerable initial variation in budding among cysts and that increasing parasite density increases the production of buds and reduces the proportion of nonbudding cysts.

Thus, initial differences in the distribution of “readiness to bud” among the cysts (as defined by the time it takes a resting cysticercus to start budding plus the time taken for a bud to become a cysticercus capable of budding) may well explain the variation of parasite loads in mice infected with apparently similar inoculums. To minimize variation in parasite loads users of ExpMurIPTcrasCistiOrf may try to presynchronize *in vitro* the cysts meant to be inoculated at 10 cysts/mL until most (80%) are already well into budding (>1 buds/cyst) before their selection and inoculation through a procedure nondisruptive of the cysts.

But now, the questions were how is crowding controlling the budding process? Are the differences in budding related

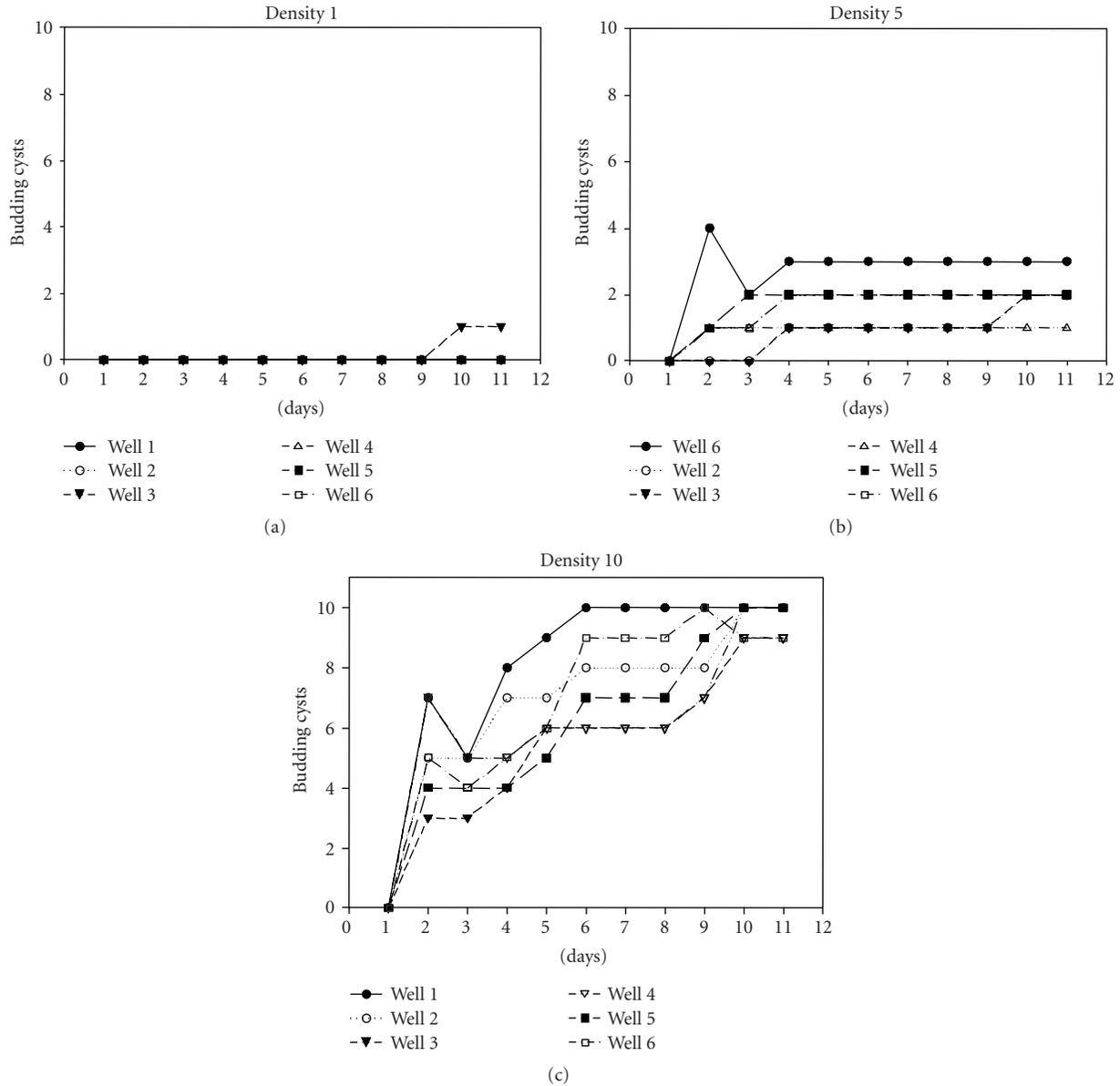


FIGURE 1: Sum of all buds in each well (\sum buds) of *Taenia crassiceps* cysticerci at three different parasite densities. The sum of buds produced in each well progressively increases with increasing parasite densities in a wave-like fashion

to and/or result from different responses to negative or positive pressures to bud existing in the over-crowded conditions in the peritoneal cavities of the donor mice and/or in the culture tubes? [10–14].

Experiment number 2 was designed to address those questions, bearing in mind that increasing density may decrease resource availability and lead the parasites to enter into stress. Accordingly, the cultured cysticerci from Donor number 2 were submitted to standard stress (heat and peroxide) or favorable conditions (addition of 17β -estradiol, insulin, glucose, insulin+glucose) and cultured in vitro as done in Experiment number 1. Figure 2 shows the budding process at the different conditions from day 0 to day 10. Table I most clearly shows that the total number of buds/cyst

produced in vitro are increased about twofold with respect to unsupplemented control values at densities 1 and 5 but not at density 10, which is in fact reduced by the supplementations. The great difference between the control values of \sum buds/cyst in Experiments number 1 and number 2 (0.17 and 3.0, resp.) speaks of there being such differences between the harvested cysticerci from the two donor mice in the cysts’ “readiness to bud,” possibly depending on the state and terms of each host-parasite relationship established with the donor mice.

It is not surprising that the supplements stimulated budding at low parasite densities because the synthesis and the role of estradiol in stimulating cysts reproduction in vivo and in vitro have been well established [7, 15]

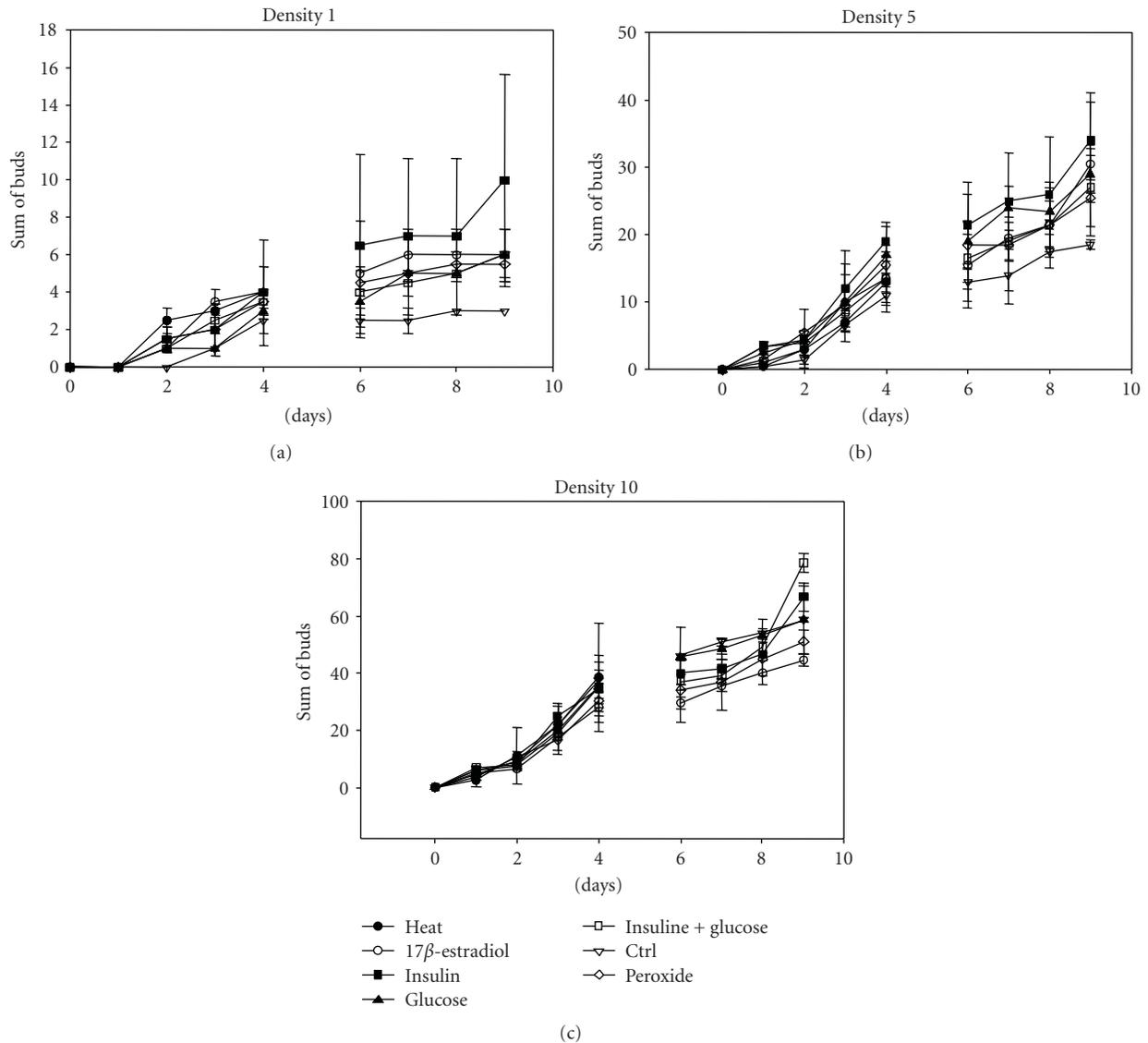


FIGURE 2: Budding process at the different conditions from day 0 to day 10. The cultured cysticerci from Donor number 2 were submitted to standard stressing (heat and peroxide) or favorable conditions (addition of 17 β -estradiol, insulin, glucose, insulin+glucose) and cultured in vitro as done in Experiment number 1. The results presented are from one experiment with its duplicate for each density. The graphic expresses the average value plus standard deviations. In $d = 1$ and $d = 5$, the budding in the presence of glucose and peroxide is significant. In $d = 10$ the budding in the presence of estradiol and insuline+glucose is significant.

and so is also the role of heat [16]. Likewise, the insulin pathway has been shown to be present in a large variety of invertebrates, including the most primitive metazoan phyla (cnidaria and sponges), and to play a central role in cell division and differentiation [17]. As other possible chemical mediators, we suspect cytokine-like substances which influence reproduction and apoptosis of heterologous cell lines, which cysticerci seem capable of producing and secreting in vivo and in vitro [18–20]. TGF- β , EGF, and insulin pathways are conserved in helminth parasites with receptor functions probably similar to those of invertebrate and vertebrate orthologs. Indeed, host-derived signals still present in the harvested cysts could have activated parasite receptors and modulated parasite development and differentiation [16, 21].

That crowding cysts at density = 10 *per se* promotes the highest budding efficiency is a novel finding which indicates that crowding is a powerful factor controlling the population of cysts. Possibly, crowding may act by the release of growth factors [19, 20] by the cysts most differentiated and ready to begin budding when placed in vitro, which then recruit those most laggard. Additionally, crowding may be mediated by adhesive molecules or membrane sensors sensitive to contact, as it has been previously reported by Haas et al. and Loverde et al., respectively [22, 23].

That the supplements did not improve, but rather lowered, the budding efficiency at density = 10 suggests that the capacity to bud has an upper limit. Such hysteresis in the system controlling budding could also explain the wave-like form in the dynamics of budding.

Overall, the results are congruent with the hypothesis that parasite inoculums composed of 1–10 apparently identical small nonbudded cysts would likely include a variety of cysts differing in their initial “readiness to bud” and thereby induce variation in parasite loads in infected mice at early times after infection when parasite loads are relatively low, followed by a progressive tendency towards uniformity at later times [13].

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Research Article

A New MAP Kinase Protein Involved in Estradiol-Stimulated Reproduction of the Helminth Parasite *Taenia crassiceps*

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MAP kinases (MAPK) are involved in the regulation of cellular processes such as reproduction and growth. In parasites, the role of MAPK has been scarcely studied. Here, we describe the participation of an ERK-like protein in estrogen-dependent reproduction of the helminth parasite *Taenia crassiceps*. Our results show that 17 β -estradiol induces a concentration-dependent increase in the bud number of in vitro cultured cysticerci. If parasites are also incubated in presence of an ERK-inhibitor, the stimulatory effect of estrogen is blocked. The expression of ERK-like mRNA and its corresponding protein was detected in the parasite. The ERK-like protein was over-expressed by all treatments. Nevertheless, a strong induction of phosphorylation of this protein was observed only in response to 17 β -estradiol. Cross-contamination by host cells was discarded by flow cytometry analysis. Parasite cells expressing the ERK-like protein were exclusively located at the subtegument tissue by confocal microscopy. Finally, the ERK-like protein was separated by bidimensional electrophoresis and then sequenced, showing the conserved TEY activation motif, typical of all known ERK 1/2 proteins. Our results show that an ERK-like protein is involved in the molecular signalling during the interaction between the host and *T. crassiceps*, and may be considered as target for anti-helminth drugs design.

1. Introduction

Cellular signaling is a crucial function for eukaryotic cells [1]. Complex molecule networks are involved in the cell's response to external signals and cross-communication among different physiological systems of the organism [2]. Transduction of cell signalling, which is mainly mediated by second messenger cascades, allows faster responses to a wide variety of stimuli [3–6], which control cellular functions such as proliferation, growth, and differentiation [2]. Among extracellular stimuli are steroid and protein hormones which are able to induce phosphorylation of membrane receptors with serine-threonine-tyrosine kinase activity which consti-

tutes the initial process that will subsequently orchestrate the whole transduction pathway [7–10].

The mitogen-activated protein kinase family (MAPK) that contain various signaling molecules have received increasing attention due to their implications on cell growth and proliferation [2]. Furthermore, MAPKs are highly conserved along the evolutionary scale [11, 12] from invertebrates to mammals [11].

MAPKs are divided into three main groups: (1) Jun NH₂ terminal kinases (JNK 1/2/3) which are mainly activated by cytokines and induce DNA damage. This MAPK has been associated with phosphorylation of c-jun. (2) p38 (p38 $\alpha/\beta/\gamma/\delta$) that switches on in response to stress stimuli and

has been related to osmoregulation and cell cycle entry. (3) Extracellular signal-regulated kinases (ERK 1/2) that can be activated by hormones associated to G-protein coupled receptors (GPCRs), growth factors, stress stimuli, cytokines, and Raf kinases, among others [2]. Two main isoforms have been described for ERK, ERK1, and ERK2, which have around 80% of homology in protein sequence [2, 13]. During cell proliferation, ERK 1/2 can activate, through specific phosphorylation of serine-proline/threonine-proline residues, several nuclear transcription factors, such as c-fos and c-jun, Elk1, c-myc, and STAT3, among others. All of these molecules are involved in cell cycle entry and regulation [14].

The role of ERK 1/2 during the molecular crosstalk between host and parasite is not clear yet, although it is present when the host synthesizes hormones and growth factors which directly benefit the parasite [15]. This bidirectional communication between the two organisms can occur due to the presence of parasitic structures that simulate the hosts' hormone receptors and ligand-binding proteins [15]. Concomitantly, little is known about the presence and function of ERK 1/2 in parasites. For instance, ERK 1/2 has been implied in the response of *Echinococcus multilocularis* to human epidermal growth factor (ERK-like MAP kinase) [16]. On the other hand, an ERK-like protein has been reported for *Trypanosoma brucei* and is involved in the parasite's growth rate [17].

Although some information is available regarding the presence of ERK homologs in parasites, not much is known on their function and possible role in mediating the parasite's response to the host's hormonal microenvironment, which occurs during an infection process, for example, in murine cysticercosis caused by *Taenia crassiceps*.

Experimentally induced murine cysticercosis represents an alternative model to study the parasite-host interaction similar to that occurring during human/porcine infection caused by *Taenia solium* [18]. Murine cysticercosis may be induced in both male and female mice by injecting the metacystode stage of the helminth parasite *T. crassiceps* into the mouse peritoneal cavity [19]. Interestingly, female mice of any strain are more susceptible than male mice and this effect is strongly related to the sex-steroid microenvironment [20]. It has been shown that 17β -estradiol (E_2) induces an increase in the parasite load by two mechanisms: tilting the hosts' immune response towards a parasite-permissive Th2 response [21] and directly promoting parasite growth and reproduction [22]. In this context, we reported that E_2 increases in vitro reproduction of *T. crassiceps* while androgens inhibit it. The proliferative effect of E_2 on the parasite seems to be mediated by the estrogen-induced AP-1 transcription factor expression in the parasite [23]. Estrogen receptor (ER) expression by cysticerci and activation of the AP-1 complex, in addition to the inhibition of proliferation by tamoxifen, support the idea that one of the proliferative mechanisms mediated by E_2 is a classical nuclear receptor-dependent pathway [22, 24].

However, the proliferative effect of E_2 on *T. crassiceps* reproduction is not fully inhibited by the antiestrogen tamoxifen suggesting that the parasite may respond to

estrogens by alternative pathways, such as those mediated by the interaction between steroid hormones and GPCRs [25]. For instance, in excitable cells from mouse reproductive tissue, estrogens can promote GPCR activity [26], triggering to the epidermal growth factor receptor activation as well as phosphorylation of the PLC-PKC pathway [27]. On the other hand, E_2 effects are not only mediated by unspecific receptors localized in the cellular membrane but also by means of the specific nuclear ER [28, 29]. In fact, the binding between E_2 and its membrane ER activates group I and II of the metabotropic glutamate receptor [30]. It should here be mentioned that ER is able to bind to SRC kinases through their highly conserved SH2 domains, which could considerably modify the effect of ERK 1/2 on the phosphorylation pattern of this transcription factor [31]. Nevertheless, information on this type of mechanisms in parasitic cells is scarce [16, 17, 32]. Therefore further studies on these mechanisms are required particularly for drug design since the knowledge obtained on the metabolic pathways that regulate parasite growth and establishment could provide with specific potential targets for therapeutic treatment. These may involve enzymes, genes, and transduction molecules which are exclusively present in the parasite. Additionally, this would also avoid host damage and nonspecific cross responses.

The aim of this work was to find experimental evidence on the functional participation of alternative molecules that can respond to the estrogenic stimulus, as may be the case of a parasite ERK-like protein, which could mediate the proliferative effects of exogenous 17β -estradiol on the helminth parasite *Taenia crassiceps*. The study of signal transduction in this parasite may potentially benefit not only the understanding of the host-parasite molecular crosstalk but also the design of drugs that specifically arrest the activity of important parasite molecules such as transduction proteins and transcription factors.

2. Materials and Methods

2.1. Harvesting and Preparing Cysticerci for Experimentation.

A new stock of *T. crassiceps* cysticerci (ORF-Kuhn2 strain) was donated to our laboratory by R. Kuhn in 2000 and was kept by serial intraperitoneal passage in BALB/c AnN female mice approximately every four months [19, 33]. Cysticerci for each experimental session were obtained from intraperitoneally infected female mice and placed in tubes containing sterile PBS (1X) supplemented with 100 U/mL of antibiotics fungizone (Gibco, Grand Island). The tubes were centrifuged for 10 minutes at 1,500 rpm and 4°C, and the supernatant was discarded. Packed cysticerci were incubated in AIM-V serum-free medium (Sigma, St. Louis, Missouri). These were then centrifuged 3 times at 1,500 rpm for 10 minutes for washing. After the final wash, the numbers of viable cysticerci (complete, translucent, and motile cystic structures) were counted under a binocular microscope. Ten viable nonbudding cysticerci of approximately 2 mm in diameter were then selected and dispensed into each well of 24-well culture plates (Falcon, Becton Dickinson Labware,

Franklin Lakes, New Jersey) in 1 mL AIM-V Medium (Gibco BRL) and incubated at 37°C and 5% CO₂. A sufficient number of culture wells were prepared to accommodate the complete experimental design to evaluate the effects of in vitro treatment of estradiol on cysticerci. Cultures were checked daily and their medium was completely replaced when necessary.

2.2. In Vitro Treatment Effects of E₂ and ERK inhibitor on *T. crassiceps* Cysticercus Reproduction. Culture grade 17- β estradiol (E₂) was obtained from Sigma. Culture grade ERK inhibitor II was obtained from Calbiochem. For in vitro tests, water-soluble E₂ was dissolved in DMEM serum-free culture medium, and ERK inhibitor II was dissolved in 3% DMSO. Each one was prepared to 100 μ M stock concentration and then sterilized by passage through a 0.2 mm millipore filter. Each of the following experimental conditions was applied to 24 parasite-loaded wells for concentration-response curves: (a) supplemented with the vehicle where hormones were dissolved (control groups), (b) separately supplemented with 0.1, 0.5, 1, and 10 μ M of E₂ and (c) separately supplemented with 0.1, 0.5, 1, and 10 μ M of ERK inhibitor II. Optimal concentrations of E₂ and ERK inhibitor II were selected from the concentration-response curves and used further on in time-response curves. Entire time of concentration-response and time-response curves was five days. The used concentrations were as follows: 0.1 μ M of E₂ and 0.5 μ M of ERK inhibitor II. The number of buds per cysticercus as a function of days in culture was assessed as the response variable. In parasites treated at the same time with estrogen and inhibitor, ERK inhibitor II was supplemented 2 hours before the addition of 17- β estradiol. Parasite reproduction was measured by counting the total number of buds in the ten cysticerci in each well. Bud count, as well as viability, was checked daily under an inverted light microscope (Olympus, MO21, Tokyo, Japan) at 4 \times and 10 \times magnification. Injury to cysticerci was recognized microscopically by progressive internal disorganization, development of whitish opaque areas on the parasite's tegument, and loss of motility. Dead cysticerci were immobile, opaque, and structurally disorganized.

2.3. Detection of ERK-Like Gene Expression in *T. crassiceps* by RT-PCR. Total RNA was isolated from hormone and ERK inhibitor II-treated *T. crassiceps* cysticerci and BALB/c AnN female mouse spleen (used as controls for specific ERK gene amplification, data not shown) by the single-step method based on guanidine isothiocyanate/phenol/chloroform extraction using Trizol reagent (Invitrogen, Carlsbad, CA). Briefly, cysticerci were disrupted in Trizol reagent (1 mL/0.1 g tissue), and 0.2 mL of chloroform was added per 1 mL of Trizol. The aqueous phase was recovered after 10 minutes centrifugation at 1,500 rpm. RNA was precipitated with isopropyl alcohol, washed with 75% ethanol, and dissolved in RNase-free water. RNA concentration was determined by absorbance at 260 nm and its purity was determined by electrophoresis in 1.0% denaturing agarose gel in the presence of 2.2 M formaldehyde. Once properly quantified, total RNA was

reverse transcribed, followed by specific PCR amplification of ERK-like gene from parasite tissue, at the same time as β -actin (control gene), as previously described [22]. Briefly, 10 μ g of total RNA were incubated at 37°C for h with 40 units of M-MLV reverse transcriptase (Applied Biosystems, USA) in 20 μ L of reaction volume containing 50 μ M of each dNTP and 0.05 μ g oligo (dt) primer (Gibco, NY). Ten μ L of the cDNA reaction were subjected to PCR in order to amplify the sequences of the specified genes. Primer design was based on the most conserved regions of sequenced genes of all species reported in the database. Sequences of primers are as follows: *Erk* sense 5'-ACAAAGTTCGAGTTGCTATCA-3' and antisense 5'-ATTGATGCCAATGATGTTCTC-3' and β -actin sense 5'-GGGTCAGAAGGATTCCTATG and antisense 5'-GGTCTCAAACATGATCTGGG. The 50 μ L PCR reaction included 10 μ L of previously synthesized cDNA, 5 μ L of 10 \times PCR buffer (Perkin-Elmer, USA), 1 mM MgCl, 0.2 mM of each dNTP, 0.05 μ M of each primer, and 2.5 units of Taq DNA (Biotechnologias Universitarias, Mexico). After an initial denaturation step at 95°C for 5 minutes, temperature cycling was as follows: 95°C for 30 seconds, 57°C for 45 seconds, and 72°C for 45 seconds during 35 cycles. An extra extension step was completed at 72°C for 10 minutes for each gene. The 50 μ L of the PCR reaction were electrophoresed on 2% agarose gel in the presence of a 100 bp ladder as molecular weight marker (Gibco, BRL, NY). The PCR products obtained were visualized by staining with ethidium bromide. In both cases, different PCR conditions were assessed until a single band corresponding to the expected molecular weight of the gene was found. The β -actin gene is a constitutively expressed gene, and it was used as internal control for differences in the amplification procedure between experiments and to stain different gels.

2.4. ERK-Like Protein Detection on *T. crassiceps* by Western Blot. Total protein was obtained from *T. crassiceps* cysticerci by conventional Tris-HCl isolation. Briefly, untreated, E₂ and ERK inhibitor II-treated cysticerci were disrupted in Tris-HCl (1 mL/0.1 g tissue), proteinase K (100 units/mL), and proteases inhibitor cocktail (Calbiochem). BW cells were used as internal control of protein extraction and integrity (data not shown). The supernatant was recovered after 15 minutes centrifugation at 1,500 rpm and the pellet was discarded. Protein concentration was obtained by absorbance at 595 nm using the Bradford-Lowry method. Total protein extracts of *T. crassiceps* cysticerci and BW cells were boiled in reducing Laemmli sample buffer, separated by SDS-PAGE (10% acrylamide), and electroblotted onto nitrocellulose membranes. The membranes were blocked overnight in TBST buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.5% Tween 20) containing 1% BSA with 3% dry milk. Then, different membranes were washed five times in TBST and separately incubated for 2.5 hours in presence of α -ERK (1 μ g/ μ L, Santa Cruz Biotechnology) and α -pERK (1 : 2000, Cell Signaling). After this first incubation, membranes were washed three times in TBST and subsequently incubated for 1 hour in presence of α -mouse IgG HRP (1 : 2000, Amersham) for pERK and α -rabbit IgG HRP (1 : 1500, Amersham) for ERK. Immediately after, the bands were visualized using

the ECL system according to the manufacturer's instructions (Super Signal ECL, Pierce). Chemiluminescent signals were captured on Kodak Bio-Max film.

2.5. Specific Detection of ERK-Like Protein in *T. crassiceps* Cysticerci by Flow Cytometry. *T. crassiceps* and mouse spleen cells were extracted by tissue disruption from cultured treated and untreated parasites. 2×10^6 cells for each treatment were incubated at 4°C for 20 minutes in presence of α -CD3, α -CD4, α -CD8, α -CD19, and α -macrophages antibodies (as the surface antibodies) and subsequently washed in sterile PBS 1X staining. Next, cells were centrifuged at 1,500 rpm for 5 minutes and incubated in GolgiPlug (BD, Biosciences) for 3 hours. Immediately, cells were washed in Perm/Wash buffer (BD, Biosciences) and centrifuged at 1,500 rpm for 5 minutes. After this, cells were separately incubated in presence of α -ERK 1 μ g/ μ L (Santa Cruz, Biotech) at room temperature for 20 minutes, and subsequently washed in PBS 1X staining. Immediately after, cells were centrifuged at 1,500 rpm for 5 minutes. Cell pellets were separately resuspended in presence of FITC-conjugated goat anti-rabbit antibody and incubated at 4°C for 30 minutes in the dark. After this, cells were washed in PBS 1X-staining and centrifuged at 1,500 rpm for 5 minutes. Cell pellets were resuspended in 500 μ L of PBS 1X staining in absence of light and analyzed by flow cytometry using an FACS Calibur (BD, Biosciences). Data were analyzed with the FlowJo software.

2.6. ERK-Like Protein Location on *T. crassiceps* Cells by Immunocytochemistry. E_2 -treated *T. crassiceps* cysticerci were washed with PBS 1X, embedded in Tissuetek (Triangle Biomedical Science), and frozen at -80°C. Parasite tissue sections (10 μ M) were fixed with 4% paraformaldehyde for 30 minutes, washed three times in PBS, and blocked for 30 minutes with RPMI medium containing 0.5% BSA and 5% FBS (Hernández-Bello et al. [34]). Cross-sections were then incubated with a 1 : 500 dilution of the polyclonal α -ERK (Santa Cruz, Biotech) for 45 minutes at 37°C, washed with PBS, and then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Zymed) at 1 : 500 dilution. Control experiments were assessed incubating the thick tissue sections in presence of only the FITC-conjugated goat anti-rabbit antibody at the same dilution. To eliminate background fluorescence, samples were contrasted with 0.025% Evans Blue for 10 minutes. After two single washings, samples were mounted in Vectashield mounting medium (Vector Laboratories Inc.) and examined with a Carl Zeiss epifluorescence microscope at 10X, 40X and 100X magnifications (Carl Zeiss, Germany) [34].

2.7. ERK-Like Protein Sequencing by 2-dimension Electrophoresis and Mass Spectrometry. Total protein from cultured *T. crassiceps* cysticerci was extracted and properly quantified as described before. Protein samples were placed in a buffer containing 8 M urea, 2% CHAPS, 50 mM DTT, IPG pH 4–7 (Bio-Rad), and bromophenol blue. Immediately after, protein samples were incubated overnight with the first-dimension gel (Amersham). Once they were properly hydrated, the first-dimension gel was isoelectrofocussed with

a constant voltage on a lineal electric gradient. After this, the gel was equilibrated in a buffer containing 6 M urea, 2% SDS, 375 mM Tris pH 8.8, 2% DTT, and 20% glycerol for 15 minutes. Next, the same equilibration process was performed using iodoacetamide 25 mg/mL instead of DTT. Once equilibrated, the gel was separated according to the molecular size of each protein in a second-dimension gel (PAGE-SDS at 12.5%). Finally, the bidimensionally separated gel was electroblotted onto nitrocellulose membranes and a α -ERK Western blot was carried out, as mentioned before. Only the immunodetected point, corresponding to the expected molecular weight and to the predicted isoelectrical point, was cut out and sequenced by mass spectrometry.

2.8. Experimental Design and Statistical Analysis. E_2 and ERK inhibitor II concentration-response and time-response curves were estimated in six independent experiments, each performed with ten cysticerci, freshly extracted from different infected donor mice, and replicated in 24 different wells. The response variable used in statistical analysis was the sum of buds in the 24 wells with each treatment along the time of exposure of each experiment. Data of the six replications of each experiment were pooled and expressed as their average \pm standard deviation. All optical densitometries as well as the mean of the fluorescence in the flow cytometry analysis was calculated for six different experiments and expressed as the average \pm standard deviation. Data were analyzed using either Student's *t*-test or one-way ANOVA and a subsequent Dunnet's Multiple Comparison Test, depending on the experimental design. Differences were considered statistically significant when $P < .05$.

3. Results

3.1. E_2 Stimulates while ERK Inhibitor Diminishes the Reproduction of *Taenia Crassiceps*. The number of buds (which directly reflects the reproductive rate in this parasite) in cultured *T. crassiceps* cysticerci clearly increased by addition of 17 β -estradiol in a concentration-dependent manner. Compared to control groups, E_2 increased the parasite reproduction rate up to 3-folds, from the lowest concentration on 100 nM up to higher pharmacologic concentrations such as 1 and 10 μ M, without affecting parasite viability (Figure 1(a)). In contrast, the ERK inhibitor showed the opposite effect on the number of cysticercus buds, although this effect was significant only at the highest concentrations (1 and 10 μ M), which suggests that the ERK inhibitor effect was also dependent on concentration (Figure 1(a)). In addition to the concentration effects, the proliferative action of E_2 on parasite reproduction was maintained throughout the culture time, reaching a 3-fold higher number of buds than that in untreated cysticerci at around five days of in vitro culture (Figure 1(b)). Interestingly, compared with control cysticerci, ERK inhibitor treatment of cysticerci had no significant effects on parasite reproduction (Figure 1(b)). However, it was clear that the ERK inhibitor completely blocked the E_2 -dependent proliferative effect (Figure 1(b)), emphasizing the importance of both E_2 and ERK-like in the reproduction of the *T. crassiceps* cysticerci.

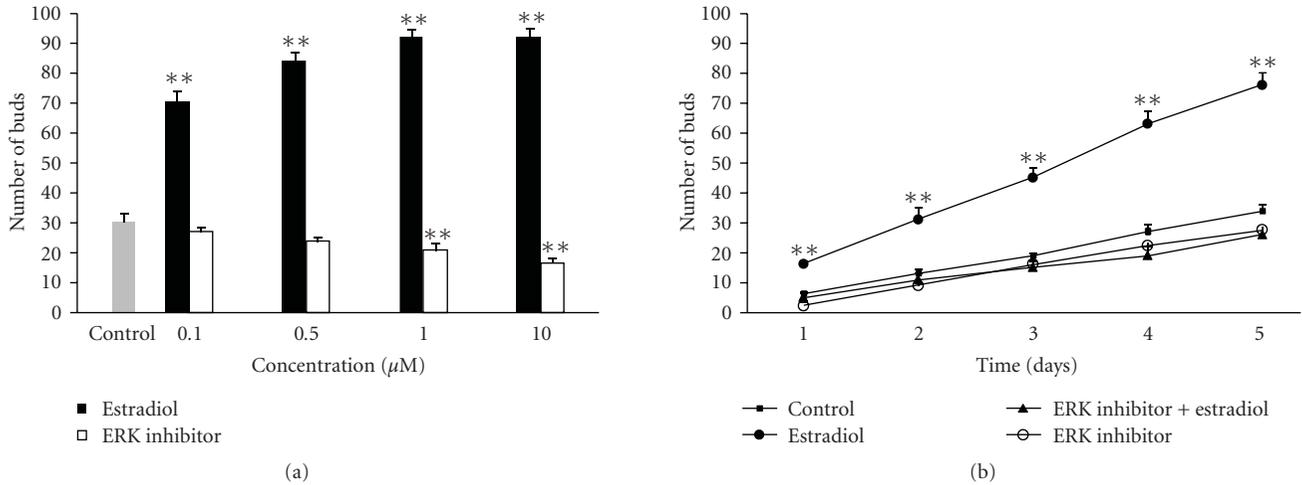


FIGURE 1: Concentration-response and response-time curves. 17β -estradiol augments the number of buds in *Taenia crassiceps* under physiologic concentrations ($0.1 \mu\text{M}$). Specific inhibition of ERK blocks the 17β -estradiol effect on the parasite, resulting in a similar number of buds between control and ERK inhibitor-treated cysticerci; (a) 17β -estradiol effect was progressively increasing during the five days of in vitro culture, whereas ERK inhibitor $0.5 \mu\text{M}$ did not modify parasite reproduction when compared to control cysticerci, even in presence of 17β -estradiol $0.1 \mu\text{M}$, and (b) Control = Parasites treated with the vehicle where hormone and inhibitors were dissolved; ** $P < .05$.

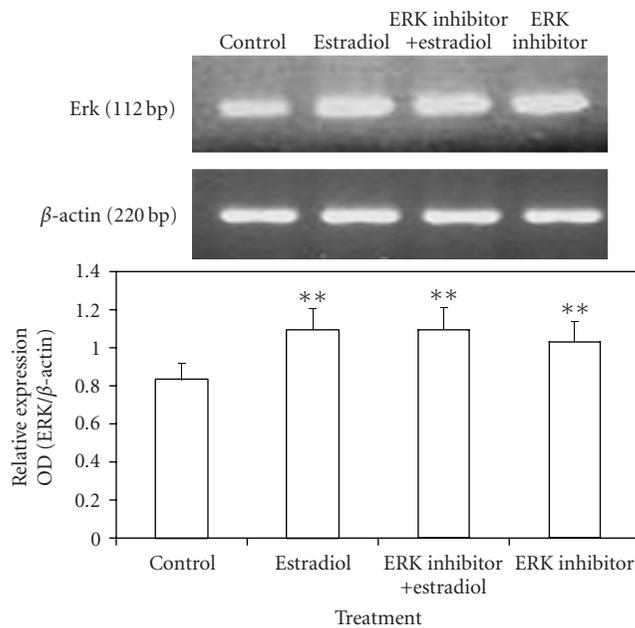


FIGURE 2: *ERK-like* expression in *Taenia crassiceps*. A single band of approximately 112 bp corresponding to ERK was detected in all in vitro treated cysticerci. Unexpectedly, either 17β -estradiol or ERK inhibitor treatment significantly induced *ERK-like* expression, related to control cysticerci. β -actin was used as control gene of constitutive expression. Optical densitometry was performed on 2% agarose gel from six independent experiments. Control = Parasites treated with the vehicle where hormone and inhibitors were dissolved; ** $P < .05$.

3.2. *ERK-Like Gene Expression in Taenia Crassiceps*. Using specific primers designed considering the most conserved sequences of all reported ERKs 1/2, a band of 112 bp was amplified and corresponds to the expected molecular

weight of the *ERK-like* gene in *T. crassiceps* and in the mouse (internal control of expression, data not shown). Interestingly, a significant increase in expression of the *ERK-like* gene was observed in response to E_2 and ERK inhibitor, even if the inhibitor was in presence of the steroid hormone (Figure 2).

3.3. *E₂ Increases ERK-Like Protein Phosphorylation but not the Total Quantity of the Native Protein*. Native ERK-like protein (55 KDa) was detected in untreated, E_2 treated, and ERK inhibitor-treated parasites. Contrary to the observations made by RT-PCR, no changes in total protein quantity were observed with any of the treatments (Figure 3(a)). Interestingly, when the phosphorylated form of ERK-like (pERK-like) protein was analyzed, it was evident that E_2 increases ERK-like protein phosphorylation 4 folds compared to untreated parasites (Figure 3(b)). Interestingly, ERK inhibitor treatment partially blocked the estradiol stimulated ERK-like protein phosphorylation, without returning it to basal levels (Figure 3(b)).

3.4. *ERK-Like Protein Is Specifically Detected in Taenia Crassiceps and Is Not a Contamination Product from Host Immune Cells*. Flow cytometry analysis firstly showed that *T. crassiceps* cells were different in size and complexity from mouse spleen cells (Figure 4). In fact, parasite cells were approximately 3 folds smaller than mouse spleen cells and exhibited poor complexity (data not shown). In addition, parasite cells showed no expression of the membrane markers CD3, CD4, CD8, and CD19, typically present in different types of mammalian leukocytes (Figure 4(a)). However, paramyosin (Ag-B, an exclusive component of the cytoskeleton of cestodes and insects) was clearly detected in *T. crassiceps* cells but not in host splenocytes (Figure 4(a)). Thus, the ERK-like protein was exclusively recognized on

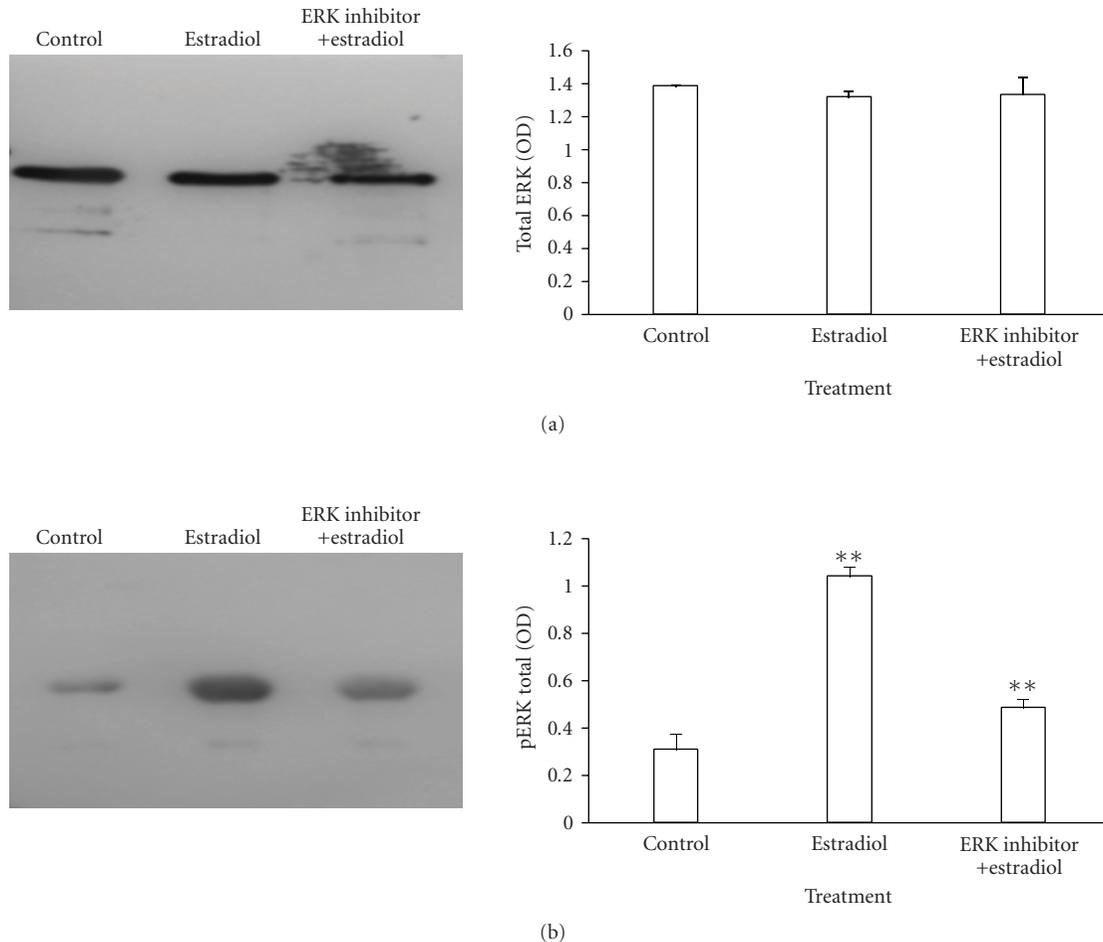


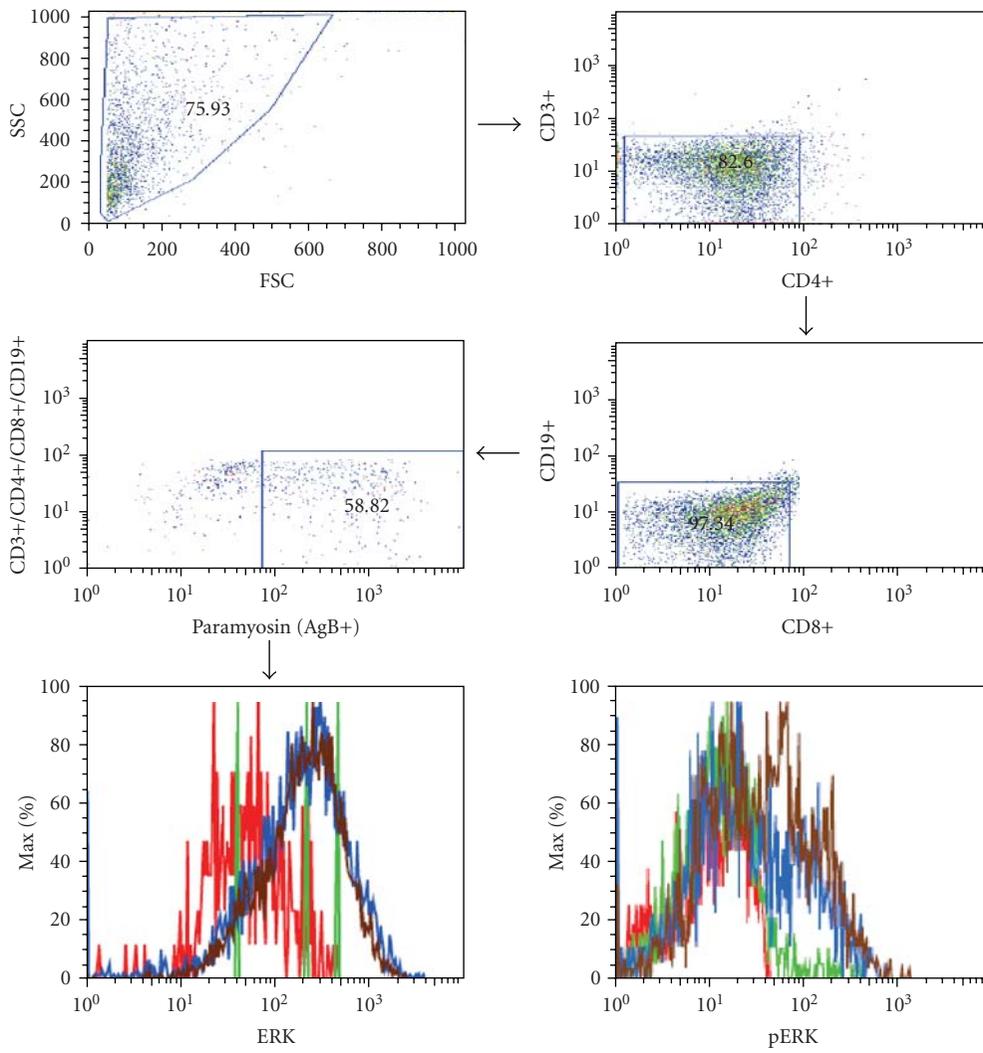
FIGURE 3: ERK-like protein detection in *Taenia crassiceps*. Total ERK-like protein in *T. crassiceps* was not modified by 17β -estradiol or ERK inhibitor treatments (a), contrary to that observed in ERK-like gene expression. Nevertheless, 17β -estradiol specifically induced phosphorylation of ERK-like by four-fold respect to control groups (b). Optical densitometry was performed on 10% acrylamide gel from six independent experiments. Control = Parasites treated with the vehicle where hormone and inhibitors were dissolved; ** $P < .05$.

T. crassiceps cells, because the FACS analysis was performed on parasite cells $Ag-B^+/CD3^-/CD4^-/CD8^-/CD19^-$. No significant differences were observed in the fluorescence intensity related to the ERK-like protein in untreated and E_2 -treated parasites (Figure 4(b)).

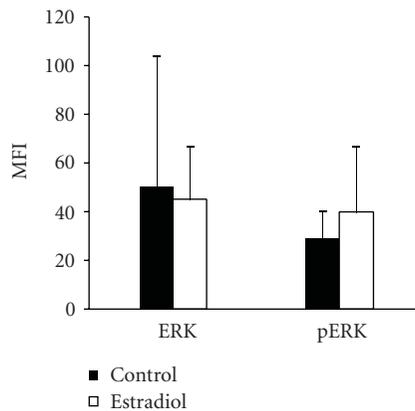
3.5. ERK-Like Protein Is Present in the Subtegument Cells of *Taenia Crassiceps Cysticerci*. Up to this point, experimental evidence indicates that the ERK-like protein is expressed in *T. crassiceps* cysticerci and that this is not a contamination product from host cells. In addition, on well-preserved *T. crassiceps* tissue (Figure 5(a)), immunohistochemistry assays were performed and the results showed that parasite cells express ERK-like protein mainly in the subtegument tissue of the cysticercus but not in tegument (Figures 5(c) and 5(d)). As expected, *T. crassiceps* tissue incubated only in presence of the secondary antibody did not give any positive signal related to ERK (Figure 5(b)), which suggests that the experimental conditions were optimal for detecting

exclusively parasite cells presenting ERK-like molecules without false positive signals.

3.6. Sequencing and Phylogenetic Analysis of the *Taenia Crassiceps* ERK-Like Protein. Total proteins from E_2 -treated parasites were separated in a pH range of 4–7, according to their isoelectrical point (IP) and molecular weight (Figure 6(a)). Moreover, a well-defined dot around pH 6.00, corresponding to the IP of most of the sequenced ERK 1/2, was recognized with high specificity by means of a single Western blot (Figure 6(b)). Interestingly, the ERK-like sequence showed high homology (60%) to ERK 1/2 from yeast, chicken, and mouse. Concomitantly, we were able to detect the activation motif TEY (threonine-glutamine-tyrosine) on the parasite's ERK-like protein sequence, which is typically present in all ERK 1/2 sequenced from several vertebrate and invertebrate organisms. Finally, the highly conserved amino acid sequence, NILT LHN VANTVMKN-PAMISKNLLR, was identified from the parasite's ERK-like



(a)



(b)

FIGURE 4: Specific detection of ERK-like in *Taenia crassiceps*. FACS assays showed that ERK-like detected in *T. crassiceps* was not a contamination product from host immune cells CD3+, CD4+, CD8+, and CD19+. As expected, ERK-like was specifically detected in paramyosin positive cells, a cytoskeleton protein only present in cestodes and some insects (a). By using the Mean of Fluorescence Intensity (MFI), no differences in total ERK-like and pERK were observed between control and 17 β -estradiol cysticerci (b). Control = Parasites treated with the vehicle where hormone and inhibitors were dissolved. Red = unstained cells; green=control parasites; blue = E₂-treated parasites; brown = ERK inhibitor-treated parasites; SSC = Side Scatter; FSC = Forward Scatter; pERK = ERK phosphorylated form; MFI = Fluorescence average.

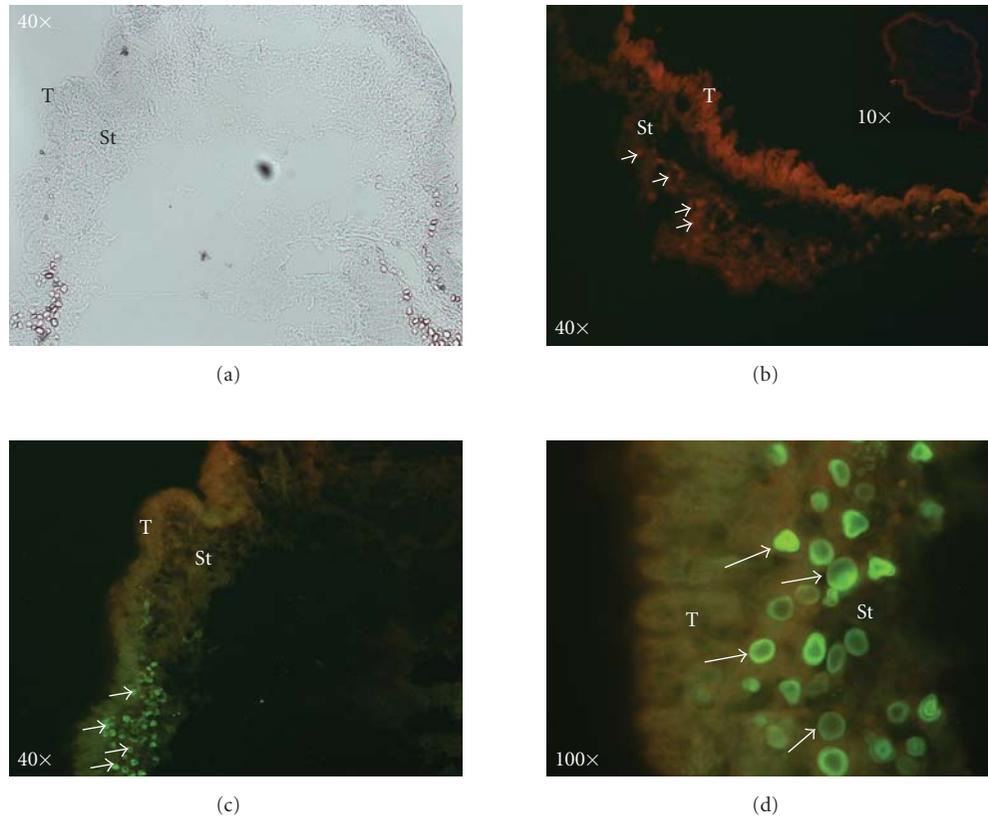


FIGURE 5: ERK-like location in *Taenia crassiceps* by immunocytochemistry. (a) Transversal section of one *T. crassiceps* cysticercus where tegument, subtegument, and cells are observed with no staining. (b) Parasite cross-section incubated with FITC-conjugated goat anti-rabbit antibody, as staining control. Nonreacting cells are shown in red at 10 \times and 40 \times . Unspecific detection of ERK-like was not observed during our experiments. (c) Specific detection of ERK-like (green fluorescent) was located in parasite cells mainly disposed along subtegument tissue, 40 \times . (d) A magnification of 100 \times shows in detail the *T. crassiceps* cells expressing ERK-like exclusively on subtegument tissue. T = Tegument tissue; St = Subtegument tissue.

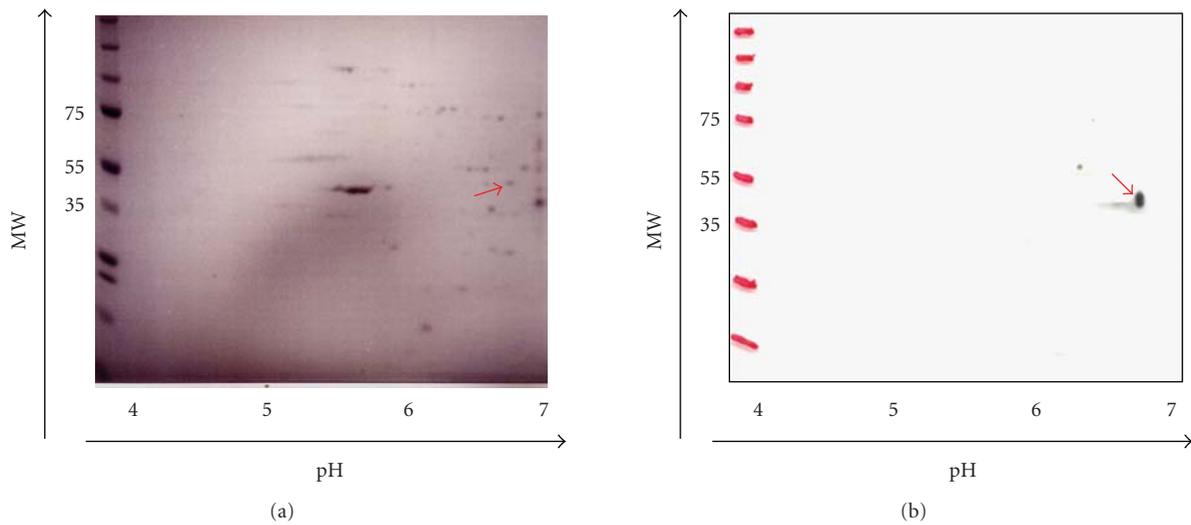


FIGURE 6: ERK-like detection by 2DE and protein sequencing. Total protein of in vitro cultured *T. crassiceps* was resolved in a double dimension gel (a). A well-defined dot (\sim 55 kDa, pH = 6.8) was detected in *T. crassiceps* and then purified from the gel for sequencing (b). MW = Molecular weight marker.

protein, which strongly suggests that this protein also conserves serine-threonine kinase activity and could influence phosphorylation of other parasite kinases (data not shown).

4. Discussion

As previously reported, 17β -estradiol exerts a direct proliferative effect on *Taenia crassiceps* cysticercus reproduction, which is not necessarily mediated by the host immune system, but through a classic nuclear receptor in the parasite [22]. However, alternative mechanisms through which E_2 can affect the parasite, securing its reproduction and establishment in an immunocompetent host, have not been completely explored yet. The aim of the present study was to investigate the participation of a signaling pathway mediated by second messenger cascades, which may be responsive to sex steroids that are highly conserved throughout the species and involved in cell proliferative processes. Thus, we explored the signaling pathway dependent on ERK-like protein, a member of the MAPK kinases which can be activated by several extracellular signals and generate rapid responses inside the cell, and the effect of the ERK inhibitor on ERK-like protein participation in parasite reproduction.

Interestingly, we found that, at progressively higher 17β -estradiol concentrations, the number of *T. crassiceps* cysticercus buds reached a maximum value. The opposite response was observed when ERK inhibitor was tested in culture. As concentration of ERK inhibitor increased, parasite reproduction progressively decreased. On the other hand, the E_2 effect was enhanced as time passed, reaching its maximum effect on parasite reproduction at day five of in vitro culture, which supports that E_2 effects are concentration dependent. Nevertheless, no time-dependent response was found when ERK inhibitor was tested, although this inhibitor completely blocked the E_2 -dependent proliferative effect, suggesting that parasite ERK-like protein is directly involved in mediating the crosstalk between the hormonal microenvironment (exogenous E_2) and parasite reproduction. These findings show a marked concentration and time-dependent pattern in the effects of E_2 on cysticercus reproduction. This observation is relevant since it suggests that sex steroids may have similar effects on mammals and parasitic cestodes, a hypothesis that evokes the wide range of steroid hormone actions not only in many different cell types but also along the phylogenetic scale, among distantly related organisms.

Moreover, determination of the actions of E_2 and ERK inhibitor on *T. crassiceps* cysticerci was important. This was approached by analyzing the ERK-like gene expression in the parasite as well as its translation to functional proteins that mediate the E_2 effects. In view of this, a band corresponding to ERK-like gene was amplified from *T. crassiceps* larval tissue using specific primers designed for the most conserved regions of these genes, previously reported in mammals such as mouse, rat, and human, as well as in yeast, birds, and amphibian. Unexpectedly, not only the ERK-like gene is expressed in the parasite, but it can also be overregulated by 17β -estradiol. However, a similar effect was observed when parasites were cultured in presence of ERK inhibitor. This

unexpected finding suggests that helminth parasites could have developed a positive feedback mechanism which is able to sense changes in the expression of very essential molecules such as MAPK kinases and maintain their activity in order to assure viability and reproduction of the parasite. On the other hand, the effects of sex steroids on parasite MAPK kinases gene expression, which are in charge of mediating the functionality of the whole pathway, have been scarcely explored. The results obtained in this study showed that this type of parasite genes is differentially regulated by exogenous sex steroids, which could be occurring in vivo from host to parasite. Also, this finding offers an alternative explanation of why *T. crassiceps* cysticerci grow better in the female mouse than in the male [20], emphasizing the molecular crosstalk between host and parasite, which is in turn differentially influenced by the hormonal microenvironment of each gender.

It is important to underline that proteins involved in signal transduction pathways, lead their functions by being phosphorylated; it was therefore crucial to determine the corresponding ERK-like protein in the parasite and to analyze its phosphorylation pattern in response to 17β -estradiol and ERK inhibitor treatments. Unexpectedly, one single band corresponding to ERK-like protein was detected in *T. crassiceps*, while most mammalian species exhibit a characteristic doublet. This finding suggests that, along many years of coevolution between the host and the parasite, several molecules, such as genes or proteins, may have been lost or "economized", passing their functions onto others that conserve similar structure and can therefore trigger the same effects due to a complex process of "molecular hypertrophy", by which a few molecules can exert many functions. The fact that the ERK-like protein differs from its homolog in mammalian cells supports two very important aspects of this study: (a) that the MAPK kinase detected in the parasite is not produced by contamination from host immune cells and (b) that, although both proteins have different characteristics, they probably conserve a high degree of similarity in their catalytic domains, which makes them recognizable with the same antibody and assures the suitable function for transducing signals in both organisms. In addition, 17β -estradiol treatment differentially stimulated the phosphorylation of ERK-like protein, which strongly supports that only parasitic ERK-like protein is expressed and regulated by exogenous estrogens but also that this MAPK kinase can be translated to a functional protein, able to be activated through E_2 -stimulated phosphorylation processes. This result suggests again that the mechanisms of action of steroid hormones on target cells are poorly diversified among species, maintaining similar and successful strategies from the simplest to the most complex organisms.

On the other hand, it was critical to determine that the detected and analyzed ERK-like protein was exclusively found in the *Taenia crassiceps* cysticercus and not a consequence of host immune cell contamination, because, as shown elsewhere, there is extremely high interaction between parasites and immune cells, which may eventually lead to leukocyte invasion into several parasitic tissues [35]. For this reason, an alternative use of flow cytometry was used to

differentiate proteins from *T. crassiceps* and the murine host by identifying exclusive molecules of the parasite which are neither synthesized nor expressed by the host. This is the case of paramyosin, a muscle protein found only in invertebrates such as *Drosophila melanogaster*, *Caenorhabditis elegans*, *Taenia solium*, and *T. saginata* [36]. The flow cytometry studies showed that presence and phosphorylation pattern of the analyzed ERK-like protein belonged specifically to the parasite, because paramyosin was only detected in *T. crassiceps* cells, where this MAPK kinase was also found and studied. In contrast, cells extracted from mouse spleen were not recognized by the α -paramyosin antibody, but they were positive for CD3, CD4, CD8, CD19, and macrophage antibodies, contrary to parasite cells.

These results demonstrate that the analyzed parasite ERK-like protein is in fact from *Taenia crassiceps* origin and not from other sources and simultaneously accentuate the potential use of flow cytometry for differential identification of molecules from organisms with extremely close contact, such as this parasite and its host.

Worth of mention is that the ERK-like protein was not only detected at messenger RNA and protein level but also localized inside the parasite cell. Interestingly, parasitic cells expressing ERK-like protein were exclusively located at the subtegument tissue, where most of the muscle and nephridium cells are found. This result suggests that the ERK-like protein is importantly involved in parasite motility and excretion, as well as in reproductive functions, which together maintain parasite viability and proliferation.

Finally, the protein sequence of the ERK-like protein showed a remarkable degree of conservation of certain segments, such as the activation domain, and functions, such as the serine-threonine kinase activity, both of them characteristic of these MAPK kinases. In fact, protein sequences from mammalian and high invertebrate ERK 1/2 are different to the parasite ERK-like protein, but closely related regarding the conserved domains, which suggests that parasites have developed similar structures as their host homologs, probably keeping only the catalytic domains in order to secure the basic functions of important proteins such as the ERK family. This hypothesis supports that development of proteins which can recognize the host's growth factors represents advantages in parasite metabolism economy, because the pathogen does not need to synthesize all molecules involved in a pathway but can take them directly from its host. This benefits the processes of reproduction, establishment, and immune evasion, among other important aspects of parasite life.

In conclusion, this work describes a functionally ERK-like protein in *T. crassiceps*. This MAPK kinase showed great capacity to transduce signals evoked by 17β -estradiol in the parasite. It is important to mention that much information about the effects of the host hormonal microenvironment on parasite physiology has been generated in recent years [15, 37–39]. Our results then contribute to understand the mechanisms by which the host microenvironment affects the parasite. Furthermore, the evolutionary origin of the molecules described herein, which facilitate exploitation of the host's hormones, is worth studying. In particular, whether those genes were acquired by the parasite through

horizontal gene transfer or evolved by mimicry, or simply from common ancestral genes, remains to be defined. Finally, our findings may help to understand, at molecular and evolutionary levels, several aspects of the crosstalk between host and parasite, of the sexual dimorphism of the immune response, and may provide information on new parasite targets for designing specific antihelminth drugs.

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Research Article

Substance P Signaling Contributes to Granuloma Formation in *Taenia crassiceps* Infection, a Murine Model of Cysticercosis

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Cysticercosis is an infection with larval cysts of the cestode *Taenia solium*. Through pathways that are incompletely understood, dying parasites initiate a granulomatous reaction that, in the brain, causes seizures. Substance P (SP), a neuropeptide involved in pain-transmission, contributes to inflammation and previously was detected in granulomas associated with dead *T. crassiceps* cysts. To determine if SP contributes to granuloma formation, we measured granuloma-size and levels of IL-1 β , TNF- α , and IL-6 within granulomas in *T. crassiceps*-infected wild type (WT) mice and mice deficient in SP-precursor (SPP) or the SP-receptor (neurokinin 1, NK1). Granuloma volumes of infected SPP- and NK1-knockout mice were reduced by 31 and 36%, respectively, compared to WT mice ($P < .05$ for both) and produced up to 5-fold less IL-1 β , TNF- α , and IL-6 protein. Thus, SP signaling contributes to granuloma development and proinflammatory cytokine production in *T. crassiceps* infection and suggests a potential role for this mediator in human cysticercosis.

1. Introduction

Neurocysticercosis is the most common parasitic disease of the central nervous system leading to seizures worldwide [1]. Humans develop cysticercosis when they ingest eggs of the tapeworm *Taenia solium* usually found in fecal-contaminated water or food [2]. Neurocysticercosis (NCC) is endemic to many parts of the world [3–7] and is becoming an increasingly important cause of seizures in the United States due to immigration from Mexico and Central and South America [8, 9]. Seizures in NCC most commonly arise as a result of the granulomatous responses to dead cysts in the brain. The granulomatous response is associated with production of several cytokines including T helper 1 (Th1) cytokines such as interferon gamma (IFN- γ), interleukin-2 (IL-2), and interleukin-12 (IL-12) [10].

T. crassiceps infection in mice is an experimental model for *T. solium* cysticercosis in man [11–15]. Intraperitoneal inoculation with 10 cysts of *T. crassiceps* results in the entire

peritoneal cavity of the mouse demonstrating granulomatous inflammation within 3–6 months. Similar to human infection, minimal granulomatous inflammation is found surrounding live parasites; rather, granulomatous inflammation is initiated when the parasite dies. The mediators contributing to development of granulomas around the dead parasite and production of proinflammatory cytokines are not completely understood.

We previously detected substance P (SP) protein within granulomas associated with *T. crassiceps* infection [16, 17]. We also demonstrated that levels of IL-2, IFN- γ , IL-4, and IL-10 protein were significantly higher in granulomas from infected WT mice than granulomas from infected SPP-knockout or the SP-receptor (neurokinin 1, NK1) NK1-knockout mice [16, 17]. In addition, we detected mRNA for IL-1 α , IL-1 β , IL-1 receptor antagonist, and TNF- α in all granulomas derived from infected WT mice [18]. However, corresponding proteins levels were not assessed nor was the contribution of SP signaling to their mRNA and protein production and to granuloma formation.

The current studies were aimed at determining if SP and NK1 contributed to granuloma development and/or to production of IL-1 β , TNF- α , and IL-6 in cysticercosis. SP stimulates production of proinflammatory cytokines such as of IL-1 β , IL-6, and TNF- α by human peripheral mononuclear cells, bronchial cells, and astrocytes [19–29]. SP also contributes to inflammatory processes associated with other infectious diseases. For example, granulomatous inflammation in murine schistosomiasis requires binding of SP to NK1 [30]. SP has been demonstrated to stimulate inflammatory cell infiltration. SP injection induced recruitment of leukocytes into the pleural cavity of mice and into the skin of humans [19–29] and stimulated the migration of human fibroblasts and peripheral blood lymphocytes in studies using modified Boyden chambers or micropore filter analysis, respectively [19–29]. In the current studies, we determined granuloma size and measured levels of IL-1 β , TNF- α , and IL-6 protein within granuloma obtained from *T. crassiceps*-infected WT and mice deficient in SPP or NK1. These studies indicate that SP signaling contributes to granuloma development and proinflammatory cytokine production in *T. crassiceps* infection and suggests a potential role for this mediator in human cysticercosis.

2. Materials and Methods

2.1. Murine Cysticercosis Model. Female mice were infected by intraperitoneal inoculation with 10 cysts of the ORF strain of *T. crassiceps*, as described in [16, 17]. Three months following infection, the mice were euthanized by cervical dislocation under anesthesia using a combination anesthetic sacrifice rodent cocktail ketamine, 25 mg/kg, acepromazine 0.8 mg/kg, xylazine 5 mg/kg intraperitoneally, at a dose of 0.5–0.7 mL/kg intramuscularly. Granulomas associated with dying cysts were removed from the peritoneal cavity of each of the infected mice that were euthanized. Three groups of mice were included in the experiments: (1) wild type C57BL/6 mice; (2) preprotachykinin or SPP-knockout mice (Jackson Laboratories, Maine, USA, bred >10 generations onto the C57BL/6 background); (3) NK1-knockout mice provided by Dr. Joel Weinstock, Tufts New England Medical Center, Boston, USA, bred >10 generations onto the C57BL/6 background. Three to 8 infected mice from each of the 3 groups were used for this study; 4–15 granulomas per mouse group were used for this study. Granulomas associated with parasites were identified visually, removed from the peritoneal cavity, and either used for quantifying cytokine proteins by ELISA or used for size determinations. This study was approved by the Animal Research Committee at Baylor College of Medicine.

2.2. Granuloma Size Determination. Intact granuloma was obtained from *T. crassiceps*-infected WT mice (2 granulomas), SPP-knockout mice (4 granulomas), and NK1-knockout mice (3 granulomas), fixed with 4% paraformaldehyde, paraffin imbedded and completely sectioned by microtome into 7 micron sections. Each section was stained with giemsa and examined microscopically. The area of granuloma within each section was measured using Image

J software (NIH). The volume of granuloma within each section was calculated by multiplying the area times 7 microns and the volume of granuloma within each section totaled to give the total granuloma volume.

2.3. Sandwich ELISA for the Detection of Cytokines. Cytokine protein levels were determined in 12–15 granulomas derived from *T. crassiceps* infected WT mice, 4–6 granulomas derived from *T. crassiceps* infected SPP-knockout mice, and 9–10 granulomas derived from *T. crassiceps*-infected NK1-knockout mice. A portion of each granuloma was homogenized in PBS, followed by centrifugation at 16,000 g. Total protein in the supernatant was quantitated using the Bradford method (cat no. 500-0006, Bio-Rad, Hercules, CA). IL-1 β , IL-6, and TNF- α protein levels were determined by sandwich ELISA (R&D Systems, San Diego, California) as per manufacturer's instruction. Results are expressed as pg cytokine/mg total protein.

2.4. Statistical Analysis. Differences between groups were compared using Student's *t*-test. Significance was set at $P < .05$.

3. Results

3.1. Granulomas from *T. crassiceps*-Infected SPP-Knockout and NK1-Knockout Mice Are Smaller than Granulomas Derived from Infected WT Mice. To begin to examine the contribution of SP signaling to granuloma formation in NCC, we measured granuloma volume in mice with normal and deficient SP signaling. The volumes of granulomas from *Taenia crassiceps* infected SPP-knockout mice ($1.8 \pm 0.45 \text{ mm}^2$) and NK1-knockout mice ($1.68 \pm 0.40 \text{ mm}^2$) were reduced by 31% and 36%, respectively, compared to granulomas derived from infected WT mice ($2.62 \pm 0.28 \text{ mm}^2$; $P < .05$ for both; Student's *t*-test; see Figure 1).

3.2. IL-1 β Protein Levels Are Reduced within Granulomas from *T. crassiceps* Infected SPP-Knockout and Infected NK1-Knockout Mice Compared to Granulomas from Infected WT Mice. IL-1 β is the primary mediator of granuloma formation in the *S. mansoni* pulmonary granuloma model [31]. Also, intratracheal injection of agarose beads coupled to recombinant IL-1 β induced pulmonary granulomas in mice [32]. To determine if decreased production of IL-1 β in SPP- and NK1-knockout mice contributed to reduced granuloma size in these animals, we measured IL-1 β protein levels in the granulomas derived from each group of mice. IL-1 β protein levels in granulomas from *T. crassiceps* infected SPP-knockout mice ($216 \pm 129 \text{ ng/mg}$ total protein) and NK1-knockout mice ($101 \pm 43 \text{ ng/mg}$ total protein) were reduced by 48% and 76%, respectively, compared to granulomas from infected WT mice ($418 \pm 278 \text{ ng/mg}$ total protein; $P < .05$ for both; Student's *t*-test; see Figure 2). Thus, SP signaling contributes to IL-1 β production within granulomas formed in response to dying *T. crassiceps* cysts. Furthermore, reduced levels of this cytokine likely contributed to reduced granuloma size in SPP- and NK1-knockout mice.

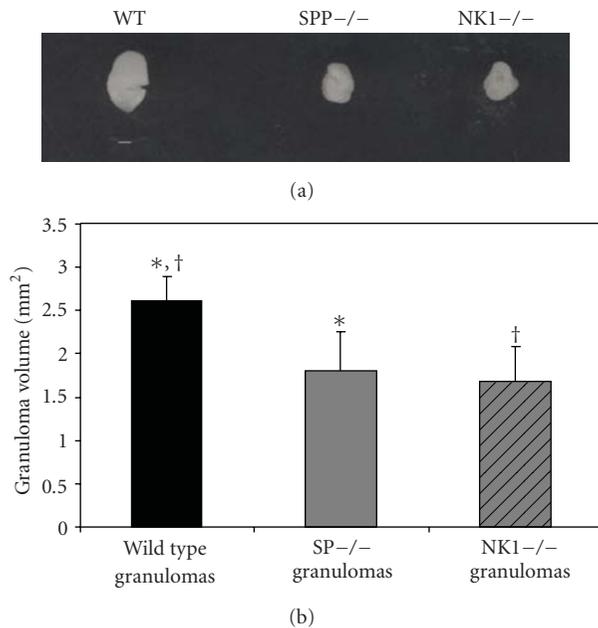


FIGURE 1: (a) Representative granuloma derived from infected WT, SPP-knockout, and NK1-knockout mice (white bar = 1 mm). (b) Volumes of granulomas derived from *Taenia crassiceps* infected, wild type ($n = 2$) versus SPP-knockout ($n = 4$) and NK1-knockout mice ($n = 3$). Data presented are mean \pm SD; * $P < .05$, size of infected, SPP-knockout derived granulomas versus WT derived granulomas; † $P < .05$, size of infected, NK1-knockout derived granulomas versus WT derived granulomas.

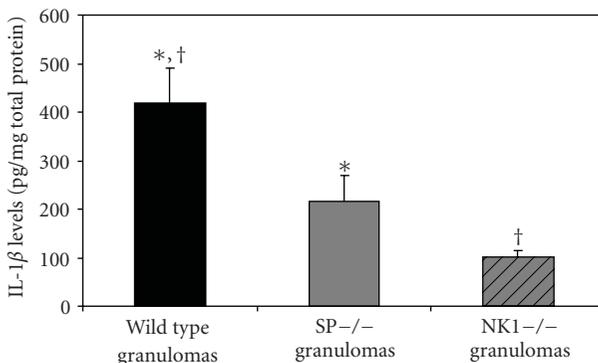


FIGURE 2: Quantitative levels of IL-1 β in granulomas derived from *Taenia crassiceps* infected, WT ($n = 15$), SPP-knockout mice ($n = 6$) and NK1-knockout mice ($n = 9$). Data presented are mean \pm SEM; * $P < .05$, IL-1 β levels in infected, SPP-knockout derived granulomas versus WT derived granulomas; † $P < .05$, IL-1 β levels in infected, NK1-knockout derived granulomas versus WT derived granulomas.

3.3. TNF- α Protein Levels Are Decreased within Granulomas from *T. crassiceps* Infected SPP-Knockout and Infected NK1-Knockout Mice Compared to Granulomas from Infected WT Mice. TNF- α is responsible for granuloma development in multiple settings. Intratracheal injection of agarose beads coupled to TNF- α induced pulmonary granulomas in mice [32]. TNF- α mediates granuloma growth in the *S. mansoni* pulmonary granuloma model [31] and is required for

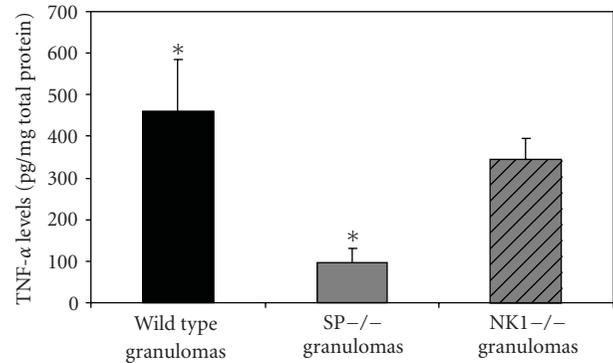


FIGURE 3: Quantitative levels of TNF- α in granulomas derived from *Taenia crassiceps* infected, WT ($n = 13$), SPP-knockout mice ($n = 4$), and NK1-knockout mice ($n = 9$). Data presented are mean \pm SEM; * $P < .05$, TNF- α levels in infected, SPP-knockout derived granulomas versus WT derived granulomas.

granuloma formation in a mouse model of tuberculosis [33]. Similar to our findings with IL-1 β , TNF- α protein levels in granulomas from *T. crassiceps* infected SPP-knockout mice (96 ± 67 ng/mg total protein) were reduced by 79% compared to levels in granulomas derived from infected WT mice (460 ± 452 ng/mg total protein; $P < .05$; Student's *t*-test; see Figure 3). TNF- α protein levels within granulomas from NK1-knockout mice (345 ± 153 ng/mg total protein) were decreased by 25% compared to levels with granulomas of WT mice; however, this difference did not achieve statistical significance. Thus, SP contributes to TNF- α production within granulomas formed in response to dying *T. crassiceps* cysts. Furthermore, reduced levels of this cytokine likely contributed to reduced granuloma size in SPP- and, perhaps, NK1-knockout mice. The failure to detect a significant difference in TNF- α protein levels between NK1-knockout and WT mice suggests the possibility that SP-mediated increases in TNF- α may occur through binding of SP to other members of the NK family, for example, NK2 or NK3.

3.4. IL-6 Levels Are Decreased within Granulomas from *T. crassiceps* Infected SPP-Knockout and Infected NK1-Knockout Mice Compared to Granulomas from Infected WT Mice. IL-6 production in human peripheral blood mononuclear cells, bronchial cells, and astrocytes is increased directly by SP through the action of nuclear factor IL-6 (NF-IL-6) and p38 MAPK, as well as indirectly in response to IL-1 β and TNF- α through the activation of NF- κ B [26, 27, 34, 35]. IL-6 mediates its acute proinflammatory effects within infected or injured tissues, in part, through upregulation of CXC chemokines, which leads to recruitment of the first wave of inflammatory cells. As we expected from the IL-1 β and TNF- α results summarized above, IL-6 protein levels in the granulomas derived from infected SPP-knockout mice (28 ± 30 ng/mg total protein) and from infected NK1-knockout mice (50 ± 16 ng/mg total protein) were reduced by 79 and 62%, respectively, compared to levels in granulomas from infected WT mice (130 ± 153 ng/mg total protein; $P < .05$ for

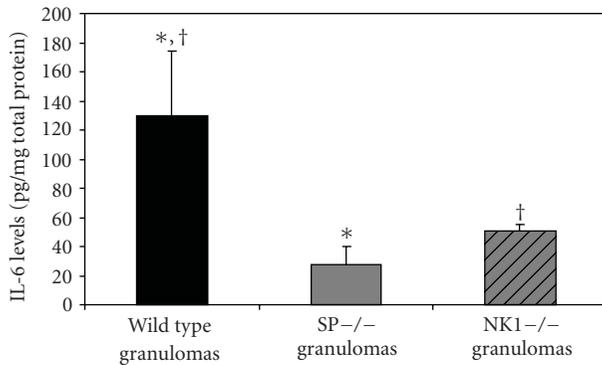


FIGURE 4: Quantitative levels of IL-6 in granulomas derived from *Taenia crassiceps* infected, WT ($n = 12$), SPP-knockout mice ($n = 6$), and NK1-knockout mice ($n = 9$). Data presented are mean \pm SEM; * $P < .05$, IL-6 levels in infected, SPP-knockout derived granulomas versus WT derived granulomas, $^{\dagger}P < .05$, IL-6 levels in infected, NK1-knockout derived granulomas versus WT derived granulomas.

both; Student's t -test; see Figure 4). Thus, SP signaling either directly or indirectly through the actions of IL-1 β and TNF- α contributes to IL-6 production within granulomas formed in response to dying *T. crassiceps* cysts.

4. Discussion

The current studies were performed to determine the contribution of SP and its specific receptor, NK1, to granuloma development and proinflammatory cytokine production within granulomas arising in mice infected with *T. crassiceps*. We demonstrated that the size of granulomas from the *T. crassiceps*-infected SPP-knockout mice and infected NK1-knockout mice were significantly smaller than granulomas from the infected WT mice. Furthermore, protein levels of IL-1 β , a key mediator of granuloma formation, were significantly lower within granuloma from SPP- and NK1-knockout mice compared to granuloma from mice. In addition, compared to granulomas from WT mice, protein levels of TNF- α , another key mediator of granuloma formation, were significantly lower in SPP-knockout mice and trended in the same direction in NK1-knockout mice. Thus, SP signaling contributes to granuloma formation, in part, through induction of IL-1 β and TNF- α , key mediators of granuloma formation.

Substance P and its high affinity receptor, NK1, are known to play an important role in inflammatory responses. Granuloma formation in response to murine schistosomiasis requires binding of SP to its specific receptor [30]. SP is known to stimulate inflammatory cell infiltration and to induce the production of proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α by human peripheral mononuclear cells, bronchial cells, or astrocytoma cells [19–29]. Our findings extend these observations and indicate that SP signaling contributes to granuloma formation and production of IL-1 β , TNF- α , and IL-6 protein within granuloma formed in response to *T. crassiceps* infection. The mechanism by

which SP stimulates the production of these cytokines may be by mediating inflammatory cell influx [19–24]. Nerves, endothelial cells, and cells of the immune system produce SP [36–38]. All of these cells have receptors for SP and are known to respond to SP [38–40]. SP is known to stimulate influx of lymphocytes, monocytes, macrophages, and other immune cells that produce proinflammatory cytokines such as IL-1 β , IL-6, and TNF- α [19–29]. Although there are various studies on the molecular mechanisms by which SP stimulates the production of IL-6 and TNF- α , there is limited information on the molecular mechanisms by which SP stimulates IL-1 β production. A study by Martin et al. determined that SP stimulated IL-1 production by astrocytes via increasing intracellular calcium. These studies demonstrated that treatment with an intracellular calcium chelator blocked SP-induced IL-1 β production [41]. Other studies have demonstrated that SP induces TNF- α and IL-6 production through NF kappa B in peritoneal mast cells [42]. Substance P production of TNF- α in peritoneal mast cells is also known to be mediated via P38 and JNK Map kinases [43]. Also IL-6 production in human peripheral blood mononuclear cells, bronchial cells, and astrocytes is increased directly by SP through the action of nuclear factor IL-6 (NF-IL-6) and p38 MAPK [26, 27, 34, 35].

Preprotachykinin protein is cleaved to form two active neuropeptides, SP and neurokinin A. Therefore, SP precursor (preprotachykinin) knockout mice produce neither SP nor neurokinin A. Consequently, our results in SPP-knockout mice can be attributed to a deficiency in either SP or neurokinin A or both. However, since NK1 binds only SP and not neurokinin A and the results in NK1-knockout mice mirror the findings in SP-knockout mice, we are confident in attributing reduced granuloma formation and proinflammatory cytokine production to the absence of SP signaling and not to reduced or absent neurokinin A signaling.

In the current studies, we demonstrated that the granuloma size and the levels of the proinflammatory cytokine, IL-1 β , are lower in the infected NK1-knockout mice compared to those of the infected SPP-knockout mice. Besides SP, peptide hormones such as hemokinin can also bind and activate NK1 at sites of chronic inflammation [44]. Therefore, although the current studies suggest that SP may be an important mediator associated with cytokine production, there may be other peptide hormones like hemokinin that also bind and activate the NK1 receptor that may be associated with granuloma and cytokine production. Therefore, it may be possible that in the NK1-knockout mice, the synergistic lack of activity of both SP and hemokinin may have resulted in lower IL-1 β levels and granuloma size as compared to SPP-knockout mice.

Granuloma formation by the host in response to agents causing chronic infections is thought to be essential for limiting and eventually clearing infection. However, recent work in zebra fish infected with *Mycobacterium marinum* suggests that granuloma formation contributes to early bacterial growth [45]. Intraparenchymal cysts of NCC are thought to die spontaneously and to elicit a granulomatous response that does not in itself contribute to the demise of

the cyst. Rather, we have previously demonstrated that early granuloma formed in response to dying cysts contributes to NCC disease manifestations by producing mediators that induce seizures [46]. Other groups have demonstrated that SP is epileptogenic [47]. The current studies demonstrating that SP signaling contributes to granuloma formation in *Taenia crassiceps* infection, together with other published observations, suggest the possibility that diminishing granuloma formation in NCC by blocking SP, which contributes to granuloma formation and epileptogenic responses, may be beneficial in the treatment of this disease.

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Research Article

Activation-Induced T Helper Cell Death Contributes to Th1/Th2 Polarization following Murine *Schistosoma japonicum* Infection

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In chronic infectious diseases, such as schistosomiasis, pathogen growth and immunopathology are affected by the induction of a proper balanced Th1/Th2 response to the pathogen and by antigen-triggered activation-induced T cell death. Here, by using *S. japonicum* infection or schistosome antigens-immunized mouse model, or antigens in vitro stimulation, we report that during the early stage of *S. japonicum* infection, non-egg antigens trigger Th2 cell apoptosis via the granzyme B signal pathway, contributing to Th1 polarization, which is thought to be associated with worm clearance and severe schistosomiasis. Meanwhile, after the adult worms lay their eggs, the egg antigens trigger Th1 cell apoptosis via the caspase pathway, contributing to Th2 polarization, which is associated with mild pathology and enhanced survival of both worms and their hosts. Thus, our study suggests that *S. japonicum* antigen-induced Th1 and Th2 cell apoptosis involves the Th1/Th2 shift and favors both hosts and parasites.

1. Introduction

The balance between Th1 and Th2 cell responses to an infectious agent can influence both pathogen growth and immunopathology. In helminth infections, the parasites have evolved the capacity to induce Th2 responses in order to protect themselves against potentially toxic Th1-dependent antiparasitic effector mechanisms [1–3]. Many factors influence the differentiation of Th1 and Th2 cells, including the antigen dose and form, the affinity between the peptide antigen and the T cell receptor (TCR) [4], the nature and degree of co-stimulation [5], the presence of antigen-presenting cells (APC) [6, 7], and the cytokine milieu surrounding the differentiating cells [8]. In addition, antigen-triggered activation-induced cell death (AICD), which is the primary form of apoptosis for clonally expanded T cells, can influence both pathogen growth and immunopathology. AICD is considered the primary mechanism for deleting mature CD4⁺ T cells in the periphery and it plays an important role during adaptive immune responses by ensuring that

a defined number of specialized T cells remain in the organism [9, 10].

Approximately 200 million people worldwide currently suffer from schistosomiasis, one of the most important human parasitic diseases. The main lesions resulting from schistosome infections are not caused by the adult worms. Instead, the lesions are caused by eggs trapped in human tissues, which stimulate immunopathological reactions including granulomas and fibrosis. Schistosomiasis is immunologically characterized by an early Th1 response that switches to a Th2-dominated response after the onset of parasite egg production [11]. Schistosomiasis has provided excellent models to study the induction and regulation of Th cell subset responses to infection. In the course of a schistosomal infection, the immune response progresses through at least three phases. (1) During the first three weeks of the infection, when the host is exposed to migrating immature and mature parasites, the dominant response is Th1-like. The response is induced by non-egg antigens, such as the cercariae, schistosomula, and schistosome worm antigens

(SWA) [12, 13]. (2) As the parasites begin to produce eggs around week four, the response alters. The Th1 component begins to decrease, and this is associated with the emergence of a stronger Th2 response, which is primarily induced by egg antigens [13, 14]. (3) During the chronic phase of infection, the Th2 response is predominant and modulated. The granulomas that form around the eggs are smaller than at earlier times during the infection [13]. This produces a situation that is optimal for parasite survival concurrent with a condition that imparts minimal self-damage to the host. In addition to the Th1/Th2 shift, it has been reported that soluble schistosome egg antigen-(SEA)-stimulated T helper (Th) cell apoptosis occurs after egg laying and continues throughout the florid and downmodulated stages of schistosome infection, suggesting that Th cell apoptosis may represent a second significant method for controlling CD4⁺ T cells that mediate the immunopathology in schistosomiasis [15, 16].

To date, there is very little data available showing that Th1 or Th2 cell apoptosis is sensitive to the stimulation of different pathogen antigens. There is also little evidence that Th cell apoptosis contributes to the Th1/Th2 polarizations observed during different stages of schistosome infection. Here, we investigated whether different schistosome antigens could primarily trigger the death of different types of activation-induced Th cells and contribute to Th1/Th2 polarization during the stages of *S. japonicum* infection.

2. Materials and Methods

2.1. Mice, Infection, and Chemotherapy. Eight-week-old C57BL/6 female mice were provided by the Center of Experimental Animals (Nanjing University, Nanjing, China). All animal experiments were performed in accordance with Chinese animal protection laws and with permission from the Institutional Review Board. *Oncomelania hupensis* harboring *S. japonicum* cercariae were purchased from the Jiangsu Institute of Parasitic Diseases (Wuxi, China).

Each mouse was percutaneously infected with *S. japonicum* by placing a glass slide carrying 12 cercariae on its abdomen for 20 minutes. Some infected mice were orally administered artesunate (a gift from professor Junfan Shi, Zhejiang Institute of Parasitic Diseases, Hangzhou, China) dissolved in ddH₂O in single 300 mg/kg doses at 7, 12, 17, 22, 27, 32, 37, and 42 days after infection. At 23, 35, and 56 days, six mice from each group were sacrificed.

2.2. Antibodies, Regents, and Antigen Preparation. Anti-CD3 (clone 145-2C11), anti-CD28 (clone 37.51), Fluorescein isothiocyanate-(FITC)-conjugated anti-CD4 (clone GK1.5), phycoerythrin-(PE)-conjugated anti-interferon-(IFN-) γ (clone XMG1.2) PE-conjugated anti-interleukin-(IL)-4 (clone 11B11), and their isotype control rat IgG1 antibodies were from eBioscience (San Diego, CA). Rabbit antimouse cleaved caspase-3 (Asp175) antibody and rabbit IgG isotype control antibody were from Cell Signaling

(Danvers, MA). The Alexa Fluor 647F(ab')₂ fragment of the goat antirabbit IgG(H+L) was from Invitrogen (Eugene, OR). Phorbol12-myristate13-acetate (PMA) and ionomycin were from Sigma-Aldrich (St. Louis, MO). The CD4⁺ T Cell Isolation Kit was from Miltenyi Biotec (Bergisch Gladbach, Germany). The BD Cytotfix/Cytoperm Plus Fixation/Permeabilization kit and Apo-Direct kit were from BD Pharmingen (San Diego, CA). The granzyme B (GrB) inhibitor Z-AAD-CMK was from Calbiochem (San Diego, CA). The caspase inhibitor Z-VAD-FMK was from BIOMOL International (Plymouth Meeting, PA).

SWA and SEA were prepared and diluted with phosphate buffered saline (PBS) to a final concentration of 10 mg/mL, as previously described [17].

2.3. Antigen Immunization. Mice were injected subcutaneously in the back with 100 μ l of a solution containing 50 μ g of SEA, 100 μ g of SWA, or PBS alone emulsified in incomplete Freund's adjuvant (IFA) [18]. Ten days after immunization, six mice from each group were sacrificed.

2.4. Cell Isolation. After infection, chemotherapy, or antigen immunization, the single cell suspension of splenocytes was prepared by teasing the spleen in PBS containing 1% fetal calf serum (FCS) (Gibco, Gaithersburg, MD) and 1% EDTA. Erythrocytes were lysed with ACK lysis buffer, and the remaining splenocytes were washed with PBS-1% FCS.

CD4⁺ T cells were purified from the splenocytes by negative selection with a CD4⁺ T Cell Isolation Kit and using a magnetic-activated cell sorter (MACS) system (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The purity of the CD4⁺ population was >97%, as determined by fluorescence-activated cell sorting (FACS) analysis.

2.5. Cell Culture. For in vitro SWA or SEA restimulation of CD4⁺ T cells from infected mice with or without chemotherapy, 5 \times 10⁷ splenocytes from each mice were incubated in 10 mL of complete RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% FCS, 2 mM pyruvate, 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, 100 U of penicillin/mL, and 0.1 mg/mL streptomycin in a 10 cm plate (Costar, Cambridge, MA). The cells were incubated at 37°C in 5% CO₂ in the presence of 20 μ g/mL SWA or 20 μ g/mL SEA. After 36 hours of incubation, CD4⁺ T cells were purified using MACS and prepared for staining with antibodies and FACS analysis.

To induce in vitro polarization and apoptosis of CD4⁺ T cells from normal mice with SWA and SEA, CD4⁺ T cells (6 \times 10⁶ cells/well) from normal mice were seeded in 12-well plates. The cells were preactivated overnight with anti-CD3 (2 μ g/mL) and anti-CD28 (1 μ g/mL) antibodies and then incubated in 3 mL of complete RPMI 1640 medium in the presence of specific antigens of 20 μ g/mL SEA, 20 μ g/mL SWA, or PBS alone for 36 hours at 37°C in 5% CO₂.

For the caspase and GrB inhibition experiments, 5 \times 10⁷ splenocytes were incubated in 10 mL of complete RPMI 1640

medium in the presence of (1) SWA (20 $\mu\text{g}/\text{mL}$) and the GrB inhibitor Z-AAD-CMK (20 μM), (2) SEA (20 $\mu\text{g}/\text{mL}$) and the caspase inhibitor Z-VAD-FMK (20 μM), (3) PBS and Z-AAD-CMK (20 μM), or (4) PBS and Z-VAD-FMK (20 μM) in a 10 cm plate (Costar, Cambridge, MA) for 36 hours at 37°C in 5% CO_2 . After 36 hours of incubation, the CD4^+ T cells in each group were purified and prepared for staining with antibodies and FACS analysis.

2.6. Intracellular Cytokines and Caspase-3 Staining. To detect intracellular cytokines and apoptotic molecules in CD4^+ T cells, purified CD4^+ T cells (2×10^6 cells/mL) were restimulated in the presence of PMA (25 ng/mL), ionomycin (1 $\mu\text{g}/\text{mL}$), and GolgiStopTM (0.66 $\mu\text{L}/\text{mL}$) in 24-well plates (2 mL/well) for 6 hours at 37°C in 5% CO_2 . Then the CD4^+ T cells were washed in PBS containing 1% FCS and stained with 0.5 mg/mL FITC-conjugated anti- CD4 in the dark at 4°C for 30 minutes, followed by fixation and permeabilization with Cytotfix/Cytoperm, according to the manufacturer's protocol. Next, the cells were stained intracellularly with 2 μL of rabbit antimouse caspase-3 monoclonal antibody or rabbit IgG isotype control antibody (mAb; 1 : 200) plus PE-conjugated anti- $\text{IFN-}\gamma$ (0.2 mg/mL), PE-conjugated anti-IL-4 (0.2 mg/mL), or PE-conjugated rat IgG1 control antibodies for 1 hour at room temperature. Finally, the cells were washed in PBS containing 1% FCS and stained at room temperature for 30 minutes in the dark with 5 μL of the Alexa Fluor 647 F(ab')₂ fragment of the goat antirabbit IgG(H+L). FACS analysis was performed using the FACS Calibur (Becton Dickinson, San Jose, CA).

2.7. Terminal Transferase dUTP Nick End Labeling (TUNEL) Assay. In the caspase and GrB inhibition experiments, apoptotic cells were labeled with the Apo-Direct kit following the manufacturer's instruction. The cells were then detected by FACS.

2.8. Necropsy and Estimation of Worm and Egg Burdens in the Liver. At 23, 35, and 56 days postinfection in animals with or without orally administered artesunate, six mice from each group were sacrificed. The animals were perfused to measure the worm and liver egg burdens. The mouse livers were removed and weighed. A sample of each liver (0.5 g) was digested overnight at 37°C with 10 mL 5% KOH. In each sample, the total adult worm and liver egg burden was measured. The residues were dissected and immediately fixed in 10% buffered formalin prior to histopathological analysis.

2.9. Liver Histopathology. Liver sections were embedded in paraffin and stained with hematoxylin and eosin (HE) prior to microscopic examination. The number of granulomas was counted in 10 random microscopic fields in each mouse liver section using a microscope (magnification $\times 4$; Olympus, Tokyo, Japan). The eggs and granulomas present in the livers were measured using a video micrometer (Olympus, Tokyo, Japan) in accordance with the manufacturer's instructions.

2.10. Statistical Analysis. For statistical evaluation of the data, two-tailed Student's *t*-tests were used, and $P < .05$ was considered statistically significant.

3. Results

3.1. Effects of Artesunate on the Parasitology and Histopathology in Mice Infected with *S. japonicum*. Adult worms were detected in the mesenteric veins of the mice 23 days post-infection (Figure 1(a)). At 35 and 56 days after infection, the adult worms had laid eggs. Egg nodes were observed in the surface of the mouse liver and tallied in the liver egg counts (Figure 1(b) and Table 1). Following administration of artesunate, the number of adult worms and fecundity were completely reduced in our study. No adult worms and eggs were detected in the artesunate-treated mice at the three time points after infection (Figures 1(a), 1(b), and Table 1).

We measured the magnitude of hepatic granulomatous inflammation, which is commonly used as an indicator of disease severity, and observed that there were significant differences in liver histology between mice that were or were not administered artesunate (Figure 1(c)). Severe hepatic granulomatous inflammation was observed around eggs 35 days after infection in mice that were not administered artesunate. However, in artesunate-treated, infected mice, no eggs were present, and there was only a mild hepatic inflammatory response observed. This mild inflammatory response may have been induced by schistosomula and early stage adult worms before they were killed by artesunate or by antigens released by destroyed parasites (Figure 1(c)).

3.2. Kinetic Analysis of Th1/Th2 Cell Polarization and Apoptosis during Different Stages of *S. japonicum* Infection. During schistosome infection of humans [19] and mice [11, 20], parasite-specific antigens (i.e., SWA or SEA) are believed to stimulate different types of responses. SWA has been shown to stimulate the type 1 response, especially during early infection, whereas SEA stimulates type 2 responses later in infection. Consistent with this, our results showed that the proportion of Th1 cells rose to 3.33% 23 days postinfection in comparison to the normal controls (1.66%). Meanwhile, the proportions of Th2 cells only slightly increased (Figure 2(a) left and upper-right panels). This demonstrates that there is a Th1-biased response due to nonegg antigens that occurs before the schistosomes lay eggs. In contrast, after eggs deposition, the number of Th2 cells rapidly increased (from 0.30% at 23 days to 0.54% at 35 days and 1.49% at 56 days). The increase in Th2 cells was more dramatic than the increase in Th1 cells (from 3.33% at 23 days to 3.94% at 35 days and 5.38% at 56 days), suggesting that the egg antigen induces a Th2-biased response.

To assess apoptosis in Th1 and Th2 cells during the different stages of schistosomal infection, CD4^+ T cells were purified and labeled for FACS analysis without further stimulation in vitro. The results in Figure 2(a) (left and upper-right panels) showed that the level of apoptosis of both Th1 and Th2 cells increased in infected mice in comparison to normal mice. During the early stage of infection (23

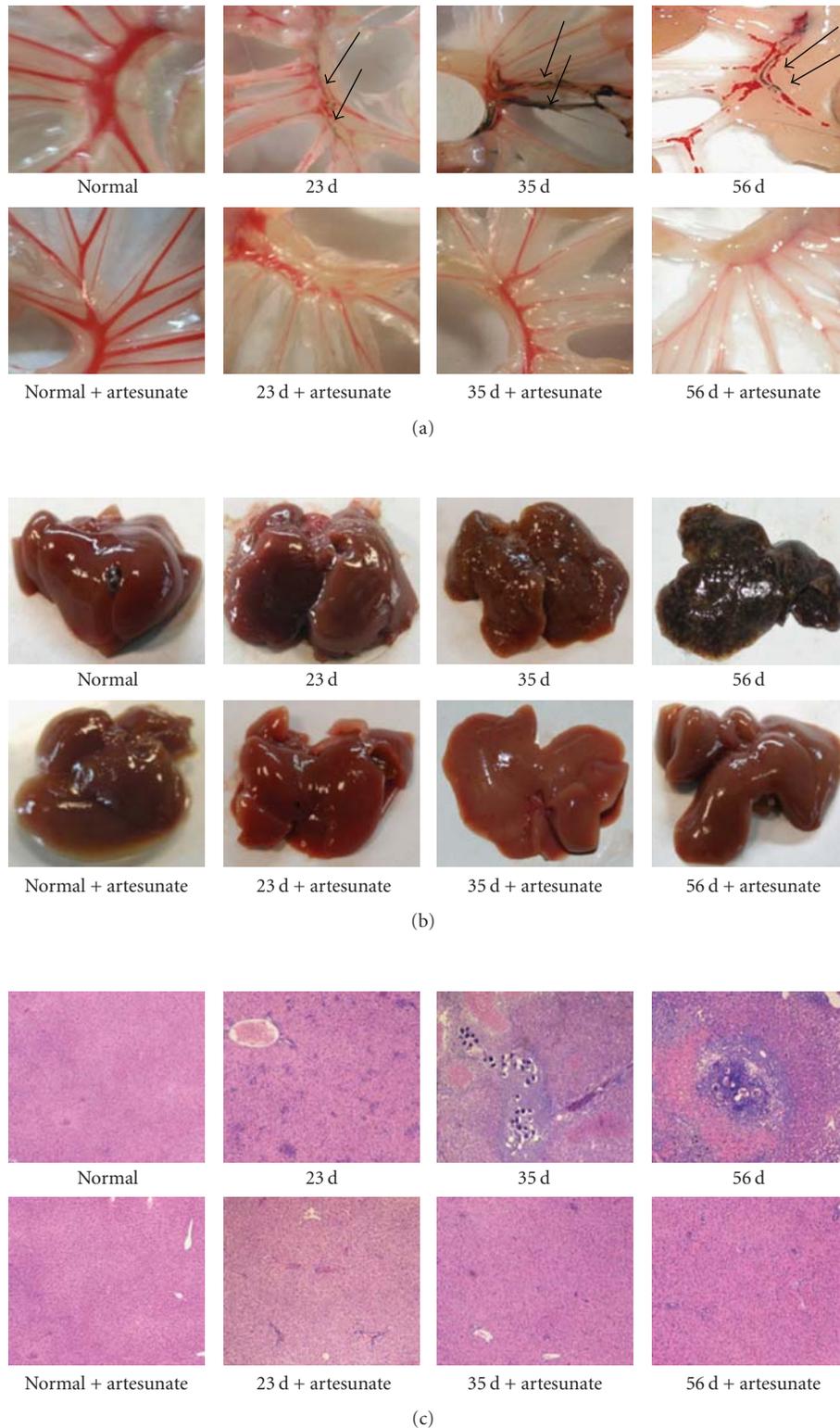


FIGURE 1: Parasitological and histopathological examination of infected mice treated with or without artesunate. In each experiment, twelve mice were percutaneously infected with *S. japonicum* and among these mice, six were orally administered artesunate dissolved in ddH₂O as described in Materials and Methods. At 23, 35, and 56 days mice were sacrificed and perfused to measure the worm and liver egg burdens and for histopathological analysis. (a) Adult worms in the mesenteric veins of infected mice. (b) Egg nodes in the surface of the livers. (c) Microscopic examination of hepatic granulomatous inflammation in liver sections (Magnification: $\times 400$). Measurements were made at 0 d and at three time points postinfection. Each micrograph is representative of three experiments.

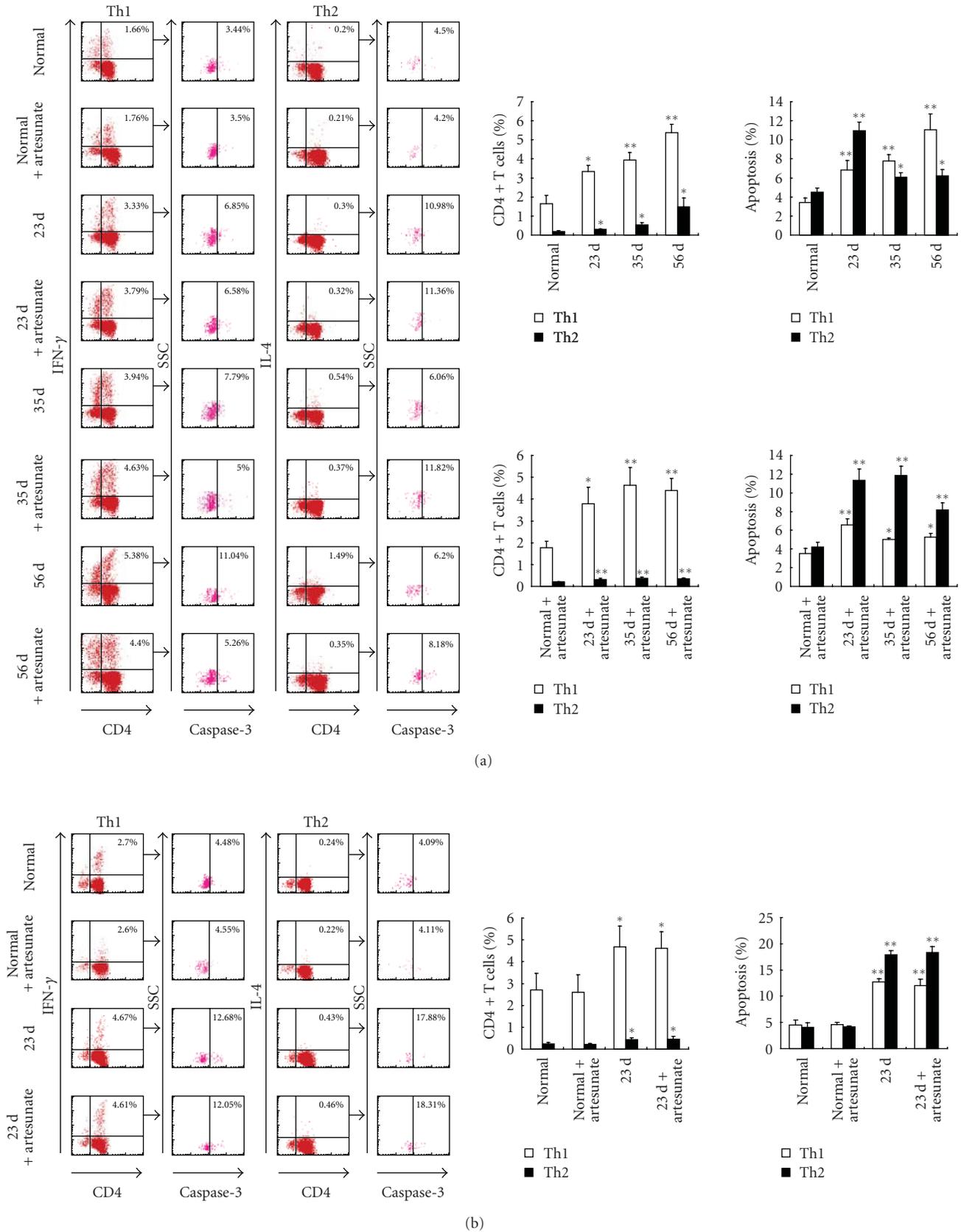


FIGURE 2: Continued.

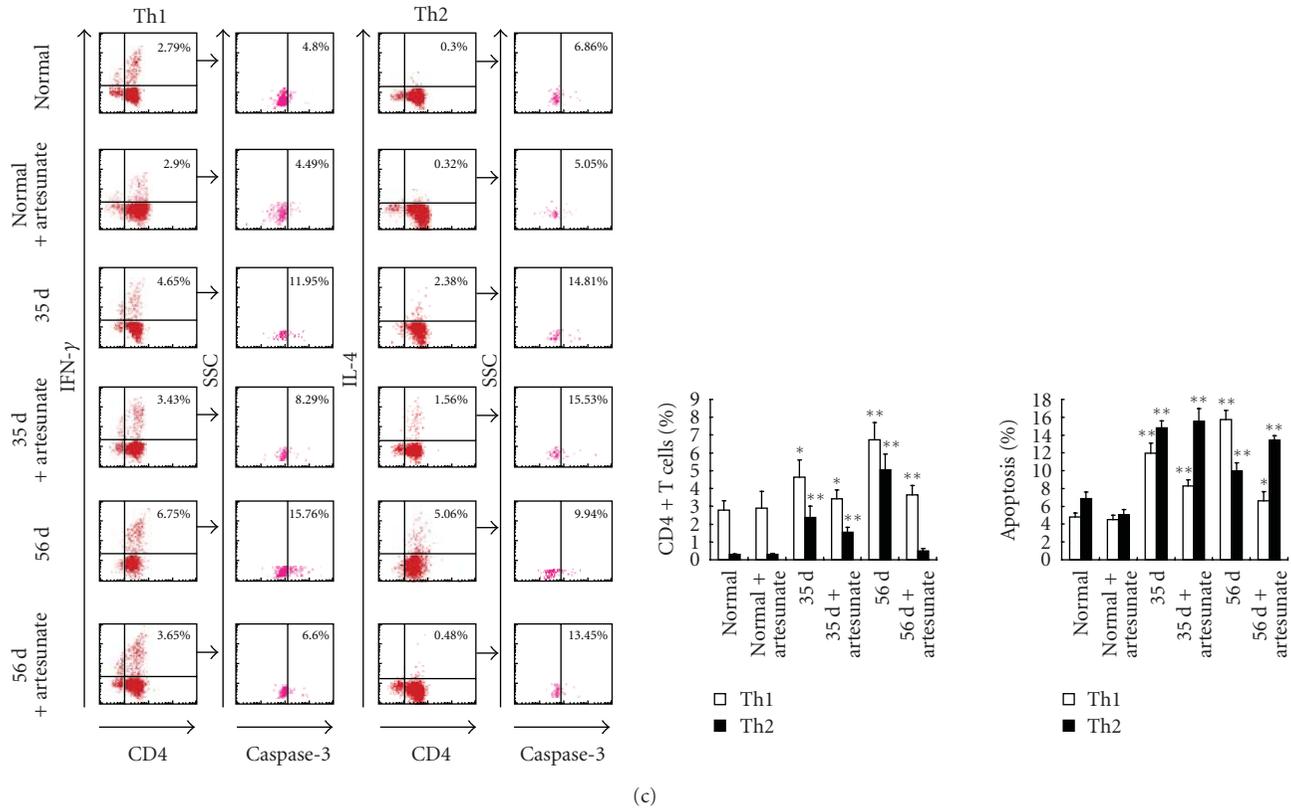


FIGURE 2: Kinetic analysis of Th1/Th2 cell polarization and apoptosis in mice infected with *S. japonicum*. In each experiment, twelve mice were percutaneously infected with *S. japonicum* and among these mice, six were orally administered artesunate dissolved in ddH₂O as described in Materials and Methods. At 23, 35, and 56 days mice were sacrificed and single cell suspension of splenocytes were prepared. (a) CD4⁺ T cells were purified from splenocytes using MACS, surface stained with anti-CD4-FITC and then intracellularly stained with rabbit antimouse caspase-3 antibody or rabbit IgG isotype control antibody plus anti-IFN- γ -PE, anti-IL-4-PE, or isotype IgG control mAbs for FACS analysis. The splenocytes were restimulated in vitro with SWA (b) or SEA (c) for 36 hours, and then the CD4⁺ T cells were purified by MACS and stained with above antibodies prior to FACS analysis. The percentage of apoptotic cells in the FACS data was derived from the CD4⁺ and IFN- γ ⁺, or CD4⁺ and IL-4⁺ cells and gated on the caspase-3⁺ population. Data are expressed as the mean \pm SD of 18 mice from three independent experiments. * $P < .05$; ** $P < .01$. Left panels: One representative experiment of flow cytometric analysis with the average percentage of Th or apoptotic cells shown in the FACS data. Right panels: The statistical analysis of 18 mice from three independent experiments.

TABLE 1: Worm burdens and eggs counts in the livers of infected mice orally treated with or without artesunate.

	0 d	23 d	23 d	35 d	35 d	56 d	56 d
			artesunate		artesunate		artesunate
Adult worms	0	9.75 \pm 0.96***	0	10.5 \pm 1.29***	0	10 \pm 1.63***	0
Liver eggs (/g)	0	0	0	26650.33 \pm 1912.95***	0	51184.58 \pm 1663.26***	0

Mice were percutaneously infected with *S. japonicum*; some were orally administered artesunate as described in Section 2. At 0 (before infection) and 23, 35, and 56 days postinfection, six mice from each group were sacrificed for worm burdens and eggs counts measurements. *** $P < .001$ compared to the artesunate groups at same time point.

days), the Th1-type polarized response induced by non-egg antigens increased. Th2 cell apoptosis increased (from 4.50% to 10.98%) slightly more quickly than apoptosis in Th1 cells (from 3.44% to 6.85%). By correlating the eggs deposition in the liver (at 35 and 56 days) and the SEA becoming the predominant stimulus, which directed a shift toward Th2 cell bias, the percentage of apoptotic Th1 cells remained higher (7.79% at 35 days and 11.04% at 56 days) than in

Th2 cells. A lower level of Th2 cell apoptosis was observed (6.06% at 35 days and 6.20% at 56 days) postinfection in comparison to the level measured 23 days postinfection. These data suggest that SEA, rather than non-egg antigens, may be responsible for the changes observed after eggs deposition.

To clarify this issue, artesunate was administered immediately after *S. japonicum* infection to reduce fecundity

and SEA-induced responses. We observed that artesunate administrated after infection resulted in strong antiparasitic activity, which stopped the infection before the adult worms laid eggs (Figure 1 and Table 1). Similar to the observations in infected mice, the nonegg antigens, whether released by live or drug-killed parasites, led to a Th1-biased response and more Th2 cell apoptosis 23 days after infection in artesunate-treated mice. At days 35 and 56, the predominant stimulus switched to SEA in the infected mice; however, in artesunate-treated mice, responses against nonegg antigens remained. In the artesunate-treated mice (at days 35 and 56), the percentages of Th1 cells (4.63%, 4.40%) remained higher, but the percentages of Th2 cells dropped to relatively lower levels (0.37%, 0.35%). Meanwhile, the level of Th1 cell apoptosis (5.00%, 5.26%) remained low, but there was significantly more Th2 cells apoptosis (11.82%, 8.18%) (Figure 2(a) left and lower-right panels). These results indicated that although both Th1 and Th2 cell apoptosis could be induced by either nonegg or egg antigens, these two subsets exhibited distinct susceptibilities to the two antigens. The nonegg antigens in *S. japonicum* triggered a Th1-biased response and more Th2 cell apoptosis. After the parasites produced eggs, the host responses developed against coexisting nonegg and egg antigens, but they were mainly induced by egg antigens, which shifted the response toward a Th2 bias. Meanwhile, more Th1 cells underwent apoptosis.

To rule out interference between nonegg and egg antigens after egg deposition, CD4⁺ T cells were isolated from mice at 23, 35, and 56 days after infection. Next, SWA and SEA were used in vitro to restimulate apoptosis in purified CD4⁺ T cells which were SWA and/or SEA antigen-primed and sensitized in vivo in infected animals [15]. SWA and SEA are well-characterized antigens that are involved in different types of Th response induction at different infection stages. When compared to the data from normal mice, restimulation with SWA resulted in an increase in both Th1 and Th2 cell apoptosis in CD4⁺ T cells derived from both artesunate-treated and untreated infected mice at 23 days; as shown in Figure 2(b), following SWA priming and sensitization, there was a greater level of apoptosis in Th2 cells (17.88%, 18.31%) than Th1 cells (12.68%, 12.05%). These results suggest that Th2 cells were more sensitive to SWA-triggered apoptosis. On the other hand, SEA was used to restimulate CD4⁺ T cells purified 35 and 56 days after infection from mice with or without artesunate administration, which were primed and sensitized by SWA alone or both SWA and SEA. As shown in Figure 2(c), after restimulation in vitro, Th1 cell apoptosis in infected mice at 35 and 56 days that were not treated with artesunate increased more markedly (from 4.80% in normal mice to 11.95% and 15.76%) than Th2 cells did (from 6.86% in normal mice to 14.81% and 9.94%). However, when compared at 35 and 56 days, much less Th1 cell apoptosis was detected in CD4⁺ T cells from the infected, artesunate-treated mice, which were not primed and sensitized with SEA in vivo (Figure 2(c)). These results further support the observation that Th1 cells are more sensitive to SEA-triggered apoptosis.

3.3. Polarization and Apoptosis of Th1/Th2 Cells in SWA and SEA Immunized Mice. To further investigate the Th cell apoptotic susceptibilities to the two different antigens, mice were immunized with SWA, SEA, or PBS as a control. After 10 days, the CD4⁺ T cells were purified from the immunized mice, labeled with antibodies, and analyzed with flow cytometry. As shown in Figure 3, compared with the control group, animals immunized with either SWA or SEA had increased percentages of Th1 and Th2 cells among the purified CD4⁺ T cells and showed both Th1 and Th2 cell apoptosis simultaneously. However, SWA immunization induced a quicker increase in Th1 cells (from 1.76% to 6.30%) than that Th2 cells (from 0.25% to 0.49%). There was also a significantly greater increase in Th2 cell apoptosis (from 5.43% to 12.24%) than Th1 cell apoptosis (from 4.63% to 6.68%). These results indicate that SWA immunization induced a Th1 polarization. Meanwhile, Th2 cell apoptosis was more susceptible to SWA than Th1 cell apoptosis (Figure 3). In contrast, SEA immunization predominantly induced a greater increase in Th2 cells (from 0.25% to 1.62%), compared to the increase of Th1 cells (from 1.76% to 3.12%). There was also a quicker increase in Th1 cell apoptosis (from 4.63% to 8.89%) than Th2 cell apoptosis (from 5.43% to 6.67%). These results indicate that SEA induced a Th2 cell polarization, and that Th1 cell apoptosis was more susceptible to SEA than Th2 cell apoptosis (Figure 3).

3.4. In Vitro Induction of CD4⁺ T Cell Polarization and Apoptosis in Naïve Mice Using SWA and SEA. After purifying CD4⁺ T cells from naïve mice, they were stimulated in vitro for 36 hours with PBS, SWA, or SEA. Flow cytometry analysis showed that in vitro stimulation of CD4⁺ T cells with either SWA or SEA increased the percentages of both the Th1 and Th2 cells in the purified CD4⁺ T cells from naïve mice as well as the number of apoptotic Th1 and Th2 cells. The data also showed that in vitro stimulation of CD4⁺ T cells with SWA preferentially increased the proportion of Th1 cells and the apoptosis of Th2 cells, but SEA preferentially increased the proportion of Th2 cells and increased the level of apoptosis in Th1 cells (Figure 4).

3.5. GrB and Caspase May Be Involved in Different Th Cells Apoptotic Mechanisms That Are Triggered by Different *S. japonicum* Antigens. Recent studies have provided strong evidence that Th1 and Th2 cells use different mechanisms to execute TCR-induced cell death (TCR-ICD) [21–25]. To investigate whether antigen AICD contributed to Th1/Th2 polarization in *S. japonicum* infection, we studied C57BL/6 mice immunized with SWA, SEA, or PBS. We also examined the effect of caspase inhibitor Z-VAD-FMK and the GrB inhibitor Z-AAD-CMK and measured cell apoptosis using the TUNEL assay considering that caspase inhibitor Z-VAD-FMK was used. As shown in Figure 5(a), SWA increased the percentages of Th1 and Th2 cells and increased the percentages of Th1 and Th2 cell apoptosis among the CD4⁺ T cells, in comparison to the controls. In the Z-AAD-

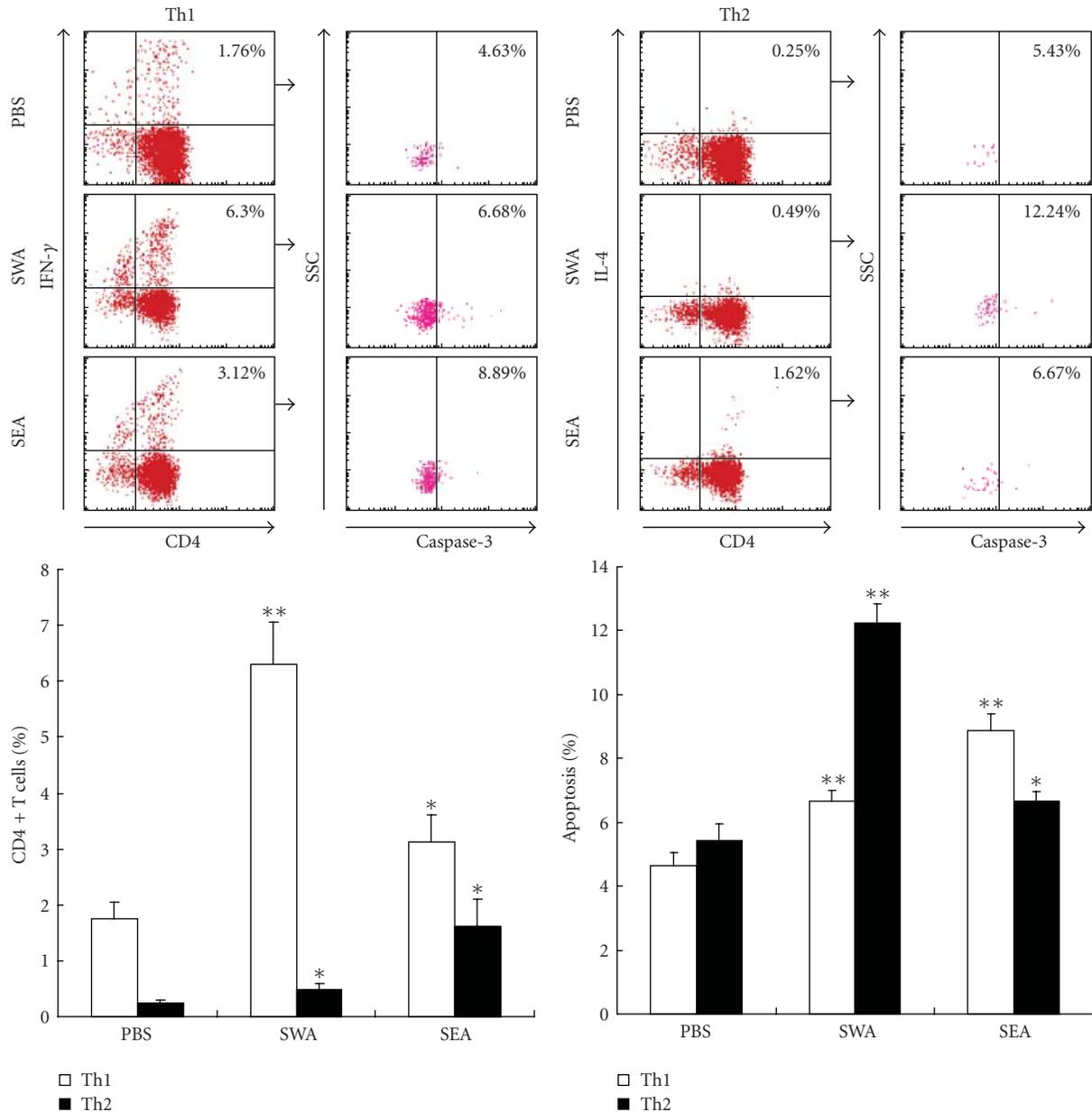


FIGURE 3: Polarization and apoptosis of Th1/Th2 cells in SWA/SEA immunized mice. Mice were injected subcutaneously in the back with 100 μ l of a solution containing 50 μ g of SEA, 100 μ g of SWA, or PBS alone emulsified in incomplete Freund's adjuvant (IFA). Ten days after immunization, six mice from each group were sacrificed. Splenocytes from SWA or SEA immunized mice were prepared, respectively, and then CD4⁺ T cells were purified from splenocytes with MACS. The cells were stained with anticaspase-3, anti-CD4-FITC, anti-IFN- γ -PE or anti-IL-4-PE mAb prior to FACS analysis as described in Materials and Methods. The percentage of apoptotic cells in the FACS data was derived from the number of cells that were CD4⁺ and IFN- γ ⁺ or CD4⁺ and IL-4⁺ and gated on the caspase-3⁺ population. Data are expressed as the mean \pm SD of 18 mice from three independent experiments. * P < .05; ** P < .01. Upper panels: One representative experiment of flow cytometric analysis with the average percentage of Th or apoptotic cells shown in the FACS data. Lower panels: The statistical analysis of 18 mice from three independent experiments.

CMK-treated groups, there was a marked decrease in SWA restimulation-related Th2 cell apoptosis and an increase in Th2 percentage; however, this was not observed for Th1 cell apoptosis and Th2 percentage. In contrast, Figure 5(b) shows that, compared to the PBS control, SEA increased both the percentages of Th1 and Th2 cells and apoptosis in Th1 and Th2 cells. However, in the Z-VAD-FMK-treated

groups, the level of Th1 cell apoptosis induced by SEA significantly decreased and the proportion of Th1 cells significantly increased, but the statistically significant decrease in Th2 cells apoptosis and the increase in the proportion of Th2 cells were not observed in both the SEA and PBS groups when compared to their respective cocultures in the absence of Z-AAD-CMK.

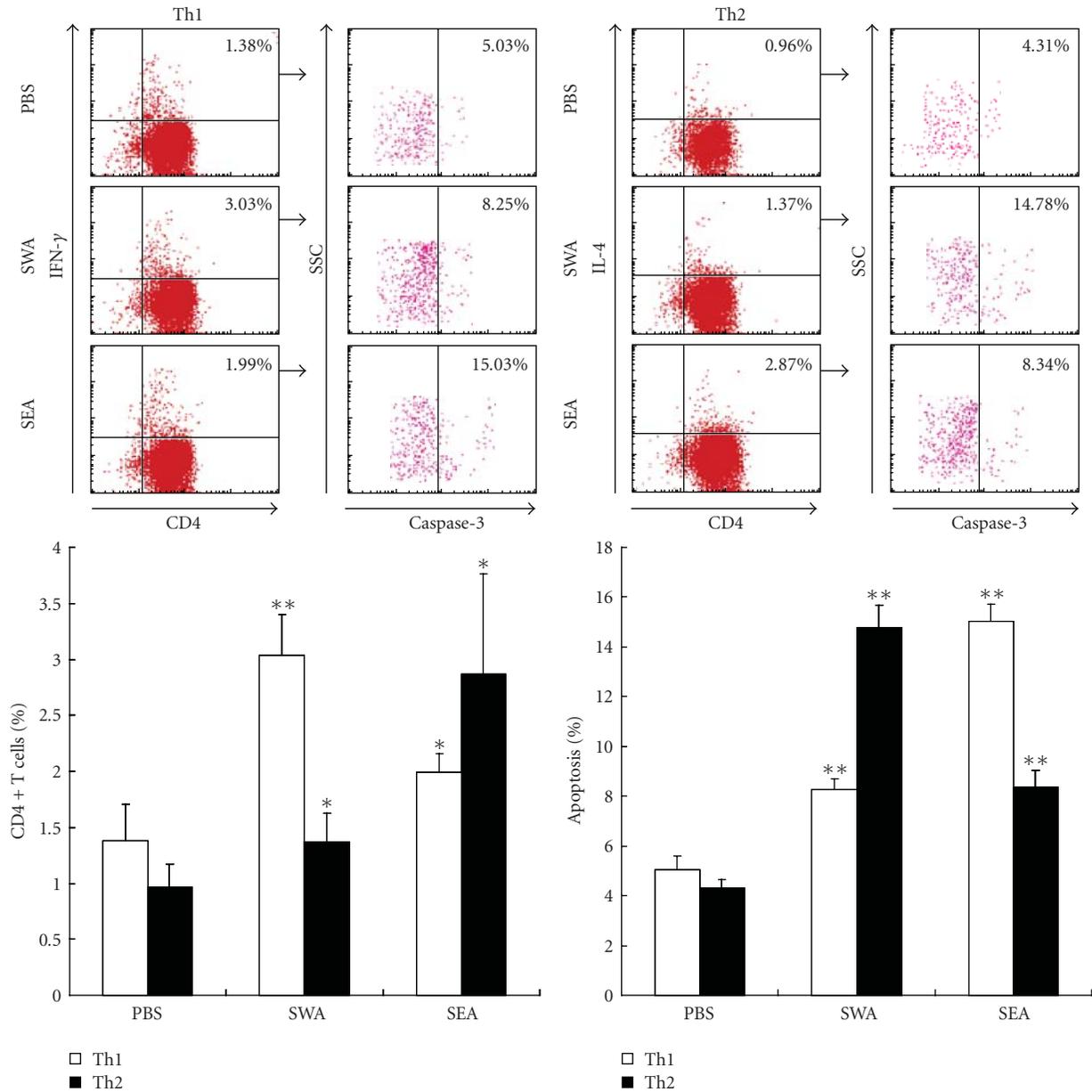


FIGURE 4: In vitro induction of polarization and apoptosis in $CD4^+$ T cells derived from normal mice with SWA and SEA. $CD4^+$ T cells were purified from normal mice by MACS and preactivated overnight with anti-CD3 ($2 \mu\text{g}/\text{mL}$) and anti-CD28 ($1 \mu\text{g}/\text{mL}$) antibodies. Then the cells were stimulated with specific antigens of SEA, SWA or PBS alone for 36 hours at 37°C in $5\%\text{CO}_2$, followed by staining with rabbit antimouse caspase-3 antibody or rabbit IgG isotype control antibody plus anti-CD4-FITC, anti-IFN- γ -PE anti-IL-4-PE mAbs, or isotype control antibodies prior to FACS analysis. The percentage of apoptotic cells in the FACS data was derived from the number of cells that were $CD4^+$ and IFN- γ^+ or $CD4^+$ and IL-4 $^+$ and gated on the caspase-3 $^+$ population. Data are expressed as the mean \pm SD of 18 mice from three independent experiments. * $P < .05$; ** $P < .01$. Upper panels: One representative experiment of flow cytometric analysis with the average percentage of Th or apoptotic cells shown in the FACS data. Lower panels: The statistical analysis of 18 mice from three independent experiments.

4. Discussion

Both Th1/Th2 shift and Th cell apoptosis are believed to benefit for both survival of parasites and reducing self-damage to the host during schistosome infection. Here, our results demonstrate that during the early stage of *Schistosoma*

japonicum infection, nonegg antigens trigger Th2 cell apoptosis via the granzyme B signal pathway, contributing to Th1 polarization. Th1 polarization is believed to be associated with worm clearance and severe schistosomiasis. Following egg-laying, the egg antigens trigger Th1 cell apoptosis via the caspase pathway, contributing to Th2 polarization, which

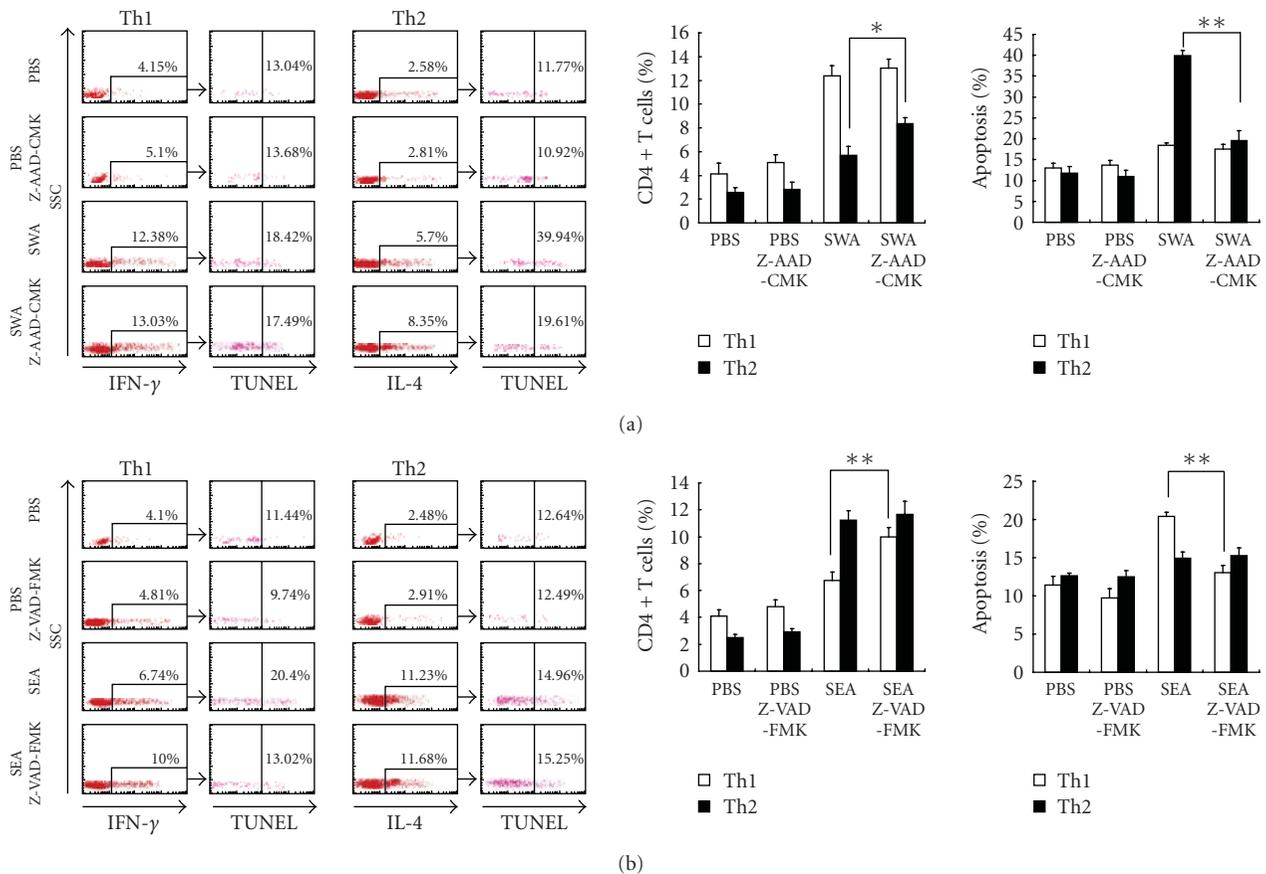


FIGURE 5: Caspase and GrB may be involved in different types of Th cell apoptotic mechanisms that are induced by different *S. japonicum* antigens. (a) Splenocytes from SWA-immunized mice were restimulated in vitro with SWA or PBS in the presence or absence of the GrB inhibitor Z-AAD-CMK. (b) Splenocytes from SEA immunized mice were restimulated in vitro with SEA or PBS in the presence or absence of the caspase inhibitor Z-VAD-FMK. After 36 hours, restimulated CD4⁺ T cells in (a) and (b) were purified by MACS and stained with anti-IFN- γ -PE or anti-IL-4-PE mAbs. Apoptotic cells were labeled with an Apo-Direct TUNEL assay kit and then detected by FACS. The percentage of apoptotic Th1 or Th2 cells among the purified CD4⁺ T cells was derived from the number of IFN- γ ⁺ or IL-4⁺ cells and gated on the TUNEL positive population. Data are expressed as the mean \pm SD of 18 mice from three independent experiments. * $P < .05$; ** $P < .01$. Left panels: One representative experiment of flow cytometric analysis with the average percentage of Th or apoptotic cells shown in the FACS data. Right panels: The statistical analysis of 18 mice from three independent experiments.

is associated with mild pathology and enhanced survival of both worms and their hosts. Thus, our study suggests that *S. japonicum* antigen-induced Th1 and Th2 cell apoptosis involves the deletion of mature Th cells in the periphery as well as a host Th response shift and polarization providing a mechanism to modulate the immune interaction between hosts and parasites.

Egg-laying is a critical event in adult schistosomes, which initiates both the formation of immunopathologic granulomas and the Th1/Th2 shift. Consistent with previous reports, we also observed that the initial oviposition occurred between 25 and 28 days after infection (unpublished data). Thus, we selected three time points (23, 35, and 56 days) postinfection as check points in our study that were representative of the response to non-egg antigen exposure, the initiation of granuloma formation in response to egg antigen exposure, and the initiation of modulation of the granuloma response.

To assess apoptosis in Th1 and Th2 cells during the different stages of schistosomal infection, CD4⁺ T cells were purified from mice at three time points mentioned above and labeled for FACS analysis without further restimulation in vitro. Our results showed that the level of apoptosis of both Th1 and Th2 cells increased in infected mice in comparison to normal mice. During the early stage of infection (23 days), the Th1-type-polarized response and Th2-biased apoptosis induced by non-egg antigens increased. At 35 and 56 days after infection, SEA became a predominant stimulus, and the Th2-type-polarized response and Th1-biased apoptosis induced by SEA increased. These in vivo data raise a question that SEA, rather than non-egg antigens, may be responsible for the changes observed after eggs deposition.

Thus, to illustrate this issue, the interference between non-egg and egg antigens after egg deposition has to be ruled out. Artesunate is a famous antimalarial drug [26, 27], which also displays activity against schistosomiasis [28, 29] through

its vermucidal effect on 7- to 35-day-old schistosomes [30]. Following administration of artesunate in our study, the worm burdens and liver egg counts significantly decreased at 23, 35, and 56 days after *S. japonicum* infection, which indicates that oral administration of artesunate after infection successfully stops infection before the adult worms lay eggs. It provides us a chance to rule out interference to the host immune responses between non-egg and egg antigens after egg deposition. Results showed that, in artesunate-treated mice, without stimulation of SEA at 35 and 56 days after infection, Th1-biased responses and Th2-biased apoptosis remained.

Then, our experiments using in vitro stimulation of CD4⁺ T cells from *S. japonicum* infected mice with SWA resulted in a preferential increase in the proportion of Th1 cells and the apoptosis of Th2 cells. In contrast, SEA preferentially increased the proportion of Th2 cells and led to increased levels of apoptosis in Th1 cells. This was further supported by our in vivo data from mice immunized with SWA or SEA, respectively. Furthermore, we in vitro stimulated CD4⁺ T cells from naïve mice where SWA preferentially increased the proportion of Th1 cells and the apoptosis of Th2 cells, but SEA preferentially increased the proportion of Th2 cells and increased the level of apoptosis in Th1 cells. All these in vitro and in vivo findings provided further support for the notion that non-egg and egg antigens from the schistosome infection regulate the types of Th immune responses and are able to induce different biased types of Th cell apoptosis.

Recent studies have provided strong evidence that Th1 and Th2 cells use different mechanisms to execute TCR-induced cell death (TCR-ICD) [21–25]. Th1 cell death was shown to be triggered by the CD95-CD95L interaction and mediated via caspase 8, which initiated the activation cascade of caspases 3 and 9, which then leads to cell death [22–24]. Th2 cell apoptosis of Th2 cells was unrelated to the TNF-family proteins, since anti-CD95L, TR6, DR5, OPG, and anti-TNF did not affect TCR-ICD in Th2 cells. Instead, Th2 cells have been shown to undergo apoptosis by GrB dependent mechanisms [23, 25]. GrB was previously implicated in CD8⁺ T cell and NK cytotoxicity and was currently also shown to be crucial for Th2 cell death [25, 31]. On one hand, GrB triggers Th2 cell apoptosis by cleaving caspase-3 and some other caspases to initiate the caspase cascade [32]. On the other hand, GrB triggers Th2 cell apoptosis also by caspase independent pathways [23, 32–34]. Thus, in the presence of complete caspase inhibition, the caspase inhibitor Z-VAD-FMK, which does not inactivate GrB, could only block Th1 cell death, while the Th2 cells were little affected [23, 32]. In contrast, Th2 cell apoptosis could only be blocked by the GrB-specific inhibitor Z-AAD-CMK, whereas the caspase inhibitor Z-VAD-FMK had no effect [23, 25, 32]. Our results indicated that at least partially, the mechanism by which the non-egg antigens triggered Th2 cells apoptosis was GrB dependent and Th2 cell apoptosis might contribute to Th1 cell polarization. Meanwhile, caspases could be critical for the mechanism by which the egg antigens induced Th1 cell apoptosis and might contribute to Th2 cell polarization.

In conclusion, our study suggests that Th1/Th2 cell apoptosis is preferentially induced by different schistosome antigens during different stages of infection. This can partially explain the host Th cell immune response shift and polarization during schistosome infections. Additionally, Th1/Th2 cell apoptosis may contribute an additional mechanism that modulates the immune interaction between hosts and parasites. Th1/Th2 cell apoptosis may be used by hosts as an efficient way to restrict immunopathology and by parasites as a survival strategy to downregulate immune resistance and maintain infection in their hosts.

Abbreviations

AICD:	Activation-induced cell death
HE:	Hematoxylin and eosin
SEA:	Soluble schistosome egg antigen
<i>S. japonicum</i> :	<i>Schistosoma japonicum</i>
SWA:	Soluble schistosome worm antigen
Z-AAD-CMK:	Z-Ala-Ala-Asp-CH ₂ Cl
Z-VAD-FMK:	Z-Val-Ala-Asp(OCH ₃)- Fluoromethylketone.

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Research Article

Anti-Inflammatory Protein of *Schistosoma japonicum* Directs the Differentiation of the WEHI-3B JCS Cells and Mouse Bone Marrow Cells to Macrophages

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Sj16 is an anti-inflammatory protein identified from *Schistosoma japonicum*. Our previous studies showed that recombinant Sj16 (rSj16) could suppress host's inflammatory responses and inhibit macrophage maturation. In the present study, the effects of rSj16 on the differentiation of the murine myeloid leukemia WEHI-3B JCS cell line and on mouse hematopoiesis were investigated. Our data demonstrated that rSj16 expressed and purified from *Escherichia coli* could suppress the proliferation of the WEHI-3B JCS cells in a time- and concentration-dependent manner, while not affect the viability of the cells. Further studies indicated that rSj16 induced macrophage differentiation of the WEHI-3B JCS cells, and arrested the cell cycle in the G1/G0 and G2/M phases. The macrophage differentiation of the rSj16-treated WEHI-3B JCS cells was confirmed by their expression of macrophage specific antigen F4/80 and phagocytic activity. Furthermore, our results revealed that rSj16 biased the colony formation of mouse bone marrow cells towards macrophage lineage.

1. Introduction

Leukemia occurs when the normal hematopoietic process fails, and the leukemic cells retain the proliferative capacity of progenitor cells and unable to spontaneously undergo terminal maturation [1]. The cell line WEHI-3B JCS is a D-subclone isolated from the WEHI 3B myelomonocytic leukemia cell line [2]. Previous studies have reported that monocytic differentiation of this cell line could be induced by phorbol myristate acetate (PMA) [2], biochanin A [3], and conjugated linoleic acid [4], and that endogenous IL-1 α , IL-1 β , and TNF- α have been shown to play an important role during these differentiation-inducing processes.

Schistosome, the causative agent of schistosomiasis in the world, is a blood born fluke (trematode) of the genus *Schistosoma*. Plenty of studies have demonstrated that the schistosome-originated substances could modulate the host's

immune system [5, 6]. For example, the prostaglandin D2 from schistosome has been well known to block the migration of langerhans cells to the draining lymph nodes, thereby inhibiting a crucial step in the initiation of immunity [7]. An IL-4 inducing factor identified from *Schistosoma mansoni* (*S. mansoni*) egg has been demonstrated to skew the immune response to Th2 [8]. Another apoptosis inducing factor produced by skin-stage schistosomula of *S. mansoni* could cause apoptosis of T cells [9]. Trottein et al. have also reported that substances from *S. mansoni* suppressed host's inflammation by activating host microvascular endothelial cells [10]. However, up to now, little is reported about the immunomodulatory effects of schistosome-derived substances on leukemia and hematopoiesis.

Recently, we have cloned a gene named Sj16 from *Schistosoma japonicum* (*S. japonicum*) and demonstrated that the recombinant Sj16 (rSj16) expressed and purified

from *Escherichia coli* (*E. coli*) could suppress the recruitment of mature macrophages into the peritoneal cavity in thioglycollate-induced inflammation in BALB/c mice [11]. In the present study, we reported the effects of rSj16 on the proliferation and differentiation of the WEHI-3B JCS and on mouse hematopoiesis.

2. Materials and Methods

2.1. Cell Line. The murine myeloid leukemia cell line WEHI-3B JCS [2] was cultured in RPMI 1640 medium (Gibco-BRL, USA) supplemented with 10 mM L-glutamine, 10% fetal calf serum (FCS; HyClone, USA), and 1% antibiotics mixture (100 unit/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B; GibcoBRL, USA). The cultures were incubated under a humidified atmosphere of 95% air, 5% CO₂ at 37°C.

2.2. Animals. BALB/c mice (female, 4–6 week), bred and kept by the Laboratory Animal Services Centre of the Chinese University of Hong Kong, were used. All animal procedures were approved by the Animal Experimentation Ethics Committee (CUHK) in accordance with the Department of Health (Hong Kong) guidelines in Care and Use of Animals. All experiments were performed under licence from the Hong Kong Special Administrative Region Government.

2.3. Expression and Purification of Recombinant Proteins. The recombinant Sj16 (rSj16) and recombinant glutathione-S-transferase (rGST) were expressed and purified from *E. coli* as we have described before [11]. The purified rSj16 and rGST were passed through a Detoxi-Gel AffinityPak polymyxin B column (Pierce, USA) to eliminate endotoxins. The absence of endotoxins in the proteins was further confirmed by *Limulus* amoebocyte lysate test (sensitivity 0.25 EU/ml, Associates of Cape Cod, USA) before the proteins were used in functional assays.

2.4. Cell Proliferation Assessment. The colorimetric 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay was employed to quantify the cell proliferation [12, 13]. Briefly, WEHI-3B JCS cells were incubated with RPMI 1640 medium containing serially diluted rSj16 or rGST in 96-well microplates (Nunc, Denmark) for 24 hours, 48 hours, 72 hours, and 96 hours. 20 µl MTT (5 mg/ml stock solution, Sigma, USA) were then added into each well and incubated for 4 hours, and the plates were centrifuged at 500×g for 10 minutes. The MTT solution was removed and replaced by 150 µl DMSO, and the plates were shaken until the formazans were thoroughly dissolved. The optical density was determined using a microplate reader (Dynatech Laboratories, USA) at a wavelength of 570 nm. Each assay was performed in triplicate.

2.5. Cell Viability. Cell viability was determined using trypan blue exclusion assay. Briefly, WEHI-3B JCS cells were incubated with RPMI 1640 medium in the presence or absence of 0.5 µg/ml rSj16 or 0.5 µg/ml rGST for 24 hours, 48 hours,

and 72 hours. The cells were then collected and mixed 1 : 1 with 0.4% trypan blue (Sigma, USA). The percentage of viable cells was recorded within 10 minutes with an inverted microscope. Greater than 300 cells per sample were examined and the data were expressed as percentage (%) viability. The experiment was performed in triplicate.

2.6. Cell Cycle Analysis. Cell cycle analysis was performed on WEHI-3B JCS cells incubated for 1–4 days with either rSj16 (0.5 µg/ml) or rGST (0.5 µg/ml). The cells were fixed in chilled ethanol overnight before staining with propidium iodide staining solution (3.8 mM sodium citrate, 50 µg/ml propidium iodide, 50 µg/ml RNase A in PBS) at room temperature for 30 minutes. Analysis was performed immediately after staining using a FACScan (Becton Dickinson, USA) and CELLFit program (Becton Dickinson, USA).

2.7. Analysis of WEHI-3B JCS Differentiation. WEHI-3B JCS cells were cultured with either rGST (0.5 µg/ml) or rSj16 (0.5 µg/ml) in the presence/absence of serially diluted neutralizing anti-IL-1α, anti-IL-1β, anti-TNF-α, or these three antibodies together (R&D Systems, USA) for up to 72 hours.

The adherent and suspension cells were collected, respectively, and counted with a hemacytometer (Bright-line, Sigma, USA) 24, 48, and 72 hours after treatment. The percentage of adherent cells was calculated. The culture supernatants after 24, 48, and 72 hours treatment were collected, stored at –80°C until cytokine measurement.

The morphology of the WEHI-3B JCS cells was studied up to 72 hours of treatment using a phase-contrast microscope (Nikon, Japan). Monocytic differentiation of the treated cells was also examined using modified Wright-Giemsa stain (Sigma, USA) after cytospin smear preparation. All experiments were independently done at least 3 times.

Expression of cell surface antigens was determined by flow cytometry 72 hours after treatment. After twice washing with FACS medium (2% heat-inactivated FCS and 0.05% sodium azide in PBS), cells were incubated at 4°C for 30 minutes with antimouse FcγR antibody (CD16/CD32, eBioscience, USA). The cells were then washed twice and incubated at 4°C for 30 minutes with affinity purified antimouse F4/80 or antimouse Gr-1 antibodies (both from eBioscience, USA). Isotype-matched control antibodies (eBioscience, USA) were used as negative controls. Unbound antibodies were removed by washing the cells twice with FACS medium. The cells were then stained with FITC-conjugated sheep antirat IgG (BD PharMingen, USA) at 4°C for 30 minutes and then washed and fixed with 1% (w/v) paraformaldehyde in PBS. Cells were analyzed by a FACScan (Becton Dickinson, USA).

Phagocytic activity of WEHI-3B JCS was determined by the modified yeast phagocytosis assay as described previously [2, 3]. Briefly, opsonized yeasts were prepared by incubating heat-inactivated yeast cells (*Saccharomyces cerevisiae*) with fresh mouse serum at 37°C for 30 minutes. 2 × 10⁷ opsonized yeasts were added to untreated, 72 hours post rSj16 or rGST treated WEHI-3B JCS cell cultures in 6-well

microplates (Nunc, Denmark). The mixtures were incubated at 37°C for 16 hours, and the percentages of phagocytic cells were counted under microscope. Cells with a minimum of three ingested yeasts were considered positive. A total of 500 cells were counted in each sample.

2.8. Cytokine Determination. Levels of IL-1 α , IL-1 β , and TNF- α in the culture supernatants were measured using DuoSet ELISA Development kit (R&D Systems, USA) according to the manufacturer's protocol. Briefly, 96-well Maxisorp multititer plates (Nunc, Denmark) were coated overnight at 4°C with appropriate capture antibodies (2 μ g/ml for anti-IL-1 α , 4 μ g/ml for anti-IL-1 β , and 0.8 μ g/ml for anti-TNF- α) in PBS at pH 7.4 and then washed three times with PBS containing 0.05% Tween 20. The plates were blocked for 2 hours at room temperature with 1% BSA in PBS, followed with three washes as described above. Serially diluted murine recombinant cytokine standards IL-1 α (1.95–1,000 pg/ml), IL-1 β (1.95–1,000 pg/ml), or TNF- α (3.91–1,000 pg/ml) and the culture supernatants were then added in duplicate and incubated for 2 hours at room temperature. After three washes, the plates were incubated for 2 hours at room temperature with appropriate biotinylated detection antibodies and then washed again. To detect biotinylated antibodies, streptavidin-linked horseradish peroxidase (1/1,000 dilution) was added and incubated for 20 minutes in the dark at room temperature. After a final washing of five times, 3, 3', 5, 5'-tetramethylbenzidine liquid substrate (Sigma, USA) was added to each well. The absorbance was read at 450 nm with the wavelength correction at 570 nm using a microplate reader (Dynatech Laboratories, USA), and the concentration of the samples was calculated using the standard curve.

2.9. IL-1 α , IL-1 β , and TNF- α Neutralization Assay (by MTT Assay). For neutralization assay, WEHI-3B JCS cells were incubated in 96-well microplates (Nunc) with RPMI 1640 medium containing 0.5 μ g/ml rSj16 in the presence or absence of serially diluted neutralizing anti-IL-1 α , anti-IL-1 β , anti-TNF- α , or these three antibodies together (R&D Systems) for 72 hours. To test whether anti-IL-1 α , anti-IL-1 β , or anti-TNF- α antibody itself affects the WEHI-3B JCS proliferation, WEHI-3B JCS cells were also incubated in 96-well microplates (Nunc) with RPMI 1640 medium containing serially diluted anti-IL-1 α , anti-IL-1 β or anti-TNF- α antibody (R&D Systems). 20 μ l MTT (5 mg/ml stock solution, Sigma, USA) were then added into each well and incubated for 4 h, and the proliferation was assessed as mentioned above. Each assay was performed in triplicate. The results of neutralization assay were expressed as proliferation index which was calculated as following: *proliferation index* = mean O.D.570 of triplicate wells containing 0.5 μ g/ml rSj16 in the presence of neutralizing anti-IL-1 α , anti-IL-1 β , or anti-TNF- α antibodies/mean O.D.570 of triplicate wells containing 0.5 μ g/ml rSj16 in the absence of neutralizing anti-IL-1 α , anti-IL-1 β , or anti-TNF- α antibodies.

2.10. Mouse Bone Marrow Cell Colony-Forming Unit Assay. Female BALB/c mice were sacrificed and wet thoroughly with

70% ethanol. The femurs were isolated, the bone marrows were flushed out, and the single cell suspension was then prepared. To measure the number of nucleated cells, 10 μ l of the single cell suspension was first diluted in 490 μ l of 3% acetic acid and then counted using a hemacytometer (Bright-line, Sigma, USA). The cell suspension was adjusted to 5×10^6 nucleated cells per ml with cold RPMI-1640 medium.

The bone marrow cell suspension was added into prewarmed RPMI-1640 medium containing 20% FCS (HyClone), 2% antibiotics mixture (GibcoBRL), and 0.33% agar (cell culture tested; sigma, USA), and mixed well (60 μ l of cell suspension per 6 ml of medium). The mixture was transferred into 24-well plates (Nunc), 0.4 ml per well, and then kept at room temperature for 15 minutes to allow the agar solidification. After solidification, 25 μ l of PBS alone, 25 μ l of PBS containing 0.5 μ g/ml of rSj16 or 0.5 μ g/ml rGST was added into corresponding wells and incubated in a humidified 37°C incubator for one hour, followed by adding 25 μ l of PBS, 25 μ l of PBS containing 0.5 ng/ml of IL-3, 25 μ l of PBS containing 36 ng/ml of M-CSF, or 25 μ l of PBS containing 12 ng/ml of G-CSF into corresponding wells and incubated for further seven days. Each semisolid agar gel was then transferred onto a cleaning glass slide, covered with 3 layers of Whatman filter papers, and dried overnight at 37°C oven. The slides were stained with hematoxylin solution (Sigma, USA), and the cell colonies of greater than 40 cells were counted. All samples were run in triplicate.

2.11. Statistics. Comparisons of data within experiments were tested for significance using the Student's *t*-test.

3. Results

3.1. rSj16 Inhibited WEHI-3B JCS Cell Proliferation. In this study, rSj16 was first expressed and purified as a rGST-rSj16 fusion protein using the prokaryotic expression vector pGEX-4T-1, and the rGST tag was then removed by thrombin digestion to release free rSj16 [11]. However, during purification of free rSj16, traces of rGST may not be absolutely excluded from the rSj16 solution. On the other hand, the rGST tag in vector pGEX-4T-1 is also *S. japonicum* originated [14]. These made rGST an ideal control protein during the rSj16 functionary assays. Thus, the rGST was purified and used as a control protein throughout the study.

The antiproliferative effect of rSj16 was evaluated by MTT assay. Data were expressed as the percentage inhibition by the ratio of each absorbance relative to the absorbance of the nontreated control, multiplied by 100%. A concentration (Figure 1(a)) and time (Figure 1(b)) dependent inhibition of rSj16 on WEHI-3B JCS cell growth was observed at 48 hours (more than 10% inhibition) and persisted until 96 h. rSj16 inhibited the proliferation of WEHI-3B JCS cells at a maximal effect of more than 40% at 72 hours, and reached this maximal effect at the concentration of 0.5 μ g/ml or higher (Figure 1(a)). Thus, in the following part of this study, 0.5 μ g/ml of rSj16 was used. rGST did not show significant effect on the proliferation of WEHI-3B JCS cells when being used as high as 5 μ g/ml (data not shown).

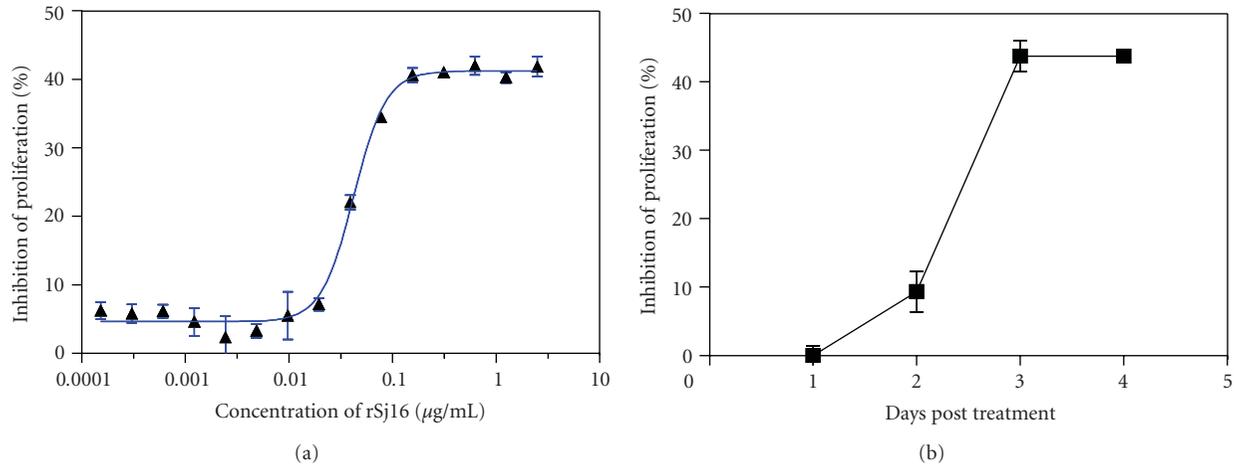


FIGURE 1: Antiproliferative effect of rSj16 on WEHI-3B JCS cells. WEHI-3B JCS cells were incubated with 0.2 ml of medium containing different doses of rSj16 for 72 hours (a) or containing 0.5 µg/ml of rSj16 for up to 96 hours (b). The proliferation was quantified by MTT assay.

In order to test whether the suppression of WEHI-3B JCS proliferation by rSj16 is due to its cytotoxic effect on the cells, WEHI-3B JCS cells were incubated with rSj16 for up to 72 hours and the cell viability was assessed by trypan blue exclusion assay. The results indicated that the viability of WEHI-3B JCS cells was not affected after 0.5 µg/ml of rSj16 treatment for 24 hours ($98.51 \pm 0.14\%$ versus $98.86 \pm 0.28\%$ viability, $P > .2$; rSj16 treatment versus untreated; $n = 4$), 48 hours ($97.80\% \pm 0.35\%$ versus $97.36 \pm 0.43\%$, $P > .2$; $n = 4$) and 72 hours ($96.48\% \pm 0.57\%$ versus $97.43\% \pm 0.51\%$, $P > .2$; $n = 4$). rGST did not show any effects on WEHI-3B JCS viability (data not shown).

3.2. Effect of rSj16 on the WEHI-3B JCS Cell Cycle. Because rSj16 exerted significant effect on WEHI-3B JCS cells proliferation (Figure 1), while it did not affect the viability of the cells (see above), we were interested in studying the pattern of cell cycle distribution of rSj16-treated cells. The cell cycle distribution was assessed by flow cytometry after treatment with rSj16 for various times.

A significant accumulation of WEHI-3B JCS cells in the G1/G0 phases of the cell cycle occurred at 24 hours (Figure 2(a)), reached the peak at 72 hours (Figure 2(c)), and retained a high level at 96 hours (Figure 2(d)), after treatment with 0.5 µg/ml of rSj16, accompanied with a concomitant decrease in the proportion of those in S phase (Figures 2(c) and 2(d)). A significant accumulation of WEHI-3B JCS cells in the G2/M phase of the cell cycle also occurred at 48 hours (Figure 2(b)), and reached the peak at 72 hours (Figure 2(c)) post 0.5 µg/ml of rSj16 treatment. After 72 h, the accumulation of G1/G0 and G2/M population did not increase anymore even exposure for longer time (Figure 2(d)). rGST had no effect on the cell cycle distribution of WEHI-3B JCS cells (Figures 2(a)–2(d)).

3.3. Morphological Study of rSj16-Treated WEHI-3B JCS Cells. The effect of rSj16 on morphology of WEHI-3B JCS cells

was studied in a time course of 72 h. The WEHI-3B JCS cells became adherent and developed pseudopodia in the presence of 0.5 µg/ml of rSj16 (Figures 3(a) and 3(b)). Aggregation of the WEHI-3B JCS cells to each other followed by the adherence of the cells to the plate was observed within 24 hours of rSj16 exposure (Figures 3(a) and 3(b)). By the time of 48 h, more than 50% of the cells attached to the plate and began to form pseudopodia (Figures 3(a) and 3(b)). After 72 hours of rSj16 exposure, 71.28% \pm 3.15% of the cells were adherent to the plate, and over 50% of the cells had pseudopodia and cytoplasmic vacuoles (Figures 3(a) and 3(b)). All of these morphological changes indicated that rSj16 may induce macrophage differentiation of WEHI-3B JCS cells, as the abilities to develop prominent pseudopodia and adhere to charged surface are typical features of normal tissue macrophages [15, 16]. In contrast, cells exposed with rGST (0.5 µg/ml) did not developed significant adhesion (only 0.02% \pm 0.007% of the cells were adherent at 72 h) and pseudopodia formation (Figures 3(a) and 3(b)).

To further characterize the morphological changes, cytopsin smear of WEHI-3B JCS cells treated with rSj16 or rGST was prepared and stained with Modified Wright-Giemsa stain (Sigma, USA). The result showed that after 72 hours exposure to 0.5 µg/ml of rSj16, the nuclear: cytoplasmic ratio of the cells reduced, the nuclear chromatin became condensed and eccentrically placed, the cytoplasmic borders were protruded, and cytoplasmic vacuoles were often present (Figure 3(e)). These morphological features were similar to those described for mature tissue macrophages [15–17]. WEHI-3B JCS cells treated with rGST did not exhibit significant morphological changes (Figure 3(d)).

3.4. Expression of Macrophage Marker on rSj16 Induced WEHI-3B JCS Cells. To confirm the macrophage differentiation-inducing activity of rSj16 on WEHI-3B JCS cells, flow cytometric analysis was carried out to examine for the expression of differentiation antigens by

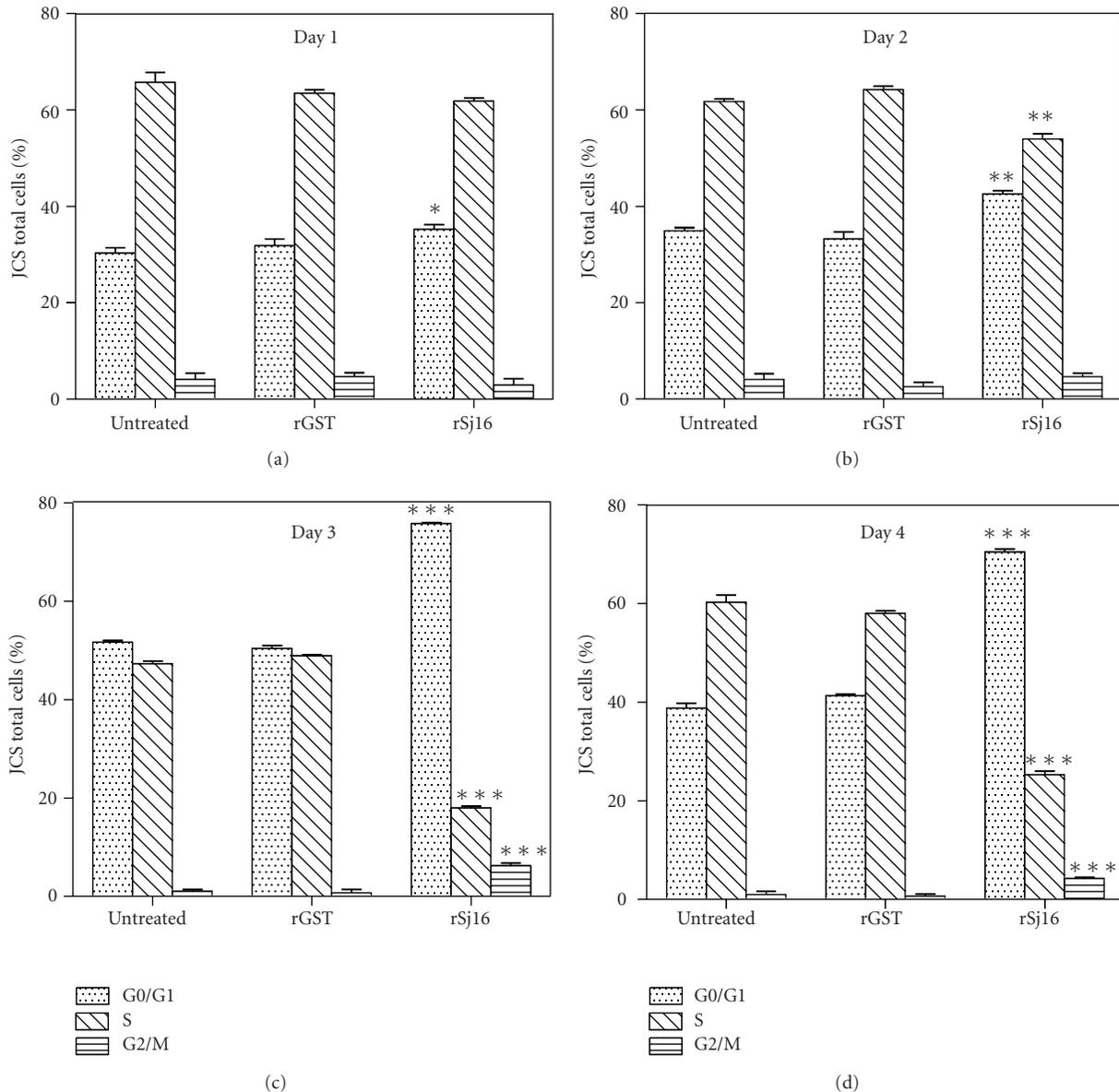


FIGURE 2: Modulation of WEHI-3B JCS cell cycle by rSj16. WEHI-3B JCS cells were cultured for one day (a), two days (b), three days (c), and four days (d) in the presence of $0.5 \mu\text{g/ml}$ of rSj16 (rSj16), $0.5 \mu\text{g/ml}$ of rGST (rGST), or medium alone (untreated), and the cell cycle status was analyzed by flow cytometry. Data indicate mean \pm SEM of three independent experiments. * $P < .1$; ** $P < .01$; *** $P < .001$ as compared with that of untreated group.

rSj16-treated cells. Untreated WEHI-3B JCS cells expressed little F4/80+ and Gr-1+ (Figure 4). 72 hours exposure of WEHI-3B JCS cells to rSj16 ($0.5 \mu\text{g/ml}$) resulted in nearly 40% increase in the expression of the antigen F4/80+, while there were no significant changes on the expression of Gr-1+ (Figure 4). rGST did not show significant effect on the expression of these antigens (Figure 4).

3.5. rSj16 Induced WEHI-3B JCS Cells Showed Phagocytic Activity. A key function of normal mononuclear phagocytes is ingestion of foreign materials. The biological function of rSj16-treated WEHI-3B JCS cells was therefore measured by their capacity of yeast phagocytosis. The results indicated

that WEHI-3B JCS cells developed the ability to efficiently phagocytose *Saccharomyces cerevisiae* after rSj16 exposure (Figures 5(a)–5(c)). More than 50% of the WEHI-3B JCS cells ingested three or more yeast cells after cultured in the presence of $0.5 \mu\text{g/ml}$ rSj16 for 3 days (Figure 5(d)). Less than 2% of the untreated or rGST-treated WEHI-3B JCS cells phagocytized yeast cells.

3.6. Up-Regulation of IL-1 α , IL-1 β , and TNF- α Expression in rSj16-Treated WEHI-3B JCS Cells. Previous studies reported that TNF- α could induce monocytic differentiation of WEHI-3B JCS cells [2], and that IL-1 α and IL-1 β were involved in the differentiation-inducing activity of TNF- α

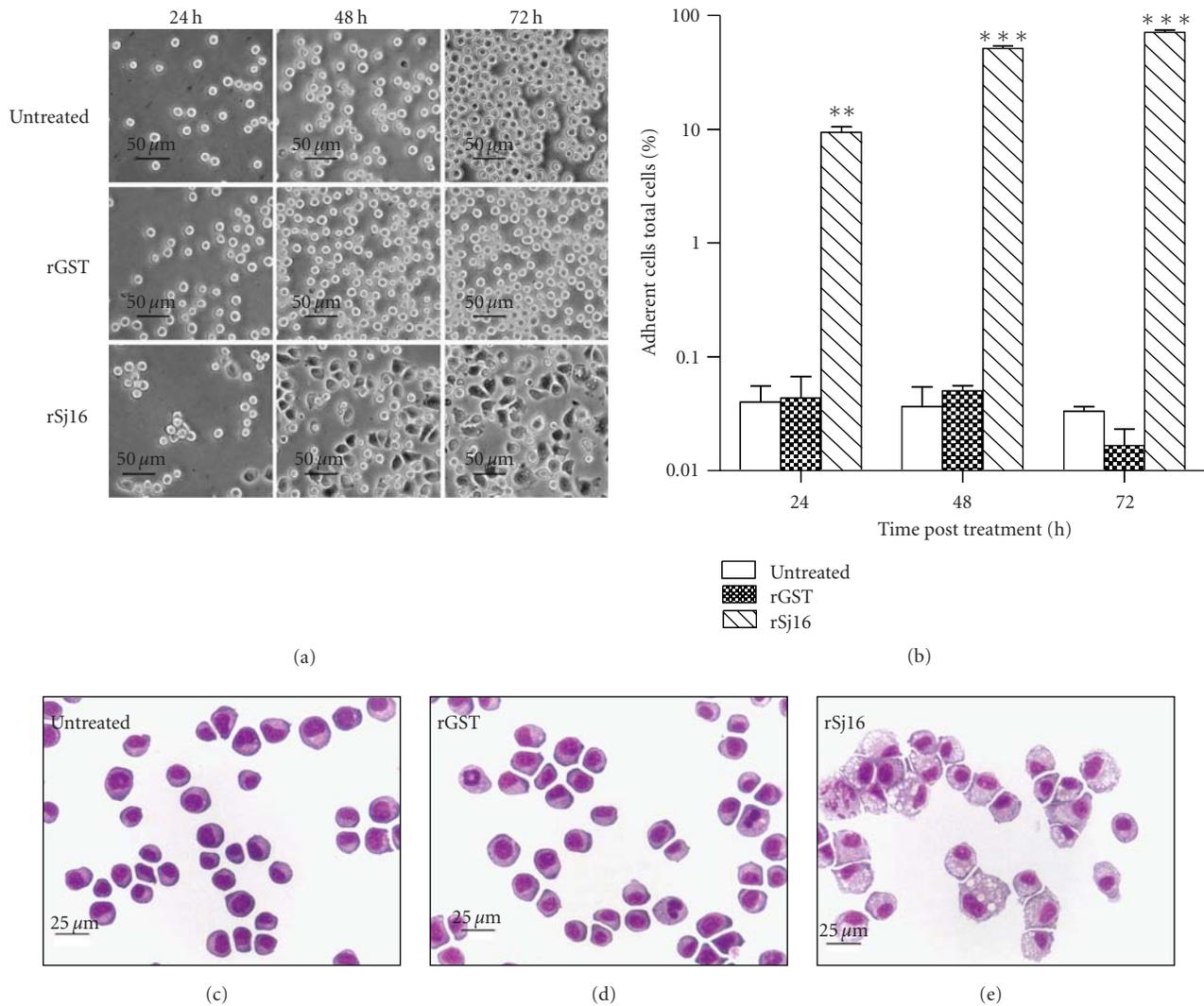
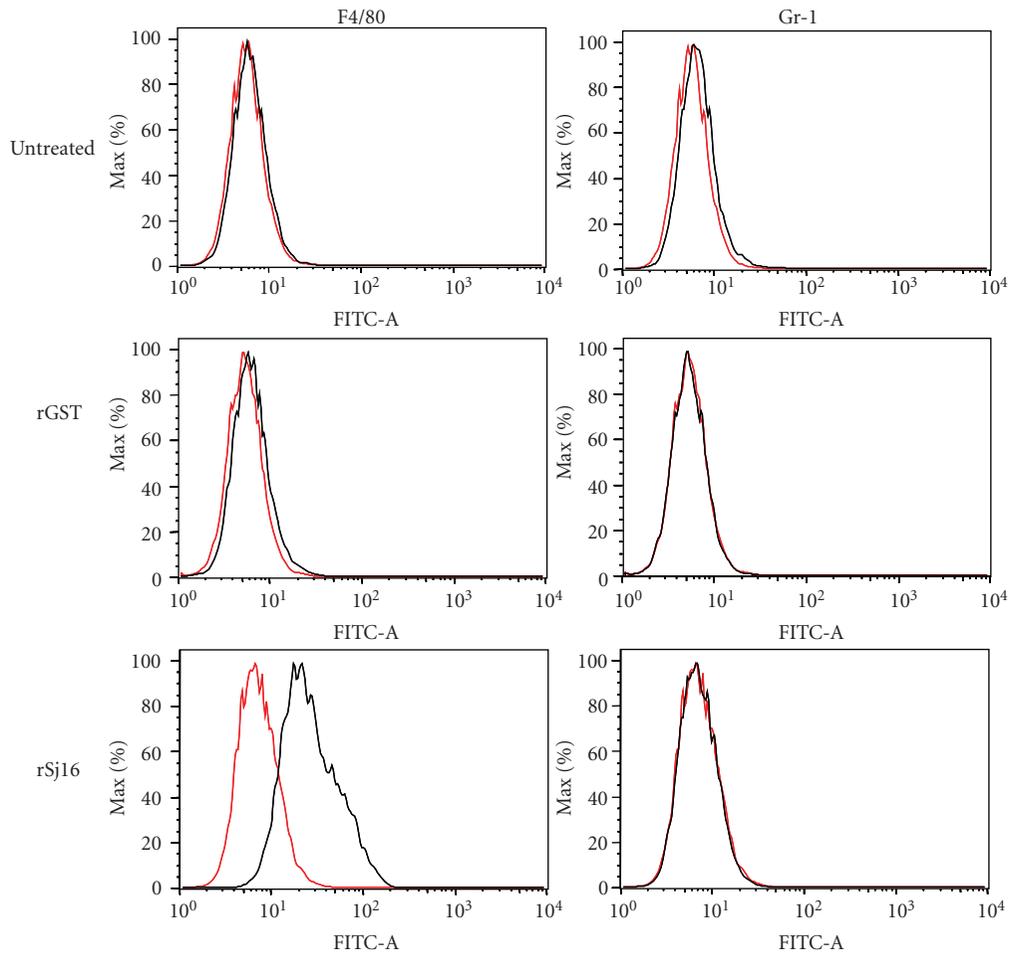


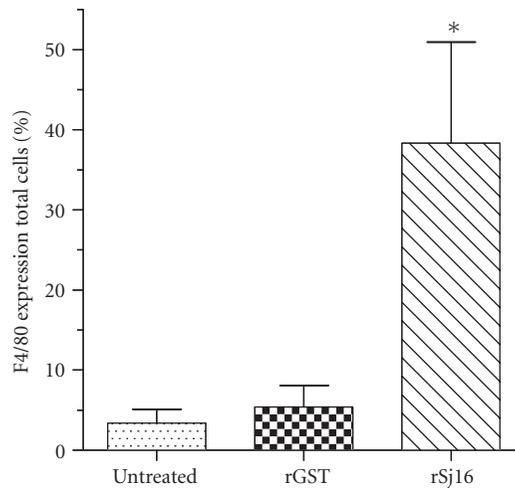
FIGURE 3: Effect of rSj16 on adherence and morphology of WEHI-3B JCS cells. WEHI-3B JCS cells were treated with $0.5 \mu\text{g/ml}$ rSj16 (rSj16), $0.5 \mu\text{g/ml}$ rGST (rGST), or medium alone (untreated) for various time periods, and morphological changes were studied using a light microscope (Nikon). (a) Time course morphological changes of WEHI-3B JCS cells (magnification, $\times 200$). Black bars represent $50 \mu\text{m}$. (b) Induction of adherence of WEHI-3B JCS cells after exposure to rSj16. The data were expressed as mean \pm SEM, $n = 3$. ** $P < .01$; *** $P < .001$ as compared with that of untreated group. (c)–(e) Modified Wright Giemsa staining of WEHI-3B JCS cells 72 hours post treatment (magnification, $\times 400$). Black bars represent $25 \mu\text{m}$. Data are representative of three individual experiments.

[18]. In addition, TNF- α has been identified as the active substance in PMA-induced macrophage differentiation of WEHI-3B JCS cells [2]. Thus, we investigated the effects of rSj16 on the expression of IL-1 α , IL-1 β , and TNF- α of WEHI-3B JCS cells. Untreated WEHI-3B JCS cells expressed little IL-1 α and IL-1 β (both less than 1 pg/ml in the culture media, Figures 6(b) and 6(c)) and expressed low level of TNF- α (Figure 6(a)). Treatment of WEHI-3B JCS cells with rSj16, but not rGST, caused marked time-dependent up-regulation of the expression of these three cytokines (Figures 6(a)–6(c)). The levels of IL-1 α , IL-1 β , and TNF- α in the culture supernatants increased to 60.77 pg/ml , 8.57 pg/ml , and 97.15 pg/ml , respectively, after 3 days exposure to rSj16 (Figures 6(a)–6(c)).

3.7. Differentiation-Inducing Effect of rSj16 on WEHI-3B JCS Cells is not Attributable to Endogenous Production of IL-1 α , IL-1 β , and TNF- α . To investigate the role of endogenous IL-1 α , IL-1 β , and TNF- α in the rSj16-induced WEHI-3B JCS cells differentiation, specific antibodies were adopted to neutralize each cytokine, respectively. The neutralizing ability of the antibodies was verified using D10.G4.1 cell proliferation assay for anti-IL-1 α and anti-IL-1 β , and using L929 cell cytolytic assay for anti-TNF- α (data not shown). Before evaluating the blocking activity of the antibodies on rSj16-mediated differentiation, an initial experiment was performed to test the effect of each antibody on the proliferation (by MTT assay) and morphological changes of WEHI-3B JCS cells, and the results indicated that the effect



(a)



(b)

FIGURE 4: Induction of macrophage marker expression on WEHI-3B JCS cells by rSj16. WEHI-3B JCS cells were treated for 72 hours in the presence of 0.5 $\mu\text{g/ml}$ rSj16 (rSj16), 0.5 $\mu\text{g/ml}$ rGST (rGST), or medium alone (untreated) and then analyzed for expression of F4/80 and Gr-1 using flow cytometry. (a) Red line indicates isotype-matched control antibody; black line, F4/80 or Gr-1. (b) Column indicates mean \pm SEM of four independent experiments. * $P < .05$ as compared with that of untreated group.

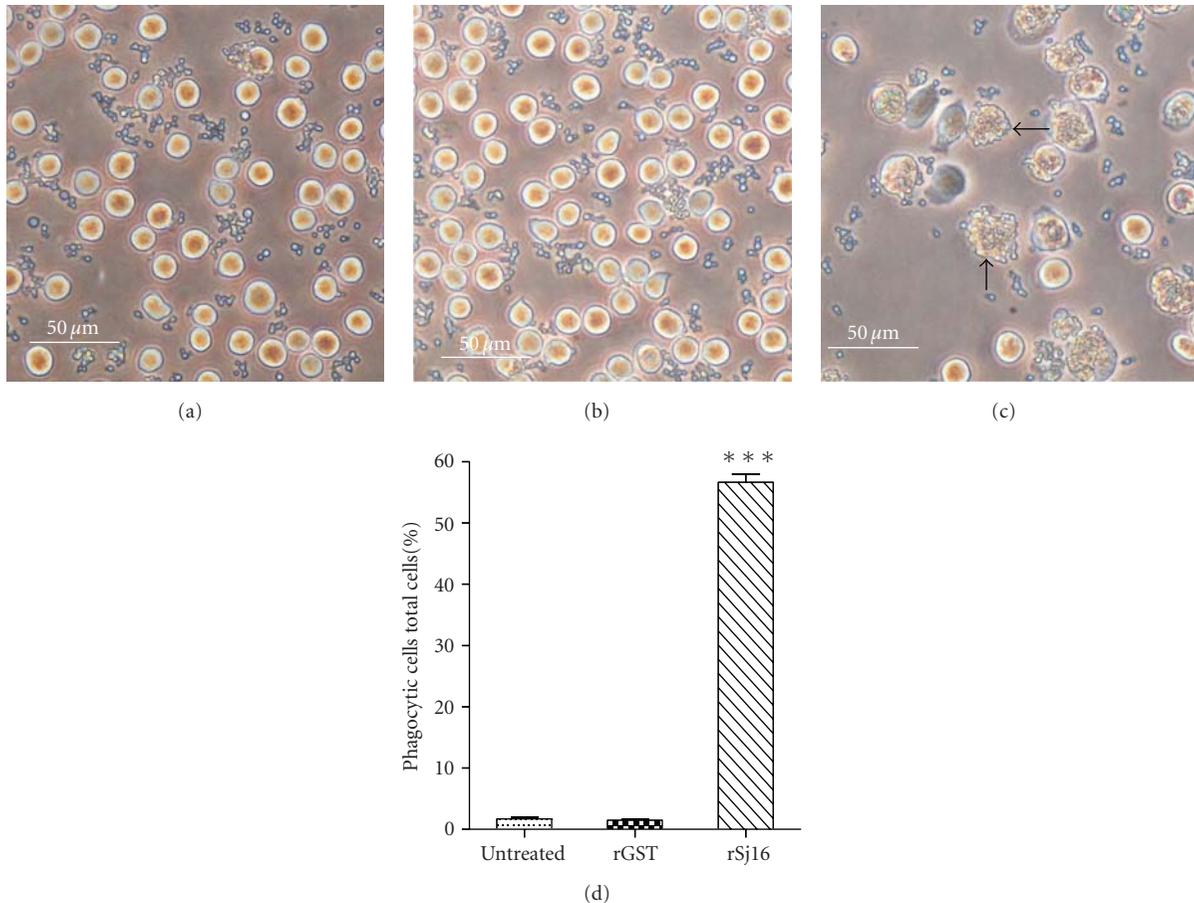


FIGURE 5: *rSj16* induced WEHI-3B JCS cells showed phagocytic activity. WEHI-3B JCS cells were treated for 72 hours with 0.5 μg/ml *rSj16* (*rSj16*, (c)), 0.5 μg/ml rGST (*rGST*, (b)), or medium alone (*untreated*, (a)) and then analyzed for the capacity of yeast-ingesting. (a)–(c) Arrows indicate the yeast-ingesting cells. White bars represent 50 μm (magnification, ×400). (d) Percentage of phagocytic cells after 72 hours treatment. The results are the mean ± SEM of three independent experiments. *** $P < .001$ as compared with that of untreated group.

of each of them neither inhibited the proliferation nor affected morphological changes of WEHI-3B JCS cells when being used as high as 10 μg/ml (data not shown). The blocking effect of the antibodies on *rSj16*-induced WEHI-3B JCS differentiation was evaluated by both MTT assay and morphological studies as described in Section 2. The results showed that none of them, no matter be used separately or combined, could block *rSj16*-mediated morphological changes and antiproliferation (data not shown) of WEHI-3B JCS cells at the concentration being used as high as 10 μg/ml. Therefore, it is suggested that *rSj16*-induced WEHI-3B JCS differentiation does not depend on the endogenous production of IL-1α, IL-1β, and TNF-α.

3.8. Effect of *rSj16* on IL-3, M-CSF, or G-CSF Induced Proliferation and Differentiation of Mouse Bone Marrow Cells. As mentioned above, *rSj16* could induce macrophage differentiation of WEHI-3B JCS cells (Figures 3–5). Since the WEHI-3B JCS cells are derived from bone marrow progenitors [3], it raised a question whether *rSj16* can also affect hematopoiesis of bone marrow cells. It is well

characterized that hematopoietic growth factors such as G-CSF, M-CSF, and IL-3 can regulate the proliferation and differentiation of bone marrow cells and stimulate related colony formation [19, 20]. To test the effects of *rSj16* on hematopoiesis, mouse bone marrow cells were cultured in semisolid agar cultures containing optimal G-CSF, M-CSF, or IL-3 in the presence or absence of 0.5 μg/ml of *rSj16*.

After treated with G-CSF or M-CSF, significant increases of CFU-G colonies (Figure 7(a)) or CFU-M colonies (Figure 7(b)) were found in the corresponding plates. Addition of *rSj16* into the culture before the treatment of G-CSF greatly reduced the colony counts of G-CSF-induced CFU-G (Figure 7(a)). It is interesting that the presence of *rSj16* in the culture did not affect the number of M-CSF-induced CFU-M colonies (Figure 7(b)) but only increased the compactness of the cells within the colonies (Figure 8(b)).

As expected, colonies CFU-M, CFU-G, and CFU-GM were significantly induced following seven days culture with IL-3 (Figure 7(c)). However, when *rSj16* was preadded into the culture one hour before the IL-3 treatment, the total colonies and the colony counts of CFU-G and CFU-GM were

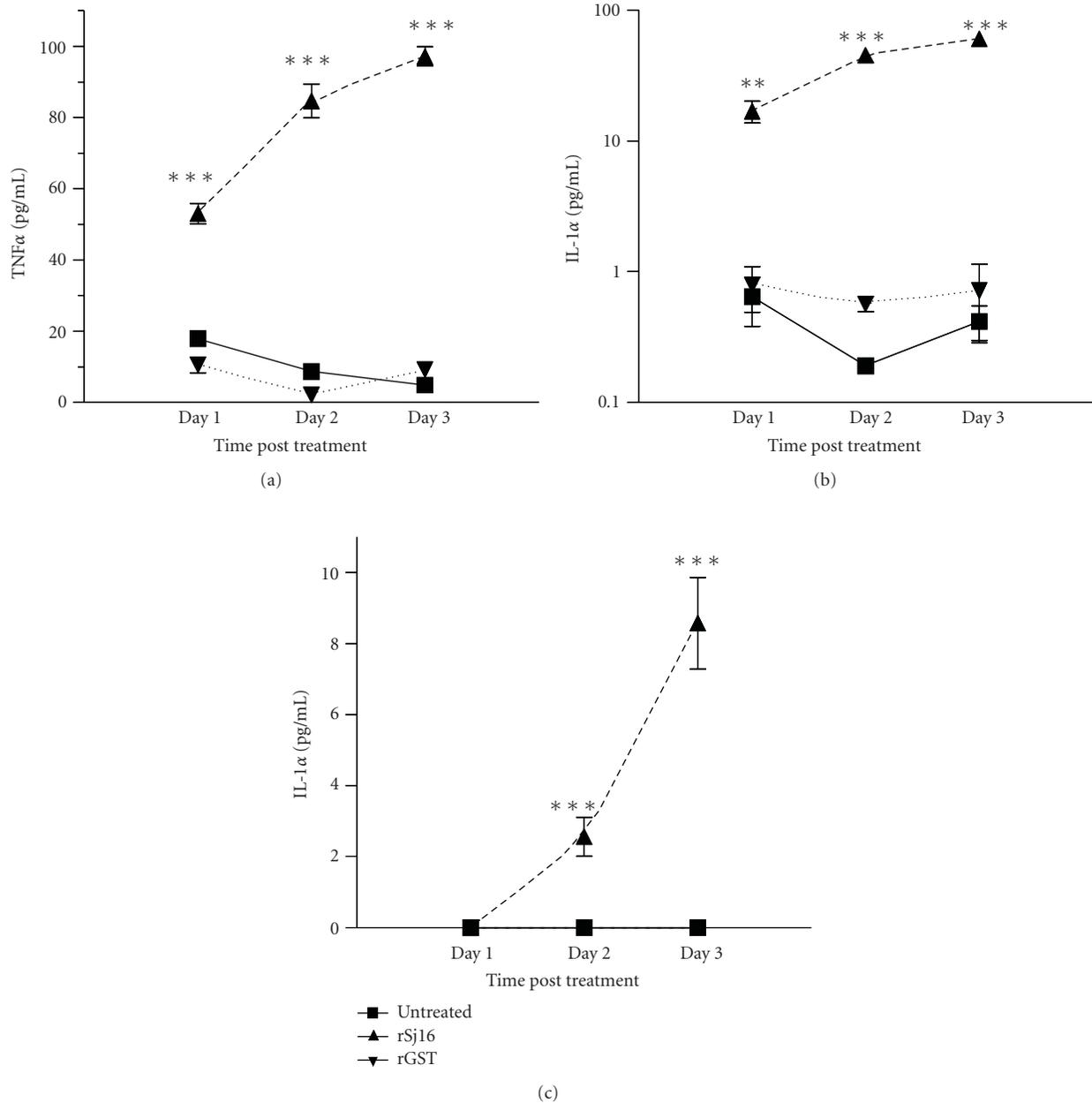


FIGURE 6: Effect of rSj16 on IL-1 α , IL-1 β , and TNF- α production from WEHI-3B JCS cells. WEHI-3B JCS cells were treated with 0.5 μ g/ml rSj16 (rSj16), 0.5 μ g/ml rGST (rGST), or medium alone (untreated), and then the culture supernatants were collected at different time as indicated for the detection of TNF- α (a), IL-1 α (b), and IL-1 β (c) by Sandwich ELISA. Values represent mean \pm SEM of three independent experiments. ***P < .001; **P < .01, as compared with that of untreated group.

significantly decreased, while the colony counts of CFU-M were increased (Figure 7(c)). Besides, just as similar as shown in Figure 8, the cells within CFU-M colonies in the presence of rSj16 also became more compact than those treated with IL-3 alone (data not shown).

There were no colonies induced when the mouse bone marrow cells were treated with PBS alone 0.5 μ g/ml of rSj16 alone, or 0.5 μ g/ml of rGST alone (data not shown).

4. Discussion

Sj16 is a secretory protein produced by *S. japonicum*. It has 100% identity with the protein sequence of the anti-inflammatory protein Sm16 which is abundantly present in the excretory secretions of schistosomulae of *S. mansoni* [21, 22]. Previous studies reported that Sm16 could suppress the host immune responses by inhibiting the antigen-induced

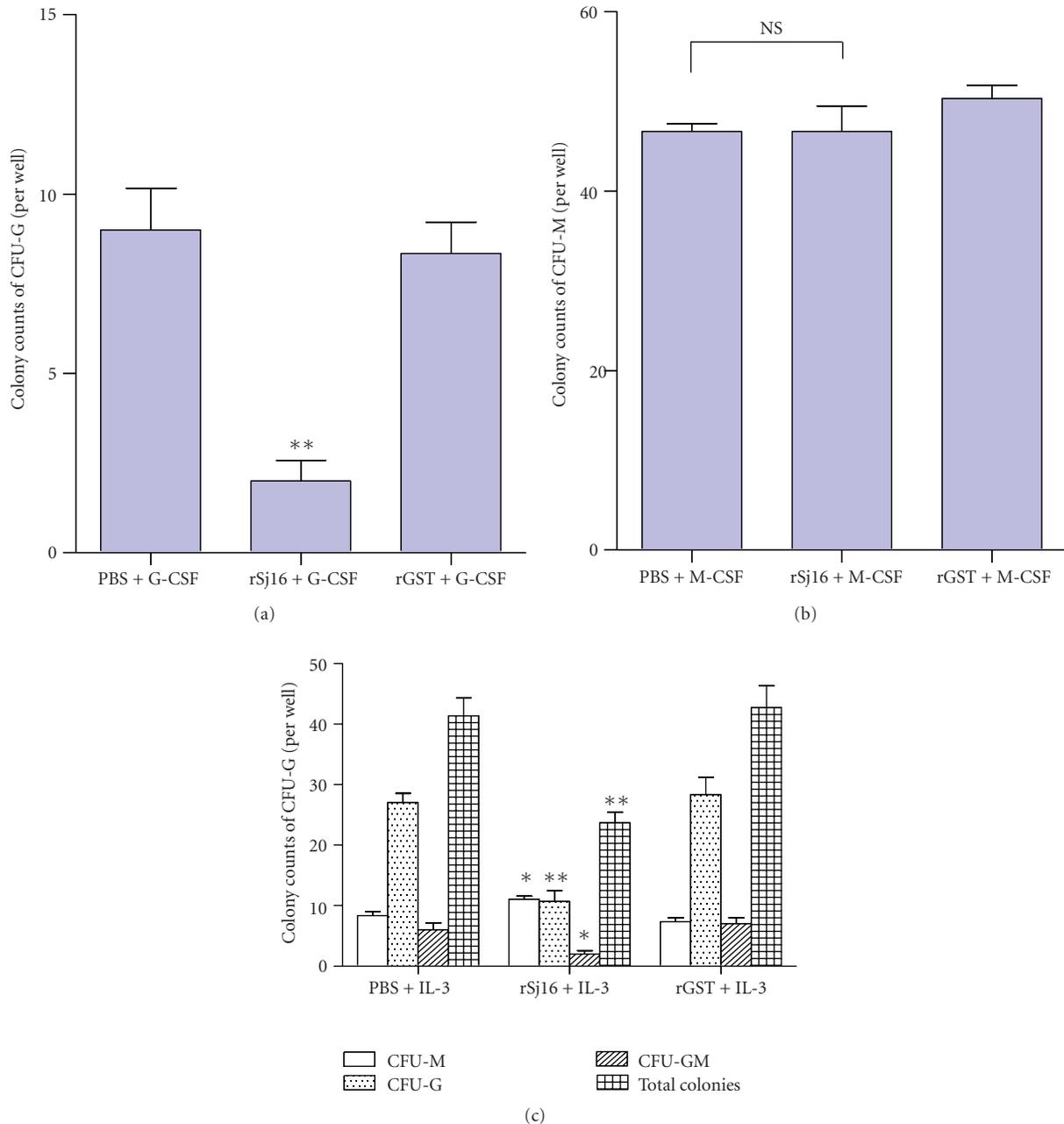


FIGURE 7: Effect of rSj16 on IL-3, M-CSF, or G-CSF induced colony formation of mouse bone marrow cells. Mouse bone marrow cells were plated in 0.33% agar cultures containing G-CSF (a), M-CSF (b), or IL-3 (c) in the presence of rSj16, rGST, or PBS in 24-well plates. The agar gels were dried and stained with hematoxylin solution following seven days of incubation. Differential colony counts were performed based on the morphology of the cells, and only colonies of greater than 40 cells were counted. Colony counts are indicated as number of colonies per well. Data are mean \pm SEM of triplicate wells and are representatives of three individual experiments. * $P < .05$; ** $P < .01$; NS, not significant versus the corresponding data in the PBS + G-CSF/M-CSF/IL-3 group.

lymphoproliferation and suppressing the IL-2 production from lymphocytes [22]. Sm16 could also suppress LPS-induced neutrophil infiltration and downregulate production of the proinflammatory cytokines [23]. In addition, a single intradermal injection of a full-length cDNA of Sm16 resulted in a significant suppression of cutaneous inflammation [24]. By using recombinant protein obtained from

E. coli, we also demonstrated that rSj16 dramatically suppressed the thioglycollate-mediated recruitment of leukocytes to the peritoneal cavity of BALB/c mice and suppressed thioglycollate-induced peritoneal macrophages maturation, accompanied by marked upregulation of IL-10 and IL-1 receptor antagonist transcripts, and down-regulation of IL-12p35, IL-1 β , and MIP-2 transcripts in peritoneal cells [11].

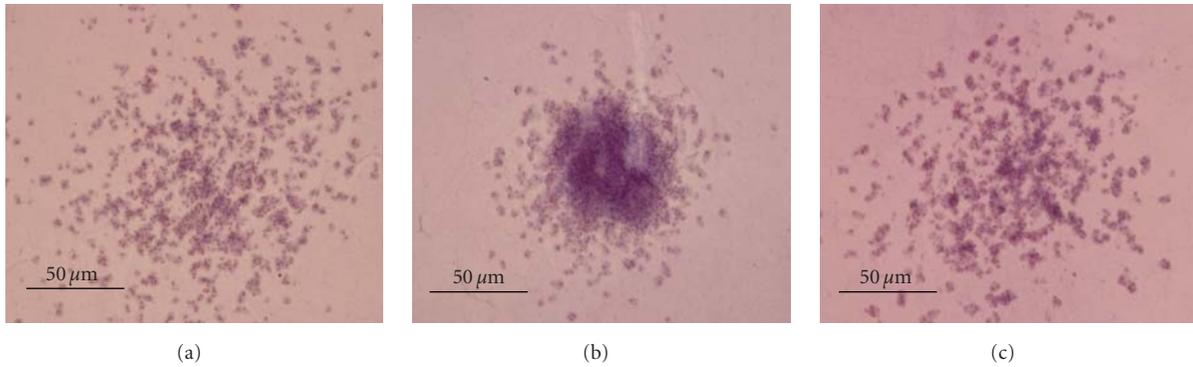


FIGURE 8: Effect of rSj16 on morphology of M-CSF-induced CFU-M colonies after hematoxylin staining. Mouse bone marrow cells were treated with M-CSF in the presence of rSj16 (b), rGST (c), or PBS (a) as described in Figure 7. The black bars represent 50 μm (magnification, $\times 100$). The photos are representatives of three individual experiments.

Further analysis revealed that rSj16 also inhibited both humoral and cellular immunity to heterologous antigens using BALB/c mouse model (Hu et al., unpublished data). Thus, it is evident that rSj16 retain an immunomodulatory function.

In this study, to extend our knowledge of the immunomodulatory function of rSj16, we first investigated the effects of rSj16 on the proliferation and differentiation of the leukemia cell line WEHI-3B JCS. Our data indicated that rSj16 could inhibit the growth of WEHI-3B JCS cells in a time- and concentration-dependent manner (Figure 1). Interestingly, it is obvious that the rSj16-mediated inhibition of WEHI-3B JCS cell proliferation is not attributable to its cytotoxic effect on the cell, since the viability of the WEHI-3B JCS cells was not affected by the rSj16 treatment. Further studies indicated that rSj16 also induced macrophage morphological changes of the WEHI-3B JCS cells (Figure 3), accompanied with markedly decrease of the cells in the S phases and concomitant accumulation in the proportion of those in G1/G0 and G2/M phase (Figure 2). These results may suggest that rSj16 induces macrophage differentiation of the WEHI-3B JCS cells.

Membrane antigens serve as excellent markers of murine macrophage differentiation *in vivo* and *in vitro* [25]. To confirm macrophage differentiation of the WEHI-3B JCS cells, we analyzed the membrane antigens by flow cytometry. Among the two antigens being analyzed, the F4/80 is expressed by a majority of mature macrophages and is the best marker for this population of cells [26], while Gr-1 is expressed by bone marrow granulocytes as well as on peripheral neutrophils [27]. As expected, our data showed that rSj16 could stimulate the expression of F4/80 on the WEHI-3B JCS cells surface but not Gr-1 (Figure 4), thus supporting the macrophage differentiation of the cells. Subsequently, this differentiated WEHI-3B JCS cells have been verified to be functional, as they acquired the yeast-phagocytizing activity after rSj16 treatment (Figure 5).

It is well known that LPS could induce macrophage differentiation of certain leukemia cells [28, 29]. In order to exclude the possible contamination of endotoxins during preparation of rSj16, the purified proteins were passed

through a Detoxi-Gel AffinityPak polymyxin B column (Pierce) to eliminate endotoxins. The absence of endotoxins in the protein samples was further confirmed by *Limulus* amoebocyte lysate test (sensitivity 0.25 EU/ml, Associates of Cape Cod). Besides, the rGST prepared as the same way as rSj16 was served as negative control in the whole study. Thus, in this study, the rSj16-induced macrophage differentiation should not due to the endotoxins contamination.

Cytokines, such as IL-1, leukemia inhibitory factor, TNF- α , and IL-4, have been shown to regulate the growth and differentiation of hematopoietic cell [30, 31]. Previous studies reported that PMA-induced macrophage differentiation of WEHI-3B JCS cells was mediated by endogenous production of TNF- α , as the differentiation-inducing effect of PMA could be prevented by neutralizing anti-TNF- α antibodies [2]. Further studies showed that macrophage differentiation of WEHI-3B JCS cells could be also induced by TNF- α , IL-1 α , or IL-1 β . We were thereby interested in whether rSj16-induced WEHI-3B JCS cells differentiation was results of endogenous production of TNF- α , IL-1 α or IL-1 β . Unexpectedly, although the production of these three cytokines has been upregulated after rSj16 treatment (Figure 6), they were obviously not involved in the rSj16-induced WEHI-3B JCS differentiation, as none of the anti-IL-1 α , anti-IL-1 β , anti-TNF- α , or these three antibodies together could prevented the activity of rSj16 (data not shown). Thus, these results indicated that rSj16 induce WEHI-3B JCS cells differentiation through some other mechanisms. However, currently we don't have information about the mechanisms. This will be addressed in our following studies.

The WEHI-3B JCS cell line is a subclone isolated from the murine myelomonocytic leukemia WEHI-3B cell line and therefore possesses some certain features of myelomonocytic progenitor cells [2]. This cell line has been widely used to be a useful model in the study of hematopoietic cell differentiation [2–4, 32, 33]. In the present study, we have demonstrated that rSj16 could induce macrophage differentiation of the WEHI-3B JCS cell (Figures 3–5). It is thus reasonable to investigate the regulatory effect of rSj16 on the mouse hematopoiesis. Our results revealed that rSj16

could suppress G-CSF-induced CFU-G colony formation (Figure 7(a)) and suppress IL-3-induced CFU-G and CFU-GM colony formation (Figure 7(c)). On the other hand, although rSj16 alone did not induce any colony formation of the mouse bone marrow cells, we demonstrated that rSj16 could increase the number of IL-3-induced CFU-M colony formation (Figure 7(c)). This result corresponded with our previous observation that rSj16 induces macrophage differentiation of WEHI-3B JCS cell (Figures 3–5). However, unexpectedly, the addition of rSj16 into the culture did not affect the number of M-CSF-induced CFU-M colonies (Figure 7(b)). A possible interpretation is that M-CSF is a potent inducer of macrophage differentiation which may mask the inducing effect of rSj16.

Furthermore, it is very interesting that the macrophages within the CFU-M colonies induced by IL-3 along with rSj16 (data not shown) and M-CSF along with rSj16 (Figure 8(b)) became more compact than those induced with IL-3 (data not shown) or M-CSF (Figure 8(a)) alone, which suggested that the differentiated macrophages after rSj16 treatment may lose the ability of migration. This result may indicate that although rSj16 induced macrophage differentiation of WEHI-3B JCS cell and increased the number of IL-3-induced CFU-M colony formation of mouse bone marrow cells, the differentiated macrophage cells may not display full functions as normal mature macrophages. As the inhibition of antigen-presenting cell migration has been proved to be a strategy for the schistosomes to escape host immune system [7], our results may further suggest that Sj16 helps the parasite to survive from host immune attack. However, further studies are needed to support this postulation and explore the potential mechanisms.

Conflict of Interest

The authors have no financial or commercial conflict of interest.

Acknowledgments

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Review Article

Immunity against Helminths: Interactions with the Host and the Intercurrent Infections

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Helminth parasites are of considerable medical and economic importance. Studies of the immune response against helminths are of great interest in understanding interactions between the host immune system and parasites. Effector immune mechanisms against tissue-dwelling helminths and helminths localized in the lumen of organs, and their regulation, are reviewed. Helminth infections are characterized by an association of Th2-like and Treg responses. Worms are able to persist in the host and are mainly responsible for chronic infection despite a strong immune response developed by the parasitized host. Two types of protection against the parasite, namely, premune and partial immunities, have been described. Immune responses against helminths can also participate in pathogenesis. Th2/Treg-like immunomodulation allows the survival of both host and parasite by controlling immunopathologic disorders and parasite persistence. Consequences of the modified Th2-like responses on co-infection, vaccination, and inflammatory diseases are discussed.

1. Introduction

Many species of helminths are parasitic multicellular organisms of medical and economic importance as they infect humans and animals and sometimes provoke fatal diseases such as schistosomiasis. They can also be responsible for economic losses due to decreased milk or meat production and the cost of anthelmintic treatments of parasitized individuals. Helminths are extremely diverse. They can be classified into 3 taxonomic groups, that is, nematodes, trematodes, and cestodes, often with very different parasitic cycles. For example, (i) they may be transmitted orally (*Strongyles*, *Fasciola sp.*, etc.) by transcutaneous route (*Ankylostoma sp.*, *Strongyloides sp.*, *Schistosoma sp.*, etc.) by an arthropod vector (*Onchocerca volvulus*, *Dirofilaria immitis*, etc.); (ii) their definitive and intermediate hosts range from mammals, birds, reptiles to fish, molluscs and arthropods, and so forth; (iii) they may be localized in organ lumen or in tissues such as gut, liver (parenchyma and bile ducts), lung, lymphatic vessels, and so forth; (iv) the successive developmental stages of parasitic species may infect different tissues and cells of different organs.

Despite this great complexity, helminths usually cause asymptomatic or subclinical chronic infection, although some parasitized individuals can suffer from severe disease which may be fatal. Indeed, worms tend to be aggregated in their distribution, with a large number of hosts harboring few parasites and few heavily infected hosts [1, 2]. This remarkable equilibrium between most hosts and parasites is the product of long-term coevolution of the two partners and particularly of the immune defence of the host and the immune evasion of the parasite. The immune responses of the hosts to helminth infection are generally characterized by a skewed Th2-like response. Helminths have developed several means of escaping these immune responses. Recently, Maizels et al. [3] called them “masters of immunomodulation”. These immunomodulatory abilities enable the worm to persist in the host and can lead to interactions with inflammatory and immune mechanisms involved in other infections or to vaccines or in allergic and autoimmune diseases. The focus in this review is on pathogenic helminths of veterinary importance, especially in *Ruminants medicine*, and includes *Fasciola spp.* and gastrointestinal nematodes.

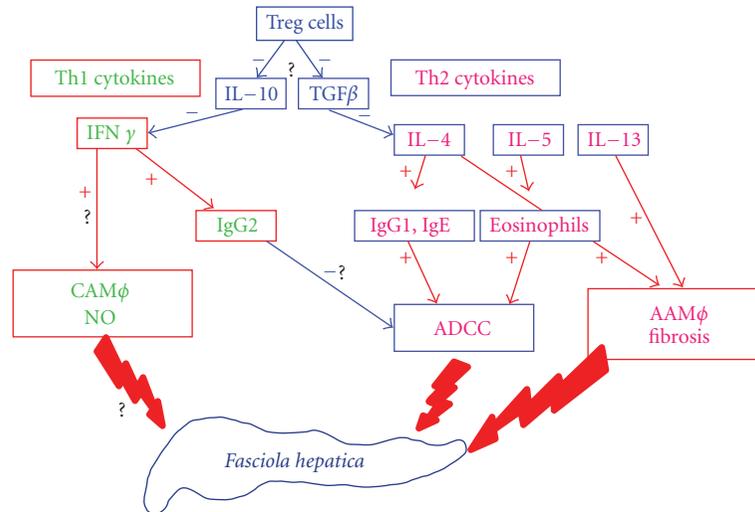


FIGURE 1: Immune mechanisms and regulation induced against *Fasciola hepatica*. Two main immune mechanisms are directed against *F. hepatica* in the liver parenchyma: (i) during the early phase of infection, classically activated macrophages may induce nitric oxide production which is toxic for the fluke. This mechanism needs to be confirmed and may be upregulated by Th1-type cytokines and downregulated by IL-10 produced by T regulatory cells. (ii) During the chronic phase of infection, antibody-dependent cellular cytotoxicity (ADCC) allows the release of toxic mediators such as major basic protein, eosinophil cationic protein, and reactive nitrogen intermediates. This mechanism is upregulated by Th2-type cytokines. T regulatory cells (Treg cells) produce IL-10 TGF β which inhibit the production and function of Th1 cytokines. They downregulate any excessive Th2 response in the immunopathogenesis of fasciolosis. Finally, alternative activated macrophage (AAM ϕ) produces molecules that are toxic to the fluke and participates in fibrosis and tissue repair.

2. Effector Immune Mechanisms against Helminths and Their Regulation

Helminth infections are typically associated with hypereosinophilia, considerable IgE production, mucous mastocytosis, and goblet cells hyperplasia [4]. These immune parameters are involved in different effector mechanisms highly depending on where the helminth is localized.

2.1. Effector Mechanisms against Tissue-Dwelling Parasites and Escape Mechanisms Developed by the Parasite. Several mechanisms against tissue-dwelling parasites have been described. These parasites are mainly larval stages, for example, of trematodes (*Schistosoma spp.*, *Fasciola spp.*) or nematodes, which migrate through tissue.

Antibody Dependent Cellular Cytotoxicity (ADCC) is dependent on eosinophils, neutrophils, macrophages, or platelets as effector cells and IgE, IgG, or IgA as antibodies. The parasitic structures covered by antibodies are destroyed by cells carrying receptors to the Fc fragment (RFc) (Figure 1). When these cells are activated by fixation of the antibodies to the RFc, they release products that are toxic to the worm (major basic protein, eosinophil cationic protein, eosinophil-derived neurotoxin, reactive nitrogen intermediates, ...). ADCCs are also able to immobilize nematode larval stages as they migrate through the gut mucosa [5–8].

A granuloma can occur around the parasite in the tissue which stops the worm migration and development. This phenomenon has been well investigated for *Schistosoma mansoni*. The granuloma is composed of eosinophils,

macrophages, and lymphocytes with an increasingly fibrotic extracellular matrix [4], which surrounds and segregates the eggs from the hepatic tissue [9]. In the long term, fibrosis may develop as the eggs die and the granuloma is resolved [9].

Finally, nitric oxide (NO), toxic to the worm, is released by the macrophages classically activated by IFN γ and TNF α (Figure 1). This mechanism has been described mainly against trematodes (*Schistosoma sp.*, *Fasciola sp.*) during acute infection, before egg production in *Schistosoma mansoni* [10–12].

Tissue-dwelling parasites have developed several mechanisms to escape to the effector response of the host (Figure 2). For example, *Fasciola sp.* escapes from the immune responses by different means as follows:

- (i) *Fasciola gigantica* produces superoxide dismutase which neutralizes superoxide radicals toxic for juveniles [13, 14].
- (ii) *F. hepatica* releases cathepsin L-protease which cleaves IgE and IgG involved in the ADCC [15].
- (iii) Juvenile flukes were found to be covered by IgM [16]. While eosinophils do not express Fc μ receptor, IgM deposition on fluke tegument could inhibit eosinophil adhesion. IgG2 produced during fasciolosis in susceptible sheep [17] has been also suspected to be a blocking immunoglobulin of the ADCC.

Furthermore, *F. hepatica* secretes several molecules able to modulate the immune response. Excretory-secretory products of *F. hepatica* (ESPFh) can depress the sheep and rat lymphocytes stimulation [18, 19] and induce eosinophil

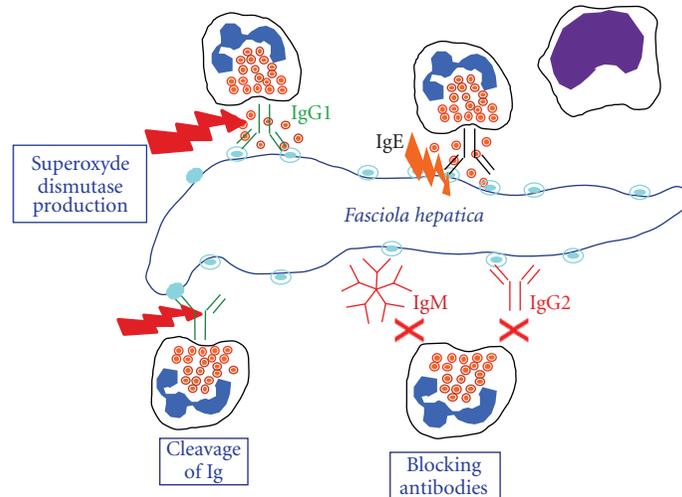


FIGURE 2: Main evasion mechanisms developed by *Fasciola spp.* against ADCC. Three main evasion mechanisms have been described: (i) production of superoxide dismutase and Glutathione S-transferase which neutralize superoxide radicals, (ii) cleavage of IgE and IgG involved in the ADCC, and (iii) production of blocking antibodies IgM and IgG2 which could inhibit eosinophil adhesion to flukes.

apoptosis [20]. Milbourne and Howell (1990, 1993) [21, 22] have shown that there is an “IL5-like” substance in the excretory-secretory products (ESPs) probably responsible in part of the local and systemic eosinophilia observed during fasciolosis. Cathepsin L-proteases induce a decrease of lymphoproliferation in sheep and of the CD4 expression on human and ovine T cells [23]. GST from *F. hepatica* induces a significant inhibition of nitrite production by rat peritoneal macrophages [18].

2.2. Effector Mechanisms against Parasites Localized in the Lumen of Ducts and Escape Mechanisms Developed by the Parasite. Intestinal anaphylaxis, with IgE-induced mast cells degranulation, is responsible for changes in the intestine physiology as well as architecture and chemistry of the gut epithelium, including stimulation of fluid, electrolyte and mucus secretion, smooth muscle contractility, increased vascular and epithelial permeability, and recruitment of immune cells such as eosinophil or mast cells [24] (Figure 3). This can lead to rapid elimination of the gastrointestinal larvae, before they reach their tissue niche, and to expulsion of the adult [25]. Furthermore, IgA on the surface of the gut mucosa helps to neutralize the metabolic enzymes released by digestive strongyles and interfere with the worm’s ability to feed [26, 27].

As for tissue-dwelling parasites, parasites localized in the lumen of ducts are able to produce immunomodulatory substances to escape to the host immune responses. For example, *Necator americanus* secretes a metalloprotease which cleaves eotaxin, a chemotactic factor for eosinophils [28]. Gastrointestinal nematodes produce also superoxide dismutase and glutathione S-transferase which neutralize toxic oxide radicals [29]. A cystatin produced by *H. contortus* and *N. brasiliensis* modulates the antigen presentation to T cells by inhibiting cysteine proteases of antigen presenting cells, involved in the processing of the antigen [30, 31].

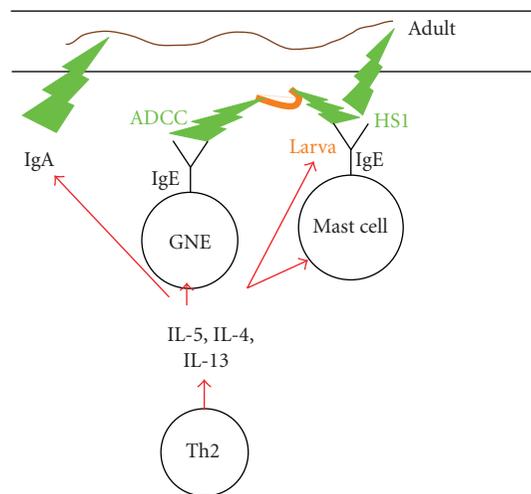


FIGURE 3: Immune response against gastrointestinal nematodes (GNE: eosinophil granulocyte, HS1: type 1 hypersensitivity). Against larva, ADCC and type 1 hypersensitivity are involved to block their migration in gut mucosa and to eliminate the parasite. Against adult in the lumen of gut, intestinal anaphylaxis responsible for muscle contractility, mucus secretion, and so forth, leads to the expulsion of the parasite. IgA neutralizes the metabolic enzymes and interferes with the worm’s ability to feed.

2.3. Regulation of Immune Responses against Helminths. All these mechanisms, except the classically activated macrophages, are regulated by Th2-like cytokines and immunomodulatory cell types (Figure 1). Interleukin-4 is involved in the IgE isotype-switched B-cell responses, IL-5 is involved in the production of eosinophils, and IL-13 has similar functions to IL-4 and is involved mainly in the effector phase of inflammation and the development of fibrosis [9]. T regulatory cells produce the suppressive cytokines IL-10 and TGF-β which have antiinflammatory effects and

could be involved in the skewed Th2-like responses. Immune deviation may also be promoted by the development of a Th2-driving dendritic cell population induced by excretory-secretory antigens from *N. brasiliensis* [32] or soluble egg antigen from schistosome [33]. Finally, IL-4 and IL-13 are able to alternatively activate macrophages (AAMPs) which have strong antiinflammatory properties, enhance Th2 cell differentiation, contribute to fibrosis, and repair at the site of injury [34]. Thus, an environment, with downregulated proinflammatory responsiveness, activated damage repair mechanisms, and a controlled development of Th2-like anti-parasite effector responses is created during infection with helminths [35].

Several proteins produced by helminths were involved in the regulation of cytokine production [36, 37]. Schistosome soluble egg antigen contains molecules as alpha-1 or omega-1 that initiate a Th2-like response [38–40]. ES-62, a leucine aminopeptidase secreted by *Acanthocheilonema vitae*, reduces CD4+ cell IL-4 and IFN γ production but promotes IL-10 production by peritoneal B1 cells [41, 42]. It also inhibits the antigen-presenting cells ability to produce IL12p70 and drives Th2-like differentiation in vitro [43, 44]. Helminths could also secrete cytokine homologues as macrophage migration inhibitory factor (MIF) which induces, with IL-4, the development of alternatively activated macrophage [45].

3. Protective and Immunopathological Effects of the Immune Response against Helminths

Despite the Th2-like response induced against helminths, these parasites are often able to persist in the host for a long time, resulting in chronic infection. However 2 types of immunity evaluated from the partial elimination of settled parasites and from host resistance to reinfection have been described, namely, premune immunity and partial immunity.

Premune immunity against helminths is very common and particularly observed against gastrointestinal strongyles. Premunition or concomitant immunity has been defined by MacDonald et al. [9] as a state wherein the host is protected from further infection with a given species by ongoing persistent infection with the same organism. Thus immune mechanisms existing concomitantly with parasites (adults and encysted larval stages) in animals infected by gastrointestinal nematodes prevent the establishment of new larvae. In contrast, the elimination of adult worms by the phenomenon of “self-cure” (spontaneous expulsion of adults by massive larval invasion during a short period of exposure) or by anthelmintic treatment results in the installation of new larvae until an equilibrium state is obtained. Premune immunity can also be expressed as a reduction in adult worm size and in female worm fertility. In contrast, the primary immune response against *Fasciola hepatica* in bovine limits the number of metacercariae which develops in adults and reduces the fertility of the females [46]. However it is unable to prevent the establishment of new parasites, which is a great difference with the premune immunity [46]. It also permits partial expulsion of adults in the bile ducts of infected cattle.

So, immune responses against *Fasciola hepatica* partially protect the host against the infection.

Effector mechanisms regulated by Th2-like responses play a major role in immune protection to strongyles. Comparison of the immune responses of different breeds of sheep to *Haemonchus contortus* revealed a greater production of Th2-like cytokines (IL-4, IL-5, IL-13) in resistant breeds [47]. Similarly, Balb/c mice, which develop a Th2-like response against *Trichuris muris*, were soon able to eliminate the parasite, in contrast to AKR mice which express Th1-like responses and chronic infection with the same parasite [48]. The protective role of Th1- and Th2-like responses during fluke infection is less clear: Th1-like responses might act on larvae migrating through the liver parenchyma whereas the chronic phase with *F. hepatica* might be due to Th2-like responses against which the fluke has developed several escape mechanisms [46]. As described during infection with *Schistosoma mansoni*, Th2-like responses are predominant during infection by *F. hepatica* but early Th1-like responses seem to be involved in protection against this parasite. Indeed, sheep susceptible to *F. hepatica* develop a mixed Th1- and Th2-like response with IFN γ and IL-10 production during the first 6 weeks of infection, whereas the immune response of sheep resistant to *F. gigantica* is Th1 like, with production of IFN γ only. This suggests that protection against *Fasciola sp.* is linked with Th1-cytokine production [49]. Similarly, vaccinal trials with cathepsin L-protease from *F. hepatica* proved that protection induced by this antigen is mediated by a Th1-like response [50].

Although the host immune reaction against helminths may control the infection, it can also be responsible for tissue lesions and symptoms which are often the primary cause of disease during worm infection. Immunopathologic phenomena have been thoroughly investigated in infections with *Schistosoma spp.* As described above for *F. hepatica* infection, acute schistosomosis is associated with Th1-like responses against adult parasites. The Th2-like responses, induced as a result of egg antigens secretion, downregulate the production and effector functions of Th1-like mediators [51, 52]. When Th2-like responses against the eggs were blocked experimentally, an exacerbated granuloma driven by Th1 and Th17 cells resulted in hepatic damage and death [53]. Granulomatous responses evolve from an early Th1- to a sustained and dominant Th2-like response [54]. Whereas tissue fibrosis stimulated by Th2-like cytokine (IL-13) promotes tissue healing, excessive fibrosis may become pathogenic with loss of hepatic functions and portal hypertension [54, 55]. It seems that during trematode infections Th1-like responses are more protective than Th2-like responses against which these parasites have developed many escape mechanisms. Although Th1-like responses are closely associated with immunopathogenesis, Th2-like responses may also contribute to inflammatory damage. Treg cells seem to regulate this detrimental immune response by suppressing the Th1-like response and by downregulating any excessive Th2-like response during granuloma formation [56].

Granuloma formation dependent on the Th2-like response is also observed during gastrointestinal nematode infection. A recent study has shown that experimental

downregulation of the Th2-like response to *Nippostrongylus brasiliensis* suppresses resistance to gastrointestinal nematode infection, pulmonary granulomatous inflammation, and fibrosis [57]. Similarly bovine ostertagiosis is associated with diarrhoea, inappetence, and weight loss. These clinical signs could result from smooth muscle contractility, increased mucus production, loss of specialized cells in the abomasal epitheliums induced by IgE-mediated immediate hypersensitivity reactions, and degranulation of mast and goblet cells and of proinflammatory mediators [24, 58].

Hence, protection against gastrointestinal nematodes and against tissue-dwelling trematodes is controlled by Th2- and Th1-like responses, respectively [59]. The migration step in tissue is considered an immunoevasive strategy due to the predominant Th2-like response during helminth infection whereas protection in tissue is mediated by the Th1-like response [59]. However, the immune mechanisms, particularly those regulated by Th1-like cytokines, are responsible for considerable immunopathological damage and for the clinical signs observed during a helminthic disease. Even if the immune responses against most of helminths are orchestrated by Th2-like cytokines, the worms are still able to persist in the host for a long time. Indeed, the immune response during the chronic phase of infection was recently reported to be a modified Th2-like response, that is, a Th2-like response associated with Treg activity and the production of antiinflammatory cytokines such as IL-10 and TGF β [60]. The induction of immunomodulatory Th2/Treg responses would allow the survival of both partners, by downregulating the host's inflammatory response and the immunopathological lesions observed during helminth infection, and also the protective immune mechanisms directed against the parasite [61, 62].

4. Helminths and Coinfection

Some helminths are able to downregulate the Th1-like response because their high immunomodulator activity allows the induction of Th2/Treg-type responses. Indeed *F. hepatica* inhibits the Th1-like response induced by *Bordetella pertussis* in mice [63]. Similarly *F. hepatica* is also able to change the predictive value of the tuberculosis diagnosis test by modifying the immune response against *Mycobacterium bovis* [64]. Helminths can affect the evolution of coinfection by making animals more resistant to pathogens in which protection is mediated by the Th2-like response and more susceptible to pathogens in which protection is mediated by the Th1-like response [9]. For example, the expulsion of *T. muris* by mice is dependent on the Th2-like response. Curry et al. [65] demonstrated that mice susceptible to *T. muris* and coinfecting with *S. mansoni* which induced the Th2-like response were able to eliminate *T. muris* by producing Th2-like cytokines. In contrast, mice infected with *S. mansoni* were more susceptible to *Toxoplasma gondii* (protected by the Th1-like response) by inducing high mortality and weak production of IFN γ and NO, compared to mice solely infected with *T. gondii* [66]. Helminths were also able to inhibit the development of protective immunity regulated by Th1-like cytokines against *Plasmodium sp.* [62].

Many analyses of helminth - *Plasmodium* coinfections have provided controversial results as the parasite burden of *Plasmodium sp.* is dependent on the helminth species used, the intensity and duration of worm infection, and the age of the individual under study [67].

Helminths influence not only host resistance to another pathogen but also the gravity of the resulting disease. Cerebral malaria is associated with an overproduction of proinflammatory cytokines. Helminth infections are able to decrease the production of these cytokines by secreting IL-10 and TGF β and thereby diminish the risk of severe disease [62]. The nematode *Heligmosomoides polygyrus* promotes an immune response regulated by Th2-like cytokines, alternative activated macrophages, and regulatory T cells, and hence, prevents the inflammatory reaction controlled by Th1 cytokines and severe immunopathological lesions observed during schistosomiasis [68]. *Trichinella spiralis* infection limits pulmonary damage induced by influenza virus in mice [69]. In contrast, *H. polygyrus* promotes intestinal lesions due to bacteria such as *Citrobacter rodentium* by alternative activation of macrophages [70].

However, other pathogens can also influence the immune response against helminths. For example, Miller et al. [71] recently showed that the production of Th1-like cytokines and classic activation of macrophages were little altered when *F. hepatica* infection preceded or succeeded *T. gondii* infections, whereas the production of *F. hepatica*-specific Th2-like cytokines and recruitment of AAMP were suppressed by *T. gondii* infection. Similarly, neutrophil-activating protein from *Helicobacter pylori* downmodulated the Th2-like response to *Trichinella spiralis* infection [72].

The effects of helminths on infections with other pathogens are complex and dependent on many factors such as the helminth species, coinfecting pathogen, protective and pathological immune mechanisms, and also the host and the individual.

5. Helminths and Vaccination

Several studies have shown that helminths can influence vaccine efficacy by modulating host immune response, in particular when Th1-like and cellular-dependent responses are required. *Schistosoma sp.* and *Onchocerca volvulus* infections decrease the efficacy of vaccine against tuberculosis or tetanus [62], and *Ascaris suum* alters the efficacy of vaccine against *Mycoplasma hyopneumoniae* [73]. In mice, *H. polygyrus* was able to downregulate the strong immunity against *Plasmodium chabaudi* induced by blood stage antigens [74]. Effects of helminth infections on vaccine efficacy must be taken into account when using vaccines and also when developing new vaccines, in particular by choosing adapted adjuvants which are able to counterbalance the immunomodulatory activities of the helminth.

6. Helminths and Allergic and Autoimmune Diseases

For several years, epidemiologic observations have shown that the prevalence of helminth infection is decreasing in

westernized countries whereas the prevalence of diseases due to immune or inflammatory disorders such as allergic or autoimmune diseases is increasing. Epidemiologic and experimental data prove that chronic infection with helminths is protective against allergy. Humans infected with worms rarely develop allergic reactions [9] and an allergic reaction against ovalbumin was inhibited in mice infected with *H. polygyrus* or *Schistosoma sp.* [62, 75]. Similarly, immunization with *Toxascaris leonina*-derived proteins was able to inhibit allergy-specific Th2-like responses to ovalbumin [76]. Treatment against gastrointestinal nematodes increases cutaneous reactivity against house-dust mites [77]. These results are paradoxical because allergy is linked to mastocyte degranulation by IgE; the production of which is stimulated by helminths. In fact, worms induce the production of large quantities of antiinflammatory cytokines (IL-10, TGF β) by the regulatory T cells which then inhibit allergic inflammation.

In the same way, helminths can protect the host against autoimmune disease or at least decrease the gravity of symptoms induced by autoimmune inflammation. For example, *S. mansoni* infection inhibits the development of type 1 diabetes in NOD mice [78] or of experimental autoimmune encephalomyelitis in mice [79]. Helminth-specific Treg cells and their antiinflammatory cytokines (IL-10, TGF β) seem to be largely implicated in the inflammatory disorders associated with allergic diseases. Several studies are currently underway to investigate the possibility of treating allergic inflammatory diseases with immunomodulatory molecules from helminths, with special focus on the molecules involved and the ways in which helminths manipulate the host response, particularly how they activate and induce the expansion of Treg cells.

7. Immunomodulatory Molecules of Helminths As New Antiinflammatory Drugs

Immunomodulatory function of helminths and their products could be used as antiinflammatory drugs. *Trichuris suis* has been tested recently to treat patients with inflammatory bowel disease and Crohn's disease with success [80, 81]. An excretory-secretory protein of *Acanthocheilonema vitae*, ES62, has been well studied for its antiinflammatory property. ES62 significantly decreases the severity of collagen-induced arthritis in mice [80] and of cutaneous hypersensitivity dependant on mast cells [81]. However, these immunomodulatory molecules could have side effects by increasing the risk of infections. Furthermore, they could be responsible for allergic reactions because they could be allergens or they could cross-react with allergens derived from pollen or another source [82].

In conclusion, helminth species have coevolved with their host for a long time. This has led to a strict adaptation which enables them to settle and persist in the host. Helminths are strong immunomodulators able to interfere with immune and inflammatory mechanisms induced by themselves and by coinfecting pathogens, inflammatory disorders, or vaccine. Immunomodulatory products from helminths are probably the antiinflammatory molecules of

the future. Effects of helminths on the host immune system are not properly known because data are partial or can not be generalized between species. Nevertheless these effects need to be taken in account when controlling helminths and the diseases induced by worms. However, an individual could be infected by several pathogens and it will be necessary to evaluate the overall immune equilibrium resulting from the immune interaction between host and pathogens in different tissues and organs, which is still difficult, for instance.

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Research Article

Are the Immunocompetence and the Presence of Metazoan Parasites in Cyprinid Fish Affected by Reproductive Efforts of Cyprinid Fish?

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Each organism has the limited resources of energy that is distributed among important life traits. A trade-off between immune response and other physiological demands of organism especially costly reproduction is expected. *Leuciscus cephalus*, the cyprinid fish, was investigated during three periods varying in reproductive investment, that is, before-breeding, breeding, and after-breeding periods. We tested whether a potentially limited investment in immunity during the breeding is associated with higher susceptibility to the metazoan parasites. Following the immunocompetence handicap and sperm protection hypotheses, males expressing more elaborated sexual ornamentation should produce better quality sperm and be more parasitized. We found that reproductive investments in fish play an important role for energy allocation into somatic condition, immunity, and reproduction. The immune parameters including respiratory burst and leukocyte count were higher in breeding; however, parasite species richness and abundance appeared low. Males investing more in spawning tubercles reached high spermatocrite and were more parasitized by digeneans.

1. Introduction

Life-history theory predicts that each organism has a limited amount of energy, which is allocated among the traits connected with maintenance, reproduction, and growth [1, 2]. One of the most important life traits involving the potential life trade-offs is the ability of immune system to defend hosts against parasites. Thus recently, the investment in immune defence is a matter of great concern in the immunoeological or evolutionary immunology studies [3].

There are several studies investigating the importance of immunological competence as a potential determinant of reproductive success and fitness predominantly using birds as a model (e.g., [4–7]). The main presumption of those studies is that the host immune system is exposed to the variety of parasite species; therefore, hosts have to develop adequately strong immune response to reduce the fitness cost due to parasitism.

Immune system of each organism could be a subject of rapid temporal changes due to limited resources of energy, especially in reproductive period, such as breeding in fish. The changes should be achieved either by trade-off between immunity and reproduction or by adaptive or nonadaptive immunomodulative action of sex hormones [8]. The costs paid for reproduction due to immunosuppression by steroid hormones were suggested for salmonid Arctic charr (*Salvelinus alpinus*), following the observation that spawning males were more susceptible to parasite infection and had smaller spleen size in comparison with the resting males [9]. A trade-off in energy allocation could also explain a negative relationship between immunocompetence (measured by spleen size) and condition factor (measurement of fish vigour and health) found in fish males, previously observed in common bream (*Abramis brama*) during breeding period [10]. The sexual ornamentation in fish males during spawning seems to reflect the potential costs of reproduction caused by

higher parasite infection although there was no effect on immunocompetence measured by spleen size.

Following the hypotheses of evolution of sexual selection, the expressed sexual traits like sexual ornamentation could be considered as a male handicap playing a key role in mate choice. The more intensive expression of sexual trait indicates the presence and effectiveness of genes for resistance (so-called “good genes”) against pathogens and parasites [11]. Many studies using different models have been conducted to investigate the associations between parasite loads and intensity of sexual ornamentation but their results are various (see [12–14]). Studies based on measurements of immune function provided stronger evidence for the Hamilton and Zuk hypothesis than those based on measures of parasite load [13]. Immunocompetence handicap hypothesis introduced by Folstad and Karter [15] is based on presumption of dualistic role of steroid hormones. On the one hand, those hormones stimulate the increase in the expression of sexual ornamentation, but on the other hand, they decrease the resistance and immune defence of individuals by immunosuppression. The role of steroid hormones, mainly in testosterone-mediated immunosuppression, was also highlighted by sperm protection hypothesis [16, 17]. Males able to tolerate the negative effect of testosterone produce more developed sexual traits and also the sperm of better quality. Therefore, they are supposed to be more susceptible to parasites because their immune response is suppressed by testosterone. This hypothesis was partially supported in roach (*Rutilus rutilus*), a freshwater cyprinid fish [18].

Leuciscus cephalus is the cyprinid fish spawning usually to sand or gravel bottom from May to June [19]. The spawning period is determined by water temperature. During the spawning period, males produce keratin-based epidermal nodules—breeding tubercles—which are invoked by several pituitary and sex hormones [20]. The breeding tubercles may have a role as a status badge or as a sign of quality [21], or they are important in mate choice indicating male’s parasite load to females [22] and resistance to parasites or pathogens [23]. Taskinen and Kortet [23] found a positive relationship between proportions of dead *Rhipidocotyle campanula* (Digenea) and sexual ornamentation in three populations of roach in accordance with Hamilton-Zuk hypothesis. Skarstein and Folstad [24] documented a negative association between lymphocyte density and sexual ornamentation (red spawning coloration) in Arctic charr which may indicate the increased investment in sexual ornamentation at the costs of immunity.

The aim of the present study was to investigate the changes in immune defence, condition status, reproduction parameters, and parasitism in selected cyprinid fish species in the periods of different reproductive investment, following the hypothesis of potential trade-offs in energy allocation. We supposed and tested whether a potentially limited investment in immunity is associated with higher susceptibility to the metazoan parasites (including especially helminths) during the period of high reproductive investment (i.e., spawning period). Finally, following the predictions of immunocompetence handicap and sperm protection

hypotheses, we tested whether males expressing more elaborated sexual ornamentation produce better quality sperm (measured by sperm density) and are parasitized by high parasite species richness due to potential immunosuppression by steroid hormones.

2. Materials and Methods

2.1. Fish Sampling. A total of 90 males of chub (*Leuciscus cephalus*, Cyprinidae) were collected in 2005 during three different periods in relation to reproduction investment. Those periods included before breeding in early May, breeding in late May, and after breeding in late June. The fish was collected from the same locality situated on the confluence of the Svitava and Svatka rivers, belonging to the Morava river basin on the periphery of Brno (Czech Republic). The fish individuals were captured using electrofishing and immediately a blood sample (340 μ L) was taken from individually electronarcotized fish. Cardiac puncture using heparinized syringes was used as an optimal sampling method in electronarcosis to collect fish blood [25]. Blood samples were collected in heparinized microtubes and mixed with heparin diluted 10x with 0.7% NaCl to retain the number of heparin units in the measured blood according to the methodology written by Kubala et al. [26]. After blood sampling, the fish were placed in a tank containing water collected from the same location and then transported to the laboratory. During the storage in the laboratory tanks, the original water temperature was maintained and a standard aquarium filter was used for water filtration. All fish individuals were killed within 24 hours. Each individual was measured (total and standard lengths in mm) and weighed (in g), and a complete parasitological dissection of the fish according to Ergens and Lom [27] was performed.

The physiological parameters, including gonad weight and spermatocrit, hematocrit, and erythrocyte counts, and the selected immune parameters, including spleen weight, leukocrit and leukocyte counts, differential leukocyte cell counts, phagocyte count, and respiratory burst activity, were measured. We calculated relative body weight (condition factor, K) using the equation: $K = \text{constant} \times \text{somatic weight (g)} / (\text{standard length (cm)})^3$. According to Bolger and Connolly [28], the assumption of the condition factor is that the heavier the fish is in relation to its length. Therefore, it is better to use the general condition rather than the weight and length particularly. The relative size of gonads, that is, gonado-somatic index, GSI, was calculated as follows: $\text{GSI} = \text{gonad weight (g)} / \text{body weight (g)} \times 100$. Spleen-somatic index, SSI, was calculated as $\text{spleen weight (g)} / \text{body weight (g)} \times 100$.

2.2. Parasite Collection and Determination. The complete dissection of fish was performed using the method of Ergens and Lom [27]. Fish individuals were investigated for all metazoan parasites. Ectoparasites (Monogenea, Hirudinea, and Mollusca) and endoparasite helminths (Digenea, Cestoda, Acanthocephala, and Nematoda) were determined using recent keys and methodology [27, 29–32]. All recorded

specimens of metazoan parasites, that is, Monogenea in glycerin-amonium picrate, Nematoda in glycerin-ethanol and other metazoan parasites (Digenea, Cestoda, Acanthocephala, and Mollusca) in 4% formalin were fixed. Digenea and Cestoda were subsequently stained using IAC carmine [33]. A light microscope (Olympus BX 50) with phase-contrast, differential interference contrast (DIC according to Nomarski), and Digital Image Analysis (Micro Image 4.0 for Windows) was used for parasite determination and measurements.

2.3. Blood Analyses. Differential cell counts were estimated in whole blood smears stained panoptically by the Pappenheim technique (May-Grünwald and Giemsa-Romanovsky) [34]. We considered 200 leukocytes and classified them using morphology into following categories: lymphocytes, monocytes, blasts, and neutrophiles (including the different neutrophiles stages, i.e., myelocytes, metamyelocytes, bands and segments) according to Svobodová et al. [34]. Erythrocyte (in $T.l^{-1}$) and leukocyte (in $G.l^{-1}$) counts were executed in Bürker's hemocytometer. Heparinized blood was diluted with Natt-Herick solution at 1 : 200 ratio in special 25 mL flask [35, 36]. Hematocrit and leukocrit (in $l.l^{-1}$) were measured using heparinized microcapillaries 75 mm long and 60 μ m in inner volume. Centrifugation was carried out with a hematocrit centrifuge at 12000 g for 3 minutes [35]. Differential leukocyte count and total count of leukocytes were used to calculate the relative count of phagocytes ($G.l^{-1}$). According to Šterzl [37] concerning neutrophiles, only metamyelocytes or older stages than metamyelocytes are considered to have phagocytic ability. Therefore, the relative count of phagocytes in our study included monocytes, neutrophilic metamyelocytes, bands, and segments.

2.4. Respiratory Burst Activity. Chemiluminescence (CL) activated with opsonised zymosan was measured in each fish sample. The chemiluminescence emission (in relative light units, RLU) was measured in five-minute intervals during 100 minutes (i.e., a total of 20 measurements were taken) to obtain the kinetic curves for each sample. The peak of the CL signal, that is, the maximal value of a respiratory burst (RB), was used for each individual. CL measurements were carried out with a Luminometer Junior (Checklight; 380–630 nm) according to methodology of Kubala et al. [26].

2.5. Sperm Quality and Sexual Ornamentation. The selected parameters potentially reflecting the quality of male individuals were measured only during the spawning period. Spermocrit (in $l.l^{-1}$), a measurement of sperm concentration expressing the percentage of a given volume of milt occupied by sperm cells was analyzed according to Kortet et al. [18]. The microcapillaries with milt samples were centrifuged for 5 minutes at 12000 g. We calculated all breeding tubercles, as secondary sexual ornaments in fish, on the head and operculum of each individual.

2.6. Data Analysis. Each parasite group was characterized by the following epidemiological parameters: prevalence

(percentage of infected host individuals in each period) and intensity of infection (number of parasites per an infected host) according to Bush et al. [38]. Moreover, abundance as a number of parasites in all investigated hosts in each period was calculated. Only parasite groups with high epidemiological values, including Monogenea, Digenea, Cestoda, and Acanthocephala, were used for statistical analyses. The differences in physiological, immune parameters, and parasitism among 3 investigated periods were tested using ANOVA. Tukey HSD post hoc test was applied for multiple pairwise comparisons. The potential relationships among the studied variables were analyzed using a Principal Component Analysis (PCA) and Pearson's correlation coefficient. Bonferroni correction for multiple tests was applied. Most of variables analyzed did not fit a normal distribution, and therefore, they required a transformation using logarithm (for GSI, SSI, respiratory burst, count of erythrocytes, and number of head tubercles), arcsine square root (for spermocrit), and square root (for parasite abundance and total parasite species richness). No transformation was necessary for condition factor and count of phagocytes. GSI and SSI instead of gonad or spleen weights were used in PCA to eliminate the effect of body weight on gonad or spleen weights. All statistical analyses were executed using Statistica 8.0.

3. Results

3.1. Physiological and Immunological Parameters. As can be concluded from the results, the significant differences were found in the measured physiological and immune parameters when comparing the three periods which varied in the investment in reproductive activity (i.e., before-breeding, breeding, and after-breeding). The mean and standard deviations (\pm SD) of total body length, body weight, gonad weight, spleen weight, and condition factor of fish within each period investigated are shown in Table 1.

We tested the differences in physiological and immune parameters among 3 investigated periods using ANOVA. The statistically significant differences were found for GSI ($F_{2,82} = 106.250$, $P < .0001$), condition factor ($F_{2,87} = 7.150$, $P = .0013$), respiratory burst ($F_{2,84} = 7.113$, $P = .0014$), erythrocyte ($F_{2,87} = 3.704$, $P = .0286$) and leukocyte ($F_{2,87} = 6.151$, $P = .0032$) counts, hematocrit ($F_{2,85} = 3.491$, $P = .0349$), and leukocrit ($F_{2,85} = 3.331$, $P = .0405$). The highest values of GSI, condition factor, and hematocrit were recorded in before-breeding period while erythrocyte count revealed the highest values in after-breeding period. The maximal values of immune parameters, including respiratory burst, leukocyte count, and leukocrit, were found in breeding period. However, no significant difference was shown for SSI and phagocyte count among three periods of different reproductive investments (ANOVA, $P > .05$). Using Tukey HSD post hoc test we compared differences between all pairs of periods. First, we detected significant differences between all periods for GSI ($P < .05$). Consequently, we found the significant differences of before-breeding period in comparison with both breeding and after-breeding periods

TABLE 1: Data on the fish investigated: *n* presents the number of males collected, total body length, body weight, gonad weight, spleen weight, and condition factor (*K*): means ± SD are shown.

Sampling period	<i>n</i>	Total length (mm)		Body weight (g)		Gonad weight (g)		Spleen weight (g)		<i>K</i>	
		Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD	Mean	±SD
Before-breeding	29	271.59	±3.25	245.37	±106.88	21.88	±10.98	0.27	±0.13	1.92	±0.22
Breeding	31	270.39	±26.93	215.97	±61.39	10.30	±3.57	0.25	±0.09	1.76	±0.17
After-breeding	30	260.52	±71.48	215.49	±63.92	10.51	±3.79	0.25	±0.08	1.60	±0.43

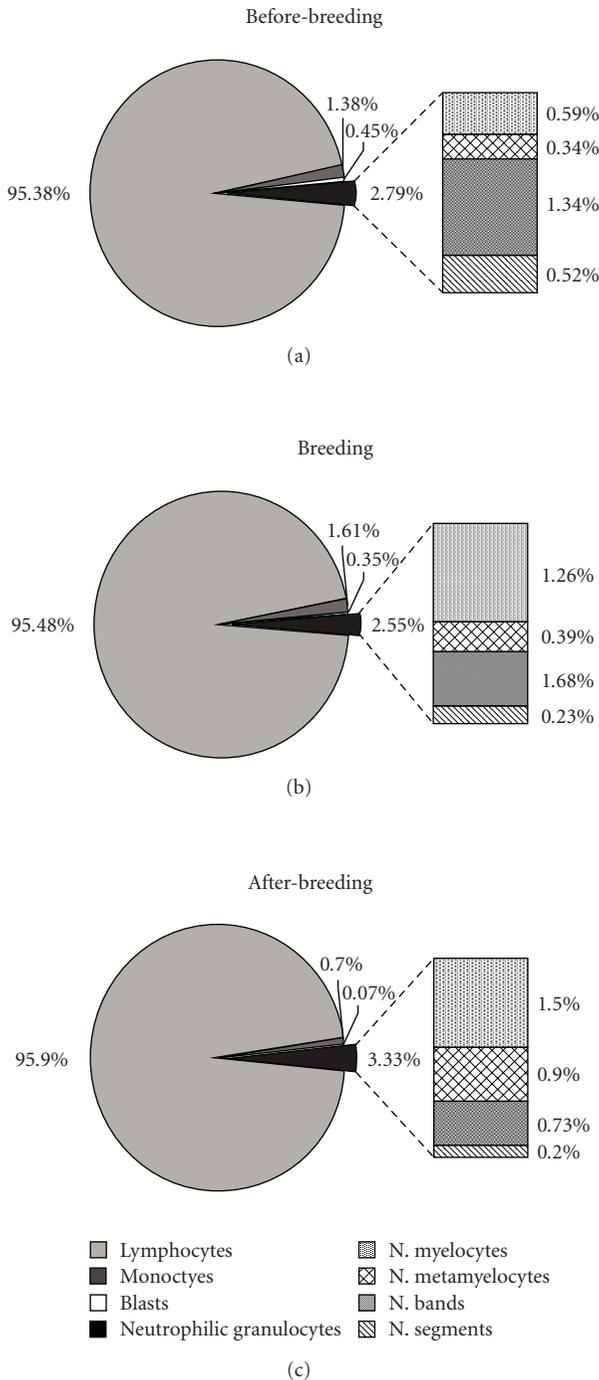


FIGURE 1: Changes in leukocyte differential count in three periods investigated.

for condition factor and leukocyte count. Further, the significant differences ($P < .05$) were also found between before-breeding and breeding periods for respiratory burst and hematocrit and between before breeding and after-breeding for erythrocyte count and leukocrit.

As revealed by comparison of differential leukocyte count among the three periods studied, only small variability in the composition of white blood cell components was found. Blood of chub showed lymphocytic character, that is, lymphocytes prevailed in the white blood cell. The proportion of lymphocytes reached the similar values in all three periods. Only a small difference was found in the proportions of monocytes, total neutrophilic granulocytes, and blasts in relation to three periods investigated (Figure 1). Nevertheless, the variations were found in proportions of the developmental stages of neutrophilic granulocytes including early ontogenetic stages, that is, myelocytes and metamyelocytes as well as late ontogenetic stages, that is, bands and segments. We recorded the low proportion of neutrophilic myelocytes and metamyelocytes and the high proportion of bands and segments in before-breeding period. On the other hand, in breeding and after-breeding periods, we found higher proportions of early ontogenetic stages (myelocytes and metamyelocytes) compared with other development stages of neutrophils. The percentage distribution of individual types of leukocytes during three periods investigated is shown in Figure 1.

3.2. *Parasite Infection.* Metazoan parasite species belonging to seven groups including ectoparasitic Monogenea, Hirudinea, Mollusca, and endoparasitic Digenea, Cestoda, Acanthocephala, and Nematoda were found. Monogenea was the species richest group, including eight species. The presence of all parasite species in three periods investigated is shown in Table 2. From a total 22 parasite species, 12 of them occurred in all three periods studied, 7 species were present only in before-breeding and after-breeding periods (i.e., those parasites were absent in breeding period), and 2 parasite species were found solely in breeding period.

When comparing the three periods, we detected a variation in epidemiological characteristics including abundance, intensity of infection, and prevalence. Those characteristics for each parasite group are shown in Table 3. ANOVA test revealed statistically significant differences in abundance among three periods investigated for three parasite groups: Monogenea ($F_{2,87} = 10.887, P < .0001$), Digenea ($F_{2,87} = 10.257, P = .0001$), and Cestoda ($F_{2,87} = 4.745, P = .0111$). The significant differences for these parasite groups were

TABLE 2: Presence (+) and absence (–) of parasite species in each sampling period. Hatching areas indicate the presence of a given species in all three periods investigated.

Parasite group	Parasite species	Sampling period		
		Before-breeding	Breeding	After-breeding
Monogenea	<i>Dactylogyrus vistulae</i>	+	+	+
	<i>Dactylogyrus fallax</i>	+	+	+
	<i>Dactylogyrus folkmanovae</i>	+	+	+
	<i>Paradiplozoon megan</i>	+	+	+
	<i>Gyrodactylus vimbi</i>	+	+	+
	<i>Gyrodactylus lomi</i>	+	+	+
	<i>Gyrodactylus prostae</i>	–	+	–
	<i>Gyrodactylus gasterostei</i>	+	–	+
Digenea	<i>Sphaerostomum bramae</i>	+	+	+
	<i>Diplostomum</i> sp. larv.	+	+	+
	<i>Metorchis xanthosomus</i>	+	+	+
	<i>Asymphylodora imitans</i>	+	–	+
	Strigeidae gen. sp.	+	–	+
Cestoda	<i>Caryophyllaeus fimbriceps</i>	+	–	+
	<i>Caryophyllaeus brachycollis</i>	+	–	+
	<i>Proteocephalus torulosus</i>	+	+	+
Nematoda	<i>Philometra ovata</i>	–	+	–
	<i>Rhabdochona denudata</i>	+	–	–
	<i>Raphidascaris acus</i>	+	–	+
Acanthocephala	<i>Pomporhynchus laevis</i>	+	+	+
Hirudinea	<i>Piscicola geometra</i>	+	+	+
Mollusca	<i>Glochidium</i> spp.	+	–	+

also found after using Bonferonni correction ($P < .05$). The highest abundance of Monogenea was recorded in after-breeding period. Digenea and Cestoda reached the maximal values of abundance in before-breeding period. Using post hoc test for pairwise comparison, we found statistically significant differences between before-breeding and after-breeding as well as breeding and after-breeding periods for abundance of Monogenea and Digenea (both $P < .05$). Further, the significant differences for Cestoda were seen between before- and breeding periods ($P < .05$).

3.3. Associations between Immunity, Physiological Parameters, and Parasite Infection. The potential correlations between immunocompetence, physiological parameters and parasitism were investigated in three periods varying in the reproduction investment. After transforming data to achieve the normal distribution, the following parameters were used for the analyses: (1) immunological data (SSI, respiratory burst, and count of phagocytes), (2) physiological data (GSI, condition factor, count of erythrocytes, spermatocrit, and number of head tubercles), and (3) parasitism. For each period two measurements of parasitism were applied, that is, total parasite species richness and parasite abundance. The second one includes abundances of the most numerous parasite groups, that is, Monogenea (all of them gill and skin parasites), *Pomporhynchus laevis* (Acanthocephala), and Cestoda (including three intestinal parasite species)

and two numerous parasite species of Digenea, that is, *Sphaerostomum bramae* and *Metorchis xanthosomus*, were treated separately because of different location (intestine or skin) and strategy (adult or larvae stages) in hosts. The graphic outputs of PCA analyses using parasite abundance as a measurement of parasite load in Figures 2–4(a), (b) and total parasite species richness in Figures 2–4(c), (d) are shown. The first three axes explain the highest proportion of total variability and therefore they are retained for graphic interpretation (see Table 4 for the variability explained by the first three axes).

In before-breeding period, the analyses showed that fish condition in before-breeding period was positively correlated to abundance of *Metorchis xanthosomus* ($R = 0.4129$, $P < .0001$) and negatively to *Sphaerostomum bramae* ($R = -0.6582$, $P = .040$) (see Figures 2(a) and 2(b)). Following PCA results, GSI is negatively associated to Monogenea abundance (Figure 2(b)). Moreover, GSI is associated to SSI in before-breeding period (Figures 2(c) and 2(d)). The total parasite species richness increased with higher SSI and lower GSI (Figure 2(c)). Nevertheless, the correlations between those variables were not significant ($P > .05$).

In breeding period, we found a positive correlation between the number of head tubercles and spermatocrit ($R = 0.4009$, $P = .038$) (see Figures 3(a), 3(b), and 3(c)). Following PCA results, GSI tends to be positively associated with both spermatocrit and number of head tubercles (Figures 3(a) and 3(c)). However, the correlations were not significant

TABLE 3: Basic epidemiological characteristics: abundance (mean \pm SD), intensity of infection (min-max), and prevalence (percentage of infected hosts) of all metazoan parasite groups.

Parasite group	Abundance	\pm SD	Intensity of infection	Prevalence (in %)
Before-breeding				
Monogenea	49.76	\pm 27.97	8–116	100.00
Hirudinea	0.10	\pm 0.41	0–2	6.90
Mollusca	0.03	\pm 0.19	0–1	3.45
Acanthocephala	3.97	\pm 5.34	0–21	75.86
Digenea	71.83	\pm 76.79	0–378	96.55
Cestoda	2.03	\pm 6.74	0–36	27.59
Nematoda	0.10	\pm 0.31	0–1	10.34
Breeding				
Monogenea	42.61	\pm 48.46	0–186	90.32
Hirudinea	0.03	\pm 0.18	0–1	3.23
Mollusca	—	—	—	—
Acanthocephala	3.52	\pm 6.52	0–34	67.74
Digenea	58.13	\pm 114.3	0–474	87.10
Cestoda	0.03	\pm 0.18	0–1	3.23
Nematoda	0.10	\pm 0.3	0–1	9.68
After-breeding				
Monogenea	92.73	\pm 59.11	1–234	100.00
Hirudinea	0.30	\pm 0.7	0–3	20.00
Mollusca	0.27	\pm 1.46	0–8	3.33
Acanthocephala	3.60	\pm 3.8	0–14	76.67
Digenea	14.17	\pm 34.04	0–180	56.67
Cestoda	0.20	\pm 0.55	0–2	13.33
Nematoda	0.03	\pm 0.18	0–1	3.33

($P > .05$). Even if there was no correlation between GSI and head tubercles, a positive correlation between gonads and head tubercles was found using gonad weight ($R = 0.3943$, $P = .042$). Moreover, spleen weight was positively correlated to spermatocrit ($R = 0.4652$, $P = .014$) (not shown in the figure). Further, count of phagocytes was positively correlated to total parasite species richness ($R = 0.4958$, $P = .009$) (Figures 3(c) and 3(d)) as well as abundance of *Pomporhynchus laevis* (Acanthocephala) was positively associated to SSI ($R = 0.4434$, $P = .021$) (Figure 3(a)) in breeding. Moreover, a positive correlation between abundance of *Metorchis xanthosomus* and spermatocrite ($R = 0.4421$, $P = .021$) (Figures 3(a) and 3(b)) and a negative correlation between total parasite species richness and count of erythrocytes ($R = -0.3822$, $P = .049$) (Figures 3(c) and 3(d)) were found.

Using PCA and Pearson's correlation for the data from after-breeding period, abundance of Cestoda was negatively correlated to fish condition ($R = -0.3768$, $P = .048$) and phagocyte count ($R = -0.4355$, $P = .021$) (Figures 4(a) and 4(b)). A negative correlation was also found between phagocyte count and abundance of *Sphaerostomum bramae* ($R = -0.4268$, $P = .024$) (Figures 4(a) and 4(b)). Finally, negative correlation between total parasite species richness and both phagocyte count ($R = -0.3871$, $P = .042$) and SSI ($R = -0.4059$, $P = .032$) were found (see Figures 4(c) and 4(d)).

4. Discussion

4.1. Differences in Immunity and Physiology. In this study, the differences in fish condition and immunity were hypothesized in relation to reproductive effort of fish. Therefore, we analyzed several immune and physiological parameters of chub, selected cyprinid fish species in three periods which varied in reproductive investment, that is, before-breeding, breeding, and after-breeding periods. The selected immune parameters are widely applied in immunoeological studies of fish. When comparing the immune and physiological variables measurements, we found significant changes in all parameters except relative weight of spleen and phagocyte count. In spite of no significant change in relative phagocyte count, the activity of phagocytes measured by respiratory burst showed significant changes among three periods investigated. The differences in proportion of developmental stages of neutrophils were also found among the periods of different reproductive investments. It may suggest that the total phagocyte activity is potentially affected by neutrophile profile rather than by total number of phagocytes. Moreover, the individual activity level of neutrophile cells is probably involved in the total signal of respiratory burst. Further, in spite of the fact that the changes in the differential leukocyte count are considered to be one of the most sensitive indicators of acute stress in fish [39], we did not record any noticeable change in percentage of lymphocytes and total

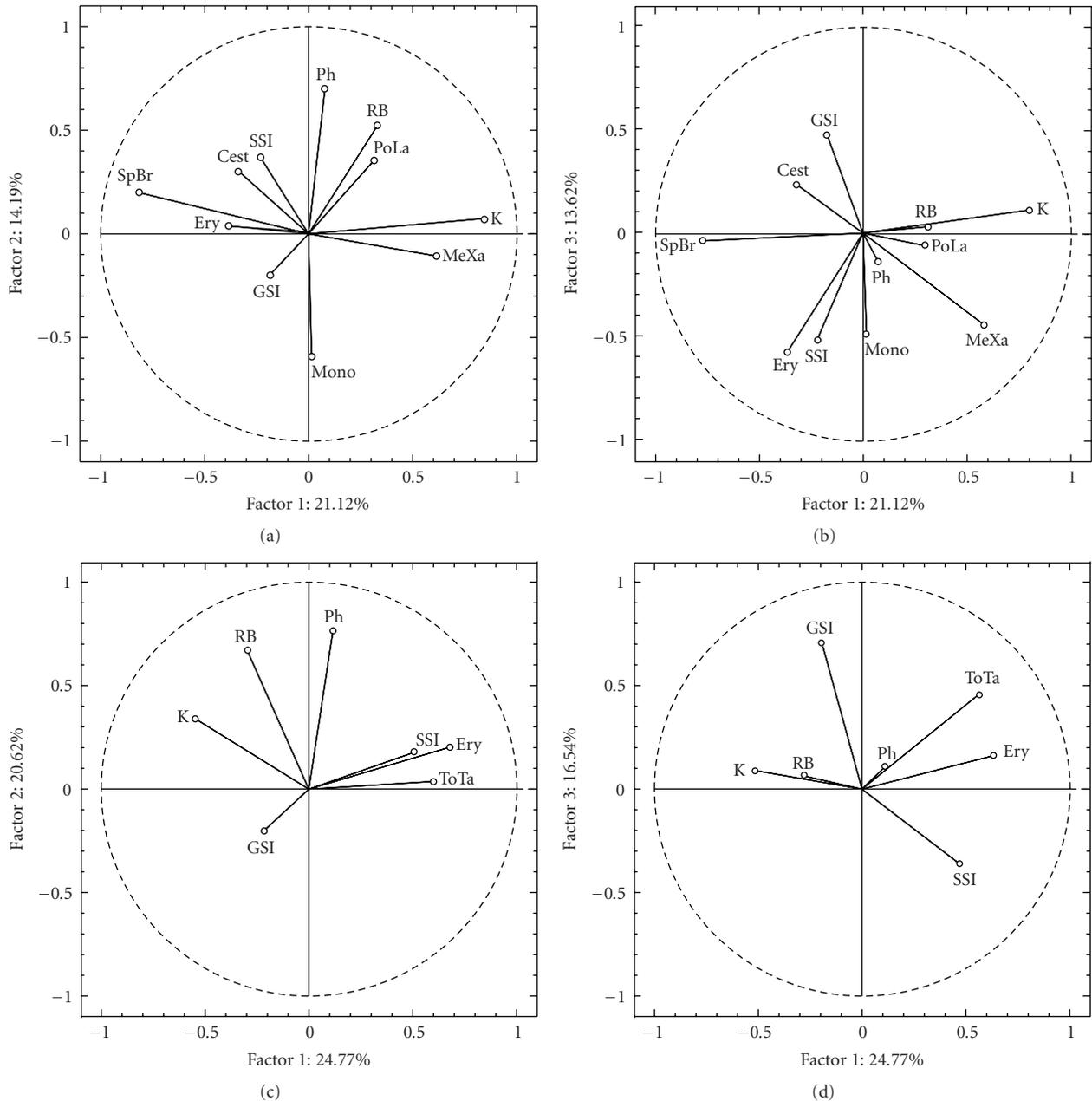


FIGURE 2: Principal component analysis (PCA) to evaluate the associations within physiology, immunity, and parasitism in before-breeding period. K: condition factor; GSI: gonado-somatic index; SSI: spleen-somatic index; RB: respiratory burst; Ph: phagocyte count; Ery: erythrocyte count; Mono: Monogenea; Cest: Cestoda; MeXa: *Metorchis xanthosomus*; SpBr: *Sphaerostomum bramae*; PoLa: *Pomporhynchus laevis*; ToTa: total parasite species richness.

neutrophils among the periods investigated. Generally, the acute stress (mainly related to the changes of glucocorticoid level) induces both neutrophilia and lymphopenia in fish [40], although rarely only lymphopenia is reported [41].

In the before-breeding period, we supposed that fish invest more energy into the gonad development. Fish males reached the highest values of GSI before breeding and GSI sharply decreased in spawning. The similar changes as observed for gonad weight measured by gonadosomatic index were observed for relative body weight measured by

condition factor among the periods of different reproductive effort. Fish were in the best condition in before-breeding period and subsequently condition factor decreased in spawning and after breeding. Spawning is considered to be physically demanding and stressful period [28, 42, 43]; therefore, we suggest that the fish accumulate its energy reserves before breeding to obtain the sufficient resources for spawning.

Moreover, in spawning period we revealed the highest values of measured immune variables including respiratory

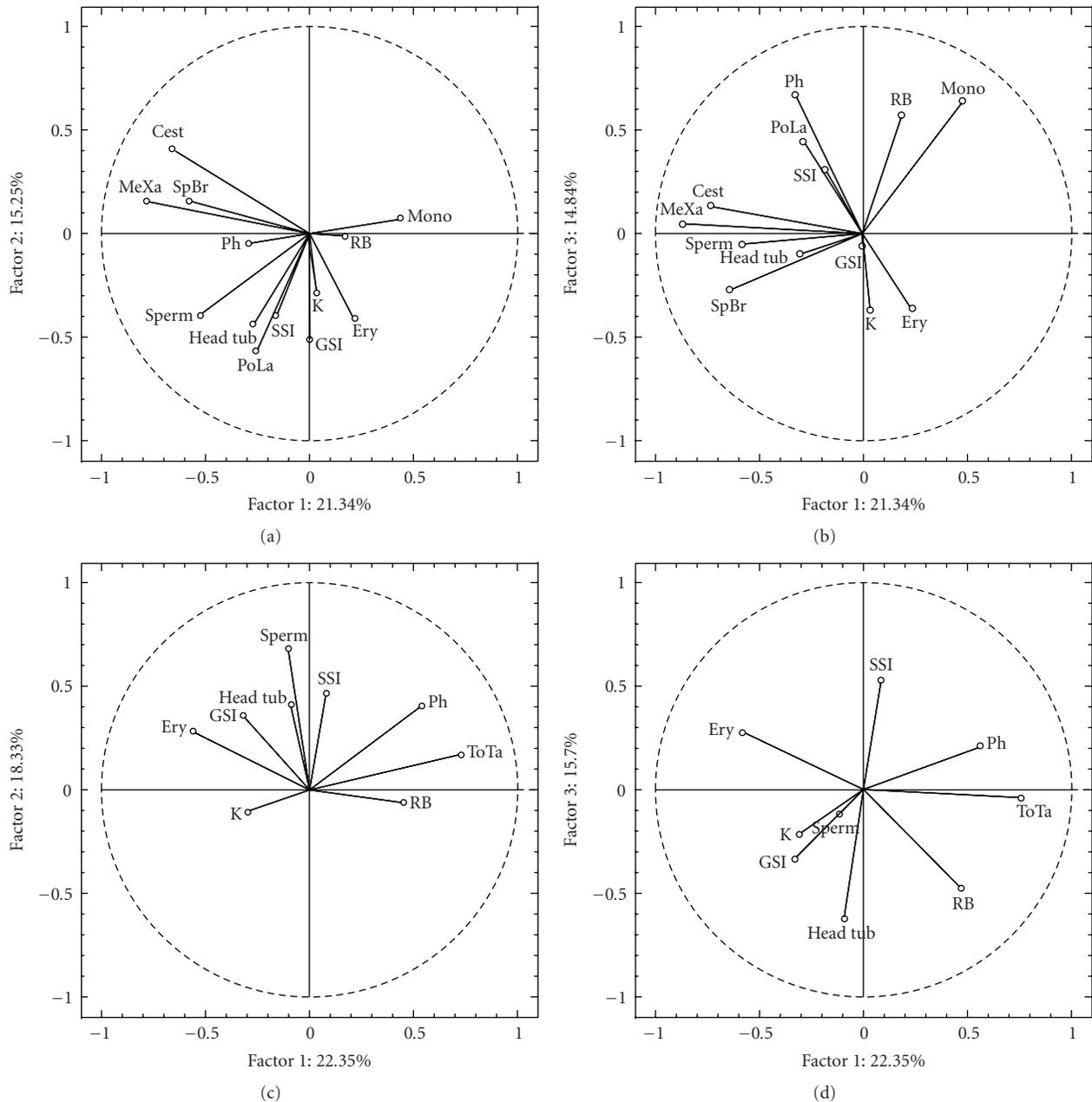


FIGURE 3: Principal component analysis (PCA) to evaluate the associations within physiology, immunity, and parasitism in breeding period. For abbreviations see legend of Figure 2.

burst, leukocyte count, and leukocrit. An increased number of leukocytes is considered to be common consequence of infection (i.e., [44]). Surprisingly, we found that total parasite diversity is lower in spawning in comparison with before-breeding and after-breeding periods. However, the high values of immune parameters may also reflect the extensive stress due to spawning or alternatively those values may be induced by pathogens, such as protozoa or viruses which were not investigated in the present study.

However, we should take into consideration that fish physiology and immunity is also affected by abiotic as well as biotic factors in each sampled period. Even if the level of

steroid hormones is considered to play a key role especially in spawning, fish physiology in a given place and time is influenced also by water temperature, quality of water and other abiotic factors as well as biotic interactions which may influence a current physiological status of individuals.

4.2. Differences in Parasite Infection and a Link with Immunity.

The parasite life cycle and real infection parameters of parasites within a given host individual or population are influenced by host physiology and immunity (e.g., [45–51]). In our study, we tried to evaluate the differences in

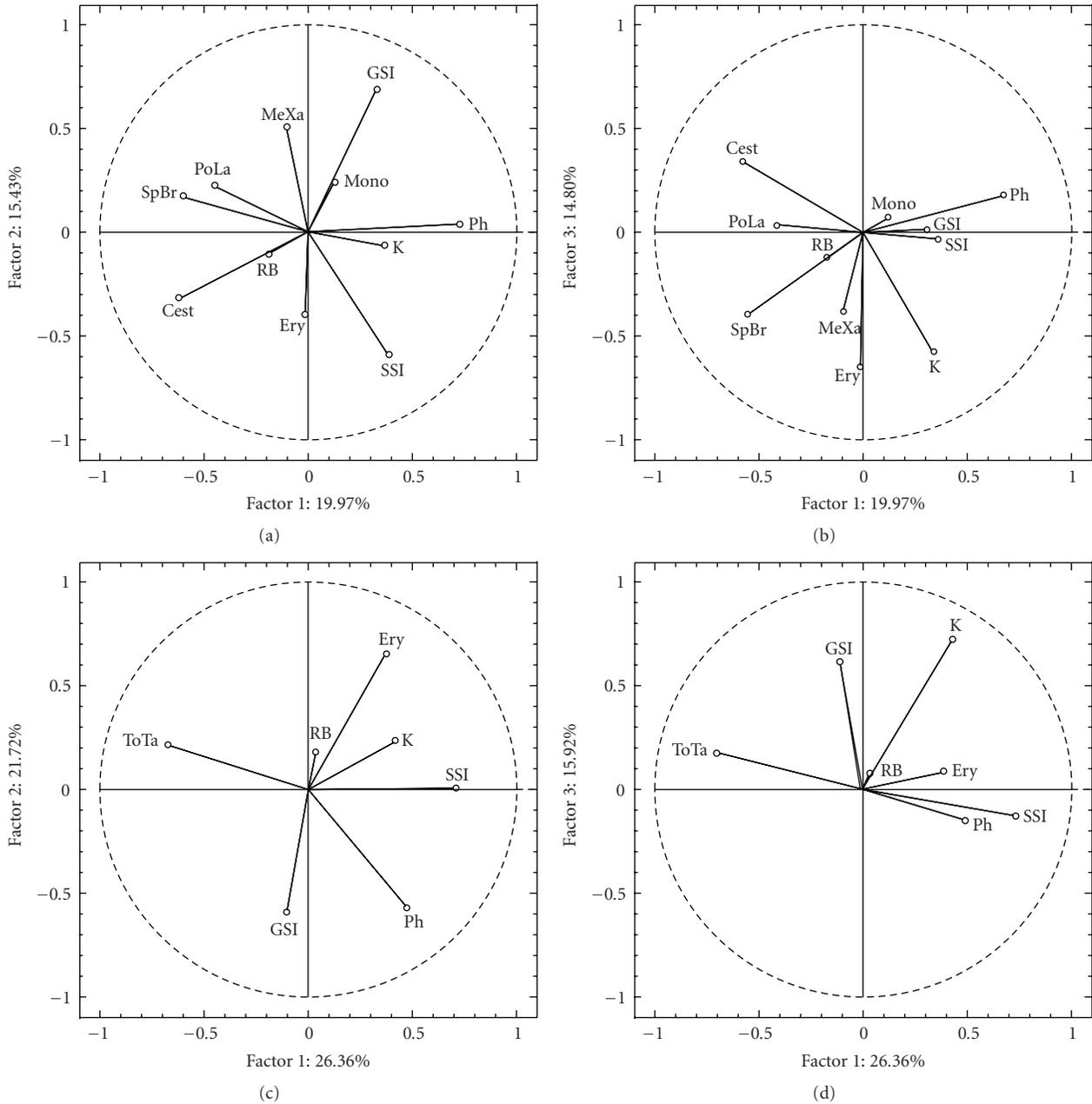


FIGURE 4: Principal component analysis (PCA) to evaluate the associations within physiology, immunity and parasitism in after-breeding period. For abbreviations see legend of Figure 2.

metazoan parasite infection in three periods which varied in the investment in fish reproduction and likewise we studied the potential associations between selected immune parameters and parasite load.

Abundance of Monogenea (a group of gill and skin parasites) reached relatively high values in before-breeding period, subsequently decreased in breeding, and finally switched over to maximal values in after-breeding period. Although we found no correlation between measured immune variables and infection of Monogenea, we suggest that it is possible that other measurements of immunity (which are not involved in recent immunoecological studies)

could reflect the changes in monogenean infection when comparing periods varied in reproductive investment. For example, the production of specific immunoglobulin against gill monogeneans in different fish species (see [49, 52, 53]) or the role of complement for parasite infection in salmonid fishes was shown in experimental studies (e.g., [54–56]). The association between infection by the gill or skin monogeneans and mucus lysozyme activity was also found [52, 57, 58]. Further, parasites dispose of various mechanisms whereby evade or cope with fish immune response. For instance, several species of monogeneans look for host sites where the immune response is not strong

TABLE 4: The axes of PCA explaining the total variability in the data set.

Period	Parasitism measure	axis 1	axis 2	axis 3
Before-breeding	Parasite abundance	21.12	14.19	13.62
	Species richness	24.77	20.62	16.54
Breeding	Parasite abundance	21.34	15.25	14.84
	Species richness	22.35	18.33	15.78
After-breeding	Parasite abundance	19.97	15.43	14.80
	Species richness	26.36	21.72	15.92

enough to kill them. Other parasites seem to have evolved and adapted their life cycles with host age or season in which the host immune system is weaker (see [59]) or the monogeneans incorporate the host molecules into their surface to evade host immune system [49].

As mentioned above, the immune parameters are also influenced by water temperature and other environmental factors (e.g., [60–66]). For instance, Poisot et al. [67] investigated the effect of several immune parameters on metazoan parasite abundance in chub after winterizing period associated with a rapid temperature increase. They did not find any correlation between mucus activity and monogeneans, the most numerous metazoan parasite group, whose infection is expected to be associated with mucous activity. However, they found a positive correlation between respiratory burst and parasitism. A positive association between respiratory burst and monogenean abundance was also found in seasonal study of chub [68], which showed a decrease for both immune and parasite variables in November and increase toward spring and summer. Although the highest values of respiratory burst in our study were observed in breeding period, surprisingly the level of infection by monogeneans was low. We could hypothesize that spawning behaviour in chub prevents transmission of gill and skin parasites with direct life cycle. However, such hypothesis should be tested in future. High increase of monogenean abundance in after-breeding period suggests that fish weakened by spawning become more susceptible to monogenean infection. The measured immune parameters in after-breeding period reached the lower values in comparison with breeding period. Therefore, we suppose that the values of measured immune parameters during the breeding reflect the stress caused by spawning rather than current parasite infection.

Concerning Cestoda, a group of intestinal parasites, the decrease in abundance was revealed in breeding period when comparing with before-breeding period. Those results could indicate the temporal presence of intermediate hosts (i.e., [69]) or reflect the changes associated with parasite life cycle. For instance, Scholz and Moravec [70] recorded reaching maximum values of prevalence and mean intensity of *Proteocephalus torulosus* in March whilst no parasites occurred in fish during summer and autumn. However, our study showed that this cestode species is present in all periods varying in reproductive investment in fish. It is possible that current infection by Cestodes is reflected in other

immune defence mechanisms than the ones investigated in our study. In the reviews by Secombes and Chappell [46] and Alvarez-Pellitero [71], the various immune mechanisms against helminths have been summarized (incl. Cestoda), that is, antibody, inflammation, or complement response, which may be caused by parasite infection. Finally, as already mentioned, no relationship between infections of Cestoda and measured immune variables may be related to parasite invasion mechanisms, especially “antigen-based strategies.” For instance, *Bothriocephalus scorpii* have the ability to bind C-reactive protein to avoid the recognition by host’s immune system [72].

Total abundance of Digenea reached the maximal values in before-breeding period and decreased toward after-breeding period. Two numerous digenean parasite species, *Sphaerostomum bramae* and *Metorchis xanthosomus*, were analyzed separately regarding the differences within host location and life strategy. Chub investigated in our study was parasitized by *Sphaerostomum bramae* adults, located in intestine, and larval stages of *Metorchis xanthosomus*, located in skin. Concerning *Sphaerostomum bramae*, we found the significantly highest abundance in before-breeding period, decreased values in breeding, and subsequently marked decrease after breeding (not shown in results). Evans [73] showed the high infection level of immature *Sphaerostomum bramae* during autumn and winter, subsequently rapid maturation of these parasites in spring, which was accompanied by decrease in infection level. Further, abundance of *Metorchis xanthosomus* also significantly changed among three periods investigated in our study reaching the high values in before-breeding period, maximal values in breeding period, and sharp decrease in after-breeding period. The larval stages of *Diplostomum* sp. represent other digenean species infecting chub. For this parasite species we observed no significant difference when comparing three periods of different reproductive investments. *Diplostomum* species belongs to the group of fish parasites that develop in immunoprivileged host tissues, that is, eye in this case, where host barriers prevent or limit the immune response [74]. This may explain why no associations with measured immune parameters were found in our study of chub.

Abundance of *Pomporhynchus laevis*, intestinal “spiny-headed” worms, was positively correlated to SSI as well as to leukocyte count (not seen in results). Although a little information is available on immune reactions to acanthocephalan parasites of fishes, helminth infections may significantly alter the number of leucocytes in the circulation as well as in lymphoid organs, such as the spleen and kidney [45, 75, 76].

4.3. Trade-Off between Immunity and Reproduction and the Role of Parasites. The central hypothesis of our study is a trade-off between immune response and other physiological demands because of limited energy resources for each organism. Therefore, we estimated the costs of reproduction paid by weakened immune response. If the immunity and reproduction are traded off, the potential associations between those traits are expected. Moreover, we could

suppose that an individual, which immunity is weakened, should be more susceptible to the infection by pathogens and parasites.

Following the prediction of energy allocation, we expected the trade-off between immunity and reproduction during the before-breeding period, considering the high investment in gonad development. Even if the correlation between GSI and relative spleen size was not significant, we found a trend for negative association between those variables. On the other hand, no trend of association between GSI and SSI was found in breeding and after-breeding periods. Using PCA, the total parasite species richness was positively associated with SSI, which suggests that hosts parasitized by wider spectrum of parasite species dispose a large spleen because they invested more energy in immune defence. The positive correlation between spleen size and species richness of nematode parasites was found in interspecies study of males birds, using comparative analysis, suggesting a causative role for parasitic nematodes in the evolution of avian spleen size [77]. Concerning fish, the positive correlation between spleen size and parasite abundance was found in interspecific comparative study of cyprinid fish for females although the correlation was lacking for males [78]. Surprisingly, using PCA and Pearson correlation we found the reverse relationships between fish condition and two most abundant digenean parasites in before-breeding period. Condition factor was negatively correlated with abundance of *Sphaerostomum bramae* and positively with *Metorchis xanthosomus*. As mentioned above, these two parasite species differ in strategy and location within fish host. *Sphaerostomum bramae* is an adult living in intestine of definitive host. On the other hand, metacercariae of *Metorchis xanthosomus* parasitizing in fish skin are long-life, resting, and intermediate stages of the parasite. Fish as intermediate host infected by metacercariae of *Metorchis* species are ingested by the definitive host (i.e., carnivores feeding in aquatic habitats). Thus, the different relationship between the abundance of those digenean parasite species and condition factor could be explained by a different parasite life strategy.

In spawning period, following the sperm protection hypothesis [16, 17], we expected and confirmed that fish males highly investing into the spawning ornamentation, that is, breeding tubercles, possess the sperm of a better quality (measured by spermatocrite) than less ornamented males. Such males should be more susceptible to the diseases regarding weakened immunity. In accordance with this prediction, we found that males with high spermatocrite values dispose of large spleen. Moreover, fish males with high spermatocrite value were parasitized by higher number of digenean species *Metorchis xanthosomus* parasitizing fish skin. Due to potential immunosuppression by testosterone as highlighted by immunocompetence handicap hypothesis [15, 79], immunity of males should be weakened when investing more extensively in spawning ornamentation, and then, those males should be more parasitized. Our results partially confirmed the immunocompetence handicap hypothesis. A significant positive correlation between number of head breeding tubercles and abundance of

digenean parasites *Sphaerostomum bramae* was found. Even if no relationship was found between head tubercles and parasite species richness or abundance, the significant positive correlation between the immune measures and parasitism was found in spawning.

In after-breeding period we expected and confirmed that condition status of fish decreased when comparing with breeding period. Fish are weakened after spawning and this may explain the increase of monogenean abundance due to relatively rapid direct life cycle. The investment in somatic condition seems to play an important role for parasite establishing. This may explain the fact that fish in worse condition are more parasitized by Cestoda. The immunity is no more suppressed by steroid hormones in this period. We found a negative correlation between phagocyte count and both parasite abundance (Cestoda and *Sphaerostomum bramae*) and total parasite species richness. Generally, the high immune response (i.e., high number of phagocytes in this case) leads to the decrease of parasite infection. However, Viney et al. [80] highlighted the fact that due to the autoimmunity, “immunologically more” may not mean necessarily “better.” Further, it was proposed that rather than the intensity of immune responses, it is recommended to determine the optimal immune response, that is, the effective protection of individuals against infection as well as measurement of individuals’ fitness.

5. Conclusion

We conclude that the reproductive investments in fish related to breeding play an important role for energy allocation among condition, immunity, and reproduction. The investments in those traits and parasitism differ among before-breeding, breeding, and after-breeding periods. The high values of immune parameters in breeding did not reflect a current infection by metazoan parasites but probably reflect the stress due to spawning or may be caused by protozoan or viral infection. A high reproductive effort associated with spawning leads to higher digenean infection in more ornamented individuals and/or males possessing the sperm of better quality.

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Review Article

Immunological and Therapeutic Strategies against Salmonid Cryptobiosis

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Salmonid cryptobiosis is caused by the haemoflagellate, *Cryptobia salmositica*. Clinical signs of the disease in salmon (*Oncorhynchus* spp.) include exophthalmia, general oedema, abdominal distension with ascites, anaemia, and anorexia. The disease-causing factor is a metalloprotease and the monoclonal antibody (mAb-001) against it is therapeutic. MAb-001 does not fix complement but agglutinates the parasite. Some brook charr, *Salvelinus fontinalis* cannot be infected (*Cryptobia*-resistant); this resistance is controlled by a dominant Mendelian locus and is inherited. In *Cryptobia*-resistant charr the pathogen is lysed via the Alternative Pathway of Complement Activation. However, some charr can be infected and they have high parasitaemias with no disease (*Cryptobia*-tolerant). In infected *Cryptobia*-tolerant charr the metalloprotease is neutralized by a natural antiprotease, $\alpha 2$ macroglobulin. Two vaccines have been developed. A single dose of the attenuated vaccine protects 100% of salmonids (juveniles and adults) for at least 24 months. Complement fixing antibody production and cell-mediated response in vaccinated fish rise significantly after challenge. Fish injected with the DNA vaccine initially have slight anaemias but they recover and have agglutinating antibodies. On challenge, DNA-vaccinated fish have lower parasitaemias, delayed peak parasitaemias and faster recoveries. Isometamidium chloride is therapeutic against the pathogen and its effectiveness is increased after conjugation to antibodies.

1. Introduction

Fish has been and will continue to be one of the major sources of animal protein for humans. It will likely become more important as the population heads towards 8 billion in about 20 years as food production (e.g., growing of crops, breeding of domestic animals) has and will continue to compete with other human activities (e.g., transportation, housing, industry) for the limited usable/inhabitable land. Besides being a more affordable animal protein many species of marine fishes have beneficial health components which include the polyunsaturated fatty acids (e.g., Omega 3). However, the capture-fishery is either stagnant or has been in decline as natural fish stocks in many parts of the world have been reduced significantly because of over and/or indiscriminate fishing and/or the destruction of spawning grounds. Many undesirable discharges (e.g., organophosphates, heavy metals) into the aquatic environments, especially from industries, are known to reduce fish survival

and reproduction. In some areas fish are no longer suitable for human consumption because of the high levels of accumulated pollutants, and no new fishing grounds have been discovered. According to the Food and Agriculture Organization, aquaculture continues to be the fastest food producing sector with about a 10% annual increase. It would be higher if not for disease outbreaks [1]. Intensive culture of freshwater and marine fishes in cages is well developed in many countries, especially in those that have large numbers of rivers and lakes and/or long coastlines (e.g., China, Chile, Norway). However, disease outbreaks become more frequent as intensive fish culture tends to facilitate the transmission of parasites between fish in cages and the acquisition of pathogens from feral fishes that are attracted to the uneaten food in cages [2].

The piscine immune system is well developed, and in many ways it is similar to that in mammals (e.g., [3]) which include a comparable set of immunocompetent cells [4]. In general, the adaptive immune response is slower to

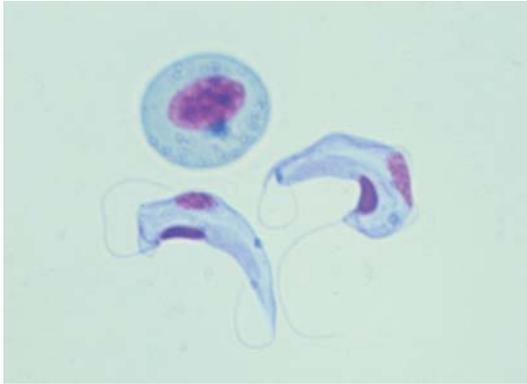


FIGURE 1: *Cryptobia salmositica* with red cell from an experimentally infected rainbow trout (reproduced from Woo [5]).

develop in fish than in mammals, and this is in part due to its lower body temperature. However, the innate immunity in fish is as well developed and is as responsive as that in mammals. The present discussion is in two parts; the first part (Section 2) is a brief review on *Cryptobia* and the pathobiology in cryptobiosis—information which are relevant to the discussion on the development of strategies against the pathogen and disease (Section 3).

2. *Cryptobia salmositica* and Cryptobiosis

2.1. The Parasite. *Cryptobia* is a parasitic flagellate that has worldwide distribution, and a few species are known to cause disease in marine and freshwater fishes. This extracellular parasite is elongated and is a little larger than a fish red blood cell. Its nucleus is close to the kinetoplast which is located at the anterior end. The parasite has an anterior flagellum and a recurrent flagellum that attaches to the body and exit as a free flagellum at the posterior end [5].

Salmonid cryptobiosis is caused by the haemoflagellate *Cryptobia* (*Trypanoplasma*) *salmositica* (Figure 1). The pathogen has been reported in all species of Pacific salmon, *Oncorhynchus* spp., along the west coast of North America [5], and outbreaks of cryptobiosis with high fish mortalities have occurred in both freshwater hatcheries and in sea cage cultures [6]. The parasite multiplies by binary fission, and the parasitaemia peaks at about 4–5 weeks after infection (e.g., [7–9]). The severity of the disease (e.g., the anaemia) is directly related to the parasitaemia and clinical signs include exophthalmia (Figure 2), general oedema, abdominal distension with ascites, a microcytic and hypochromic anaemia, positive antiglobulin reaction (or positive Coombs' test) of red cells (Figure 3), and anorexia [7, 10, 11]. Anorexia is a double-edged sword—it is beneficial to the host in that it reduces the severity of the disease by lowering plasma proteins and subsequently the parasitaemia but it is also detrimental to the fish in that it contributes to the immunodepression [11, 12]. During acute disease the haemolytic activity of complement is significantly lowered [13]. In addition, plasma thyroxine (T3 and T4),



FIGURE 2: Exophthalmia in rainbow trout with an acute experimental cryptobiosis (reproduced from Woo [5]).



FIGURE 3: Positive antiglobulin reaction; red blood cells from an experimentally infected rainbow trout (reproduced from Woo [5]).

protein, and glucose are reduced along with depletion of liver glycogen [14]. The metabolism and swimming performance of infected rainbow trout are also significantly reduced [15], and the bioenergetic cost of the disease in juvenile fish is considerable. These are contributing factors to the retarded growth as there are significant reductions in food consumption, dry weight and energy gained, energy concentration, and gross conversion efficiency. However, the attenuated vaccine strain (Section 3.3.1) has no detectable bioenergetic cost to juvenile fish [16].

2.2. A Cysteine Protease and a Metalloprotease. Two proteases have been identified in the pathogen [17]. The cysteine protease consisting of four polypeptide bands (49, 60, 66, and 97 kDa) is a metabolic enzyme while the metalloprotease (200 kDa) is a histolytic enzyme. The metalloprotease has been isolated and purified (Figure 4). Its proteolytic activity is inhibited by excess of zinc ions [17–19]. The purified enzyme lyses red blood cells [20] by digesting erythrocyte membranes [18]. Consequently, it is an important contributing factor to the anaemia which is a very consistent clinical sign of the disease. Also, the purified metalloprotease readily degrades types I, IV, and V collagens (Figure 5) and laminin [18]. It is secreted by the parasite in fish [21] and in culture [19], and it contributes significantly to the development

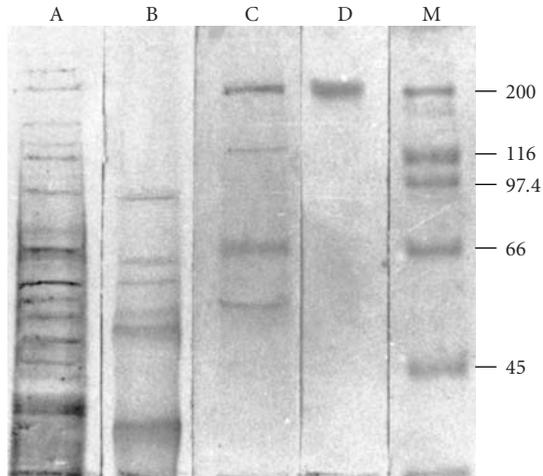


FIGURE 4: Purification of cysteine protease and metalloprotease from *Cryptobia salmositica*. Lane A: crude cell lysate; lane B: partially purified cysteine protease from a DEAE-agarose column; lane C: partially purified metalloprotease from a DEAE-agarose column; lane D: a single band of purified metalloprotease from a Sephacryl S-300 column; lane M: molecular markers (kDa) (reproduced from Zuo & Woo [18]).

of the disease and histopathological lesions in infected fish [22].

Briefly, metalloprotease activities can be neutralized by either a monoclonal antibody (Section 3.1) or a natural antiprotease (Section 3.2.2) or the antibody against the DNA vaccine (Section 3.3.2); this neutralization essentially “disarms” the pathogen so that the host immune system can more readily control the infection.

3. Strategies against *Cryptobia* and Cryptobiosis

3.1. Serological. A murine IgG1 monoclonal antibody (mAb-001; Figure 6) has been produced against the 200 kDa glycoprotein (Cs-gp200). The antibody is therapeutic when injected intraperitoneally into infected fish—it significantly lowers the parasitaemias in fish and this is similar to the effects of the inoculation of antisera from fish that had recovered from cryptobiosis. Also, mAb-001 has prophylactic effects in fish [23]; however, it does not fix complement but agglutinates live parasite. In vitro exposure of the parasite to mAb-001 reduces its survival and infectivity when inoculated back into fish [24]. The monoclonal antibody also inhibits parasite multiplication and its aerobic respiration [25], and it completely neutralizes the activity of the metalloprotease [26]. The Cs-gp200 epitope consists of carbohydrate determinants and conformational polypeptide with internal disulphide bonds. It is hydrophilic and is secreted by the parasite [27]. The epitope has its asparagine-bound N-glycosidically linked hybrid-type carbohydrate chain with the minimum length of a chitobiose core unit. It has a phosphatidylinositol residue which anchors the conformational polypeptide (with disulphide bonds) to the surface of the pathogen. The

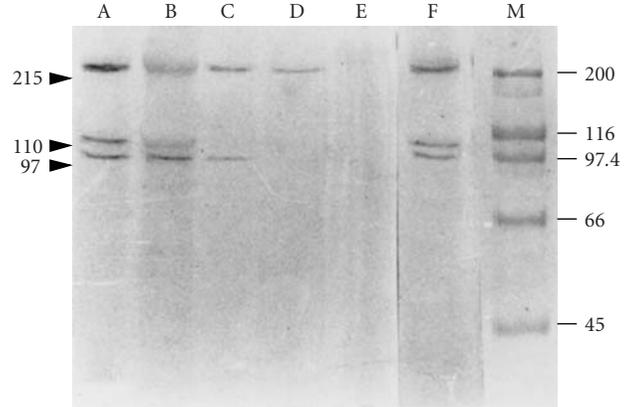


FIGURE 5: In vitro proteolytic degradation of collagen type V by purified metalloprotease from *Cryptobia salmositica*. Lanes A–E: collagen incubated with metalloprotease for 0, 2, 4, 6, and 8 hours respectively; lane F: collagen incubated under same conditions as lanes A–E, but without metalloprotease (control); lane M: molecular markers (reproduced from Zuo and Woo [18]).

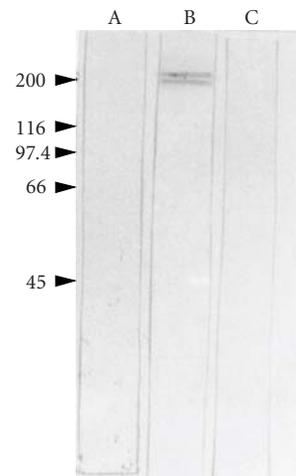


FIGURE 6: Immunoblot using mAb-001 on *Cryptobia salmositica* lysate. Lane A: mAb-001 after immunoabsorption with live parasites; lane B: mAb-001 without immunoabsorption; lane C: hybridoma culture medium. The numbers on the left are molecular mass (kDa) of protein standards.

molecule is extensively posttranslationally modified [28], has high mannose components, and appears as a doublet in the pathogenic strain and as a single band in the attenuated vaccine strain [29].

3.2. Innate (Natural) Immunity. In the present discussion, a distinction is made to distinguish between the two forms of natural immunity—the first is absence of disease in an infected fish (pathogen-tolerant fish) and the second is resistance to infection by a fish (pathogen-resistant fish).

3.2.1. Cryptobia-Resistant Fish. Some laboratory/hatchery raised brook charr, *Salvelinus fontinalis*, cannot be infected

with *C. salmositica*, and this is innate resistance to infection. Resistance to *Cryptobia* infection is inherited by progeny and it is controlled by a single dominant Mendelian locus. *Cryptobia*-susceptible brook charr are homozygous recessive while the *Cryptobia*-resistant fish are either homozygous or heterozygous dominant for the locus [30]. Consequently, we can now breed resistant fish by initially testing the freshly collected plasma from the brood fish for cryptobiocidal effects. Briefly, under in vitro conditions fresh plasma from *Cryptobia*-resistant charr lyse the parasite via the Alternative Pathway of Complement Activation [31]. There is no detectable difference in the immune responses of both *Cryptobia*-tolerant (Section 3.2.2) and *Cryptobia*-resistant charr to other antigenic stimulations including a commercial vaccine [32]. Not much is known about this type of resistance and its inheritance by progeny; consequently, further studies on innate resistance to infections in other animals would be most rewarding because it can be a good strategy against some pathogens.

3.2.2. *Cryptobia*-Tolerant Fish. Parasitaemias in some infected brook charr are just as high as those in *Oncorhynchus* spp.; however they do not suffer from cryptobiosis (*Cryptobia*-tolerant fish). *Cryptobia*-tolerant brook charr are resistance to disease because the metalloprotease secreted by *C. salmositica* is neutralized by the $\alpha 2$ macroglobulin (a natural antiprotease) in the blood. The amount of $\alpha 2$ macroglobulin is higher in brook charr than in rainbow trout prior to infection and it remains high (about 40%) even at peak parasitaemia while that in trout drops to about 12% [21, 33]. Parasitaemias in both infected rainbow trout and brook charr peak at about 4–6 weeks after infection and as antibodies are produced the parasitaemias decline; however, the parasitaemia fluctuates in rainbow trout while that in infected *Cryptobia*-tolerant charr rapidly declines after peak parasitaemia [32]. Neutralization of the metalloprotease by $\alpha 2$ macroglobulin was demonstrated under both in vivo and in vitro conditions [18, 21, 33]. Since *Cryptobia*-tolerant charr do not suffer from clinical disease, the immune system readily controls the infection and the fish recover much more rapidly than trout from the infection [32].

An obviously option to control cryptobiosis in salmon is to consider producing transgenic *Cryptobia*-tolerant salmonids. It is expected that the transgenic salmon will maintain high levels of $\alpha 2$ macroglobulin in their blood, essentially to neutralize the metalloprotease secreted by the pathogen—the additional $\alpha 2$ macroglobulin will eliminate or at least reduce the severity of the disease. Since the disease is absent or less severe, the fish immune system can more effectively control the infection. This proposal is a novel approach to the management of an infectious disease in animals and it perhaps needs further discussions. An obvious “downside” with this approach is that it may increase the pool of reservoir animals (with infections but no disease) in the population but one very obvious advantage is that no further human interventions (e.g., vaccination,

chemotherapy) are required once the transgenic animal is produced.

3.3. Adaptive (Acquired) Immunity. Adaptive immunity has also been exploited to protect the susceptible *Oncorhynchus* spp. from cryptobiosis. Two distinctly different experimental vaccines (a live attenuated vaccine and a metalloprotease-DNA vaccine) have been developed. Fish inoculated with the live attenuated *Cryptobia* vaccine are protected from infection when challenged with the parasite. However, the metalloprotease-DNA vaccine does not prevent an infection in vaccinated fish but antibodies produced in the vaccinated fish neutralize the disease-causing factor secreted by the pathogen. Although the DNA-vaccinated fish is infected, it does not suffer from cryptobiosis, and it essentially turns the pathogenic *Cryptobia* into a nonpathogenic flagellate as in the case of the *Cryptobia*-tolerant brook charr (Section 3.2.2).

3.3.1. Live Vaccine. *C. salmositica* was attenuated by prolonged in vitro culture and the strain has been cloned. The attenuated parasite is maintained in tissue culture medium and it has remained avirulent since 1990. It produces a low infection in rainbow trout, does not cause disease, circulates in the blood for at least 6 months, and is protective when the fish is challenged with the pathogen [34]. The vaccine strain is smaller in size and has lost a few polypeptides. It is adapted to in vitro culture and hence multiplies much more readily than the pathogenic strain in tissue culture medium [35, 36]. A single injection of the strain protects 100% of vaccinated fish and consequently it is used routinely as an experimental vaccine to study the development and mechanism of protective immunity in salmonids and the pathobiology of the disease. The vaccine has no detectable bioenergetic cost to juvenile rainbow trout [16], and it protects various species of juvenile and adult salmonids from the pathogen (e.g., [8, 9, 37–41]).

Rainbow trout vaccinated in fresh water and transferred to sea water are also protected on parasite challenge [42]. A single dose of the vaccine protects rainbow trout for at least 24 months [8]. Vaccinated fish are partially protected if they are challenged at 2 weeks postvaccination (wpv) while all vaccinated fish are protected (e.g., no drop in packed cell volume and virtually no detectable infection after parasite challenge) at 4wpv. Protection is via the production of complement fixing antibodies and under in vitro conditions activated macrophages from head kidneys of vaccinated fish show antibody-independent and antibody-dependent cytotoxicities. Also, in the presence of antiserum macrophages are very efficient in engulfing living parasites (Figure 7).

The complement fixing antibody titres (e.g., [8]) and cell-mediated response (e.g., [40]) in vaccinated fish rise significantly soon after parasite challenge (classical secondary responses), and the responses in vaccinated and challenged fish are similar to those in naïve fish at 6 weeks after infection. Humoral and cell-mediated immunity are important

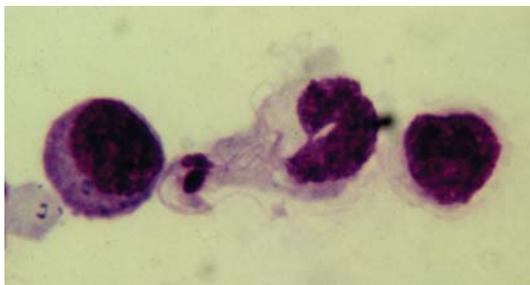


FIGURE 7: Peritoneal macrophage in the ascites of an experimentally infected rainbow trout, *Cryptobia salmositica* in the process of being ingested (reproduced from Woo [7]).

components of the protection against *C. salmositica* in both vaccinated and recovered fish (e.g., [8, 9, 40, 43]).

3.3.2. Metalloprotease-DNA Vaccine. As indicated earlier (Sections 3.1 and 3.2.2) the disease causing metalloprotease (200 kDa glycoprotein) can be neutralized. This is accomplished either by the $\alpha 2$ macroglobulin (a natural antiprotease) in *Cryptobia*-tolerant brook charr [18, 21, 33] where the parasitaemia does not fluctuate and the fish recovers rapidly [32] or by an antibody (mAb-001) produced against the 200 kDa glycoprotein [26]. The monoclonal antibody (mAb-001) agglutinates the parasite and reduces its survival and infectivity [24]. Neutralization of the metalloprotease by antibodies in vaccinated fish is the basis of our current DNA vaccine.

Briefly, the metalloprotease and cysteine protease genes of *C. salmositica* were sequenced [45, 46] and inserted into plasmid vectors (pEGFP-N) to produce a metalloprotease-plasmid vaccine and a cysteine-plasmid vaccine [47]. Rainbow trout and Atlantic salmon, *Salmo salar*, injected intramuscularly with the metalloprotease-plasmid vaccine consistently had lower packed cell volume (as metalloprotease was secreted into the blood) than controls (fish inoculated either with plasmid alone or with cysteine-plasmid vaccine) at 2–4 wpv. However, the packed cell volume in metalloprotease-vaccinated fish returned to normal by 5 wpv—this was because the metalloprotease was neutralized as antibodies were produced. Agglutinating antibodies against *C. salmositica* were detected 5–7 wpv in the blood (and not before 5 wpv) in metalloprotease-vaccinated fish, but not in fish injected with either the cysteine-plasmid or plasmid alone injected fish. Fish were challenged with the pathogen at 7 wpv and the metalloprotease-vaccinated fish had lower parasitaemia, delayed peak parasitaemia, and faster recovery than control infected fish. In a recent review on the use of DNA vaccines in aquatic organisms Kurath [48] confirms that this is the “... first published demonstration of protective effects of a fish parasite DNA vaccine in fish.”

Many protozoa that are of medical and economic importance (e.g., *Trypanosoma* spp., *Leishmania* spp.) have metalloprotease and cysteine protease [49]. Some of these pathogens also modify their surface coats to evade the host immune response (e.g., antigenic variations as in the

Glossina-transmitted mammalian trypanosomes in Africa); consequently vaccines based on surface membrane epitopes are not effective. However, an enzyme-based vaccine may be worth serious considerations as enzymes are quite conserve. Also, an enzyme-based vaccine will most likely protect against all isolates of the pathogen including those from different geographical locations which may have different surface membrane antigens. Enzymes are generally quite conserve and this is true even among different pathogens; for example, sequences of PCR-derived fragments of the metalloprotease gene of *C. salmositica* [45] are similar to those in other kinetoplastids, such as *Leishmania chagasi*, *L. donovani* [50], *Trypanosoma cruzi* [51], and *T. brucei* [52]. Major surface protease (MSP) also known as GP63 or leishmanolysin is a highly abundant zinc metalloprotease present on the cell surface of *Leishmania* spp. The NCBI-conserved domain search shows that the alignment for the metalloprotease of *C. salmositica* has 78.3% similarity with peptidase_M8, leishmanolysin domain [45].

3.4. Chemotherapy and Immunochemotherapy. Chemotherapy is essentially differential toxicity; that is, the drug is more toxic to the target organism than it is to the host. Severity of the side effects of chemotherapy is dependent partly on tissue damage and adverse reactions by the host to the drug. However, the drug can be directed more specifically to the pathogen if it is conjugated to an antibody specific for the target organism. Immunochemotherapy will obviously increase costs and is generally not meant for routine use; it may however be a useful tool under certain circumstances as it reduces the drug dosage and its side effects. For example, it can be used to treat infected brood fish as about 50% of brood fish annually die from cryptobiosis in some hatcheries on the west coast of North America [6]. It is expected the side effects and accumulation of drug residues in host tissues will be reduced in immunochemotherapy, and this may also lower the risk of the development of drug-resistance by the pathogen. Reduction in drug residue in host tissues is also an important consideration if the fish are for human consumption.

3.4.1. Chemotherapy. In tropical Africa isometamidium chloride (Samorin) is widely used against trypanosomiasis in domestic animals [53], and it is also used as a prophylactic drug against bovine trypanosomiasis [54]. In fish, Samorin (1.0 mg/kg weight) reaches peak level in the blood 2–3 weeks after intramuscular injection [55]. The drug is therapeutic against *C. salmositica* in rainbow trout during pre- and post-clinical phases of the disease. However, it is not effective during acute disease partly as we believe that the drug “modifies” surface epitopes of the parasite so that they are not lysed by complement fixing antibodies [56]. The drug is more effective in infected Atlantic salmon, and at a higher dose (2.5 mg/kg) the infection is eliminated in about 30% of adult fish and significantly reduces the parasitaemias in remaining fish. Also all infected juvenile chinook salmon, *Oncorhynchus tshawytscha*, treated with isometamidium chloride (1.0 mg/kg) survived the disease

TABLE 1: Infectivity of *Cryptobia salmositica* to chinook salmon after in vitro exposure to isometamidium chloride to polyclonal antibodies from a recovered fish (reproduced from Ardelli and Woo [59]).

Weeks After Infection	Group 1 PAIC	Group 2 Drug	Group 3 PAI	Group 4 Antibody	Group 5 Untreated Controls
1	1/10 • 0.30 ± 0.95 ■	7/10 1.80 ± 2.78	0/10 0	0/10 0	8/10 6.60 ± 4.72
2	1/10 0.30 ± 0.95	6/10 8.10 ± 5.28	2/10 1.80 ± 3.91	0/10 0	10/10 48,750 ± 45,814
3	2/10 0.50 ± 1.27	7/10 33,250 ± 38,207	2/10 1,950 ± 4,310	0/10 0	10/10 35,625 ± 29,978
4	2/10 0.30 ± 0.675	8/10 302,000 ± 254,142	3/10 2,500 ± 5,270	3/10 5,558 ± 16,665	10/10 5,487,500 ± 5,439,838
5	2/10 0.60 ± 1.58	10/10 9,395,000 ± 16,925,911	4/10 54,740 ± 112,499	4/10 556,944 ± 1,500,001	10/10 3,175,000 ± 3,639,196
6	1/10 3,750 ± 11,858	10/10 13,475,000 ± 15,298,624	4/10 503,750 ± 1,030,810	4/10 2,600,000 ± 5,577,465	10/10 3,243,750 ± 5,416,196
7	1/10 11,250 ± 35,575	10/10 18,305,000 ± 52,500,000	4/10 928,860 ± 1,326,575	4/10 44,383,334 ± 129,150,260	10/10 27,051,250 ± 56,209,714

• Number of infected fish/number of fish inoculated.

■ Mean parasitaemia ± standard deviation, determined by HCT or haemocytometer.

while 100% of untreated infected fish died with massive parasitaemias. The drug also has prophylactic value, and it does not seem to affect fish growth, food consumption, blood complement levels, or haematocrit values in fish [57].

Samorin accumulates rapidly in the kinetoplast of the parasite [44], causes condensation of its kinetoplast DNA, forms vacuoles, and swells the mitochondrial cristae (Figure 8). Although the parasite normally undergoes aerobic respiration [25], it also has glycolytic enzymes sequestered in microbodies called glycosomes [58]. The in vitro oxygen consumption and carbon dioxide production decrease significantly after drug exposure with very significant increases in secretion of glycolytic products (lactate and pyruvate) as the parasite switches from aerobic respiration to glycolysis after its mitochondrion is damaged by the drug [44]. Also, in vitro exposure to sublethal levels of the drug reduces infectivity of the parasite to fish and changes the surface glycoprotein antibody-receptor sites of the parasite. This alteration of surface epitopes explains the protection of some parasites from lysis by complement fixing antibodies when rainbow trout with acute infections were treated with the drug [56].

3.4.2. Immunochemotherapy. Ardelli and Woo [59] conjugated isometamidium chloride to polyclonal antibodies (from “recovered” fish) and the monoclonal antibody (mAb-001, Section 3.1). The conjugated drug is on the entire parasite while the unconjugated drug accumulates only in the kinetoplast (Figure 9). Before drug conjugation both antibodies agglutinate living parasites but they react differently after drug conjugation. Polyclonal antibodies-conjugated drug (PAIC) lyses most of the parasite and it no longer agglutinates the parasite. In contrast, the mAb-00-conjugated drug does not lyse *C. salmositica* but agglutinates it. After in vitro

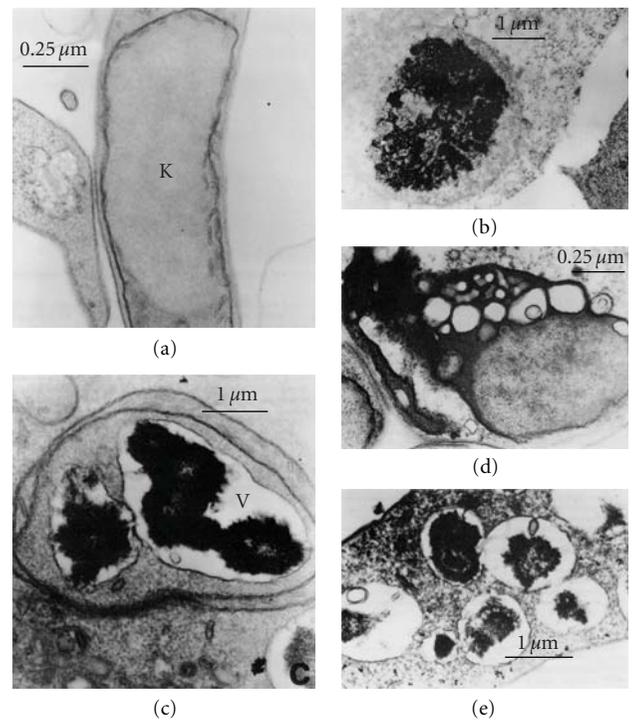


FIGURE 8: Ultrastructural lesions in *Cryptobia salmositica* after in vitro exposure to isometamidium chloride. (a) Parasite kinetoplast (K) not exposed to the drug; (b) condensation of kinetoplast DNA after exposure to the drug; (c) vacuole (V) formation after drug exposure; (d) swelling of mitochondrial cristae (C) after drug exposure; (e) vacuole formation in cytoplasm after drug exposure (reproduced from Ardelli and Woo [44]).

exposure to PAIC the infectivity of the parasite and subsequent parasitaemias in inoculated fish were significantly

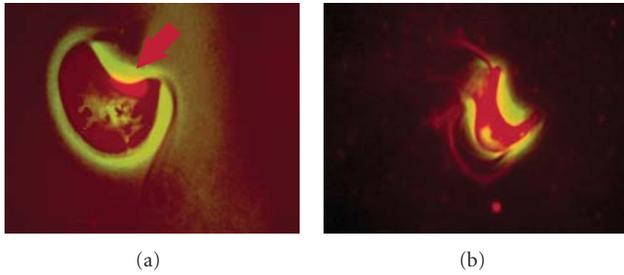


FIGURE 9: Phase contrast and fluorescent microscopy of *Cryptobia salmositica* after exposure to isometamidium chloride. (a): Exposure to drug only, note accumulation of drug (in red) in the kinetoplast; (b): exposure to drug conjugated to polyclonal antibodies from a recovered fish, note that the drug (in red) is throughout the organism (reproduced from Ardelli and Woo [59]).

lowered. Fish survival (Table 1) was much higher in juvenile chinook salmon infected with parasites exposed to the polyclonal antibodies-conjugated drug (PAIC) than to drug alone (Drug) or to polyclonal antibodies alone (Antibody) or to drug plus polyclonal antibody (PAI). Also, preliminary studies indicate the drug-antibody conjugate to be effective when injected into infected fish. The results are encouraging and further studies are needed, for example, to determine dosages needed, refinement of the approach (e.g., stage of infection, species of salmonids).

4. Conclusions

Our concerted efforts to better understand the biology of *Cryptobia* and the mechanism of the disease have allowed us to develop more rational strategies against the pathogen and disease. We have been relatively successful in exploiting the piscine immune system to protect salmonids against *C. salmositica* and cryptobiosis. This is an ongoing and evolving research program and there is obviously a great deal of work that needs to be conducted. However, I hope our research will be of interest and perhaps be useful to colleagues who are also developing control measures against similar pathogenic organisms. The research has been both challenging and fascinating, and I would like to think the best is yet to come.

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Review Article

Dendritic Cells in the Gut: Interaction with Intestinal Helminths

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The mucosal environment in mammals is highly tolerogenic; however, after exposure to pathogens or danger signals, it is able to shift towards an inflammatory response. Dendritic cells (DCs) orchestrate immune responses and are highly responsible, through the secretion of cytokines and expression of surface markers, for the outcome of such immune response. In particular, the DC subsets found in the intestine have specialized functions and interact with different immune as well as nonimmune cells. Intestinal helminths primarily induce Th2 responses where DCs have an important yet not completely understood role. In addition, this cross-talk results in the induction of regulatory T cells (T regs) as a result of the homeostatic mucosal environment. This review highlights the importance of studying the particular relation “helminth-DC-milieu” in view of the significance that each of these factors plays. Elucidating the mechanisms that trigger Th2 responses may provide the understanding of how we might modulate inflammatory processes.

1. Introduction

Dendritic cells (DCs) were first identified around 140 years ago. Nevertheless their role in immunology was discovered more than 100 years later, in 1973; and, since then, ongoing research on their localization, phenotypic characterization, and functions is still taking place [1]. DCs in the intestine show two major paradoxical roles. On one hand they are responsible for the generation of immunological tolerance in absence of infection or danger signals, albeit the presence of a myriad of intestinal commensal microorganisms and alimentary antigens; and, on the other hand, they must be able to react and induce specific immune responses against pathogens [2, 3]. Intestinal helminths constitute a cause of high morbidity and chronic infections in humans [4]. Chronicity is probably associated with immunomodulation of the intestinal immune response. In this context, current evidence indicates that helminth products are capable of inhibiting *in vitro* generated DCs [5–7]. While there is a vast amount of studies on DCs and gut bacteria [8, 9], only few publications describe the interaction of intestinal

parasites and these cells. Since DCs from intestine have inherent characteristics regarding their ability to induce Th2 responses [2, 3, 10], the study of this particular interaction is of fundamental importance. The cross-talk between intestinal helminths or their excretion/secretion (E/S) products and the different DC subsets present in the gut, which result in the induction of Th2 responses and regulatory T cells (T regs) in that milieu, is reviewed.

2. Intestinal Immunity

The first line of defense against pathogens and exogenous antigens present in the gastrointestinal (GI) tract includes the physical barrier provided by epithelial cells (enterocytes), the tight junctions formed between them, the ciliary movements, the mucus secretion by goblet cells on the apical portion of the mucosa, microfold (M) cells, the cytotoxic activity of natural killer cells, as well as the production of antibacterial peptides by Paneth cells that include defensins, lysozyme, and secretory phospholipase A2. The adaptive immune system in the GI tract includes the GI-associated lymphoid tissue

(GALT), comprising Peyer's patches (PPs), isolated lymphoid follicles (ILFs), and diffuse lymphoid cells present along the lamina propria (LP) in the small intestine. In the large intestine, caecal and rectal patches are similar to individual follicles of the PPs. Cryptopatches (CPs), aggregates of lymphocytes and DCs should also be considered part of the immune system, since they are present throughout both the small and large intestines, in the LP between the crypts [11]. CPs have been shown to develop into ILFs recruiting B cells, in response to Gram-negative commensal bacteria through recognition of NOD-1 (nucleotide-binding oligomerization containing domain 1) and toll-like receptors resulting in generation and maturation of B cells, respectively. In addition, alterations in gut microflora composition occur in mice that lack NOD-1 showing reciprocal regulation of the intestinal microflora and ILFs which in turn affects intestinal homeostasis [12]. These findings can significantly contribute to development of novel treatments for pathologies involving intestinal inflammation through the modulating capacity of commensal by-products. The gut epithelium, lamina propria, and intraepithelial lymphocytes (IELs) represent the lymphoid effector sites, while mesenteric lymph nodes (MLNs), PPs, and ILFs are considered inductive sites where immune responses are initiated. Immune cells migrate from LP and PPs via efferent lymphatics to MLN and finally reach effector sites, traveling through the thoracic duct and blood circulation.

PPs consist of collections of closely associated lymphoid follicles. The epithelium that covers these aggregates, termed follicle-associated epithelium, is characterized by the presence of specialized cells, M cells that lack cilia and take up antigens releasing them at the basal surface, where associated antigen presenting cells start their processing and latter presentation to lymphocytes. Just beneath the follicle-associated epithelium, PPs consist of a dome region, populated by T and B cells, macrophages, and different subsets of DCs. B cells that switch to IgA producing plasma cells that prevail in the germinal centers and TCR $\alpha\beta$ CD4⁺ T cells, which represent the predominant phenotype, are found in interfollicular regions accompanied by interstitial DCs. TCR $\gamma\delta$ and CD8⁺ T cells are also present. PPs contain both naïve and memory T and B cells whereas in the LP only memory cells and fully differentiated plasma cells are present. The spatial cell organization of these lymphoid organs makes them suitable to deal with both harmless and hazardous stimuli from incoming antigens. DCs represent one of the cell types in charge of differentiating between these two completely opposite phenomena and regulating the outcome of the immune response [2, 13].

Afferent lymphatics drain the epithelium and LP from both the small and large intestines to MLNs which are organized along the mesenteric side of the intestine and drain succeeding sections of the gut. Like other lymph nodes, MLN consist of B cells that are organized as lymphoid follicles in the cortex, while T cells and DCs shape the paracortical areas. Efferent lymphatic vessels leave the lymph node from the medulla and drain to the thoracic duct and finally to the systemic circulation. Stromal cells in MLN are important in imprinting antigen primed T cells with the capacity to

express $\alpha 4\beta 7$ integrin and chemokine receptor 9 (CCR9) that are essential molecules in gut homing. Expression of these two molecules is dependent on retinoic acid and stromal cells from MLN, but not from peripheral LN [14, 15]. In addition, MLNs have been shown to be important in the regulation of IgA responses since removal of these organized lymphoid structures results in an enhanced IgA response to orally administered antigens [16]. Thus, MLNs contribute to gut homeostasis and effector mechanisms by providing regulatory environmental factors.

Besides the organized tissues, cells in diffuse compartments, that is, cells in LP and IEL, are important players in mucosal immunity. Memory/effector cells home to the LP and plasma cell differentiation is accomplished in this compartment. LP stromal cells support IgA secreting plasma cells generation from B220⁺IgM⁺ lymphocytes [17]. The majority of IELs are T lymphocytes expressing CD3 and the T cell receptor, either $\alpha\beta$ or $\gamma\delta$. Most IELs in the small intestine are CD8⁺ and are able to express regulatory properties and secrete IL-10 and TGF- β . In addition, IELs are able to secrete cytokines and chemokines as well as cytotoxic molecules during inflammatory conditions contributing to the dual function of mucosal immunity in tolerance and defense [18].

3. DC Subsets

Several hematopoietic DC subsets have been described in mice and are often classified on the basis of the surface receptors they express, developmental origin, anatomical localization, migratory and functional properties, and their maturation state. Such heterogeneity makes a single classification of DCs rather difficult; nonetheless, some common phenotypical and functional characteristics are used to define DCs, besides their prominent dendritic appearance, both in situ and in vitro: (i) CD11c expression, (ii) endocytic ability, (iii) high expression of MHC-II molecules, and (iv) strong capacity to activate naïve T cells [19]. Hematopoietic DCs are subdivided in two main subsets: plasmacytoid DCs (pDCs) and conventional DCs (cDCs) [20]. A distinctive feature of pDCs is their rapid and very high production of type I IFN when properly stimulated (virus, DNA, etc.). When activated, expression of MHC-II and costimulatory molecules is upregulated. Compared to conventional DCs, pDCs are less efficient as antigen presenting cells, although they are able to stimulate naïve T cells in addition to cells from the innate immune response (e.g., natural killer cells). pDCs express intermediate amounts of CD11c⁺ and are B220⁺. These DCs mature in the bone marrow, are found in all lymphoid organs and blood, and are capable of recirculating [21]. On the other hand, cDCs are also bone marrow derived but emerge with an immature phenotype characterized by low expression of MHC-II and costimulatory molecules and are present in different organs and lymphoid tissues. cDCs can be further subdivided in migratory and resident DCs. Migratory DCs include Langerhans cells in epidermis and interstitial DCs in all organs and the gut, mainly in their immature state. Upon encountering antigen they drain to regional lymph nodes acquiring a mature phenotype, and in the case of the gut, they travel into T cell areas of MLN. The second subset

of cDCs is the resident DCs that are the only type present in spleen and thymus and account for ~50% of the DC population present in lymphoid organs probably emerging from blood precursors and differentiating in situ [3, 20, 21]. Until they encounter pathogens, resident DCs express an immature phenotype. These cells apparently do not exit lymphoid tissues and can be further subdivided depending on surface marker expression. All these cDC subsets express both CD11c and MHCII [2, 20].

As mentioned above, both pDCs and cDCs are able to modify their phenotype and functional capabilities in response to external stimuli resulting in a further classification of DCs in immature (naïve) and mature DCs. These two different conformations result in the differential expression of surface markers. Immature cells express very low levels of costimulatory molecules such as CD80, CD86, and CD40. In this state, DCs are highly endocytic and express C-type lectin receptors such as Langerin/CD207 and DEC 205, involved in antigen capture and internalization. In addition, innate receptors involved in antigen recognition such as Toll-like receptors (TLRs) that recognize pathogen-associated molecular patterns (PAMPs) are also present and are differentially expressed in the various subsets. In their mature state, DCs upregulate costimulatory surface antigens which are important for T cells interaction and activation [22, 23].

4. Intestinal DCs

Along the small and large intestines, DCs are present in different locations. Both pDCs and cDCs are present in PPs, MLN, as well as in the small intestinal and colonic LP and ILFs. Various DC subpopulations expressing different surface markers and showing diverse functional properties can be identified (Table 1). Surface antigen expression can be associated with specific locations within the lymphoid tissue and importantly with functional characteristics. For example, in PPs, DCs present underneath the follicle-associated epithelium are mainly immature cells expressing low MHC-I and -II and costimulatory molecules and are highly macropinocytic and phagocytic in order to sample the environment and process antigens that have passed through M cells. In addition, these immature DCs express Toll-like, C-type lectin, and scavenger receptors which recognize PAMPs that, in the presence of microbial products and danger signals released by damaged tissue, are able to activate and induce their maturation. Mature DCs expressing high levels of MHC-II, CD80, CD86, and CD40 lose their endocytic ability and migrate from the subepithelial dome to T cell areas within the PPs or to the regional lymph nodes, where they can efficiently present antigen and activate CD4⁺ T cells [2, 3].

Several examples of the unique functional properties and specific location of particular DC subsets can be cited. For instance, CD8 α^+ DCs are mainly found in the interfollicular region and preferentially secrete IL-12, while CD11b⁺ DCs produce IL-10 upon stimulation and are localized mainly in the subepithelial dome of the PPs. Moreover, CD11b⁺ DCs induce T cells to secrete IL-4 and IL-10, and thus, are

important in regulating immunity as well as facilitating IgA-mediated humoral responses [10, 24]. Additionally, CD11b⁺ DCs in PPs and MLN internalize secretory IgA. Neither CD8 α^+ nor DCs isolated from peripheral lymph nodes or spleen show this particular feature [25].

The majority of cDCs found in the LP are CD11b⁺, although small numbers of CD8 α^+ and double-negative cells (CD8 α^- CD11b⁻) are present. Although CD11b⁺ cells are able to upregulate costimulatory molecules and prime specific T cells, when they are adoptively transferred into recipient mice, they induce a lower response and express mRNA for IL-10 and IFN- β . It is important to clarify that LP DCs were isolated from small intestine devoid of PPs but containing ILFs; thus, these DCs do not represent true LP DCs [26]. In a different study, a CD11c⁺ CD11b⁺ DC subset that produces retinoic acid and is able to induce differentiation of Th17 cells and IgA producing plasma cells in the LP, upon activation of TLR5, was identified [2, 27]. DCs isolated from LP could be contaminated with DCs present in ILFs; thus, immunohistochemical analysis should be performed to confirm which subsets are present in these two diffuse compartments. Interestingly, a CD103 expressing DC subset which constitutes ~25% of the total LP DC populations constitutively expresses IL-10 and favors the maintenance of tolerance by inducing, through TGF- β and retinoic acid dependent mechanisms, the generation of FoxP3⁺T regs in the intestine. These “regulatory DCs” are able to migrate to MLN in a CCR7 dependent manner [28–31]. Retinoic acid produced by CD103⁺ DCs imprints primed T cells and IgA expressing plasma cells to express gut homing receptors such as the integrin $\alpha 4\beta 7$ and CCR 9 [32]. Accordingly, this DC subset expresses retinal dehydrogenase 2 (RALDH2), a key enzyme involved in the formation of retinoic acid from retinaldehyde [27]. In addition, some LP DCs are able to form tight-junction-like structures with enterocytes and project their dendrites to sample antigens in the intestinal lumen, both in normal conditions and during infection; hence, their name is “periscoping DCs” [33]. As cDCs in mucosal sites, pDCs in the GALT are able to induce differentiation of T reg cells and upon CpG-induced maturation retain their regulatory capacity, suggesting the necessity of a strict regulatory environment in the gut [34].

Recently, a different population of DCs arranged as a planar network has been identified after a sheet separation technique that results in two layers: one facing the lumen and the other one the serosa. These DCs were localized in the muscularis layer close to the serosa and have surface antigens characteristic of an immature phenotype. Upon systemic or oral stimulation with LPS or bacteria, they are able to acquire a mature phenotype even though they are not close to the intestinal lumen. In addition, a higher frequency of these muscularis DCs was detected in the large intestine, where the concentration of commensal bacteria is higher, suggesting a possible association with microbial external stimuli and a possible immunoregulatory role [23]. Concerning the large intestine, DCs in the colon are very similar to the subpopulations present in MLN and reside mainly in the subepithelial region and in the isolated lymphoid follicles. These DCs express IL-10 in a constitutive manner, again

TABLE 1: Intestinal DC subsets.

DC subset	Organ	Functional characteristics	References
*CD11b ⁺ DCs (1) **CD8 α ⁺ DCs (2) ***DN DCs (3)	Peyer's patches	(1) During infection recruited to FAE from subepithelial dome. (2) Produce IL-10. (3) Produce IL-12 and drive IFN γ by T cells.	[2, 3, 19]
*CD11b ⁺ DCs **CD8 α ⁺ DCs ***DN DCs CD8 α ^{int} CD103 ⁺	Mesenteric lymph nodes	CD11b ⁺ cells show higher capacity to secrete IL-10 and prime Th2 cells. CD103 ⁺ DC migrate from LP in a CCR7 dependent manner.	[3, 28, 35]
CD11b ⁺ CD8 α ^{int} CD103 ⁺	Lamina propria	Constitutive expression of IL-10 and IFN- β . Extend their dendrites to sample bacteria through epithelial cell tight junctions.	[26]
CD11b ⁺ DCs CD8 α ⁺ CD103 ⁺ DCs CD8 α ⁻ CD11b ^{lo} CD11b ⁺ CD103 ⁺	Colon	Constitutive expression of IL-10. Maintenance of T cell homeostasis. Concentrated in isolated lymphoid follicles (ILFs) few in lamina propria.	[2]
MHCII ⁺ CD11c ^{mid} Dec 205 ⁺ , Dec 207, CD14, CD16/32	Muscularis layer	Not known. Possible correlation with increasing number of commensal bacteria.	[23]
CD11c ⁺ , MHCII ⁺ (Lymphatic or "veiled" DCs)	Lymphatic vessels draining the intestine	Transport of apoptotic bodies from intestinal epithelial cells to regional lymph nodes.	[36]
CD11c ^{mid} B220 ⁺ (plasmacytoid DCs)	Peyer's patches, Mesenteric Lymph nodes, and Lamina Propria (small intestine and colon)	PPs: production of type I IFN regulated by IL-10, TGF- β , and prostaglandin E ₂ . MLN: induction of T regs.	[34, 37]

*CD11c⁺ CD11b⁺ CD8 α ⁻ (CD11b⁺).

**CD11c⁺ CD11b⁻ CD8 α ⁺ (CD8 α ⁺).

***CD11c⁺ CD11b⁻ CD8 α ⁻ (double negative: DN).

suggesting a role in peripheral tolerance mechanisms [2]. Thus, DC subsets with immunomodulatory properties are well represented along the GI tract.

5. Intestinal DCs and Infection

Helminth infections affect more than three billion people worldwide, mostly in underdeveloped countries. The study of the immune response against geohelminths has revealed the importance of these parasites not only in the ability to induce Th2 responses, but also in the modulatory role of the parasite or its products on the immune system of the host. The protective host's immune response induced by helminths is mediated by Th2 cells involving IL-4, IL-5, IL-10, and IL-13 secretion, IgE, IgG1 production, and activation of effector cells such as mast cells, eosinophils, and basophils [5, 38, 39]. In addition, some helminths are able to persist in their hosts for many years by creating an antiinflammatory environment favorable to their survival and maintenance. DC modulation represents a potential mechanism by which helminths may exert their effects on the immune system.

In spite of increasing numbers of studies involving helminth products and DCs generated in vitro, few studies cope with the interaction of these parasites in the intestinal

milieu (see below). The significance of studying these interactions is highlighted by the fact that there is rising research aimed on the use of helminth infections or their products as therapy for autoimmune, allergic, or inflammatory diseases [6]. In particular, intestinal DC subsets have unique characteristics and the effects helminths may exert upon them probably depend on several parameters, which include (i) the DC subset, (ii) the helminth species, (iii) the excretory/secretory (E/S) products, and (iv) the regulatory environment in the gut. These interactions will result in the different immunomodulatory mechanisms, such as downregulation of costimulatory or antigen-presenting molecules, IL-10 and TGF- β secretion, and/or generation of T regs, depending on the biological players involved (Table 2).

The role of DCs and the phenotype induced during infection is essential in order to get insights of the cellular and molecular interactions in vivo, which include intestinal DC subsets within the appropriate environment, as well as the timing of production of E/S molecules by the established parasites. In mouse infections with *Trichuris muris*, an animal model for the human geohelminth, *T. trichiura*, the adult worms reside in the caecum and colon. IL-4 and IL-13 promote protection against the murine nematode in

TABLE 2: Effect of intestinal helminth infections or their products on DC function.

Intestinal helminth product	DC subset	DC response	References
<i>Ascaris suum</i>	High molecular weight components of adult worms	CD11c ⁺ MLN DCs	Downregulation of CD 40, CD 80, CD86, and MHCII induced by immunization. Production of IL-10. [40]
<i>Heligmosomoides polygyrus</i>	Infection	CD11c ⁺ Spleen and MLN DCs	Activation with upregulation of CD80 and CD86. Production of IL-10. Inhibition of protective response against bacteria and exacerbated colonic inflammation (passive transfer of Hp-DCs). [41, 42]
	E/S products	Bone marrow DCs	Inhibition of cytokine production. Inhibition of upregulation of CD40, CD86, and MHCII by TLR engagement. Induction of IL-10 producing T cells (regulatory T cells). [43]
<i>Necator americanus</i>	E/S products	Human monocyte-derived DC	Downregulation of CD86, CD1a, HLA-ABC, and HLA-DR resulting in a diminished capacity to induce T cell proliferation. [44]
<i>Nippostrongylus brasiliensis</i>	E/S products	Bone marrow DCs	Upregulation of CD86 and Ox40L. No effect on CD80 and MHCII. Production of IL-6, IL-10, and IL-12 p40. Inhibition of LPS-induced IL-12 p70. [45]
	Infection	CD11c ⁺ CD8 α ^{int} CD103 ⁺	Reduction of this subpopulation during infection. Reduction of CD86 and Dec 205. Reduced production of IL-12 and increase in IL-6, TNF α , and IL-10. In <i>H. polygyrus</i> infection reduction of this DC subset is more pronounced. [46]
<i>Trichuris muris</i>	Infection (E-J isolate)	CD11c ⁺ MLN DCs	Expansion of DCs correlates with worm expulsion. Production of IL-4, IL-13, and IL-10. [47–49]
	E/S products (S, J, E isolates)	Bone marrow DCs	S isolate induces higher levels of IL-6 and IL-10. Upregulation of CD 40 by all three isolates. [50]
	Infection	CD11c ⁺ MLN DCs	Thymic Stromal Lymphopoietin (TSLP) produced by intestinal epithelial cells (IECs) interacts with DCs inhibiting LPS-induced IL12/23p40 and increasing IL-13 production. [51, 52]

contrast to Th1 cytokines such as IFN γ , IL-12, and IL-18 that help maintain a chronic infection depending on the mouse strain [53, 54]. Resistance to infection has been associated with mobilization of colonic DCs to the epithelium since there is an increased number of DCs in resistant mice that developed a mature phenotype, compared to susceptible strains [55]. Accordingly, in a different study, the kinetics of DC numbers after *T. muris* infection revealed a correlation between increased numbers of CD11c⁺ B220⁻ DC in MLN and worm expulsion from the gut with the concomitant production of IL-4, IL-13, and IL-10 [47, 48]. IL-10 has been shown to be essential for the expulsion of *T. muris* from the gut, since IL-10 deficient mice fail to expel worms, resulting in a chronic infection [56]. CD11c⁺ DCs from PPs and MLN were shown to be responsible for the production of this cytokine although particular DC subsets were not

identified [49]. In contrast, in a different study, IL-10 and IL-6 production by bone marrow-derived DCs after activation with E/S was correlated to the capacity of a particular isolate of *T. muris* to survive in its host [50]. Production of IL-10 by DCs is also observed in MLNs of mice infected with the intestinal helminth *H. polygyrus* that induces a chronic infection in most inbred strains of mice [41]. Therefore, IL-10 production by DCs can induce worm expulsion or survival and this different outcome might be dependent on presence of other cytokines that might influence the Th1/Th2 balance. A very recent study analyzed the DC populations present in MLN during the absence or presence of infections with both *Nippostrongylus brasiliensis* (short-lived infection) and *Heligmosomoides polygyrus* (chronic infection). *N. brasiliensis* infection induces a reduction on CD8 α ^{int} CD11b⁻ subset and decrease in CD40 and

CD86 expression. The decline of this DC subset is more pronounced during *H. polygyrus* infection and could result from a lack of migration of this subset from LP. This subset represents the subpopulation that expresses the highest levels of CD103 and CD205. Concomitantly, cytokine production is altered; IL-12 production in response to LPS was reduced whereas IL-6, TNF α , and IL-10 secretion was upregulated [46].

6. DC Activation versus the “Default Hypothesis”

Generation of Th1 responses through activation of DCs by viral or bacterial products is much better understood than Th2 induction. It is known that binding of those products to TLRs through activation of MyD88 initiates signaling cascades that result in enhanced expression of MHCII (signal 1) and costimulatory molecules such as CD80, CD86 and CD40 (signal 2) with concomitant production of IL-12 (signal 3). In turn, for Th2 responses signals 2 and 3 have not yet been identified. Indeed lack of costimulatory molecules, upregulation, and IL-12 induction have led to propose a “default hypothesis” [57]. E/S products of helminths are able to affect DC maturation in different ways, either through their activation (upregulation of MHC-II and costimulatory molecules) or through the inhibition of their maturation (“default response”). This response is characterized by a diminished gene expression and no upregulation of classical costimulatory molecules, both types of responses (activation and inhibition of DC) results in a type 2 phenotype [39, 58]. For example, adult worm components from *Ascaris suum* have been studied for their ability to suppress the immune response. DC upregulation of MHC-II and costimulatory molecules is inhibited and consequently there is no lymphoproliferation. IL-10 was shown to play a crucial role in this phenomenon since the inhibitory effect was not observed in IL-10 deficient mice, suggesting that helminth components are able to affect the inductive phase of the immune response, probably through the generation of IL-10, compromising antigen presentation and T cell proliferation [40, 59]. Since this effect was shown in inguinal and peritoneal CD11c⁺ DCs of subcutaneously immunized mice, which might not be reflecting an actual infection in the intestine, it is necessary to study the role of IL-10 production by intestinal DCs in response to *A. suum* infection. *Necator americanus*-infected individuals also show significantly downregulated expression of CD86, CD1a, HLA-ABC, and HLA-DR, which reflects an immature phenotype that results in diminished ability to induce T cell proliferation [44]. Lack of DC maturation by E/S form *H. polygyrus* has also been reported. These DCs are able to induce the generation of CD4⁺CD25⁺ IL-10-producing T cells in vitro, thus inhibiting T cell proliferation and IFN- γ production. The authors suggest that E/S might suppress both Th1 and Th2 responses via the generation of regulatory T cells. In many cases helminth DCs are programmed to synthesize IL-10. The induction of T reg differentiation is an important mechanism by which, intestinal DCs, exposed to helminths, are able to

modulate immune responses [43, 60]. In fact, Foxp3⁺ T reg cells develop in the intestine driven by the LP DCs [30]. Products of other non intestinal helminths such as soluble egg antigen from *Schistosoma mansoni* and ES-62, the immunomodulatory filarial glycoprotein, similarly fail to induce DC activation [61, 62].

Several studies, where genomic and proteomic approaches were used to analyze Th2 polarizing DCs exposed to helminth parasites, have shown that few genes or proteins are expressed after stimulation [7, 62]. Although the general picture favors the “default hypothesis” with a limited gene expression, there are examples where, depending on the DC subset exposed, either to E/S products or to the parasite, the response varies (Table 2).

Interestingly, and in contrast to the effect of *H. polygyrus*, the E/S products mentioned above, which downmodulate maturation of in vitro generated bone marrow DCs, spleen and MLN-derived DCs obtained from *H. polygyrus*-infected mice, were shown to upregulate costimulatory molecules and synthesize IL-10 and IL-4 [41, 42]. Also, E/S products from *N. brasiliensis* are able to upregulate costimulatory molecules such as CD86, CD40, and OX40L and induce production of IL-6 and IL-10 [45]. These two examples contradict the “default hypothesis” and highlight the necessity of analyzing better these interactions, taking into account the intestinal microenvironment and the different pathways activated by the different DC subsets. Concomitantly, helminth stimuli involved should be considered, since different helminth parasites and their individual products may use different and possibly multiple mechanisms to induce modulation of DC functions.

Indeed, increasing evidence shows that C-type lectins are likely to play a role in conditioning DCs towards Th2 responses leading to the “alternate pathway model” [57]. Scavenger receptors are also pattern recognition receptors involved in pathogen recognition and facilitation of immune responses expressed in macrophages and DCs [63]. Scavenger receptor-A engagement results in inhibition of LPS-stimulated production of IL-12 which might contribute to induce a Th2 phenotype by DCs counteracting with the ability to respond to TLR ligands and induce Th1 responses [64]. In accordance, calreticulin from *H. polygyrus*, a secreted protein that has been implicated in the host-parasite interactions, stimulated robust Th2 responses in mice immunized with the protein devoid of extrinsic adjuvant and stimulated IL-4 and IL-10 secretion in T cells from infected animals. Furthermore, *H. polygyrus* calreticulin interacted with, and was internalized by, scavenger receptor type A on DCs [65]. Concerning costimulation, recently, OX40L upregulation by thymic stromal lymphopoietin (TSLP) has been implicated in generation of Th2 responses [66]. As mentioned above, E/S products from *N. brasiliensis* upregulate this TNF family receptor. Thus other molecules besides the classical Th1 costimulators might be involved in inducing Th2 phenotypes (Figure 1).

Additional candidates may include inducible T cell costimulator (ICOS) and its ligand (ICOSL) that is expressed in DCs. ICOS is able to transmit reverse signals through its ligand to DCs, enhancing antigen presentation in vitro, in

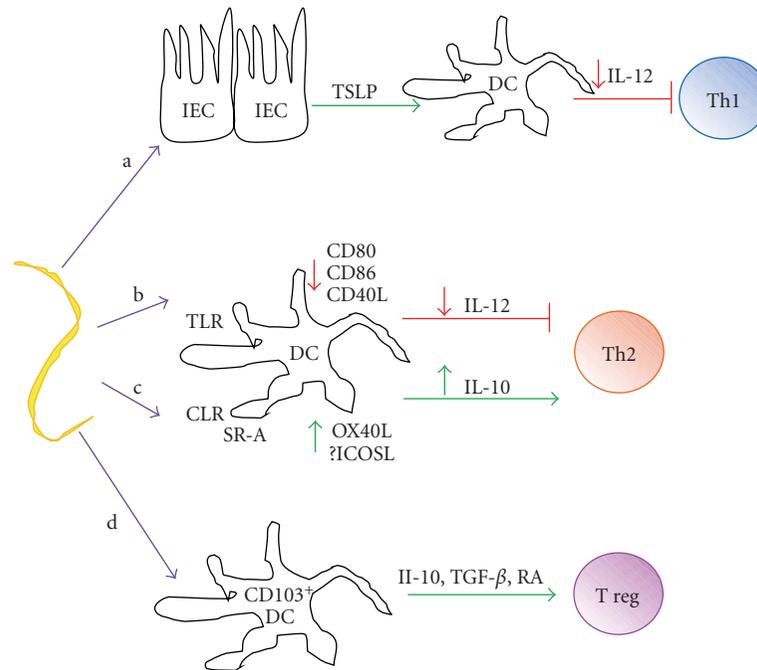


FIGURE 1: Helminth E/S products are able to act on intestinal epithelial cells (IECs) inducing TSLP that engages with the TSLP receptor on DCs and inhibits IL-12/p40. (a) Some E/S products are able to induce signaling through TLRs directly on DCs, resulting in the inhibition of this Th1-promoting cytokine. (b) On the other hand, Th2 responses could be promoted through interactions of helminths with other pattern recognition receptors such as C-type lectins (CLR) or scavenger receptor-A (SR-A) and induction of costimulators such as OX40L that result in Th2 promotion. (c) Finally, interaction with CD103⁺ DCs may result in the induction of T regs through TGF- β and retinoic acid (RA) production. (d) The question of whether different subsets are responsible for these phenomena remains to be determined.

addition to its role in expanding Th2 responses and antibody production [67]. Both ICOSL and ICOS deficient mice have been shown to be defective in early T cell IL-4 production [68]. In a recent study the Th2 response and IL-4 production in the draining MLN were studied during infection with *H. polygyrus*. IL-4 producing cells were present in germinal centers with a typical T follicular helper cell phenotype expressing high levels of ICOS [69].

7. “Helminth-Educated DCs”

In addition to the direct effect of intestinal helminths or their products on DCs, “helminth-educated DCs” can influence immune responses, both Th1 and Th2 types. For example, DCs obtained from mice infected with *H. polygyrus* exacerbated the bacterial inflammation and colitis after adoptive transfer to recipient mice infected with *Citrobacter rodentium*, a murine bacterial pathogen that induces Th1 protective responses. The helminth primed DCs were shown to secrete IL-10 that modulates the bacteria-induced Th1 response reducing IFN- γ production by MLN cells and stimulated IL-4 production by the colonic mucosa, resulting in a Th2-biased response, and thus a more severe bacterial infection due to less inflammatory components [41]. Regarding Th2 responses, adoptively transferred bone marrow-derived DC pulsed with *N. brasiliensis* E/S products induced IL-4, IL-5 and IL-10 by in vitro activated lymph node cells [45].

8. Other Cells Affecting DC Function

Although DCs are capable of directly responding to helminth stimuli, the importance of the interactions of DCs with other cells in the intestinal mucosa and how this cross-talk might influence mucosal immunity require special attention. Intestinal epithelial cells are critical regulators of DCs and of CD4 T cells in the GI tract. Expression of TSLP by epithelial cells limits proinflammatory cytokine production by DCs, inhibiting nonprotective Th1 responses after *T. muris* infection and promoting the pathogen-specific Th2 responses that result in clearance of infection. Furthermore, TSLP limits expression of IL12/23p40 and TNF α by DCs and CD4⁺ T cell-derived IFN γ and IL-17, and thus prevents intestinal inflammation. TSLP expression was shown to be dependent on the activation of the NF κ B pathway in the GI tract [51, 52]. Recently, it has been shown that TSLP does not affect Th2 responses after *H. polygyrus* and *N. brasiliensis* infection. E/S products from these two helminths inhibit the CD-40 and LPS-induced production of IL-12p40 by DCs directly, thereby attenuating Th1 responses and enhancing protective Th2 immunity. In contrast, E/S products from *T. muris* are unable to affect DC function; expulsion of this parasite is dependent on TSLP that inhibits IL-12 p40 during infection [70]. These data demonstrate that different strategies are able to result in attenuation of IL-12/p40 production by DCs and concomitant Th2 activation (Figure 1). In another example both differentiated and undifferentiated intestinal

pig epithelial cells stimulated *in vitro* with low doses of *T. suis* E/S products predominantly synthesized IL-6 and IL-10. These two cytokines were produced within the first 24 h of stimulation, suggesting that they might be part of the *in vivo* primary immune response to this helminth [71]. In addition, mucosal epithelial cells are involved in the recruitment of DCs through the secretion of specific chemokines such as CCL5 and CCL20 [55].

9. Concluding Remarks

Intestinal DCs are *sui-generis* cells as indicated by the different subpopulations, degrees of maturation, specific characteristics, and properties, such as being periscopes of the intestinal lumen. Moreover, survival of intestinal helminths is associated with local DCs which usually generate an antiinflammatory milieu. Despite the increasing number of studies analyzing the effects of helminth products on *in vitro* generated DCs, very few studies have been designed to try to understand the interaction of intestinal DC subsets with helminths. The importance of this kind of studies is highlighted by the fact that the biological function of each subset of DCs present in the gut is well adapted to its microenvironment and that these cells show different properties compared to DCs in other locations. The mechanism by which DCs induce Th2 responses is still not completely understood. The influence of the regulatory milieu in the intestine should not be disregarded since different signals that act upon DCs might influence the outcome, both locally and systemically. Helminths might prove useful tools as models to understand the mechanisms that are turned on during type 2 responses and might help in developing new strategies to manipulate hazardous immune responses that arise during inflammatory, allergic, or hypersensitivity reactions and probably to diminish the risk for cancer. DCs are known to respond not only to PAMPs, but also to different bioactive metabolites and factors secreted by other cells in their vicinity. The combination of these stimuli might result in the different activation conditions that have been reported for DCs when stimulated by helminths to generate Th2 responses. Thus, the study of the interaction of parasites and host's DCs should include especially *in situ* studies of microenvironmental conditions, as well as the intrinsic characteristics of intestinal DCs that differ in some ways from DC from other locations or from *in vitro* generated DCs.

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Review Article

Modulation of Dendritic Cell Responses by Parasites: A Common Strategy to Survive

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Parasitic infections are one of the most important causes of morbidity and mortality in our planet and the immune responses triggered by these organisms are critical to determine their outcome. Dendritic cells are key elements for the development of immunity against parasites; they control the responses required to eliminate these pathogens while maintaining host homeostasis. However, there is evidence showing that parasites can influence and regulate dendritic cell function in order to promote a more permissive environment for their survival. In this review we will focus on the strategies protozoan and helminth parasites have developed to interfere with dendritic cell activities as well as in the possible mechanisms involved.

1. Introduction

Dendritic cells (DCs) control the development of adaptive immune responses due to their remarkable ability to integrate signals coming from the environment, deliver this information to naive T cells, and in turn activate them inducing the appropriate response for the initial stimuli [1, 2]. During infection, the responses induced by DC are critical to control and eliminate the invading agent, the infection itself being the inducer of DC activity. DC maturation and activation involves upregulation of several molecules that play main roles in costimulation and antigen presentation to T cells such as CD80, CD86, CD40, and major histocompatibility complex II (MHC-II), along with the release of cytokines that influence the type and intensity of the immune response [2–4]. Mature DCs are fully potent antigen-presenting cells (APCs) that will prime naive T cells inducing their differentiation and proliferation [1, 5]. Protozoan parasites can activate and induce the maturation of different DC subsets and in most cases the activity of these cells leads to a response that is effective in controlling the infection [6–8]. The case of helminthes is more complex

since most reports show a partial maturation of DC in response to these parasites or their antigens that does not contribute with parasite elimination [9–11]. In any case, it has been shown that both, helminthes and protozoan, are capable to interfere with DC activity promoting a more permissive environment for their own survival inside their host [11–14]. Different interactions of parasite-derived molecules with receptors on DC such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and others seem to be the key event in the mechanisms that ultimately will lead to the altered function in these cells [15–18]. In this review we will focus on some important protozoan and helminth parasites and their ability to modify DC function, the implications of such modulation, and the possible mechanisms involved. We will discuss the differences and similarities in the interference with DC activity between these two distinct parasite groups.

2. Protozoan Parasites

2.1. *Plasmodium*. It has been shown that different species of *Plasmodium sp.* can modulate the response of DC

[6, 13, 19–22]. While infection by some nonlethal strains seems to induce the activation, maturation, and secretion of important proinflammatory cytokines such as IL-12 by DC [6, 22], infection not only with lethal strains such as *P. yoelii* YM or *P. berghei* but also with the nonlethal *P. vivax* can impair DC function. Modified DC function by *Plasmodium* species can be achieved through varied mechanisms. Some of these mechanisms involve a decrement in total DC numbers as well as an altered ratio of myeloid versus plasmacytoid cell subsets, the latter being probably involved in the induction of a regulatory phenotype mediated by regulatory T cells (Tregs) and IL-10 production [12]. Other studies have reported null or reduced capacity of *Plasmodium*-exposed DCs to prime T cells [6, 21–24], situation that could be related with the inability of these DCs to establish prolonged interactions with naïve CD4⁺ T cells [23]. Interestingly, DCs from infected mice with lethal or no lethal strains show an impairment in their response to TLR stimulation, as a marked reduction in IL-12 secretion is observed upon stimulation with LPS, CpG, or poly:IC, resulting in some cases, in the inversion of the IL-12/IL-10 secretion pattern displayed by DC [6, 21, 24]. Since these changes can occur as malaria infection progresses [21], altogether these data suggest that *Plasmodium* has the ability to switch an aggressive immune environment into a more permissive one for its survival. Other effects that *Plasmodium* has on the function of DC have been reported. These effects can be attributed to the invading species, the parasite life stages, or the products they release. For example, a preferential increment in CD8⁻ DC in spleen is observed in the acute phase of *P. chabaudi* infection. Importantly, only CD8⁻ dendritic cells induce proliferation of merozoite surface protein- (MSP1-) specific T cells, and since these T cells produce considerable levels of IL-4 and IL-10, it is suggested that the change from a Th1 to a Th2 response that takes place in *P. chabaudi* infection [25] can be attributed to these already modified DC populations [13].

Maturation of human monocyte-derived DC (MDC) through CD40L can be also prevented by *P. falciparum* merozoites. These DCs show a diminished IL-12p70 secretion but enhance IL-10 production and prime CD4⁺ naïve T cells to produce higher levels of IL-10 and lower levels of IFN- γ . These effects by merozoites on DC seem to involve the activation of the extracellular-signal regulated kinase (ERK) pathway [26]. In contrast, in the same study, DC exposed to *P. falciparum* infected erythrocytes respond to CD40 signaling secret proinflammatory cytokines that lead to a proinflammatory response by naïve CD4⁺ T cells, and this time, p38 mitogen-activated protein (MAP) kinase plays an essential role. These evidences indicate that *P. falciparum* uses distinct kinase pathways to modulate the activity of DC [26]. Moreover, those findings also indicate a different activity on DC function between the whole parasite and its products.

DC maturation can also be compromised in vivo during the last stage of *P. yoelii* infection and in vitro when DC are cultured in the presence of *P. yoelii*-infected erythrocytes [27]. Interestingly, the presence of the complete parasite

is not necessary to interfere with DCs function and by-products of hemoglobin degradation mediated by *P. falciparum* such as hemozoin can also modulate the activity of DC. This malarial pigment inhibits the differentiation and maturation of MDC, reducing the expression of major MHC-II and the costimulatory molecules CD83, CD80, CD54 and CD40 [28, 29]. T cells activated in the spleen by hemozoin-containing DC, are not completely functional since they can not secrete cytokines or migrate to B-cells follicles [19].

More, recently it was shown that soluble factors released by *P. yoelii* or *P. falciparum*-infected erythrocytes can inhibit LPS induced maturation of DC and change their cytokine secretion profile resulting in a failure to produce IL-12 [27]. Induction of regulatory DC that in turn promotes a Treg response is another mechanism by which malaria parasites may subvert host immune systems. Surprisingly, this effect appears to be mediated by recognition of the parasite's molecules by TLR9 [12, 30, 31].

Considering the importance of IL-12 and the Th1 response along with the full activation of DC to prime efficiently T cells in the immune response against *Plasmodium* species, the modulatory effects over DC function discussed above, seem to be an effective evasion strategy actively induced by *Plasmodium* parasites that certainly can influence the outcome of the infection.

There is evidence supporting the interaction of *Plasmodium* parasites with several receptors expressed by DC. For example, among the different surface antigens expressed on *P. falciparum*, the *Plasmodium falciparum* erythrocyte membrane protein 1 (PfPRBC-1) seems to play a main role [32]. This protein can mediate adherence to DC through the interaction with the Scavenger receptor CD36 [33] but also through the proteoglycan molecule, chondroitin sulfate A (CSA) [34, 35]. However, the interactions of PfPRBC -1 seem not to be related with the interference of *P. falciparum* with DC function, in fact; in vitro studies have shown that modulation of DC responses might not need contact between these cells and the infected erythrocytes [35], opening the possibility for parasite-soluble factors playing a major role. This is well exemplified by the interaction of *Plasmodium* parasites and TLR2, TLR4 and TLR9 which can indeed recognize infected erythrocytes-derived products [36–38]. Soluble extracts from *P. falciparum* and hemozoin (by presenting malaria DNA) can activate DC in a TLR9 dependent fashion, inducing up-regulation of costimulatory molecules and proinflammatory cytokines and chemokines, some of them involved in host resistance against *P. falciparum* [36, 37, 39]. Interestingly, it was recently shown that *P. yoelii* uses the interaction with the same receptor on DC to in turn induce Treg cells, a response that is associated with *P. yoelii*-immune evasion [30]. This finding indicates that different *Plasmodium* species may induce different responses through their interaction with TLR9. However, there is still little information about the receptors and ligands involved in the DC downregulation consistently observed in *Plasmodium* infections or in response to their products. See Table 1.

2.2. *Leishmania*. Clearance and resistance to *Leishmania* infections is dependent on the development of a Th1 response and the production of IL-12 [40–42], and in this regard, DCs play a critical role [43]. It has been shown that during *Leishmania* infections, engulfment of amastigotes and promastigotes by DCs leads to their activation and a consequent IL-12 and TNF- α production that can contribute with host resistance [44, 45]. However, more accurate is the fact that the effect of *Leishmania* infection on DC maturation and cytokine production could vary for the different *Leishmania* species and strains; for example, while the uptake of *L. major* promastigotes and amastigotes results in DC maturation and IL-12 production [46, 47], promastigotes of *L. amazonensis* can interfere with DC responses affecting their differentiation and decreasing their ability as APC [48]. Both stages of this specie can reduce CD40 and CD83 surface expression and IL-12p40 production by bone-marrow-derived dendritic cells (BMDCs) in a mechanism that requires the activation of the MAP kinase ERK [7, 49]. Additionally, IL-12p40, IL-12p70, and IL-6 are downregulated and IL-10 secretion increased when infected DC are treated with LPS [7]. Lack of activation and a reduced IL-12 production by DCs also observed after *L. mexicana* infection along with a delay in their apoptotic process [50, 51]. *L. donovani* can also inhibit DCs maturation and modify their CCR7 expression, interfering with their migration to the periarteriolar lymphoid sheath [52, 53]. Interestingly, some of the regulatory effects that *L. donovani* has on DC depend on the phosphoglycans present in the parasite [52]. In fact, several *Leishmania* products can also impair DC activity as it has been shown that parasite culture media or lipophosphoglycans (LPGs) from *L. major* inhibit the motility of murine splenic DC [54] or the migration of Langerhans cells [55], suggesting that *Leishmania* products may interfere with antigen transportation. *L. major* phosphoglycans (PGs) are involved in downregulation of IL-12p40 production by DC and in the ability of these cells to induce a Th1 response since these molecules favor IL-4 and IL-10 but not IFN- γ production [56]. Moreover, excreted-secreted antigens of *L. donovani* or *L. major* induce a slight decrease in the surface expression of CD40, CD86, human leukocyte antigen-DR (HLA-DR), and cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) on DC concomitant with a downregulation on IL-10 and IL-12p70 secretion [57]. In the case of *L. mexicana*, the activity of LPGs on DC function also includes a reduction in IL-12 production that may depend on the impairment on NF- κ B nuclear translocation induced by parasite's antigens [51]. Since the maturation state of DC seems to determine the type of immune response induced in *Leishmania* infections, where an immature state promotes a Th2 response which does not control the infection and a mature phenotype induces a Th1 response with the subsequent host resistance [58], the interference of these parasites and their products in the maturation process of DC may be critical for parasite survival.

Some studies have shown that activation and production of IL-12 and IFN- α/β by myeloid DC (mDC) and plasmacytoid DC (pDC) after stimulation with *L. major*, *L. brasiliensis*,

or *L. infantum* is strictly dependent on TLR9 signaling [59, 60] and the maturation of splenic DC is improved by the myeloid differentiation primary response gene 88 (MyD88)-signaling pathway, indicating an important role of TLRs in activation of DC by *Leishmania* [61]. In addition, others found that TLRs such as TLR2 and TLR4 are involved in the response to *Leishmania* parasites and their products, like LPGs that might signal through TLR2 inducing a TNF- α response [62–64]. Interestingly, a noncharacterized soluble product from *L. brasiliensis* activates DC in a MyD88-independent fashion, suggesting that receptors other than TLRs may play a role in the responses induced by *Leishmania* products on these cells [65]. But even when interaction of *Leishmania* sp. parasites or their products with TLRs and probably other receptors can induce the activation of DC, it is still unknown which kinds of molecules, receptors, and pathways are involved in the impairment of function that has been observed in DC exposed to *Leishmania* parasites, *Leishmania* LPGs, or *Leishmania* excreted-secreted products. Therefore, deeper studies in this field should improve our knowledge on *Leishmania*'s strategies to survive the immune response. See Table 1.

2.3. *Trypanosoma*. Infection of MDC with *Trypanosoma cruzi* induces functional changes in these cells. For example, secretion of IL-12 and TNF- α is impaired and the maturation process induced by LPS is affected, observing a marked reduction in proinflammatory cytokines secretion as well as on the expression of HLA-DR and CD40. Moreover, the same effects are observed when DCs are cultured in the presence of *T. cruzi*-conditioned medium, indicating a modulatory activity of the molecules released by these parasites [66]; in fact, the exposure of DC to *T. cruzi*-derived GIPL (glycoinositolphospholipids) can also affect the LPS-induced cytokine secretion and expression of costimulatory molecules in similar way that the whole parasite does [67]. Using also in vitro studies, Poncini and colleagues showed that trypomastigote stage of *T. cruzi* fails to activate DCs, these cells become in TGF- β and IL-10 producers and are not efficient as lymphocyte stimulators. For these reasons, the authors classify these cells as regulatory DCs [68]. Importantly, in vivo experiments also robustly show the interference of *T. cruzi* with DC function. At day 14 of an active infection, there is a progressive fall in the number of splenic DCs and this is accompanied with a low expression of the costimulatory molecule CD86, therefore suggesting an immature phenotype. These DCs are unable to migrate to the T cell area in the spleen or undergo maturation upon LPS stimulation [69]. Interestingly, only the infection with *T. cruzi*-virulent strain RA but not the low-virulence strain K98 affects MHC-II expression on splenic DCs and their capacity to prime T cells [70], indicating a relation between virulence and a more effective strategy to avoid immunity. This is further supported by the evidence that the infection of the susceptible strain of mice (BALB/c) with *T. cruzi* induces a downregulation of splenic DC activity. These cells display a diminished CD86 and CD40 expression and a reduced potential to present alloantigens. Importantly, these changes are not observed

TABLE 1: Protozoan interactions and their regulatory effects on DC.

Parasite	Product	Putative DC Receptor ¶	Effect on DC	Effect on DC upon TLR stimuli	Ref.
Plasmodium					
<i>P. berghei</i>	Infection	N.D.	↓ Tcell priming	↓ IL-12p70, endocytic activity	[22]
				↑ IL-10	
	Infection	TLR9	Immature ↓ T cell priming, shorter DC/T cell interactions	↓ CD40, CD86, MHCII, IL-12p70	[6, 23, 24, 30]
				↑ IL-10	
<i>P. yoelii</i>			↑ Treg*		[21, 31]
	Soluble Factors	N.D.	↓ IL-12	↓ CD40, CD86, MHCII, IL-12	[27]
<i>P. vivax</i>	Infection	N.D.	↓ mDC:pDC ↑ Treg*	N.D.	[6, 12]
<i>P. chabaudi</i>	Infection	N.D.	↑ CD8- DC Th2*	N.D.	[13]
<i>P. falciparum</i>	Merozoite	N.D.	↓ IL-12p70, IFN γ * ↑ ERK,IL-10, IL-10*	N.D.	[2, 20]
	Hemozoin	N.D.	Immature ↓ Differentiation Migration	N.D.	[19, 28]
	Soluble extracts	N.D.	Immature ↓ IL-12	↓ IL-12	[27]
Leishmania					
<i>L. major</i>	Promastigote Amastigote	N.D.	Immature ↓ IL-10	N.D.	[55]
	LPGs	TLR2	↓ Motility Migration	N.D.	[54]
	PGs		↓ IL-12p40, IFN γ * ↑ IL-4*, IL-10*	↓ IL-12p40	[56]
	ES	N.D.	Immature ↓ DC-SIGN, IL-10, IL-12p70	N.D.	[57]
<i>L. amazonensis</i>	Promastigote	N.D.	Immature ↑ ERK ↓ IL-12p40	IL-12p40, IL-10 ↓ IL-12p70, ↑ IL-6	[45, 48, 49, 65]
<i>L. mexicana</i>	Promastigote	N.D.	Immature ↓ IL-12 Apoptosis	N.D.	[50]

TABLE 1: Continued.

Parasite	Product	Putative DC Receptor ¶	Effect on DC	Effect on DC upon TLR stimuli	Ref.
<i>L. donovani</i>	LPG	N.D.	IL-12	N.D.	[51]
	Promastigote	N.D.	↓ NFκB Immature Migration, CCR7,	N.D.	
	ES	N.D.	↓ CD11b, CD51, CD86, IL-12p70 Immature ↓ DC-SIGN, IL-10, IL-12p70	N.D.	[52, 53] [57]
Trypanosoma					
<i>T. cruzi</i>	Infection and Soluble Factors	N.D.	Immature DC number,	Migration, IL-12, TNFα, ↓ IL-6, HLA-DR,	[66, 69, 70]
	Trypomastigote	N.D.	↓ T cell priming, IL-12, TNF-α Immature ↑ TGF-β, IL-10	CD40, CD86 TNF-α, IL-12p70, IL-6, ↓ CD40, HLA-DR,	
	GIPLs	N.D.	IL-12, TNFα, ↓ IL-10	↑ IL-10 TNF-α, IL-12, IL-10, ↓ CD83, CD80, CD86,	[67]
	Sialyted structures	Siglec-E	N.D.	CD40, HLA-DR ↓ IL-12p40, IL12p70 ↑ IL-10	
Toxoplasma					
<i>T. gondii</i>	Infection	N.D.	N.D.	↓ IL-12p40	[87, 91]
	Tachyzoites	N.D.	Immature ↑ Motility Migration	IL-12p40, ↓ TNF-α, MHCII	
	Soluble Factors	N.D.	↓ T cell priming Immature ↑ DC attraction	N.D.	[91, 93]
	Endogenous LXA ₄ parasite-induced	FRPL-1 AhR	↑ SOCS2 ↓ IL-12	N.D.	[16]
Giardia					
<i>Giardia lamblia</i>	parasite extracts	N.D.	CD80, CD86, ↑ CD40 IL-12, TNF-α, ↓ IL-10	↑ IL-10 ↓ IL-12, MHCII, CD80, CD86	[94]

¶ Involved in modulatory effects on DC.

*T cell response.

in the C57Bl/6 mice, a strain capable to survive the acute phase of this infection [71]. Together, these two studies indicate that modulation of certain DC features by *T. cruzi* depends on both host and parasite genetic backgrounds. They also remind us that even when targeting of DC functions by parasites is an important strategy for immune

evasion, it can also unbalance the delicate host/parasite relationship in such a way that infection could be lethal for both.

There are different ways for an intracellular pathogen such as *T. cruzi* interacts with elements of the innate immune system. Macrophages and DCs are cells that

can be infected by this parasite; nevertheless, when these cells become activated by TLR signaling, through either the adaptor molecules MyD88 or TIR-domain-containing adapter-inducing interferon- β (TRIF), both cell types are less prone to allow parasite replication with IFN- β playing an important role in parasite clearance [72]. Different molecules derived from *T. cruzi* can be recognized by TLRs. Glycosylphosphatidylinositol (GPI) anchors from this protozoan parasite trigger TLR2-dependent antiparasite responses in macrophages [73] while *T. cruzi* DNA can induce their activation by interaction with TLR9 [74]. Indeed, the combination of TLR2 and TLR9 seems to account for all the protective IFN- γ production generated by MyD88-dependent signaling pathways in macrophages exposed to live trypomastigotes in vitro [74]. More importantly for this review's topic, the Tc52-released protein from *T. cruzi* signals via TLR2 and induces DC maturation and production of the inflammatory chemokines IL-8, MCP-1, and MIP-1 α [75]. On the other hand, the bradykinin B₂ receptors (B₂R) in DC might be also involved in the activation and maturation of these cells, and in turn in the generation of protective responses such as IL-12 production and the development of Th1 immunity [76]. As for other protozoan parasites, the interaction between DC receptors and *T. cruzi* molecules that lead to downregulated responses in these cells remain poorly understood; however, recent evidence has brought some insights to this phenomena. Erdmann and colleagues demonstrated that sialylated structures from *T. cruzi* pathogenic Tulahuen and to a less extent the less pathogenic Tehuantepec strain interact with the inhibitory sialic acid-binding protein Siglec-E [17]. This lectin receptor is expressed on DC and interestingly its ligation suppresses the LPS-induced production of IL-12 by these cells. The subsequent T cell response is also affected since a marked decrease of IFN- γ production is observed when Siglec-E-ligated DCs are used as APCs. This response could play a role in the immunosuppression observed during *T. cruzi* infection as well as determining the distinct pathogenicity that these two different strains display [17]. In addition, this finding suggests that the interference with DC function induced by *T. cruzi* could be achieved through the interaction of lectin receptors with parasite products. See Table 1.

2.4. *Toxoplasma*. Either live tachyzoites or a soluble tachyzoite extract from *Toxoplasma gondii* injected into mice induces a rapid and strong production of IL-12 from splenic DC [77, 78]. This IL-12 response is unusually potent since not even stimuli like LPS or CpG can reach the IL-12 levels induced by *T. gondii* antigens [79]. Such reaction is mediated by the specific recognition of *Toxoplasma*-derived profilin by TLR11 expressed specifically on CD8 α + DC [80], although other molecules and other TLRs could be also involved [81, 82]. Nevertheless, other reports indicate that the potency of the *T. gondii* to induce IL-12 can be achieved thanks to the engagement of CCR5 by cyclophilin-18 (C18), a chemokine mimic of MIP-1 β derived from the soluble extract of the same parasite [83]. It is, however, important to highlight that the production of high levels of IL-12 secreted by DC in the presence of *T. gondii* antigens quickly reaches baseline level

(24 h post stimulation) and DCs become refractory to any further exposure to *T. gondii* in a phenomenon called "DC paralysis" [84]. It is believed that these dramatic changes in the DC response are due to a parasite strategy that assures the control of a potentially lethal parasite growth and prevents a cytokine-mediated immunopathology, as both scenarios could be deleterious for the host and in turn to the parasite [79]. A line of evidence has further identified a sophisticated *T. gondii*-induced modulation of the immune response. The parasite-induced production of the eicosanoid lipoxin A₄ (LXA₄) has been linked to the downregulation of IL-12 production by DC [85] in a mechanism that seems to involve the binding of LXA₄ to the receptors formyl peptide receptor-like 1 (FRPL-1) or aryl hydrocarbon receptor (AhR) on the same cells [16]. This interaction triggers the expression of the suppressor of cytokine signaling-2 (SOCS-2) that in turn helps to control the proinflammatory and otherwise potentially lethal immune response against *T. gondii* [16]. In line with this idea, in the absence of the AhR receptor (AhR knock out) mice succumb rapidly to ME49 *T. gondii* infection, a situation that is associated with the higher proinflammatory response observed in these animals [86].

In contrast with the evidence discussed above, there are some reports that show that invasion of immature DC by *T. gondii*-living parasites does not induce their activation and renders them also resistant to activation by TLR ligands or CD40L [87, 88]. The differences in DC responses upon *T. gondii* exposure could be explained by the identification of different DC subpopulations that are indeed involved in the initial response to *T. gondii*; among those are CD8 α +, CD11b+, and, surprisingly, pDC [8]. It is noteworthy to consider the findings from Diana and colleagues who reported that soluble factors released from low-virulent strains of *T. gondii* can inhibit DC maturation [89] while other reports have demonstrated that *T. gondii*-soluble extract can induce a marked IL-12 production by DC [77]. Despite the fact that all these apparently contrasting findings may suggest that different soluble molecules from this parasite can provoke varied responses in DC, this is probably due to different soluble antigens interacting with distinct types of receptors.

T. gondii is capable of exploiting parasitism in a remarkable way by using DC to invade other tissues [88, 90] or other immune cells [91], promoting in turn the spreading and establishment of the infection. This may be achieved by the early target of these cells by *T. gondii* tachyzoites, an interaction that seems to modify the DC motility, trafficking, and migration properties as well as their cytokine effector function following TLR stimulation [88–90, 92]; the last one probably renders them unable to activate direct and indirect killing pathways. In addition, *T. gondii* may use the NK-cell mediating targeting of infected DC to disseminate, allowing viable parasites to enter in NKs that later appeared not to be efficiently targeted by other NK cells [91].

Different mechanisms could be related with the change in trafficking and migration behavior of DC exposed to *T. gondii*; however, it seems that most of these parasite-induced alterations are mediated by G-protein signaling pathways [83, 89, 92]. It has been shown that soluble

factors in excreted-secreted *T. gondii* antigens appear to possess a chemokine-like activity that attracts immature human [93] and mouse DCs [83] while other factors from a parasite extract can even trigger DC migration towards the chemokine MIP-3 β [93]. In both human and mouse cells, the chemokine-like activity might be achieved by the *T. gondii*-derived chemokine mimic C-18, although other parasite factors could be involved [83, 93]. In any case, it is likely that this DC attraction might enhance the chances for DC infection while the alteration in DC trafficking and migration properties could participate in parasite dissemination. See Table 1.

2.5. Other Protozoan Parasites. There are several other species of protozoan parasites including *Trichomonas sp.*, *Giardia sp.*, *Entamoeba sp.*, and *Cryptosporidium sp.* Unfortunately, these parasites have generally been poorly studied as far as their interactions with DC concern probably due to the fact that they have less morbidity and mortality among human populations. Nonetheless, recently it was shown that DCs incubated with *Giardia lamblia* live parasites or its extracts, display enhanced levels of CD40, CD80, and CD86 indicating their mature state. These cells produce small amounts of IL-6 and TNF- α and no IL-10 or IL-12 is detected [94]. Importantly, coinubation of DC with parasite extracts and TLR ligands only enhance IL-10 production while markedly reducing IL-12 secretion and MHC-II, CD80, and CD86 levels. The mechanism of DC inhibition involves the activity of the phosphoinositide 3-Kinase (PI3K) since specific inhibition of this enzyme restores IL-12 production by these cells [94]. It is hypothesized that the ability of *Giardia lamblia* to inhibit IL-12 and enhance IL-10 production may contribute the maintenance of an antiinflammatory environment in the gut.

It is also known that lipopeptidophosphoglycan (LPPG) molecules from *Entamoeba histolytica* establish interactions with TLR2, and TLR4 leading to the release of IL-10, IL-12, and TNF- α [95]; and additionally, DNA from this parasite can be recognized by macrophages through TLR9 triggering TNF- α production [96]. With regard to DC, LPPG and also the parasite's surface Gal-lectin can activate these cells and induce their maturation along with IL-12 production, resulting in the induction of Th1-type responses [97, 98], although no findings regarding DC downregulation are available for this parasite.

TLR receptors are also involved in recognition of *Cryptosporidium parvum* and *Trichomonas vaginalis* but these interactions are normally between the parasites and host cells other than DC [99–102]. See Table 1.

3. Helminth Parasites

In marked contrast to protozoan parasites, helminthes are much larger hence they do not occupy intracellular spaces or are engulfed, having their interactions with immune system through the attachment of some immune cells to their surface or mainly by the recognition of diverse antigens released from the parasite. However, as for any parasite,

their survival will depend on their capacity to cope with immune system's attacks. In this sense, interfering with DC activity is again one of the main ways to induce a permissive environment that allows their development inside the host. As noted earlier, during development and differentiation DC can be exposed to different stimuli which significantly influence their function and maturation, and helminth-derived antigens are not the exception to this rule.

3.1. Nematodes. Semnani and coworkers demonstrated that when monocytes undergoing differentiation to DC engulfed *B. malayi* microfilariae antigens they display an inhibited production of IL-12p40, IL-12p70, and IL-10 in response to *Staphylococcus aureus* Cowan antigen (SAC) and SAC plus IFN- γ , suggesting an interference with TLR and IFN- γ signaling induced by this nematode [10]. Cell viability or expression of costimulatory molecules, including MHC-I and MHC-II is not altered; however, DC exposed to these filarial antigens induce lymphocyte activation to a lesser degree than DC that were not exposed [10]. The same group recently reported a reduced TLR4, TLR3, and MyD88 mRNA expression in HMDC, as well as an impaired cytokine response to poly:IC and LPS upon exposure to live *B. malayi* microfilaria (mf) [103]. This indicates that this parasite can alter not just DC maturation but also their TLR responses affecting in turn T cell activation. In addition, MDC exposure to mf led to an enhancement in SOCS1 and SOCS3 mRNA transcripts [103], which are molecules known for their modulatory activity on cytokine production [104]. Furthermore, mf induces apoptosis in HMDC but not in macrophages [103]. Thus, the interaction of this helminth parasite and its antigens with DC clearly interferes with the function of these cells at different levels, from downregulating proinflammatory receptors to the induction of molecules involved in the suppression of cytokine production.

Another nematode, *Nippostrongylus brasiliensis*, secretes in vitro diverse glycoprotein named excretory-secretory products (NES). These antigens polarize the immune response towards a Th2 type without requiring live infection of the mice. Interestingly, BMDC pulsed with NES can upon transfer to naive recipients prime them to a Th2 response [105], suggesting that the Th2 driving properties of this extract occur through DC. In fact, NES upregulates DC markers associated with Th2 promotion, including CD86 and OX40L. In addition, the high levels of IL-12p70 induced by LPS are suppressed in DC that have been preincubated with NES [105]. This situation may contribute to the Th2 polarized response observed either during the infection with *N. brasiliensis* or in response to its antigens.

In in vivo studies, adult *Ascaris suum* high-molecular-weight components (PI) inhibit MHC-II and costimulatory molecules in CD11c+ LN cells from OVA-PI-immunized mice compared with those immunized with OVA-CFA [9]. In line with an immature phenotype, these CD11c+ are poor inducers of proliferation, phenomenon that is IL-10 dependent. Interestingly, OVA+PI was administered in CFA (Freund's complete adjuvant), suggesting that PI could inhibit the inflammatory effect of this adjuvant [9]. In addition,

peritoneal CD11c⁺ cells recruited by pseudocoelomic fluid (PCF) from *A. suum* show basal levels in CD86 expression while BMDCs exposed to the same extract exhibit low increase in CD40 expression and are refractory to LPS stimulus, displaying once again an immature phenotype and a dose-dependent reduction in IL-12 production. [106]. Moreover, glycosphingolipids from *A. suum* containing phosphorylcholine (PC) downregulate IL-12p40 and TNF- α secretion from DC, as well as a MHC-II, CD40, CD80, CD86, and CD54 in response to LPS. This modulation is due to native glycosphingolipids and PC-removed glycosphingolipids, suggesting that a molecule other than PC possesses immunomodulatory properties over DC, although this one has not yet been elucidated [107].

ES-62 is another molecule that contains PC; it is derived from excreted/secreted products of the nematode *Acanthocheilonema vitae* and has been shown to display a variety of immunomodulatory activities [108]. ES-62 exerts its immunomodulatory effects on an array of cells of the murine immune system, including macrophages and DC [108–110]. In particular, exposure of immature DC to ES-62 lead to an unexpected increment in the expression of costimulatory molecules such as CD40, CD80, and CD86 and also, unlike other nematode molecules, ES-62 induce low but significant levels of IL-12p40 and TNF- α production in a MyD88-dependent manner. Interestingly when DC are stimulated with ES-62, there is an up-regulation of TLR4 expression; however, ES-62 had overall inhibitory effects on IL-12 and TNF- α production induced by TLR ligation. This suppressive effect is abrogated in TLR4 knockout but not in C3H/HeJ mice-derived BMDC, suggesting the use of a coreceptor in this ES-62-TLR4-dependent signaling [108]. ES-62 is also capable of shifting BMDC into the “DC2” activation state [108]; when these DC2 cells present an ovalbumin peptide to naïve CD4⁺ T cells from OVA TCR transgenic mice, an increase of IL-4 and a decrease of IFN- γ production by lymphocytes are observed. In addition, the switch to a Th2 response is not affected by differential regulation of CD80 or CD86 and it is achieved even in the presence of IL-12 [110]. Importantly, despite the fact that ES-62 is a high Th2 inducer, this molecule is incapable to overcome and bias the immune response to a Th2 type in the context of some inflammatory Th1 conditions such as *T. gondii* infection and *O. volvulus* synthetic protein [111, 112].

Other important data regarding DC activity modulation have come from studies with *Heligmosomoides polygyrus* excreted-secreted-derived products (HpES) and the adult worm homogenate (AWH). None of these antigens induce BMDC maturation and IL-12, TNF- α or IL-10 is not found in culture supernatants [113]. Alike other helminth-derived extracts, HpES alters TLR-induced cytokines and chemokines, since limited IL-12, TNF- α , MCP-1, and RANTES production is elicited by stimulation with CpG, LPS, and poly:IC. Interestingly, the production of the immunoregulatory cytokine IL-10 is also impaired. In addition, costimulatory molecules including CD40, CD86, and MHC-II but not CD80 are drastically downregulated also in response to TLR stimuli [113]. Even more, studies in vitro with a model of CD4⁺ OVA restricted activation, show that

DC treated with HpES not only attenuate but also IL-4 and IFN- γ production by CD4⁺ T cells but enhance IL-10 leading to Treg induction [113]. The findings for HpES correlate with an in vivo study where spleen DC isolated from *H. polygyrus* infected mice have an increased IL-10 production and a moderate up-regulation of CD80 and CD40. These DC are poor inducers of CD4⁺ T cell proliferation and have the ability to decrease IFN- γ and enhance IL-4 production [114]. Thus, it is likely that the responses induced by *H. polygyrus* in DC, participate in providing a safer environment for parasite establishment and survival. Importantly, the impaired cytokine effect observed in DC exposed to *H. polygyrus* antigens is TLR2 and TLR4-independent [113] while data are available showing that these same receptors recognize molecules from other helminthes such as ES-62 from *A. vitae* [11, 15, 108, 115]. These findings imply that the recognition and activity of helminth-derived molecules occurs through different receptors. This hypothesis has been further supported by a study that shows that calreticulin (CRT) present in *H. polygyrus* antigens lead to a Th2 response in vivo and this same molecule can be recognized by BMDC through Scavenger receptor A (SR-A) [116]. However; more studies are required to determine the phenotype that such recognition may confer to DC, and whether this phenotype is involved in the polarization toward Th2-type responses observed in the previous studies. Importantly, since CRT is preserved across the helminth parasites [86, 117–121], it is likely that this molecule might represent a potential pathogen-associated molecular pattern (PAMP) with a Th2-polarizing activity. See Table 2.

3.2. Trematodes. The most studied helminthes by far have been *Schistosomes* and in particular *S. mansoni*. They produce a great variety of glycosylated proteins and lipids to which mainly humoral immune responses are directed. In the case of *S. mansoni*, proteins, glycans and lipoconjugates can induce Th2-type responses. Several groups have shown that schistosome soluble egg antigens (SEA) contain molecules that drive the polarized CD4⁺ Th2 response [122, 123]. Many involved mechanisms have emerged and in this regard DC seem to have a critical role. For example, some glycans found in SEA such as core α -3-fucose, β 2-xylose and Lewis X, have been shown to play an important role in the changes observed in DC activity [18]. DC pulsed with fractions containing a motif of α 3-fucosylation of a GlcNac or of β -xylosylated core sugar drive strong Th2-cell responses in mice [124]. When egg-derived glycoconjugates are captured, processed, and presented to naïve T lymphocytes by DC, the immune response is again skewed to a Th2-type response. Periodate treatment reverses this effect and CD1d is apparently crucial to this phenomenon, indicating that SEA glycolipids may be involved in the Th2 polarization by DC [124]. SEA activity on DC is even more profound, for instance; immature DC pulsed with this antigenic extract do not show an increase in expression of costimulatory molecules or cytokines while their LPS-induced activation, including expression of MHC-I and costimulatory molecules as well as IL-12 production is also suppressed. In addition, SEA inhibits the ability of CpG, poly:IC and hyaluronic

TABLE 2: Helminth products interactions and their regulatory effects on DC

Parasite	Product	Putative DC Receptor¶	Effect on DC	Effect on DC upon TLR stimuli	Ref.
Nematodes					
<i>B. malayi</i>	Microfilarie alive	N.D.	Immature TLR4, TLR3 ↓ IL-8, RANTES, ↑ TNF- α , IL-1 α , IL-1 β , SOCS1, SOCS3 DC apoptosis	↓ MIP-1, IL-12p70, IL-1 α , IFN- α , IL-12p40, MyD88, NF κ B (p50-p65)	[103]
	Microfilarie Ag	N.D.	↑ CD80, CD40, MHCI	↓ IL-12p40, IL-12p70, IL-10	[10]
<i>N. brasiliensis</i>	NES	N.D.	↑ OX40L, CD86Th2*	↓ IL-12p70	[105]
<i>A. suum</i>	PI	N.D.	Immature	↓ IL-12p40	[9]
	PCF	N.D.	Immature	↓ IL-12, CD40, CD86	[106]
	Glycosphingolipids (PC contained but PC independent)	N.D.	Immature	↓ IL-12p40, TNF α , CD86	[107]
<i>A. vitae</i>	ES-62 (PC contain)	TLR4	TLR4, CD80 * ↑ IL-12p40 (low), TNF α (low) Th2*	IL-12, TNF α ↑↑ IL-10	[108, 110]
<i>H. polygyrus</i>	ES	N.D.	Immature IFN γ *, IL-4* ↑↑ IL-10(Treg)*	IL-12, TNF α MCP1, RANTES, ↓ MHCII, CD40, CD86	[113, 114]
	Calreticulin	SR-A	N.D.	N.D.	[116]
Trematodes					
<i>S. mansoni</i>	SEA	DC-SIGN, MR, MGL	Immature DC-SIGN, MR, ↑ DCIR, MGL, Jagged 2, TLR4 Th2*	IL-12p40, IL-12p70, ↓ TNF α , IL-6, MHCII, CD80, CD86	[18, 124– 126]
	LNFP III	TLR4	Immature ↑ ERK, NF κ B, Th2*	↑ IL-10 N.D.	[15, 128]
	Lysophosphatidyl-serine	TLR2	↑ ERK, c-fos, Th2*, Treg*	↓ IFN γ *	[11, 115]
Cestodes					
<i>E. granulosus</i>	AgB	N.D.	IRAKp, NF- κ B, ↑ TNF- α ^{low} , IL-10 ^{low} , IL-6 ^{low} , Th2*	IL-12p70, ↓ TNF α , IL-6, IL-10, HLA-DR CD80, CD86	[14]
<i>T. crassiceps</i>	ES (carbohydrate dependent)	N.D.	↑ MHCII, Th2*	IL-15, IL-12p40, ↓ IL-12p70, TNF α , CD80, CD86, CD40, CCR7, IFN γ *	

¶ Involved in modulatory effects on DC.

* T cell response.

acid (HA) to induce production of IL-12 and up-regulation of MHC-II, CD80, and CD86 on DC. Even though IL-10 production is augmented in the presence of SEA, not all the SEA effects on DC depend on this cytokine [125, 126]. SEA also suppresses the LPS-induced expression of 46 genes in DC, many of which are proinflammatory, and it also prevents the LPS-induced downregulation of 37 genes that may be involved in the changes observed in DC function upon SEA exposure [125]. Thus, SEA appears to have a profound effect on TLR ligand-induced DC maturation/activation, suppressing inflammatory events associated with development of Th1-type responses. SEA probably also affects the antigen-processing pathway as DC exposed to SEA show a difference in antigen processing by segregating SEA to a different compartment when compared with a bacterial antigen (from *Propionibacterium acnes*). Since these two antigens are handled very differently, this could explain the contrasting responses they induce in these cells [125].

In contrast with other helminthes, molecules from *S. mansoni* have been more fully characterized and important data regarding them have been reported (reviewed in [127]). Lipids from *S. mansoni*, particularly those containing phosphatidylserine, act as Th2-promoting factors through the blockade of IL-12 production by DC while promoting the development of IL-10-producing Treg cells [11]. It has been shown that these molecules can be recognized through TLR2 and interestingly antibody blockade of TLR2 diminishes their ability to induce a Treg response but not the Th2 polarization [11], suggesting another pathway and probably another receptor involved in the DC Th2-driving activity. New data show that these lysophosphatidylserine molecules can increase ERK but not p38 phosphorylation on DC and induce an up-regulation in c-Fos transcription; in contrast, Delta4 (Notch ligand) is strongly downregulated. These combined factors may ultimately lead to the development of a Th2 response [115]. According to this report, the glycoconjugate LNFPIII (also *S. mansoni* egg derived) leads to ERK1/2 phosphorylation but not p38 in DC and possesses Th2-biasing ability through the induction of DC2 in a TLR4-dependent manner [15]. NF- κ B alternative activation through p105 degradation is necessary for these LNFPIII-mediated effects [128]. However, recent evidence shows that the Th2 polarizing effect of SEA is maintained in TLR2 or TLR4 knockout mice and MyD88 is also unnecessary [126]. This finding may rule out the participation of TLR in the effects induced by the molecules present in SEA. These data also open the possibility of the involvement of many other receptors such as CLR, other lectin or Notch receptors in the recognition and modulatory activity of the *S. mansoni* antigens over DC. According to this idea, DC can recognize carbohydrates present in SEA through CLR like mannose receptor (MR), macrophage galactose-type lectin (MGL) and DC-SIGN while exposure of the same cells to SEA can upregulate the expression of these same CLR and the one of DC-immunoreceptor (DCIR) [18]. In addition, there is recent evidence showing that DC may recognize helminth products through molecule-grabbing nonintegrin receptor 1 (SINGR1). This CLR has been shown to bind SEA in vitro,

however studies using SINGR1 knock-out mice have not found any significant differences in the kinetic of immune responses after *S. mansoni* infection [129]. SEA can also enhance expression of Jagged 2 in DC, but downregulation of Jagged 2 using RNAi does not affect the ability to prime Th2 responses [130]. This situation could reflect the fact that different receptors present in DC may have similar effects [131]. Due to the diverse molecules found in SEA and indeed in other different parasite products (crude extracts or excreted/secreted products), many receptors could be involved in the recognition of the different molecules present in these extracts, in such a way, that the lack of one receptor might not affect the immune response induced by these antigens. Therefore, helminth parasites probably possess redundant molecules to escape from immunity, unfortunately so far, only few of them have been characterized and their interaction with DC is still far from being understood. Even when CLR or Notch receptors could be involved in DC recognition of helminth products, more studies are necessary to establish whether these interactions participate in the changes observed in these cells and in turn in the systemic effects they induce. See Table 2.

3.3. Cestodes. Studies regarding the interaction of this class of helminthes and DC are far less extensive when compared with trematodes and nematodes. Nevertheless, most of them point to similar pathways of alteration of DC function by cestode-derived molecules. For example, Rigano and colleagues demonstrated that *Echinococcus granulosus* hydatid cyst antigens affect DC in different stages of maturation [14]. Purified antigen B (AgB) and sheep hydatid fluid (SHF) affect host DC differentiation from monocyte precursors by reducing the number of cells that differentiate into immature DCs (iDCs) inhibiting as well the up-regulation of CD1a while increasing CD86 expression. When these cells are stimulated with LPS, there is a significantly lower expression of CD80, CD86, and HLA-DR and lower quantities of TNF- α and IL-12p70. In addition, *E. granulosus* antigens interfere in the maturation process of these iDCs, inducing only a slight up-regulation of CD80, CD86 and a small TNF- α , IL-10, and IL-6 production ablating IL-12p70 completely. In response to LPS, DC previously exposed to *E. granulosus* antigens, express fewer CD80 and CD86 molecules and show reduced TNF- α and IL-12p70 production. When DC matured in the presence of *E. granulosus* antigens are used as APCs, the majority of naïve T lymphocytes differentiate into IL-4-producing cells, suggesting the potential of these antigens to skew the response to a Th2 [14]. However, neither the receptors on DC nor the type of molecules derived from *Echinococcus* that are involved in DC modulation has been identified in this model.

Another cestode that has recently been proved to interfere with DC activity is *Taenia crassiceps*. Carbohydrates present in soluble antigens of this parasite are responsible for Th2 polarization in vivo [132] and induction of myeloid suppressor innate cells [133]. We have found that DC exposure to excreted/secreted products of high molecular weight from *T. crassiceps* metacystodes (TcES) induce an increment in MHCII expression but not in the costimulatory

molecules CD80, CD86 or CD40 or in cytokine production. In line with findings for other helminthes, DC exposure to TcES results in an abrogated response to TLR ligands such as CpG, LPS and *Toxoplasma* soluble antigens; these include the inhibition of IL-15, IL12p40, IL12p70, TNF- α secretion and costimulatory molecules but no MHC-II expression. In addition, the chemokine receptor CCR7 is also downregulated, suggesting the reduced ability of DC to migrate to rich T cell areas as another possible mechanism used by *T. crassiceps* to evade the immune response. When TcES-stimulated DC are tested in an allostimulation assay, these cells are weak inducers of T cell proliferation and the presence of TcES also downregulate the strong proliferation expected by addition of LPS or CpG. Of particular interest is that periodate-treated TcES are unable to inhibit the production of proinflammatory cytokines elicited by different TLR ligands and in the same way, carbohydrates in TcES are necessary to polarize the immune response towards a Th2 type in a CD4+ OVA transgenic model. This situation opens the possibility for a role of carbohydrate receptors in the modulation of DC function by *T. crassiceps*-antigens (C, Terrazas, manuscript under review). Interestingly, the limited responses observed in DC of BALB/c mice (a susceptible strain for *T. crassiceps* infection) upon TLR stimulation is not observed when DC derived from the resistant mice C57Bl/6 are exposed to TcES [134], indicating that modulation of DC response by *T. crassiceps*-products is a key element in the outcome of this infection. See Table 2.

4. Parasites and the Interference with DC Function: Is There a Common Pathway in a Common Strategy?

The evidence discussed in this review clearly show that by targeting the principal sentinels and directors of the immune system, DC, parasites actively evade immune attack. DC pleiotropy allows protozoan and helminth parasites to interfere with different aspects of the immune response. In most cases, either protozoan or helminth parasites, are able to impair the maturation process and the proinflammatory cytokine production of DC [9, 14, 19, 52, 66, 113], but other effects like a modified migration or an inability to fully differentiate are also found on DC exposed to these parasites or their products [14, 28, 55, 92]. The consequences of these different effects on DC function are diverse, but ultimately it is believed that they provide a less aggressive environment for parasite development inside their host. Several findings indicate the recognition of parasites and their molecules through TLRs [11, 36, 65, 108]. However, it has been more complicated to define whether these interactions lead to the altered phenotype and function that is observed in DC exposed to protozoan and helminth parasites. For the protozoan parasites, most data indicate that recognition and signaling of the whole parasites or their products through TLRs trigger activation pathways in DC that ultimately confers resistance against infection [36, 75, 82]. However, it is important to point out that some of the same molecules

that act as TLR ligands and induce a protective response, can also interfere with the DC function by inhibiting their maturation and production of the proinflammatory cytokines associated with parasite clearance [28, 37, 51, 62]. The mechanisms involved in DC “inactivation” are far from being completely understood. One possible explanation for this is the recognition of protozoan-derived molecules by other receptors than TLRs that in turn might interfere with TLR function. According to this rationale, in the last years new evidence has implicated CLRs as important players in intracellular signalling rather than only phagocyte receptors and more importantly, these receptors are also capable of inhibit TLR-mediated signalling [131]. Of special interest is to note, that impairment of TLR-induced response has turned out to be a common and a main feature in the modulation of DC induced not just by protozoan, but also by helminth parasites [66, 87, 103, 113]. Interference with TLR signalling may explain most of the downregulatory effects that these parasites have on DC, since MyD88-dependent pathways are involved in DC maturation and secretion of key cytokines such as IL-12 [61, 135]. However, evidence shows that other receptors and hence other signalling pathways may be involved in DC downregulation. An example of this is the phenomenon of “DC-paralysis” observed after the peak of IL-12 production in response to *T. gondii* parasites or its antigens. This is achieved by the parasite-induced production of lipoxin A₄ (LXA₄) [85, 136], which in turn is recognized by the FRPL-1 or AhR receptors on DC [16], triggering SOCS2 and turning off IL-12 production [137]. Likely, similar mechanisms could promote the low response observed on DC exposed to other protozoa and even helminthes since *B. malayi* microfilariae can also up-regulate expression of SOCS1 and SOCS3 [103]. Alternatively, TLR-stimulation could trigger different responses from DC, probably by becoming tolerogenic [30] or exhausted [84, 138, 139] (Figure 1).

For helminthes, the interaction with TLRs on DC lead, in general, to a “immature state” characterized by an absence or a moderate expression of costimulatory molecules together with no proinflammatory cytokines secretion, features that in turn seem to induce the development of a Th2 immune response [140]. Once again, the exact mechanism implicated in DC proinflammatory cytokine downregulation in response to TLR stimulus and the signals elicited by helminth products that condition these same cells to induce a Th2 response still remain unclear [140, 141]. Interestingly, not all the costimulatory molecules are downregulated in DC exposed to helminth products. For OX40L, a molecule important for an optimal Th2 response, there is an up-regulation in presence of some helminth products [105, 142]. In this regard, it would be important to determine if other molecules can be expressed selectively after helminth antigen recognition and this knowledge could lead to a definition of a truly Th2 polarizing DC phenotype. Other features have been investigated in an effort to define the phenotype and characteristics of helminth pulsed DC. While most of the evidence shows these DC are in an “immature state”, recent data indicate that SEA can induce phagosome maturation, a characteristic that is associated with DC maturation. In this

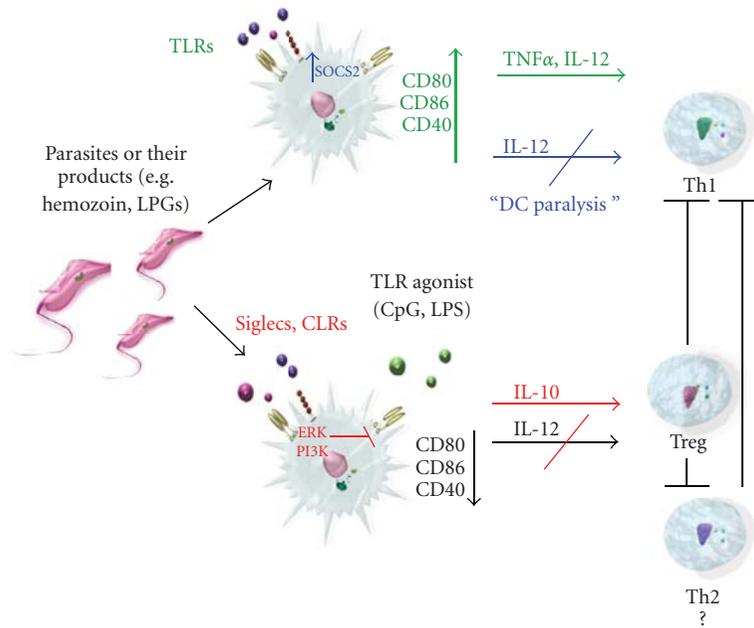


FIGURE 1: Overview to protozoan and DC interactions. Protozoan parasites and their products interact with TLRs on DC leading to their activation and release of proinflammatory cytokines and up-regulation of costimulatory molecules promoting a Th1 responses and the control of the infection. However, in some cases (*T. gondii* infection), this response can be later impaired by the same parasites through mechanisms that involve enhancement of SOCS proteins expression and downregulation of IL-12 production. In addition, interactions of parasite molecules with Siglecs and CLR receptors may be responsible of maintaining DC in an immature state and refractory to TLR stimuli, diminishing their proinflammatory response likely by using ERK and PI3K-dependent pathways. These DC may lead to activation of Treg responses that presumably favour parasite survival.

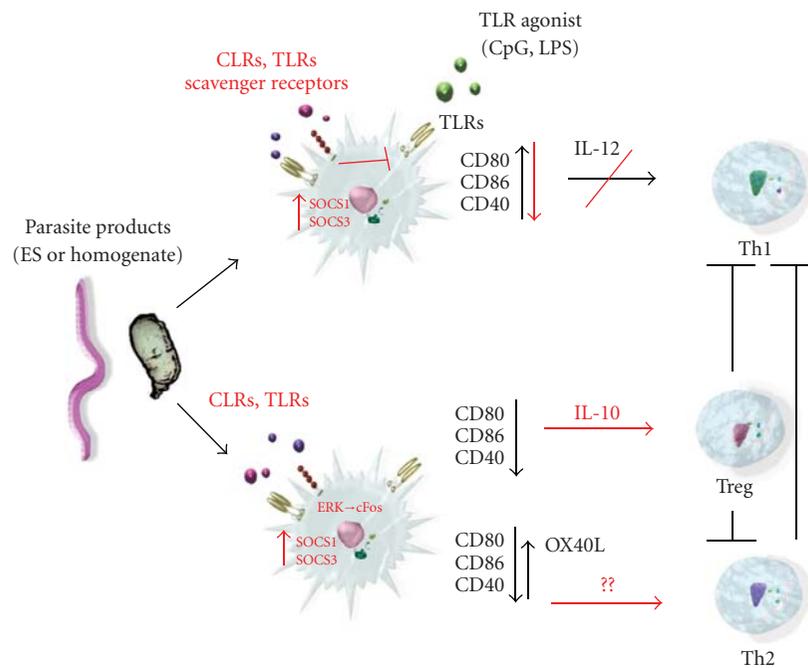


FIGURE 2: Overview to helminth and DC interactions. Helminth products are recognized by receptors such as TLRs, CLR receptors or Scavenger receptors. These cells remain in an immature state and unresponsive to further TLR stimuli probably due to interaction and signalling of parasite molecules through CLR receptors. Signalling pathways that implicate ERK phosphorylation, c-Fos up-regulation and expression of SOCS proteins may play a role in downregulation of DC responses particularly by suppressing IL-12 production. Once again, interactions of helminth molecules with CLR receptors but also with TLRs may be involved in these inhibitory effects. Finally, these helminth-conditioned DC induce a Th2 or Treg lymphocyte responses.

study, LPS and SEA-stimulated DC have similar phagosome acidification and proteolytic activity and this effect is MyD88 independent disregarding the activity by most TLRs [143]. Thus DC exposed to helminth products could exhibit an immature phenotype along with some features of a mature phenotype (Figure 2).

As mentioned earlier in this review, ligation of some CLRs might lead to the impairment of TLR-mediated signalling [131]. For instance, ligation of DC-SIGN on the DC can preferentially induce IL-10 production upon LPS addition [144] and the interaction of this receptor with pathogens such as *Mycobacterium sp.*, HIV-1 and *Candida albicans*, affects TLR-4-mediated immune responses by the same cells [145, 146]. Interestingly, DC-SIGN binds SEA and this antigen extract and other glycoconjugates derived from *S. mansoni*, can alter DC responses after TLR-stimulation [18].

Other lectin receptors might also possess the ability to interfere with TLR-induced response in DC. Recently, it has been shown that sialylated structures from *T. cruzi* bind the inhibitory lectin, Siglec-E. Of special interest, is that this interaction likely account for the suppression of LPS-induced IL-12 production and the enhanced levels of IL-10 observed in DC exposed to *T. cruzi* parasites [17]. Supporting this idea, other studies have shown a downregulation of immune cells responses after ligation of Siglecs [147, 148].

Signalling pathways involving activation of the kinases ERK and PI3K have been implicated in the negative regulation of DC maturation and IL-12 synthesis as well as in the enhanced production of IL-10. It is believed that pathogens use these signals to preferentially evoke Th2-type immune responses or at least impaired their opposing party [141, 144, 149–151]. In this regard, it is important to note, that among protozoan and helminth parasites, activation of ERK and PI3K seem to play indeed, a main role in the interference of DC function. Different examples of ERK activation implicated in impaired DC maturation, downregulation of IL-12 production or polarization of the immune response to Th2 have been reported for both types of parasites [26, 49, 115, 128], whereas the protozoos *G. lamblia* and *L. major* can also inhibit IL-12 synthesis in a PI3K-dependent pathway [94, 151]. Interestingly, pathways ERK and PI3K-dependent can be activated by engagement of CLRs [144, 152],

We believe that interaction of protozoan and helminth molecules with CLRs and probably other lectin receptors, along with the consequent activation of ERK and PI3K-dependent pathways, are key events that might determine the way a DC respond, either to direct parasite stimuli or to heterologous TLR ligands. This mechanism may explain, the induced immature phenotypes observed in DC exposed to these parasites and their products as well as the decreased proinflammatory cytokine production (IL-12 mainly). The enhanced IL-10 synthesis and Treg or Th2 polarization activity of DC exposed to protozoan and helminthes antigens, may also be a consequence of such interactions. Therefore, even when more studies are necessary to determine the exact mechanisms involved in downregulation of DC function by parasites, after all, there is maybe a common pathway that

helminth and protozoan parasites use to achieve it (Figures 1 and 2).

5. Concluding Remarks

During evolution, parasites have developed several sophisticated strategies in order to avoid or deal with the attacks of immune system. These studies have shown that interfering with the activity of DC can be one of the most effective ways to induce a safer environment to parasite development. Interestingly, despite the phylogenetic distance between protozoa and helminthes and even among helminthes, there seems to be a remarkable similarity in the way all these parasites modulate DC responses. Interaction with different receptors, particularly CLRs, may induce the downregulation in DC function although several other molecules like, Scavenger, lectins or G-coupled signaling receptors could also be involved. Understanding the different mechanisms that these parasites use to interfere with DC will certainly answer important questions that will increase our understanding of these pleiotropic cells and help us to design targeted therapeutic strategies based on this knowledge.

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Review Article

Similarity and Diversity in Macrophage Activation by Nematodes, Trematodes, and Cestodes

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This review summarizes current knowledge of macrophages in helminth infections, with a focus not only on delineating the striking similarities in macrophage phenotype between diverse infections but also on highlighting the differences. Findings from many different labs illustrate that macrophages in helminth infection can act as anti-parasite effectors but can also act as powerful immune suppressors. The specific role for their alternative (Th2-mediated) activation in helminth killing or expulsion versus immune regulation remains to be determined. Meanwhile, the rapid growth in knowledge of alternatively activated macrophages will require an even more expansive view of their potential functions to include repair of host tissue and regulation of host metabolism.

1. Introduction

Since the discovery in the late 1980s that T helper cells exhibit distinct cytokine profiles, the unique immunological profile associated with helminth infection has been explained by the activation of the Th2 cell pathway. In particular, the dramatic increase in numbers of eosinophils, mast cells, and IgE could be directly explained by cytokines produced by the Th2 subset. More recently, we have come to appreciate that in addition to eosinophils and mast cells, “alternatively-activated” macrophages (AAM Φ) are a characteristic feature of the polarized Th2 response. Macrophages at helminth infection sites were termed AAM Φ because they exhibited specific characteristics, such as arginase 1 production, that had been observed when macrophages were exposed in vitro to IL-4 [1–3]. Siamon Gordon and colleagues described this as an alternative activation pathway that contrasted with the “classical” activation by LPS and IFN γ [4, 5]. With further exploration of the in vivo phenotype, it has become apparent that AAM Φ express a whole range of molecules that distinguish them from classically activated macrophages (CAM Φ) [6–9].

What has been remarkable is the consistency of the findings between the diverse laboratories and infectious

models in which these cells have been studied, with identification of the same key molecules (arginase 1, resistin-like molecule [RELM] α , Ym1, etc.) expressed during helminth infection (see table in Reyes & Terrazas review [10]). This is remarkable because “helminths” represent an enormously diverse range of pathogens with entirely different phylogenetic origins and life histories. Indeed, the AAM Φ phenotype seems to occur in any strong Th2 environment including allergy and some chronic microbial infections [10–12]. The commonality of these findings has, perhaps erroneously, suggested to us that we could define a broad function for these cells analogous to microbial killing for CAM Φ . However, despite an ever-broadening definition of AAM Φ and their associated markers and characteristics, we are still essentially ignorant of their in vivo function. Perhaps it is time to explore the many differences between the models used to study AAM Φ and consider that these cells may function differently depending on context.

2. Helminths and Th2 Immunity

2.1. Helminth Phylogeny. Helminth parasites are routinely used as models to study T helper cell polarization and

as a result, our understanding of Th2 subset development and control has become increasingly sophisticated [13–17]. However, it is important to appreciate that to draw broad conclusions from experiments with schistosomes and then apply these to nematode parasites (or vice versa), is potentially misleading. Despite the common terminology, the only shared biological features of many “helminthes” are their metazoan origins and the ability to infect mammals. Schistosomes are part of the platyhelminths that include the cestodes (tapeworms) and other trematodes (flukes or flatworms). The phyla Nematoda (roundworms) include hookworms, whipworms, and filarial parasites. The split that led to Platyhelminthes and Nematoda occurred over 1 billion years ago, long predating the split between vertebrates and invertebrates [18]. Nematodes are the most abundant animal on earth both in terms of total numbers and numbers of species. Within this group of animals, parasitism has independently evolved many times [19] and parasitic nematodes represent an enormous burden in terms of human, animal, and even plant health. In terms of human disease, platyhelminths infect fewer numbers but are responsible for higher levels of morbidity and mortality [20].

2.2. Th2-Biased Immunity. The utility of helminths as models to study Th subset bias stems from the striking feature that, despite their phylogenetic diversity, they all induce profound Th2 responses, characterized by CD4⁺ T cells producing IL-4, IL-5, IL-9, IL-10, and IL-13 among others. However, recently it has become apparent that even the Th2 subset itself is enormously complex, with T cells that specifically function to provide B cell help and produce IL-4 but not the other signature Th2 cytokines (follicular helper cells) [15], specific IL-9 producing cells [21], and other T helper subsets that produce Th2 cytokines such as the recent discovery that IL-10 is produced by Th1 cells [22]. In an adaptive immune response, macrophages ultimately respond to T cell derived cytokines, and a sustained alternative activation phenotype absolutely requires CD4⁺ Th2 cells [23]. Thus, differences in Th cell cytokine profiles in different tissues and in response to distinct parasites will determine differences in macrophage activation.

2.3. Nematodes, Trematodes, and Cestodes and the Induction of the Th2 Response. Although virtually all helminths induce Th2 cytokines, the pattern and magnitude of these responses differ widely due to not only the vast differences in the biology of the pathogens as mentioned above, but also their broadly different migration and eventual host niche. Nematodes typically drive strong type 2 cytokine responses from the onset of infection. Indeed, within hours of infection innate activation of the Th2 pathway can be detected [23–26]. However, even within the nematode phyla the intensity of this early type 2 response varies, perhaps reflecting the differential ability of nematodes to inhibit the type 1 inducing cytokine, IL-12 [27].

Eggs released by the schistosome parasites are believed to be the strongest known inducers of Th2 cytokines in mammals, and yet the invasive cercariae induces only

a moderate Th2 response that is matched by a Th1 response of similar magnitude [28]. This initial mixed response is swamped by the extraordinarily high Th2 response generated to the eggs produced when the adult pairs reach sexual maturity [29]. Because of its importance as a cause of human morbidity and the availability of excellent mouse models, far more is known about the immunology of schistosome infection than other trematodes. However, *Fasciola hepatica*, a liver fluke that predominantly infects sheep and cattle, has also been studied in mouse models. Consistent with indicators of type 2 immunity in cattle, *F. hepatica* infection of mice results in a dominant Th2 response that has the capacity to suppress Th1 responses [30].

Cestodes are also dramatically understudied despite their capacity to cause severe disease in animals and people. Nonetheless, the data is consistent with the general helminth literature in that cestodes by and large have a strong propensity to drive Th2 immune responses. Similar to responses to schistosomes, peritoneal implantation of BALB/c mice with *Taenia crassiceps* metacestodes results in an initially weak mixed Th1/Th2 response that becomes strongly Th2 dominated as infection progresses to the chronic phase [31, 32]. However, there are resistant mouse strains that expel the parasite in the acute phase due to the dominance of IFN γ production [33]. Responses to peritoneal infection with *Echinococcus granulosus* protozoa are unusual in that the initial Th2 responses that dominate early (week 1) become more mixed, with emergence of IFN γ production as infection progresses (week 4) [34].

2.4. Protective Immunity against Nematodes, Trematodes, and Cestodes. In addition to the differences in the kinetics and magnitude of Th2 induction, the role of Th2 immunity in host protection varies substantially between these different parasites. Indeed, the paradigm that Th2 immunity is acting to destroy or expel worms is by no means universal.

The scenario in which there is an absolute requirement for Th2 immunity in host protection is that of the gastrointestinal nematodes. Expulsion of all GI nematodes studied to date is exquisitely dependent on Th2 cells. However, the specific cytokines (IL-4, IL-5, IL-9, IL-10, IL-13 among others) and the effector cells on which they act (epithelial, smooth muscle, mast cell, macrophage, nerve) vary tremendously depending on the location of the parasite, as well as its invasive properties. The situation with nematodes, such as the filariae, that live entirely in the tissues is somewhat different. Although Th2 responses are required for worm killing [35], Th1 immunity and particularly IFN γ , rather than inhibiting the anti-parasite Th2 response, act synergistically with IL-5 to kill the adult stage of the parasite [36]. In the case of these tissue-dwelling nematodes, our increased understanding of the cytokine pathways required for parasite destruction has still left us in the relative dark as to the actual killing mechanism(s).

During schistosome infection, Th2 responses are essential for host survival but this has little to do with detrimental effects on the parasite. This is largely due to the fact that pathology is the result of the egg deposition stage and

it appears that in the absence of a Th2 response (or a Th1 response for that matter), the egg is highly tissue destructive. Death can occur either due to overwhelming gut inflammation and sepsis or liver damage [37]. Additionally, Th2 responses to the egg promote fibrosis that itself can be a major cause of morbidity [38].

Cestodes provide an unusual perspective, in that despite inducing a potent Th2 response, protective immunity can require Th1 cells [33, 39]. Indeed, dominance of Th2 responses during infection with *T. crassiceps* leads to susceptibility to infection mainly through the suppression of Th1-driven nitric oxide (NO) production, a key effector molecule against these parasites [32, 39]. However, cestodes are also unusual in that there is little consistency in immunological mechanisms that protect against infections within this group. In this respect, resistance to *E. granulosus* actually increases in the absence of NO production [40], possibly due to the absence of the considerable suppressive effects on proliferation that this molecule exerts in echinococcosis [41].

3. The Molecular Profile of AAM Φ

AAM Φ are becoming increasingly recognised as a key effector arm of the Th2 immune response, but their actual function in different helminth infections has yet to be unravelled (see below) and is likely to be as diverse as the role of Th2 immunity itself. The concept of “alternative macrophage activation” was introduced by Siamon Gordon and colleagues in the early 1990s to describe the in vitro response of macrophages to the Th2 cytokines IL-4 and IL-13 [4, 5]. Significantly, the term AAM Φ was coined to highlight the activated nature of these cells that distinguished them not only from macrophages classically activated by microbial products and Th1 cytokines (CAM Φ), but from deactivated macrophages in which costimulatory molecules and class II expression are suppressed by down-regulatory cytokines such as IL-10. The two features that distinguished AAM Φ in vitro were the expression of arginase 1 and the mannose receptor. The requirement for IL-4 and/or IL-13 was subsequently confirmed in vivo, using gene-deficient mice [6, 11, 42, 43].

Realization that the AAM Φ described in vitro were a feature of helminth infection came from studies of *Schistosoma mansoni* and *Brugia malayi* [6, 43]. Both studies verified IL-4 dependent arginase 1 expression by macrophages in vivo, but the *B. malayi* study additionally identified novel IL-4 dependent genes associated with this phenotype including Ym1 and RELM α /FIZZ1 [6]. The highly unique profile was rapidly confirmed across the full range of helminth infections [7, 44–48]. Although Ym1 and RELM α were discovered in vivo, the direct induction of these genes by IL-4 and/or IL-13 was also demonstrated in vitro [2, 3]. It should be noted here that IL-4 and IL-13 both utilize the same signal transducing receptor chain, the IL-4 receptor α (IL-4R α), which explains the considerable overlap in function of these cytokines. Which of these cytokines is more important for alternative activation of macrophages in vivo remains to be fully determined, however, a recent report using mice

deficient for the IL-13 receptor α 1 subunit suggests that IL-13 is dispensable for expression of Ym1 and RELM α but not arginase in the liver during *S. mansoni* infection [49].

A molecular signature for AAM Φ (defined here as an IL-4/IL-13 dependent phenotype) has arisen that is represented by the three most abundant IL-4/IL-13 dependent gene products: Ym1, RELM α , and arginase 1. Ym1 is a member of the family 18 chitinases but has no chitinolytic activity and is thus referred to as a chitinase-like molecule [50]. Other members of this family in mice include Ym2 and acidic mammalian chitinase (AMCase), the latter functioning as a true chitinase. Ym2 and AMCase are also IL-4/IL-13 inducible proteins and the similarity between Ym1 and Ym2 is so high that most studies do not actually distinguish between them. All antibodies to date recognize both, and most PCR methods do not distinguish them, although this is possible with careful primer design. Thus, unless a study clearly identifies a specific Ym protein, it might be appropriate to use the more ambiguous designation Ym1/2. RELM α was first described in a lung asthma model, where it was described as FIZZ1 [51], but was subsequently identified as a member of a family of cysteine-rich molecules related to resistin, a hormone involved in glucose metabolism [52]. Arginase 1 is the best studied of these proteins and has well-established roles in regulating NO production by competing with iNOS for their common substrate L-arginine [1], as well as inhibition of T cell responses through L-arginine depletion [53]. The arginase pathway additionally leads to the production of proline and polyamines, which contribute to tissue repair and fibrosis [54]. Subsequently there has been identification of numerous other markers associated with the alternative activation phenotype [7, 9] and this number is likely to grow as more extensive transcriptomic and proteomic analyses are undertaken [55].

Macrophages with an AAM Φ phenotype characterized mainly by arginase 1 production also arise in protozoan (reviewed in [10]) and certain bacterial infections [56]. In cutaneous leishmaniasis (*Leishmania major*), this AAM Φ phenotype is dependent on signaling through the IL-4R α chain [57] as in helminth infection models [6, 11, 42]. However, a STAT6-independent pathway also leads to arginase 1 expression during *Mycobacterium tuberculosis* and *Toxoplasma gondii* infections, which in the former is dependent upon TLR signaling [56]. The main effect of arginase 1 expression in all of these settings appears to be an increase in susceptibility to infection through diversion of L-arginine from production of the reactive nitrogen intermediates that kill these pathogens [10, 56].

As interest in these cells grew, the term “alternatively-activated” came to include any cell displaying an alternate phenotype to CAM Φ . Subdivision of the M1 and M2 terminology has helped to address this issue with M1 equating with CAM Φ while M2 includes M2a, M2b, and M2c. M2a most closely reflects the IL-4/13 dependent phenotype originally associated with AAM Φ , while M2b includes activation by other modulators such as immune complexes that lead to high IL-10 production and M2c reflecting the more deactivated phenotype associated with IL-10 treatment in vitro [58]. Nonetheless, the difficulty in

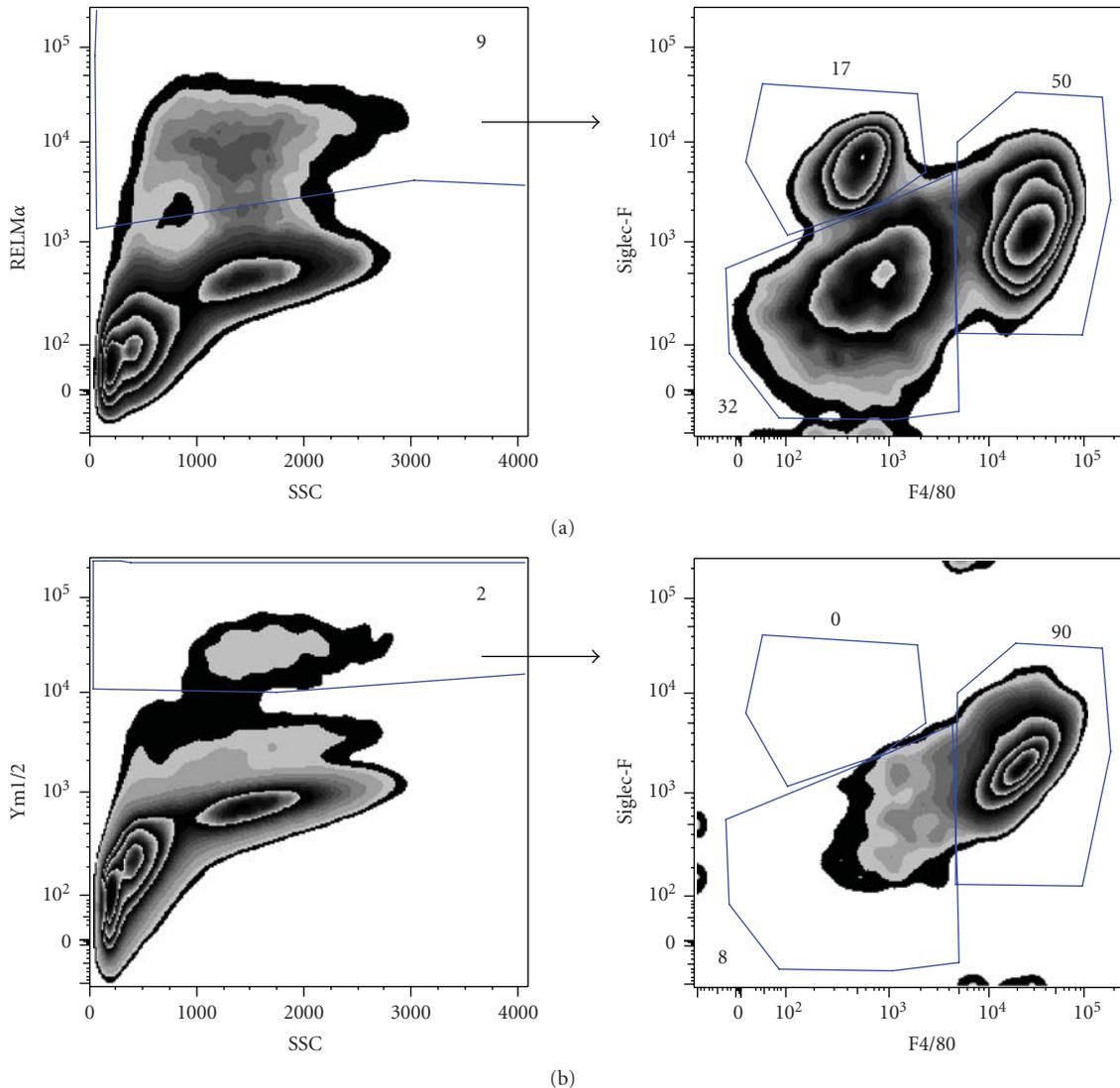


FIGURE 1: RELM α and Ym1/2 expression in the pleural cavity during *L. sigmondontis* infection. *Left-hand plots*: Flow cytograms depicting side scatter (SSC) versus RELM α (a) or Ym1/2 (b) of pleural cavity cells 12 days post infection with *L. sigmondontis*. The gates for RELM α ⁺ and Ym1/2⁺ cells were set using isotype control staining. The proportion of cells positive for RELM α and Ym1/2 in naïve mice was 1.5% and 0.05%, respectively. *Right-hand plots*: Siglec-F versus F4/80 expression on Ym1/2⁺ or RELM α ⁺ cells. Numbers in italics represent percentage of cells within the neighbouring gate.

finding appropriate terms is a reflection of the enormous diversity in macrophage phenotype found both in vivo and in vitro [59], as well as their capacity to rapidly alter their expression profile in response to a new set of environmental signals [60].

A current difficulty in delineating the functions of AAM Φ is that many of the “signature” AAM Φ molecules are not restricted to macrophages. The availability of good antibodies for intracellular staining and fluorescence microscopy, the creation of mice that report gene expression, and the ability to sort cell subsets prior to gene expression analysis have greatly increased our knowledge of the range of cells that show an “alternative-activation” phenotype, as well

as the comparative breadth of expression of the different AAM Φ markers. For example, in liver granulomas from mice infected with *S. mansoni*, the main producer of RELM α appears to be eosinophils rather than macrophages [61], whilst in lung granulomas induced by i.v. injection of schistosomula, RELM α ⁺ cells are comprised of macrophages, eosinophils, and airway epithelial cells [62]. In the serous cavities of mice infected with *Litomosoides sigmondontis* or *B. malayi*, we have observed a similarly broad pattern of RELM α expression, with mature macrophages (F4/80^{hi} Siglec-F⁻), eosinophils (Siglec-F^{hi} F4/80^{lo}), and F4/80^{lo-intermediate} Siglec-F⁻ cells that include DC, all capable of making this protein (Figure 1(a) and data not shown). Expression of Ym1/2 is

markedly different, being almost exclusively restricted to F4/80^{hi} Siglec-F⁻ mature macrophages (90%), and with no expression detectable in eosinophils (Figure 1(b)). However, Ym1/2, like RELM α can also be expressed by epithelial cells in the lung [61, 63, 64] and both Ym1/2 and RELM α appear to be a feature of many types of antigen presenting cell found in the lymph nodes draining helminth infection sites [46]. Thus, it is now apparent that many cell types can show an “alternative-activation” phenotype, with chitinase- and resistin-family members prominent. Epithelial cells in particular express not only Ym1/2 and RELM α during Th2 immune responses but related family members including the true chitinase, AMCase [63] and RELM β [65]. Of the three most abundant AAM Φ markers [6], arginase 1 appears to have a more macrophage-restricted expression profile. This was demonstrated by Reese et al. using mice that contain an IRES-YFP knock-in allele that reports arginase 1 expression, in which extra-hepatic arginase 1 expression was macrophage-restricted in the lung or peritoneum of *Nippostrongylus brasiliensis* infected or chitin injected mice, respectively [66].

Another major difficulty has been efforts to translate our understanding of murine AAM Φ to humans, not only because some of the mouse defined AAM Φ markers are not present in the human genome, but because the relevant tissues cannot be readily accessed. An example of this problem has been the argument over whether human macrophages express arginase 1, strongly reminiscent of earlier arguments about NO production [67]. It may be that arginase 1 expression is more limited in human macrophages or that we have just not yet identified the right tissues. Indeed, arginase 1 can be induced in human macrophages by IL-4 [68] and can be observed in monocytes of filarial-infected individuals [69].

Even more problematic has been the realization that Ym1 is not even present in the human genome. However, distribution of the family 18 chitinases (including AMCase and Ym1/2) between different mammalian species is a fascinating puzzle in itself. Mammals have two genes encoding active chitinases that represent an ancient gene duplication event and show high sequence homology to chitinases of lower organisms. The mammalian chitinase-like proteins (CLPs) that include Ym1, appear to represent more recent gene duplication events with subsequent loss-of-function mutations [70]. Thus all mammals express the highly conserved active enzymes, chitotriosidase and acidic mammalian chitinase (AMCase) but additionally express a broad range of diverse CLPs, with each mammalian species exhibiting a different complement of CLPs [70]. In mice these include Ym1, Ym2, and YKL-40/BRP-39, which have all been strongly implicated in Th2 conditions [50, 71]. Humans express YKL-40 but also a distinct CLP, YKL-39 [70]. Because no two mammals express the same set of these proteins and CLPs appear to be undergoing remarkably active evolution, no animal model can fully represent the human genes. Studying mice should nonetheless be informative as one can presume that despite species differences a common theme lies behind the evolutionary forces driving the divergence of CLPs.

4. Functional Roles of AAM Φ

As the molecular definition of AAM Φ becomes more refined, our hope has been that an understanding of function would follow. However, the functions of gene products associated with alternative activation, such as RELM α and Ym1 remain elusive and our full understanding of the contribution of macrophages during helminth infection is an increasingly active area of investigation. Considering the diversity of helminth infection and the complexity of the associated Th2 response, a single well-defined role for AAM Φ is unlikely to emerge.

4.1. Do M Φ Promote Helminth Killing or Expulsion? The depletion of macrophages using clodronate-loaded liposomes has provided a powerful tool by which to analyse the function of these cells during helminth infection. This technique has provided evidence that macrophages play a central role in nematode expulsion during intestinal infection, both in the memory response to a secondary infection with *Heligmosomoides polygyrus* [72], and in expulsion of primary *N. brasiliensis* infection [73]. In both these settings, parasite clearance is dependent upon a strong Th2 response, which acts to rapidly recruit immune cells including macrophages to the infection site and to stimulate their expression of Arginase 1, RELM α , and Ym1/2 in a STAT-6 dependent manner. Critically, blocking recruitment of macrophages via depletion of monocytes resulted in prevention of worm expulsion, whilst the Th2 response and recruitment of other inflammatory cell populations were left intact. Our understanding of macrophage function in filarial nematode attrition is more limited. However, observations that worm survival during murine peritoneal infection with either *Brugia pahangi* or *malayi* L3 larvae is enhanced following injection of carbon particles or carrageenan [74, 75] imply an effector function for peritoneal macrophages. Consistent with a role in filarial killing, macrophages make up significant proportion of the granulomas that encase dying *B. malayi* and *L. sigmodontis* worms but the conundrum is: do granulomas cause worm damage or form because the worms are already damaged?

While there is evidence for macrophage effector function during nematode infections, it is still unknown whether this occurs via direct or indirect mechanisms. Macrophages greatly increase the hypercontractility of intestinal smooth muscle during *N. brasiliensis* infection, providing a potentially indirect effector mechanism [73]. Because filarial nematodes are restricted to tissue sites during infection, it is likely a distinct though overlapping array of effector mechanisms is required to act against these nematodes. Perhaps a more likely role for macrophages in these infections is to recruit other Th2 effector cells important in nematode attrition. In this respect, eosinophils have a well-documented role in vivo, acting against larval stages of both *B. malayi* and *L. sigmodontis* [76, 77], and recent data demonstrates that recruitment of eosinophils to the peritoneal cavity following *N. brasiliensis* infection or injection of chitin is dependent on macrophages [66, 78]. An attractive possibility for a direct anti-nematode effector function is the association of AAM Φ

with chitinases and chitinase-like molecules [50], which in principle have the capacity to act on chitin-containing stages of the parasite. However, as of yet, there is no direct evidence to support this.

4.2. Is Alternative Activation Required for Anti-Worm Effector Function? Whilst macrophages can perform as anti-nematode effector cells, the question remains whether they need to alternatively activate to exert this function. Anthony et al. [72], showed that, like macrophage depletion, an inhibitor of arginase, S-(2-boronethyl)-L-cysteine, could impair worm expulsion during secondary *H. polygyrus* infection [72]. Using the same technique, arginase I expression was also implicated as mediating expulsion of *N. brasiliensis*, although experiments were inconclusive since treatment only prevented worm expulsion in 60% of the mice despite parasite egg production and host smooth muscle hyper contractility being greatly impaired [73]. The broad-acting nature of this treatment (it blocks both arginase I and II and could potentially act directly on worms in addition to other non macrophage host cell sources) makes it hard to draw firm conclusions. A stronger case against alternative activation driving these macrophage effector mechanisms, is provided by two earlier studies both of which used mice on the same resistant BALB/c background as Zhao et al. [73]. These demonstrated that IL-4R α need not be expressed on macrophages/neutrophils or indeed any hematopoietic population in order for efficient expulsion of *N. brasiliensis* [79, 80]. Using the same macrophage/neutrophil-specific IL-4R α -deficient mice, it has also been shown that alternative activation of macrophages is not required for expulsion of another intestinal nematode *Trichinella spiralis* [81]. Other potential effector functions of “Th2-associated” macrophages may also be independent of alternative activation state, as for example, macrophage-dependent-recruitment of eosinophils in response to chitin injection is STAT6-independent [66]. It is quite conceivable that in Th2 infections, macrophage effector function could be completely independent of AAM Φ -associated molecules or that expression of arginase 1 or other AAM Φ -associated molecules could be induced by an IL-4R α -independent mechanism, for example via a TLR-dependent event [10, 56] such as exposure to gut flora. Unfortunately, the expression of either arginase 1 or other AAM Φ associated markers was not investigated in the intestinal tissues of *N. brasiliensis* or *T. spiralis* infected M Φ /neutrophil-specific IL-4R α -deficient mice [79, 81]. Comparative analysis of the susceptibility of mice which lack, in macrophages specifically, either IL-4R α or “alternative activation” proteins such as arginase 1 would help considerably to resolve the issue of the function of alternative activation “per se” in intestinal nematode infections. Interestingly, a study with such mice has shown that arginase 1 expression by AAM Φ has no host protective effect against primary infection with the trematode *S. mansoni* [82].

4.3. Can CAM Φ Act against Helminths? In contrast to the ambiguity surrounding alternative activation in immunity to

nematodes, it is clear that the reactive oxygen or nitrogen species can damage most types of helminth parasites [39, 83–86]. However, only in cestode infection do reactive nitrogen species and CAM Φ appear to function against the parasite in vivo. In murine cysticercosis (*T. crassiceps*) blocking of iNOS using the inhibitor L-NG-monomethyl arginine leads to increased parasite burdens [39]. Consistent with this, induction of Th2 responses and STAT-6 signaling underlie susceptibility to infection, whilst Th1 responses and STAT-4 signaling underlie resistance [32, 33]. However, as mentioned above, this is not a requirement in immunity to all cestodes. Indeed, NOS2 deficient mice, which are incapable of making iNOS, are actually less susceptible to infection with the cestode parasite *E. multilocularis* [40]. In this infection CAM Φ appear to have a pathological effect, most likely due to the direct immunosuppressive effect of NO on cell proliferation [87].

Given the divergence of the helminth parasite phyla and the host tissue sites they have chosen to infect, it is perhaps unsurprising that diverse effector mechanisms are required for immunity to different infections [44, 88]. However, a common thread is that macrophages can act against both nematode and platyhelminth infections, and there is still no published evidence of any infection in which macrophages can be dispensed at no cost to resistance. The mechanisms employed by the macrophages though are seemingly disparate. As discussed below, AAM Φ do play an important role in protecting the host in schistosomiasis by limiting parasite-mediated tissue damage rather than mediating killing [79]. Indeed as we struggle to identify direct antihelminth effects of AAM Φ , the evidence builds that the macrophage products most associated with alternative activation such as arginase 1 and RELM α have profound inhibitory effects on host immunity, including the Th2 response itself [61, 62, 82]. This raises the possibility that the alternative activation state of macrophages does not function primarily as an effector arm but has critical regulatory or parasite disposal (rather than killing) roles.

4.4. AAM Φ Are Potent Suppressors of Cellular Proliferation. One property of activated macrophages that is consistently observed in a wide variety of systems is the ability to block the proliferation of cells with which they are cocultured. This feature has been well described for CAM Φ in which the antiproliferative properties of NO are responsible [87]. Myeloid cells derived from helminth infected animals also exhibit similar antiproliferative properties [60, 89–91]. Importantly, it can be replicated in vitro by treatment of macrophages with IL-4 or IL-13 [2, 60] and in vivo is reliant on IL-4 and/or IL-13 in certain settings [89]. Indeed, the ability to inhibit cellular proliferation is a defining characteristic of AAM Φ . Despite the near-universal finding that AAM Φ suppress cellular proliferation ex vivo, the in vivo significance is not known. Understanding the relevance of this proliferative suppression has been complicated by the fact that, unlike CAM Φ , a single mechanism for proliferative inhibition has not been identified. Instead a multitude of pathways have been found that differ depending on the infection context (reviewed in [44]) and include Programmed

death ligand (PD-L) interactions [92, 93], TGF- β production [94], lipid mediator release [95], IL-10 production [96, 97], and L-arginine depletion [82]. There appear to be three categories of proliferative suppression generally observed during helminth infection: contact and IL-4 dependent, contact dependent and IL-4 independent, and finally IL-4 dependent and contact-independent. No doubt the target cells will also differ depending on the pathways involved, with some mechanisms, such as the PD-L pathway seen during infection with the platyhelminths, *T. crassiceps*, and *S. mansoni* [92, 93], affecting predominantly T cells. Other mechanisms have a broader target including even tumor cells that typically have no restriction on cell division [89].

One cannot overemphasize the diversity of suppressive mechanisms observed. For almost every mediator identified as critical for AAM Φ -mediated suppression of T cells, there is another study that finds that mechanism dispensable. This disparity could be due to the distinct biological mediators released by these vastly different parasites, which presumably all favour an immuno-suppressive environment. However, many other factors could account for this diversity, from differences in the magnitude and bias of the Th cell response to tissue localization. Of interest, proliferative suppression is also a feature of myeloid-derived suppressor cells (MDSC), which share many features with AAM Φ but are associated with cancer and other immune suppressive environments rather than helminth infection [98]. T cell suppression by MDSC is mediated by both iNOS-driven production of NO and arginase 1-driven depletion of L-arginine [53]. L-arginine is essential for T cell activation [99] but L-arginine depletion could also lead to production of suppressive reactive oxygen intermediates [95, 100]. This is similar to recent data showing that macrophage-derived arginase 1 is required to suppress the proliferation of T cells from *S. mansoni*-infected mice [82] but also during non-healing *Leishmania major* infection, which is associated with AAM Φ [1]. Although arginase 1 is emerging as one of the most important mediators of proliferative suppression, it is not the full story. Chemical blockade of arginase 1 had only a small impact on suppression mediated by AAM Φ from the peritoneal cavity of *B. malayi* implanted mice, and full IL-4-dependent suppressive capacity was maintained when arginase expression was reduced by LPS/IFN γ treatment [60].

Finally, it is important to consider that NO mediated suppression, although most strongly associated with microbial infection, also has a role to play during helminth infection. As already mentioned, NO can act as an effector molecule during infection with the cestode *T. crassiceps* [39]. However, within the same infection model [95], and infection with *E. multilocularis* [41], NO mediated suppression by peritoneal cells has been observed. Even in filariasis, where the IL-4 dependent AAM Φ suppressive phenotype has been well described, NO-mediated suppression can play a role [101].

4.5. AAM Φ as Antigen Presenting Cells. In line with the immuno-suppressive effects of AAM Φ described above, one

of the most consistent findings in human studies is that individuals infected with helminth parasites exhibit profound defects in lymphocyte proliferation [102–105]. One popular hypothesis has been that monocytes or macrophages from infected individuals were somehow defective in their antigen presentation capacity. However, as the discovery of alternative activation emerged and their capacity to actively block cellular proliferation was revealed the expectation shifted somewhat. Further, by definition AAM Φ are activated and thus might be expected to express good levels of class II and costimulatory molecules. Not surprisingly, the analysis of macrophage APC activation state during helminthiasis has been shown to vary considerably with infection. However, expression of antigen presentation-associated molecules is frequently intact or elevated, consistent with an “activation” profile. Mice carrying schistosome infections show marked up-regulation of MHCII but not CD80 or CD86 by splenic macrophages [93]. Transient up-regulation of co-stimulatory molecule and MHCII expression on lung macrophages occurs during the period *N. brasiliensis* larvae migrate through the lung but is quickly lost thereafter [106]. Following peritoneal implant of adult *B. malayi*, macrophages exhibit relatively high levels of MHCII, CD80, and CD86 expression compared to thioglycollate elicited M Φ , but not compared to LPS-stimulated cells [60]. Perhaps the strongest activation is seen in *T. crassiceps* infected mice, where MHCII, CD40, and CD86 but not CD80 are greatly up-regulated over an 8-week period [91]. However, this is by no means a feature of cestode infection, since the one documented parasitic helminth that leads to a reduction in activation state compared to naïve M Φ is *E. multilocularis* although only expression of CD40 is reduced whilst CD80 and CD86 remained unchanged [107].

A number of labs have investigated M Φ expression of B7 family members PD-L1 and PD-L2, with a diversity of findings in nematode, trematode, and cestode models. Independent of parasite species, Loke et al. defined PD-L2 as a marker for AAM Φ , specifically up-regulated by IL-4 in a IL-4R α /STAT-6 dependent manner and PD-L1 as a Th1-associated ligand [108]. However, neither PD-L1 or PD-L2 are up-regulated on peritoneal AAM Φ elicited by the nematode *B. malayi* [60]. In contrast, both ligands are up-regulated in the lung following but not during *N. brasiliensis* larval migration [106]. Similar dichotomy exists in the response to platyhelminths, with only PD-L1 up-regulation in response to *S. mansoni* infection [93], yet PD-L1 and PD-L2 up-regulation in response to *T. crassiceps* [92]. Significantly in these two settings, PD-L1 and/or PD-L2 act to potentially block the proliferation of T cells and are thus at least in part responsible for the contact-dependent proliferative suppressive effect of AAM Φ discussed above.

How then do AAM Φ perform as APC? Given that AAM Φ exhibit a profound ability to suppress cell division and fail to induce naïve T-cell proliferation, it was a surprise when initial experiments showed that AAM Φ from *B. malayi* infected mice were strong inducers of Th2 cytokine production when cocultured with naïve T-cells [109]. This ability is also shared with AAM Φ from chronic late-stage *T. crassiceps* infection [91]. Interestingly, the capacity to

drive Th2 cytokine production correlated with alternative activation, as adherent peritoneal cells from early-stage infection induce more of a mixed Th1/Th2 response while showing much lower expression of RELM α and Ym1/2 [91]. It remains to be determined whether the ability to drive Th2 cytokine production is a shared function of AAM Φ from all helminth infections. The difficulty in extracting AAM Φ in sufficient quantity from tissues, such as the gut lamina propria, has so far prohibited analysis of APC function in many settings, particularly intestinal infections.

It is tempting to draw a parallel to dendritic cells (DC) obtained from schistosome infected mice or exposed to helminth products *in vitro*. These exhibit a muted activation phenotype, with little change in expression of costimulatory molecules, and limited up-regulation of MHCII. However, they also efficiently promote Th2 polarisation and cytokine production [110–112]. Furthermore, DC can exhibit an alternative activation phenotype *in vitro* [46, 113] and during infection [46] or allergy [114], up-regulating expression of Ym1/2 and RELM α in an IL-4/IL-13 dependent manner [46, 113, 114]. Indeed, experiments looking at the ability of “alternatively activated” DC to drive Th2 responses *in vitro* and *in vivo* have identified Ym1/2 as a key molecule involved in the process [113, 114]. Ym1/2 appears to exert this effect by binding to 12/15-lipoxygenase and blocking production of PPAR γ ligands [114], which are thought to have immunoregulatory effects on macrophages and T cells [115]. Given the large quantities of Ym1/2 produced by AAM Φ it is quite possible they also influence Th2 priming via this molecule.

4.6. AAM Φ as Negative Regulators of Th2 Immunity

4.6.1. RELM α . The discovery that two novel proteins (Ym1 and RELM α) were secreted in abundance by macrophages activated during helminth infection [6] led rapidly to the speculation that these would be effector molecules against the metazoan invaders. This was supported by the realisation that Ym1 was a member of a family of chitinases with presumed defensive roles against chitin-containing pathogens such as nematodes. More direct (but still circumstantial) evidence came with the recognition that RELM β , another resistin family member, was abundantly secreted by epithelial cells in the intestines of nematode infected mice and bound directly to the chemosensory structures of the parasite [65]. The expectation naturally followed that similar anti-parasite roles would be identified for macrophage-derived RELM α . However, two recent papers utilizing RELM α -deficient mice have turned that idea on its head and instead identified RELM α as a critical regulator of Th2 immunity [61, 62]. Using models of *S. mansoni* and *N. brasiliensis* infection, and schistosome egg-induced lung granuloma formation, RELM α was shown to limit Th2-mediated immune pathologies by suppressing Th2 but not Th1 cytokine production. Importantly, this was mediated at least in part by a direct suppressive effect of RELM α on cytokine production by Th2 cells, as RELM α bound to Th2 cells and could exert this suppressive effect on T cells cultured

alone *in vitro* [62]. RELM α could also be detected bound to other cells, including macrophages and DC (but not Th1 cells) suggesting other non T cell mediated functions for this molecule. It is worth noting that macrophages appeared to be only a minor source of RELM α in the lung and liver in these studies, perhaps explaining why Th2 responses remain normal during *S. mansoni* and *N. brasiliensis* infections in macrophage/neutrophil-specific IL-4R α deficient mice [79].

4.6.2. Arginase 1. Given that one of the downstream products of arginase-mediated L-arginine catabolism is a major component of collagen, it has been widely assumed that AAM Φ would promote the fibrotic pathologies associated with chronic Th2 stimuli. However, a recent elegant study using mice in which macrophages were deficient in arginase 1 expression has demonstrated that in fact, arginase 1 negatively regulates Th2 responses and actually suppresses Th2-mediated fibrosis [82]. In contrast to the effects of RELM α documented by Nair et al. [62], arginase 1 expression by macrophages impaired IFN- γ production by T cells in addition to down regulating output of Th2 cytokines. T cell proliferation in the draining lymph node was also exaggerated in the absence of arginase 1 expression by macrophages [82]. Importantly, this data confirms an *in vivo* role for arginase 1 in proliferative suppression mediated by macrophages, but extends this to show that macrophages also exert an inhibitory effect on cytokine production. Critically, they demonstrate that macrophages exhibit an overall inhibitory effect on fibrosis during schistosomiasis via their production of arginase 1.

4.7. Summary of AAM Φ as Regulators of Th2 Immunity. One caveat to the conclusion that AAM Φ have a critical function in the regulation of Th2 cytokines in both nematode and platyhelminth infections is the fact that Th2 generation in both the secondary lymphoid organs and the infection site (in which AAM Φ are present in greatest numbers) appeared unaffected by either the absence of IL-4R α signaling in macrophages or the depletion of macrophages, during *S. mansoni*, *N. brasiliensis*, and *T. spiralis* infection, and *H. polygyrus* and *N. brasiliensis* infection, respectively, [72, 73, 79, 81]. It cannot be ignored that because the role of macrophages in Th2 generation was not the main focus of these studies, the methodology for assessing the quality and quantity of the responses was not as thorough as that described for the studies on the function of RELM α [61, 62], arginase 1 [82], and Ym1/2 [113, 114]. It is possible though, that the removal of macrophages, or their alternative activation state, takes away both negative (RELM α and arginase 1) and positive (Ym1/2) regulatory signals such that the net effect on Th2 responses is nil. Whilst an *in vivo* role for arginase 1 production specifically by macrophages in Th2 regulation during schistosome infection cannot be denied, we await confirmation that RELM α and Ym1/2 production by these cells plays a major role in regulation of Th2 cytokine production *in vivo*. It may well be that “alternatively activated” DC and cells such as basophils play the greater role in Th2 response induction, maintenance, and

regulation. With the recent recognition that basophils are a critical APC in promoting Th2 cell activation [14, 116], it would be of interest to know whether Ym1/2 is produced by these cells.

4.8. AAM Φ as Cells That Repair Damage to the Host. Much of the data described above suggests that AAM Φ act as anti-inflammatory down-regulatory cells, consistent with previously proposed functions for macrophages during helminth infection [117, 118]. Additionally AAM Φ are important sources of TGF- β and IL-10 [60, 109, 119], as well PGE2 [91] and the IL-1 receptor antagonist [119, 120]. The chemokine expression profile is also strongly associated with a non-inflammatory role [58] and with specific down-regulation of key proinflammatory cytokines by IL-4 [6, 120]. It may seem counter-intuitive that an activated cell population manifests such profoundly suppressive features. However, this could be in part explained if one sees tissue repair or wound healing as a fundamental function associated with AAM Φ . Effective tissue repair can only proceed if inflammation has been stopped [119, 121] and thus all these anti-inflammatory features may contribute to their role in repair. Early reviews on AAM Φ ascribed them a wound healing phenotype based on the production of arginase 1 [1] and angiogenic factors [1] as well as extracellular matrix components and fibronectin [122]. However, the specific role of IL-4/IL-13 in this healing phenotype versus glucocorticoids or IL-10, which the authors also considered alternative activators, was not immediately apparent. Furthermore, the relevance to helminth infection was not obvious.

Two recent papers have provided evidence that there is indeed very strong relevance to helminth infection. While investigating the kinetics of alternative activation in a model whereby *B. malayi* parasites are surgically implanted into the peritoneal cavity of mice, we noted that control animals who underwent only sham surgery exhibited transient up-regulation of Ym1/2, RELM α , and arginase 1 in a strictly IL-4R α manner [23]. However, only when both the nematode and Th2 cells were present was this alternative activation response sustained. This suggested that the induction of the signature molecules of AAM Φ was in fact an innate response to direct injury. One feature all these helminths have in common is the capacity to injure tissue in the course of their migration through the host, providing a possible evolutionary explanation for the association of Th2 immunity and wound healing [123]. The strongest evidence to date from helminth models that AAM Φ have a combined anti-inflammatory/wound healing function is in a study of *S. mansoni* infection in mice that lack the IL-4R α specifically on macrophages and neutrophils and thus completely lack AAM Φ but have otherwise intact Th2 responses [79]. Following *S. mansoni* infection, these mice died from overwhelming inflammatory responses in the intestine and leakage of bacteria into the blood. Although not conclusive evidence, the data strongly suggests that in the absence of AAM Φ , these mice were unable to repair the damage caused by egg migration through the intestinal wall. Further supporting a direct role for AAM Φ in wound healing, RELM α has angiogenic properties [124] and Ym1/2

has the ability to bind extracellular matrix [125]. The specific roles these proteins play in the complex orchestra for tissue repair and remodeling are still to be established.

5. Summary

Mast cells, basophils and eosinophils have long been considered the serious cellular players in the host response to helminth infection. Previously ignored, the macrophage is now taking center stage in this cellular family as one of the most important targets of Th2 immunity. This is fully appropriate when we consider that macrophages are frequently the most abundant cell type recruited to the site of helminth infection. However, it is only since the discovery of AAM Φ in vivo less than 10 years ago that a focus on these cells in helminth infection has begun. As a result, we have a long ways to go before we attain the extensive knowledge associated with CAM Φ . The challenge is to define key roles for AAM Φ while accepting that these may differ radically depending on infection stage, site, and parasite species. Macrophages are the workhorse of the immune system, and as such, can radically alter their phenotype to adapt to environmental signals [55, 59, 60]. In turn, they can actively regulate the inflammatory environment to which they are recruited or the tissues in which they reside. Using the tools available to modern scientists we can now begin to define the environmental codes that alter the AAM Φ expression profile, understand the function of the products they produce, and decipher their communication with other cells. Recent discoveries that AAM Φ are central to the regulation of host metabolism [126] mean this cross-talk is not only between cells of the immune system but with the entire organism. Unravelling this amazing complexity will keep helminth immunologists busy for years to come.

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Review Article

Nitric Oxide and Respiratory Helminthic Diseases

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Nitric oxide (NO) is a very simple molecule that displays very important functions both in helminths (mainly those involved in respiratory pathology) and in mammalian hosts. In this paper we review four issues related to interaction of NO and lung helminthic diseases. Firstly, we evaluated data available on the NO synthesis and release by helminths and their biological role. Next, we summarized the effect of antigens obtained from different phases of the biological cycle on NO production by host mammalian cells (mainly from human sources). Thirdly, we revised the evaluation of NO on the biological activities and/or the viability of respiratory helminths. Lastly, the deleterious consequences of increased production of NO during helminthic human infection are detailed.

1. Introduction

Many helminths are frequently in contact with pulmonary cells inducing lung injury (Box 1) which has different clinical manifestations (Table 1). During the helminthic lung infection there are many molecular interactions between inflammatory cells and helminths. Among them, nitric oxide (NO) is an important molecule involved in the pathogenesis of these diseases (Box 2).

The interaction between NO and helminths could be dissected in different issues that are reviewed in this paper: firstly, the NO production by different stages of the biological cycle of these parasites; secondly, the effect of helminth antigens on NO release by host cells; thirdly, the effect of NO on some helminths; and lastly, the pathological consequences derived from the increased NO production in helminthic diseases.

2. Do Helminths Produce NO?

There is fragmentary information available about the NO production by different helminths. This is due to the different techniques utilized in these studies (e.g., immunohisto-

chemistry, histochemical techniques (NADPH diaphorase), functional studies (use of inhibitors)) together with the absence of systematic studies in all helminth species.

NO-Synthase (NOS) enzymes or NO-activity-derived products (nitrites or nitrotyrosine) have been detected in different locations of adult worms. This is the case of neural NOS (nNOS) which it has been mainly found in the nervous tissue of *Schistosoma mansoni* [1] and, in the same way, inducible NOS (iNOS) in the parenchyma of *Schistosoma mansoni* and in the subtegument of *Schistosoma japonicum* [2]. Additionally, NO release in living schistosomes has been demonstrated by Kohn et al. [3], suggesting a functional role for the NO in the schistosome biology.

The presence of NOS has been also demonstrated in other helminths; for example, its functional activity has been detected in different nervous structures (central, peripheral, and enteric) and the hypodermis of the nematode *Ascaris suum* [4–6]. Similar distribution appears in *Toxocara canis* [7]. NOS is located in the muscular wall from adult worms in *Brugia malayi*, *Dirofilaria immitis* and *Acanthocheilonema vitae filariae* [8, 9]. Expression of endothelial NOS (eNOS) has been detected in the cuticle and stichocytes from *Trichinella britovi* [10]. Nitrites have been detected in the

Respiratory system is formed by two components: Lungs (principally responsible for the respiratory function) and chest wall, which allows adequate ventilation. The lungs are constituted by a system of conduction (bronchia and bronchioles) and a system of gaseous exchange (alveoli), assembled by connective tissue and surrounded by the visceral pleura. The alveoli are the structures responsible for the gaseous exchange, located in the wall of the respiratory bronchioles or in the peripheral region of the alveolar sacs. Alveoli are not isolate structures but are found physically joined among themselves, to the bronchioles and to the visceral pleura and connected to other pulmonary structures by Köhn's pores and Lambert's canals which communicate the alveolar sacs to the terminal bronchioles. Structurally two cell types form the alveolar region, type I and type II neumocytes. Although the numerical proportion between both is 1 : 2, the type I neumocytes cover an area 25 times greater than that of type II neumocytes. Participation in the gaseous exchange is the principal function of the type I neumocytes, whereas the type II neumocytes are responsible for the production of surfactant and cellular regeneration after aggression to the alveolar region. The alveolar macrophages are the principal defensive element of the alveolointerstitial region. In their surface are expressed receptors related to adhesion (CD11a/CD18, CD29/CD49, CD54), to the capture of antigens joined to immunoglobulins, complement, or proteins (CD14, CD16, CD32, CD64, Cd11b/CD18, CD11c/CD18), to the capture of nutrients (CD71), and related to the response to cytokines (CD25, CD115-130) and molecules related to the antigenic presentation (HLAII). Alveolar macrophages could be activated by different stimuli (e.g., γ IFN) and their response involved release of lysosomal enzymes, production of free oxygen radical, and generation of nitric oxide.

Box 1

TABLE 1: Clinical-biological pattern in pulmonary helminthic diseases.

Clinical-biological patterns	Subtypes	Syndrome	Parasites
Pulmonary mass or nodule	No extrapulmonary	Löffler's Syndrome	<i>E. granulosus</i>
			<i>D. immitis</i>
Pulmonary infiltrates	Extrapulmonary	Tropical eosinophilia	<i>Ascaris</i> sp.
			<i>A. duodenale</i>
		Katayama's Syndrome	<i>N. americanus</i>
			<i>W. bancrofti</i>
Visceral larva migrans	<i>B. malayi</i>		
	<i>B. timori</i>		
Miliar pattern/Pulmonary hypertension		Hyperinfection Syndrome	<i>Schistosoma</i> sp.
			<i>Schistosoma</i> sp.
Pleural effusion			<i>Paragonimus</i> sp.
Inflammatory myopathy			<i>Trichinella</i> sp.

hydatid liquid of fertile *Echinococcus granulosus*, although NOS expression has not been identified yet [11]. Finally, expression of iNOS and nNOS has been detected in the parenchyma and nervous structures of the filariform larvae from *Strongyloides venezuelensis* (*unpublished data*). Moreover, NOS expression has also been demonstrated in other phases, such as eggs, sporocysts, and cercariae of *Schistosoma* sp. [2] and other structures as oocytes, spermatozoids, and embryonic forms of *Brugia malayi* [8].

NO is essential in different biological functions of some helminths; among them neurotransmission at the nervous and muscular levels is the best characterized [1, 4, 12]. NO is involved in muscular relaxation and is a mediator of a variety of neuropeptides on their activity in the ionic channels (e.g., of potassium) [13]. The detection of

NOS in embryonic stages of different helminths and the role of this mediator in other species (e.g., *Drosophila*) suggest that NO plays an important role in the control of proliferation and differentiation during embryogenesis [8]. In this sense, helminths seem as other invertebrates (i.e., *Drosophila* spp, *I obsolete*) in the involvement of NO as an essential development signal [14–16]. Finally, NO plays an important role in the detoxification of free radicals of oxygen at least in two types of helminths: firstly, the hemoglobine from *Ascaris lumbricoides* functions as a deoxygenase, using NO to eliminate oxygen [17, 18]; secondly, NO stimulates the haemooxygenase activity in the nurse cell of *Trichinella britovi*, as an useful strategy to control the “oxidative burst” that takes place after the invasion of the muscular cells [10].

Nitric oxide (NO) is a very simple molecule that performs different biological functions, both in the intra- and extracellular space. This molecule is generated from the amino acid L-arginine, by the action of the nitric oxide synthase (NOS), enzymes which, in the presence of oxygen produces L-citrullin and nitric oxide. These enzymes require three substrates for their action: arginin, NADPH, and oxygen and five cofactors: haem group, tetrahydrobiopterin, calmodulin, FMN (flavin mononucleotide), and FAD (flavin adenine dinucleotide). The study of the metabolism of NO could be performed using inhibitors of the NOS. The main inhibitors used in the practice are analogues of arginine, such as L-NAME (N-nitroarginin methyl ester) and L-canavanin with an irreversible effect and L-NMMA (N^w-monomethyl-Larginine) with a reversible action. Four types of NOS are described: neuronal NOS (nNOS), endothelial NOS (eNOS), mitochondrial NOS (mNOS), and inducible NOS (iNOS). Constitutive enzymes (nNOS and eNOS) requires for activation a calcium dependent union to calmodulin. In the case of iNOS, the union between the enzyme and calmodulin is not calcium dependent. The synthesis of iNOS is stimulated by bacterial substances, for example, lipopolysaccharide (LPS) and by different cytokines released by macrophages or Th1 lymphocytes. The main physiological actions of NO are (i) the control of vascular tone (arterial vasodilation and inhibition of adhesion and aggregation of platelets), (ii) neurotransmission (learning and memory at the central nervous system and relaxation of visceral smooth muscle in the peripheral nervous system), and (iii) pathogenesis and control of infectious and parasitic diseases. NO can be measured directly or indirectly. The direct type of measurement is difficult to perform, since it is a molecule with a very short half life and rapidly diffuses to the tissues to perform its action. The indirect methods more employed for the NO detection are Griess technique and NOS expression. Nitrites and/or nitrates can be detected as products of its metabolism by Griess technique. NOS detection can be performed by immunocytochemistry or Western Blot and gene expression by RT-PCR or real-time PCR.

Box 2

3. Do Helminths Induce NO Production by the Host Cells, and if So, How They Do It?

There are several pieces of evidence indicating that helminths induce NO production by the host cells. Firstly, the presence of products derived from this molecule (mainly nitrites and nitrates) has been directly measured in the sera of humans affected by different parasitic infections, such as hydatidosis and schistosomiasis where it has been shown an increase in the production of nitrites [19, 20]. Specifically, a positive correlation was found between NO production and severe clinical data in hydatidosis [21].

A second group of studies was constituted by the detection of different NO related products in experimental models of parasite infection. Thus, mice infected with *E. multilocularis* demonstrated an increase in iNOS expression in peritoneal macrophages [22]. Furthermore, in a hepatic schistosomiasis model induced by eggs of *S. japonicum*, an increase on the expression of iNOS has been detected in inflammatory cells (e.g., neutrophils, macrophages, Kupffer cells), and hepatocytes [23]. Also, an increase in the concentration of nitrites in serum, accumulation of nitrosilated products (nitrotyrosine) and expression of iNOS in inflammatory hepatic cells has been detected in experimental toxocariosis [7]. Expression of transmurial iNOS in jejunum has been observed in experimental infection with *Trichinella spiralis* larvae [24] and expression of iNOS has been also observed in cells of inflammatory infiltrates around larvae in skeletal muscles of *Trichinella spiralis*-infected mice [25]. In vivo administration of *S. mansoni* antigens (e.g., p38) plus IL-2 gives rise to granulomas in which an increase in the expression of iNOS is detected [26]. Our research group studied the concentration of nitrites in the urine of

mice experimentally infected with *Strongyloides venezuelensis*. Mice infected with *S. venezuelensis* had high values of nitrites at the second postinfection day, corresponding with the passage of the larvae through the lungs (*unpublished data*).

Thirdly, the stimulation of NO production induced by parasite antigens has been evaluated by cell cultures. The results obtained by our research group demonstrated three well-differentiated aspects, according to the helminth species, the type of antigen from the same parasite, and the kind of inflammatory cell utilized in the experiment (Figure 1). Firstly, there are opposed effects on the NO production according to the groups of helminths studied. We compared the effect of *Echinococcus granulosus*- and *Echinococcus multilocularis*-defined metacestode structural and metabolic antigens on the NO production by rat alveolar macrophages. Our results showed that none of these antigens could stimulate macrophage NO production. Moreover, some *Echinococcus* antigens inhibit in vitro NO production when cells were previously exposed to lipopolisaccharide (LPS) stimulation. This inhibitory effect was also seen when *Echinococcus multilocularis* laminated-layer or cysts wall soluble components from both species were used in the experiment [27]. On the other hand, different effects were observed when macrophages were incubated with antigens from *Paragonimus mexicanus* and *Schistosoma bovis*. We have observed that excretory-secretory products from *Paragonimus mexicanus* adult worms trigger NO production from alveolar macrophages in vitro in a specific and concentration-dependent manner [28]. Our results also demonstrated that the stimulation of NO production by alveolar macrophages was accompanied by an increase in iNOS mRNA detection. In this study, we demonstrated

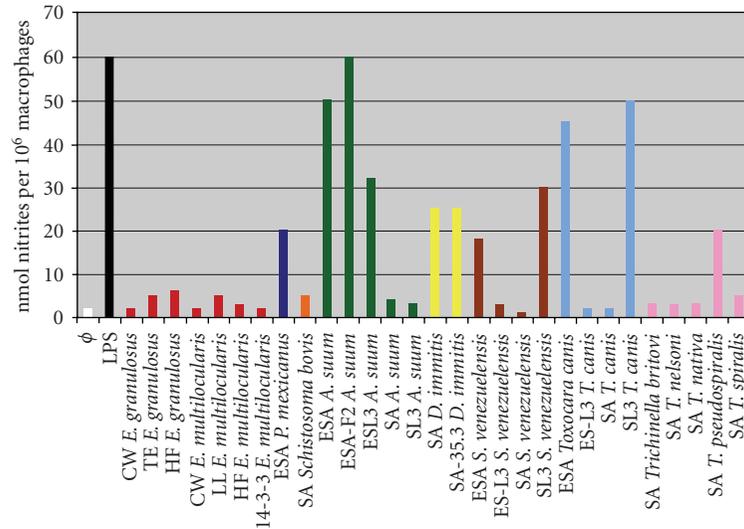


FIGURE 1: Effect of NO production by alveolar macrophages stimulated with different helminth antigens. Nonstimulated (ϕ), LPS-stimulated macrophages (LPS), cyst wall (CW), total extract (TE), hydatid fluid (HF), soluble laminated-layer (LL), E14t recombinant 14-3-3 protein (E14t), excretory-secretory adult (ESA), and somatic adult (SA), excretory-secretory larvae (ESL3), somatic larvae (SL3). Helminths are represented in different colours.

that specific excretory-secretory antigens from *Schistosoma bovis* adult worms did not induce in vitro NO release from alveolar macrophages. This could be explained because some of these parasites specifically localize in the lungs (e.g., *Paragonimus sp.*), whereas others migrate through the lungs and definitively settle in other organs (e.g., *Schistosoma sp.*).

Secondly, there are different effects in species of parasites from the same genus. We investigated the stimulatory/inhibitory role of L1 antigens from four encapsulated (*Trichinella spiralis*, *Trichinella britovi*, *Trichinella nelsoni* and *Trichinella nativa*) and one nonencapsulated (*Trichinella pseudospiralis*) species of *Trichinella* on NO production. Our results demonstrated that encapsulated and non-encapsulated *Trichinella* species differ in their ability to stimulate the NO secretion from the host macrophages [29]. These differences could be related to the complete and incomplete formation of the nurse cells in trichinellosis. Finally, we have studied the effect of different antigens, somatic and excretory/secretory of larval and adult worms of nematodes, on NO production from rat alveolar macrophages. We have observed that somatic antigens from the third stage larvae and excretory/secretory antigens from adult worms in *Toxocara canis* and *Strongyloides venezuelensis* stimulated the NO production from alveolar macrophages ([30]; unpublished data); similar data were found with *Ascaris suum* antigens [31].

We tried to define specific parasite molecules responsible for the stimulation of NO by nematode antigens. Thus, we assayed 10 protein fractions purified from *Ascaris suum* excretory/secretory adult worm antigens. The fraction-2 included three protein bands with molecular weights of 46, 44, and 37 kDa. The MALDI-peptide mass fingerprinting

analysis showed similarities with two glycolytic enzymes (enolase and phosphoglycerate kinase) and with proteins involved in catalysing the elongation of peptide chains in protein biosynthesis (the elongation factor *Tu*). These proteins are crucial for survival and key components of the protein synthesis machinery [28]. We also analyzed different parasite proteins to define the specific *Dirofilaria immitis* somatic adult-worm antigens involved in host cell NO secretion. We identified a parasitic specific component (DiID35.3) which does not belong to the endosymbiont *Wolbachia* and which is intimately related to proteins of the Immunoglobulin Superfamily (ISP) group, triggering NO release from macrophages in a dose-dependent and specific manner [31].

The cellular receptor of antigens, and the intracellular transduction pathways, that trigger NO production have been precisely identified [32]. Our research group studied the cytoplasmic signalling pathways involved in the NO production after stimulation with adult excretory/secretory antigen of *Toxocara canis* [33]. Our results suggested that both phospholipase A2 and phospholipase C macrophage pathways play an essential role in activating the production of NO triggered by this antigen. This suggests that NO production could be due to an increase of intracellular calcium and activation of the arachidonic acid pathway.

4. What Is the Biological Effect of NO Production on the Helminths?

The role of NO in the defence against the helminths is sustained by various types of studies, such as the utilization of NO donors in different experiments (both in vivo and

in vitro) and the evaluation of their action on the stages of the biological cycle of the helminths. This strategy has been used to study the effect of this molecule on *Echinococcus granulosus*, *Brugia malayi*, and *Trichinella spiralis*. SNAP donors produced lesions and cell death in *Echinococcus granulosus* [34]; and similarly, SIN-1 had cytostatic effects in vitro on microfilariae and adult worms of *Brugia malayi*, being the adults more resistant [35]. Moreover, the use of different donor (DAE/NO) in an experimental model of filariasis inhibits the development of adult worms and alters its motility [36]. On the other hand, it has been observed that different donors of NO increased worm recovery in a trichinellosis experimental model [37, 38]. Moreover, induction of NO synthesis may have a negative effect on the host's immune response in a local environment. This, together with downregulation of mannose receptor expression, could participate in the survival strategy of the *Trichinella spiralis* in the host [39].

Our research group studied the effects of two NO donors, SIN-1 and SNOG, on *Toxocara canis* larvae at different concentrations. The results showed that none of the concentrations used from both donors or in combination with oxygen free radicals exerted any cytotoxic effect on *Toxocara canis* larvae [40]. Therefore, the stimulation of NO production by *Toxocara canis* larvae antigens does not seem to play any host-defensive role, in contrast to the deleterious effects attributed to this molecule upon other helminths, for example, filarial nematodes. We also utilized NO donors (with short, middle and long half life) to evaluate their effect on larvae and *Strongyloides venezuelensis* female adult worms where we could demonstrate a dose-dependent cytotoxic effect (*unpublished data*).

Interaction with oxygen free radicals or immunoglobulins may be considered to evaluate combined effect in host cells. Coculture of *Echinococcus multilocularis* protoescolices with prestimulated macrophages leads to the destruction of the parasite, being NO the mediator involved [41]. Moreover, peritoneal macrophages stimulated with γ -IFN cause damage in *Echinococcus granulosus*, similar to the damage produced by NO donors [34]. A similar effect occurs when *Brugia malayi* and *Onchocerca lienalis* microfilariae are cocultured with macrophages activated by γ -IFN [42]. Finally, other cells like *Biomphalaria* haemocytes are capable to destroy *Schistosoma mansoni* sporocysts [43].

NO synthesis inhibitors have been used in vivo to evaluate their effect on parasitic infection. This strategy has mainly been used in experimental models of filariasis by *Brugia malayi* [36], toxocarosis [40], trichinellosis [44], and strongyloidiasis (*unpublished data*). The results obtained are divergent, since the use of aminoguanidin diminishes the lesions in toxocarosis, whereas it increases the parasite load in filariasis and strongyloidiasis. Moreover, mice treated with aminoguanidine at the beginning of muscle phase of the infection inhibits the reduction of muscle larvae number [44] and cells of inflammatory infiltrates did not show any specific iNOS reaction [25]. A decrease in eggs in faeces and reduction of larvae in the lung and females in the intestine have been observed in experimental strongyloidiasis. A model of immunosuppression was developed in mice

infected with *Strongyloides venezuelensis* administrated with dexametasone. Our results demonstrated the importance of NO in the defence against *Strongyloides venezuelensis*, since the immunosuppressed mice treated with aminoguanidin presented a very significant increase in both, the egg count and the larvae and female recoveries from the lung and intestine. Moreover, the use of antihelminthics (praziquantel, ivermectin and diethylcarbamacine) is associated with an increase in serum concentration of nitrites and nitrates. It has been interpreted that the antiparasitic activity of these drugs depends in part on NO release [45, 46].

As conclusion, it is important to highlight that the use of different experimental methods leads to contradictory results. For example, whereas in vitro studies demonstrated the role of NO in the destruction of schistosomules [47], in vivo studies did not support these results [48], probably because schistosomules are protected from the effect of NO by haemoglobin from red blood cells. Moreover, some authors suggested that NO is involved in the control of lymphatic filariasis [36, 42], whereas other question the role of this molecule in the clearance of microfilariae in *knock-out* mice model. [49].

5. What Pathological Consequences Are Derived from the Production of Nitric Oxide in the Host?

NO production by the host cells in response to helminth infections can cause adverse effects. Direct lesions or tissue functional alterations, immunosuppression, and carcinogenesis are the principal consequences. Trichinellosis [50–52], schistosomiasis and dirofilariasis [3] are the main examples of structural and functional direct lesions. It has been demonstrated that NO released in response to some helminths leads to immunosuppression, with low proliferative response of splenocytes or apoptosis induction of CD4 lymphocytes. Infections by *Echinococcus multilocularis* [22, 53] and filariasis [54] are the best characterised examples. Finally, the release of NO in response to the infection by *S. haematobium* seems to play an important role in the bladder cancer, through modifications of the p53 protein [55].

Our research group studied the influence of the inhibition of the NO production in a toxocarosis experimental model. The results clearly suggested that in vivo inhibition of the NO synthesis by iNOS decreases the deleterious effects of the parasite upon the host, especially the lung vascular alterations. We could show that in vivo NO production induced by infection with *Toxocara canis* results in direct damage to the host. This induction constitutes an evasion/adaptation mechanism of the parasite [40]. Moreover, *Toxocara canis* excretory/secretory adult antigen also stimulated alveolar macrophages to produce prostaglandin E₂ (PGE₂). The addition of L-canavanine decreased the release of PGE₂, which suggests that NO mediates the production of this molecule [30]. These results indicate that *Toxocara canis* can stimulate the release of vasodilatory mediators by host macrophages.

It is also important to indicate that in some helminthic diseases both excess and lack of NO can have deleterious effects for the host [56–58]. Excessive production of NO in schistosomiasis unleashes an acute response with direct hepatotoxicity and formation of granulomas with scarce fibrosis. However, a low NO production of this mediator is associated to chronic evolution of schistosomiasis with the development of intense fibrosis and granulomas of great size.

6. Conclusions and Future Perspectives

Nitrites or NOS has been detected in helminths at different stages of their biological cycle and in distinct anatomical structures. Stimulation of cells involved in the defence of the respiratory system (alveolar macrophages) with antigens from different biological stages of helminths has demonstrated that (i) antigens of cestodes inhibit the NO production, (ii) antigens of trematodes have different effects on its production, and (iii) antigens of nematodes stimulate the production of this mediator. It has been demonstrated that somatic antigens of larvae and excretory/secretory of adult worms induce the NO production in nematodes such as *Toxocara* or *Strongyloides*. Moreover, there are differences between the cystic and noncystic species of *Trichinella* in their capacity to stimulate the production of nitric oxide. These differences could be related to the complete and incomplete formation of the nurse cells in trichinellosis.

In an attempt to advance in the study of the molecules responsible for the stimulation of NO production, two glucoytic enzymes (enolase and phosphoglycerate kinase) and a protein that catalyses the elongation of peptide chains named *Tu* elongation factor have been identified in *A. suum*. A protein of *D. immitis* has also been recognised which is related to the Immunoglobulin Superfamily Protein (ISP).

The information obtained regarding the biological effects that NO production unleashes on the parasite or on the host is contradictory. NO is not harmful on *Toxocara canis*, whereas it kills larvae and adults of *Strongyloides venezuelensis*. In addition, NO plays an important role in the defence against strongyloidosis; its absence might even play a role in hyperinfection syndrome caused by this parasite. Furthermore, NO production constitutes an evasion mechanism in toxocarosis.

Finally and given that (i) VEGF (vascular endothelium growth factor) and FGF2 (fibroblastic growth factor) are important molecules in angiogenesis, performing other essential functions in inflammation (e.g., chemotaxis), (ii) alveolar macrophages, and specifically human alveolar macrophages, express VEGF, (iii) NO and VEGF are molecules with an intimate functional relationship, (iv) angiogenesis plays an important role in some helminthic diseases (schistosomiasis, onchocercosis, trichinellosis), and (v) the role of the helminths in the production of these factors is not well known, the study of the production of angiogenic factors in the respiratory helminthic diseases and the analysis of their biological effects would be of great interest to the knowledge of the pathogenic mechanisms of these infections.

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Review Article

Peroxisome Proliferator-Activated Receptor (PPAR): Balance for Survival in Parasitic Infections

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Parasitic infections induce a magnitude of host responses. At the opposite ends of the spectrum are those that ensure the host's needs to eliminate the invaders and to minimize damage to its own tissues. This review analyzes how parasites would manipulate immunity by activating the immunosuppressive nuclear factor, peroxisome proliferator-activated receptors (PPARs) with type 2 cytokines and free fatty acids from arachidonic acid metabolism. PPARs limit the action of type 1 immunity, in which classically activated macrophages act through the production of proinflammatory signals, to spare the parasites. They also favor the development of alternately activated macrophages which control inflammation so the host would not be destroyed. Possibly, the nuclear factors hold a pivotal role in the establishment of chronic infection by delicately balancing the pro- and anti-inflammatory signaling mechanisms and their ligands may be used as combination therapeutics to limit host pathology.

1. Introduction

Infection is the outcome of a contest between a pathogen and its host. The host responds to an invasion by activation of inflammation and launching of innate and specific immunity. The goal is to eliminate the pathogen. On the other hand, the pathogen seeks to proliferate and spread to a new host when one is destroyed. The task of parasites is particularly challenging because they need to strike a balance between their own and the host's defense so they can continue to take advantage of the host to survive. It is well-known that parasites are mostly immunosuppressive; the mechanisms they use to penetrate immune defense may be unique or shared by many species. Among the many means of surviving host immunity, one that has recently emerged is the activation of PPAR. This strategy promotes parasite survival by increasing energy metabolism and suppressing inflammation to allow invasion and avoid host destruction. The goal of this article is to review our current knowledge on how activation of PPAR may affect the survival of parasites and their hosts.

2. Peroxisome Proliferator-Activated Receptors

PPARs are ligand-activated transcription factors of the nuclear hormone receptor superfamily that consists of over 48 transcription factors. There are three PPAR isoforms—PPAR α , PPAR β/δ , and PPAR γ also known as NR1C1, NR1C2, and NR1C3, respectively, and they differ in tissue distribution and function [1, 2]. PPAR α is expressed in a variety of tissues involved in fatty acid oxidation, mainly the hepatocytes, cardiac myocytes, and proximal tubular epithelial cells of the kidney. It was so named because it was found to induce proliferation of peroxisomes in rodent livers (but not humans) when activated. Whereas PPAR α has limited tissue expression, PPAR β/δ is expressed ubiquitously and plays an important role in energy homeostasis. PPAR γ is a master regulator of adipocyte differentiation and is an important determinant of insulin sensitivity. Two subtypes are known in mice and four in humans [3, 4]. They are most highly expressed in adipose tissue, the colonic mucosal epithelium, and cells of the immune system.

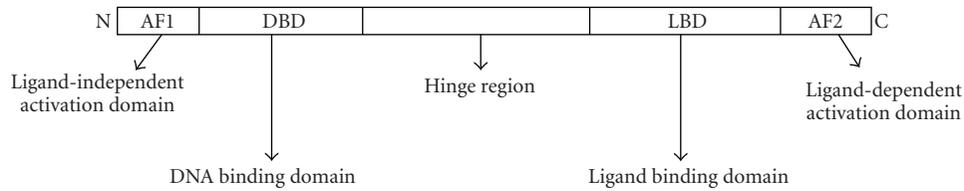


FIGURE 1: *Domain structure of PPARs.* PPARs contain the following functional regions: a N-terminal domain with AF-1 domain (ligand-independent activation domain), a DNA binding domain (DBD) with two zinc fingers, a hinge region, and a C-terminal ligand binding domain (LBD) and AF-2 domain (ligand-dependent activation domain).

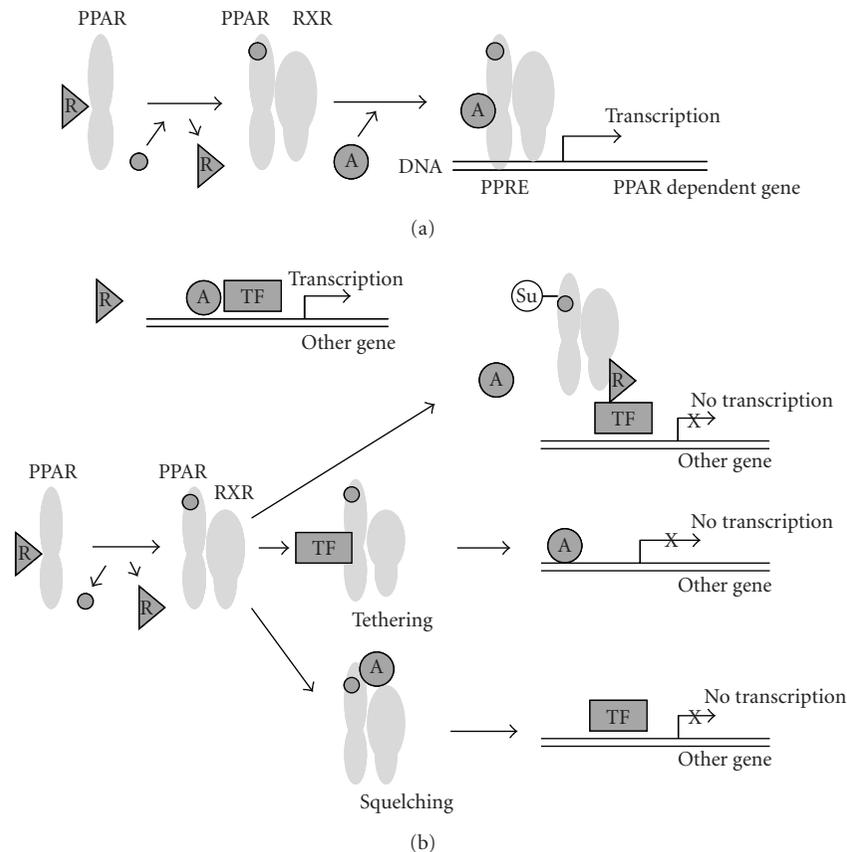


FIGURE 2: *General Schematics of PPAR function.* General mechanisms of (a) genomic expression and (b) transrepression of gene expression by PPAR/RXR heterodimers. In (a), upon ligand binding PPAR associates with RXR and coactivator to turn on target genes via the PPAR response element (PPRE). In (b), blocking the dissociation of corepressor from transcription factor at the gene locus, association of transcription factor to PPAR/RXR by tethering, and association of coactivator with PPAR/RXR by squelching, inhibit the transcription of different genes. R = corepressors, filled circle = ligand, A = coactivators, TF = transcription factor. Su indicates modulation by small regulatory protein (sumoylation).

Structurally, PPARs have a DNA-binding domain, a ligand-binding domain, two activation function sites, as well as sites for phosphorylation and dimerization (Figure 1) [2, 5, 6]. Activation is primarily ligand dependent and heterodimerization with the retinoid X receptor (RXR) is necessary. Figure 2 shows a simplified scheme of PPAR function in genomic expression and transrepression. Endogenous ligands for the PPARs include free fatty acids. Eicosanoids and 9-cis-retinoic acid bind to the PPAR-RXR complex to cause conformational changes that dissociate the corepressor, setting the complex free to interact genomically with the

PPAR response element (PPRE), AGGTCANAGGTCA (two AGGTCAs separated by one nucleotide), located at the 5' end of target genes (Figure 2(a)).

In contrast to genomic binding, PPARs also interact with other transcription factors through nongenomic transrepression. In this process, they inhibit transcription by preventing dissociation of corepressors or sequestering the co-activators necessary for the binding of the transcription factor to DNA [7, 8]. Both PPAR α and γ suppress inflammation and immunity by inhibiting the activation of major transcription factors that control the expression of cytokines.

For example, PPAR α can tether on to the nuclear factor-kappa B (NF κ B) and prevent its transcription of interleukin-6 (IL-6); sumoylated PPAR γ can bind nuclear receptor corepressor (NCoR)-histone deacetylase 3 (HDAC3) complex to prevent it from dissociating from NF κ B and thus preventing the gene expression of inducible nitric oxide synthase (iNOS) (Figure 2(b)) [9].

3. Immune Defense against Parasitic Infections

Many parasitic infections are controlled by cell-mediated immunity. For host protection, controlling parasite growth relies on a type 1 response with activation of the classically activated macrophages (caMac, also known as M1), T helper-1 (Th1), Th17, the production of proinflammatory mediators, such as IL-1 β , IL-6, tumor necrosis factor α (TNF α), IL-12, IL-23, IL-27, interferon γ (IFN γ), and generation of nitric oxide. On the contrary, a type 2 response with Th2 cells that produce IL-4 and IL-13 would render the host susceptible to infection. These cytokines activate PPAR γ , which suppresses production of proinflammatory cytokines, are critical for the formation, activation, and maintenance of a subpopulation of macrophages, alternatively activated macrophages (aaMac, also referred to as M2) [10]. AaMac are found during parasitic infections and have been associated with downregulation of type 1 immunity as well as the survival of both protozoa and helminthes. This emerging subject of investigation has been reviewed recently by Reyes and Terrazas [11] and other investigators [12, 13]. AaMac not only inhibit T cell proliferation, but also promote resolution of inflammation and fibrogenesis. They actively express a set of genes enabling them to regulate anti-inflammatory processes, induce tolerance and wound healing. These anti-inflammatory regulatory mechanisms can act as a counterbalance to limit disease severity and protect the host from detrimental effects of an excessive type 1 response.

4. PPARs Regulate Immune Responses

Given the role of PPARs in metabolism and adipocyte differentiation, it is intriguing that lipid-activated transcription factors such as PPARs have a role in human immune cell regulation. Initial documentation of PPAR activation in the immune system occurred in 1994 [14]. PPARs were originally described in monocytes and macrophages, neutrophils and peripheral blood lymphocytes. They have since been reported to exist in other immune cell types of hematopoietic origin, including T lymphocytes, B lymphocytes, NK cells, dendritic cells, eosinophils, and mast cells. The extensive expression by many immune cell types suggests that this nuclear receptor may play a very important role in the regulation of immune responses [15–20].

Immune regulation through the activation of PPARs can occur in response to various long chain unsaturated fatty acids generated from the cyclooxygenase and lipoxygenase pathways [21]. The eicosanoid 15-deoxy-Delta^{12,14}-prostaglandin J₂ (15d-PGJ₂) produced during arachidonic acid metabolism can activate PPAR γ during inflammatory

responses. Additional known ligands for include leukotriene B₄, 8(S)-hydroxyecosatetraenoic acid (HETE), 15-HETE, and 13-hydroxyoctadecadienoic acid (13-HODE). Both 15-HETE and 13-HODE can be produced via the action of 12/15 lipoxygenase. Cytokines through induction of cyclooxygenase and lipoxygenase can regulate the expression and activity of PPAR γ ; for example, IL-4 induces 12/15 lipoxygenase and production of 15-HETE and 13-HODE whereas IL-13 enhances COX-2 expression and production of PGE₂ and 15d-PGJ₂ [22–24].

PPAR activation is generally known to result in an anti-inflammatory environment and may exert an effect on the immune response by an array of mechanisms [9, 25–28]. PPAR α and γ exert anti-inflammatory activities through their ability to antagonize other signaling pathways, in part through the interaction with other transcription factors, including NF κ B, activator protein-1 (AP-1), and signal transducers and activators of transcription (STATs). PPAR γ agonists inhibit cell-mediated immunity by suppressing the production of inflammatory cytokines like TNF α , IL-6, IL-1 β , and IL-12 [29]. They exhibit suppression on effector mechanisms of classically activated macrophage—inhibiting induction of inducible nitric oxide synthase (iNOS), matrix metalloproteinase-9 (MMP-9), and scavenger receptor A [17]. In mouse thioglycolate-elicited macrophages, PPAR γ inhibits recruitment of macrophages to the sites of inflammation by repressing the transcription of monocyte chemoattractant protein-1 (MCP-1) and its receptor CC chemokine receptor 2 (CCR2) in macrophages [29].

With respect to T cells, PPARs affect activation and effector mechanisms via binding and blocking the action of nuclear factor of activated T cells (NFAT), a transcription factor. Specifically, 13-HODE, an endogenous PPAR γ agonist, prevents NFAT association with the IL-2 promoter, thus inhibiting clonal T cell proliferation [20]. PPAR α has also been shown to repress production of IFN γ and IL-17 by CD4⁺ T cells, and PPAR γ ligands modulate dendritic cell function to elicit the development of anergic CD4⁺ T cells.

Whereas PPARs downregulate type 1 immunity they upregulate type 2 responses. A study by Dasgupta et al. [30] showed that gemfibrozil, a PPAR α agonist, increases the activity of the transcription factor GATA-3 and inhibits expression of the transcription factor T-box expressed in T cells (T-bet) to cause increase in IL-4 production by Th2 cells. In a positive feedback manner, IL-4 has also been shown to simultaneously increase the expression of PPAR γ and 12,15-lipoxygenase, the enzyme involved in the generation of 13-HODE [22].

5. Activation of PPARs in Parasitic Infections

By rendering the host less capable of an inflammatory burst, PPAR activation might favor the establishment of a chronic parasitic infection, making symbiotic survival between host and parasite more likely. *Plasmodium falciparum* and *Schistosoma mansoni* infection produce hemozoin, which induces the release of endogenous ligands lipoxin A₄ (LXA₄), 5,15-diHETE, and 15-HETE, that can activate PPAR. Production of IL-4 and IL-13 in *Leishmania* and *Toxoplasma* infections

also favors the activation of aaMac whose development is dependent on PPAR γ .

5.1. Hemozoin. The *Plasmodium* protozoa are parasites that cause malaria. During disease pathogenesis, rapid growth of merozoites within red blood cells leads to host cell rupture and as the disease progresses, reinvasion of released parasites heightens parasitemia. As red blood cells and hemoglobin are destroyed, the malaria parasite produces hemozoin, a molecule formed via heme-catalyzed lipoperoxidation as a detoxification product and released together with other cell debris [31].

Plasmodium hemozoin reacts with membrane phospholipids to generate hydroxy-polyunsaturated fatty acids, which are ligands of PPAR γ . The fact that hemozoin activity is dependent on PPAR γ has been demonstrated with the PPAR agonist ciglitzone, the antagonist GW9662, and the PPAR γ ligand 15-HETE. Hemozoin has long been known to be a potent modifier of myeloid cells. It modulates phagocytosis, activation by inflammatory cytokines, and generation of the oxidative burst in monocytes. It also inhibits granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-4-mediated differentiation of human monocytes into immature dendritic cells. Furthermore, hemozoin-loaded immature dendritic cells are also unable to differentiate into mature dendritic cells. In these cells, the PPAR γ mRNA level increases, whereas the expression of MHC class II, costimulatory molecules CD83, CD80, CD54, CD40, CD1a, and CD83-specific mRNA are lowered [32, 33].

In addition to *Plasmodium*, the metazoan parasite *Schistosoma mansoni*, commonly known as blood fluke, also produces hemozoin. The adults of this snail-transmitted parasite feed on red blood cells in humans during part of its life cycle and biomineralizes dimeric heme into an inert crystalline pigment that is structurally identical to *Plasmodium falciparum* hemozoin. The lipid coat of native schistosome hemozoin is a complex mixture of both neutral lipids and polyunsaturated fatty acids. It can generate, by nonenzymatic catalysis, large amounts of lipoperoxidation products, such as monohydroxy derivatives of arachidonic (HETE) and linoleic (HODE) acid and 4-hydroxynonenal (HNE), which are natural ligands of PPAR. Murine RAW 264.7 macrophage-like cells that have phagocytized schistosomal hemozoin are also decreased in lipopolysaccharide-stimulated nitric oxide production [34–36].

Interestingly, the kissing bug *Rhodnius prolixus*, which is the vector for *Trypanosoma cruzi*, the etiological agent of Chagas' disease, also crystallizes heme into a dark brown pigment. Since this insect feeds on blood meal, a link may be extrapolated between blood-feeding and hemozoin formation. Production of this PPAR activating molecule can be a strategy commonly acquired by blood feeding parasites and blood taking vectors to suppress host immune response [37].

5.2. Polyunsaturated Fatty Acids (PUFA). The protozoan *Toxoplasma gondii* infects feline epithelium and grows intracellularly in different mammalian nucleated cells, but it does not feed on or destroy red blood cells and does not produce hemozoin. However, it induces the production of natural

ligands for PPAR from platelets. Infection induces platelets to release LXA₄, 5,15-diHETE, and 15-HETE at very high levels, two orders of magnitude greater than those used to resolve inflammation [38]. Toxoplasmosis is a chronic disease that is contained by immunity. The disease is only manifested in severely immunocompromised individuals and detrimental to fetus in early gestation. Henderson Jr. and Chi [39] have reported that other PPAR activating lipids, for example, 13-HODE at 10 nanomole concentration has cytotoxicity against the parasite (however, 12-HETE at 1 μ M does not). Currently, the role of PPAR γ in toxoplasmosis remains to be elucidated. However, it is possible for it to have a role in maintaining a balance that supports host and parasite in dormant, chronic infections.

5.3. Interleukin-4. The pivotal role of PPAR γ on IL-4 and aaMac-mediated susceptibility has been demonstrated in *Leishmania* pathogenesis. Leishmaniasis is transmitted by the sandfly vector carrying parasitic protozoa of the genus *Leishmania*. Clinically the disease can be manifested in three major types, cutaneous, mucocutaneous, and visceral, each caused by different species of the protozoan genus. In the murine model of the cutaneous infection, BALB/c mice, the *Leishmania major* susceptible strains, have a T helper 2 (Th2) response whereas the C57BL/6 mice, resistant strains, have a T helper-1 (Th1) response [40]. AaMacs are found in all Th2 cytokine environments. Interestingly, BALB/c mice are more supportive of aaMac maturation than C57BL/6 mice, which are resistant to leishmaniasis [41].

PPAR γ expression is strongly associated with maturation of aaMac. Our laboratory investigated visceral leishmaniasis and found that *Leishmania donovani* induces PPAR γ expression in residential macrophages, liver and spleen of BALB/c mice. In addition, oral administration of the PPAR γ agonist, curcumin further increases PPAR α and PPAR γ expression, and the increase is associated with a heavier parasite burden (Figure 3) [42].

Consistent with our findings, cutaneous *Leishmania* infection is less severe in mice whose PPAR γ gene has been knocked out in their macrophages (Mac-PPAR KO). Mac-PPAR KO mice had significantly less footpad swelling 5–7 weeks after injection of *L. major* promastigotes. Lesions in Mac-PPAR KO started to stabilize after 7 weeks, but the footpads of wild type mice continued to enlarge and rapidly underwent necrosis. The loss of PPAR γ is associated with loss of aaMac, as cells that express their phenotypic marker, arginase I, are decreased. The production of nitric oxide and IL-6, correspondingly, is increased as well [41].

PPAR γ and PPAR δ regulate expression of the arginase I gene, a key marker, in aaMac. The PPAR γ agonist GW7845 and PPAR δ agonist GW0742 activate arginase in murine macrophages, and this activation only occurs in wild type macrophages that have PPAR γ or PPAR δ , but not in cells where either of their genes has been knocked out [43]. Arginase decreases the amount of arginine, the substrate for NOS to produce nitric oxide. Since intracellular killing of amastigotes requires nitric oxide, both cutaneous and visceral infections are unrestrained in iNOS knockout mice [44–46].

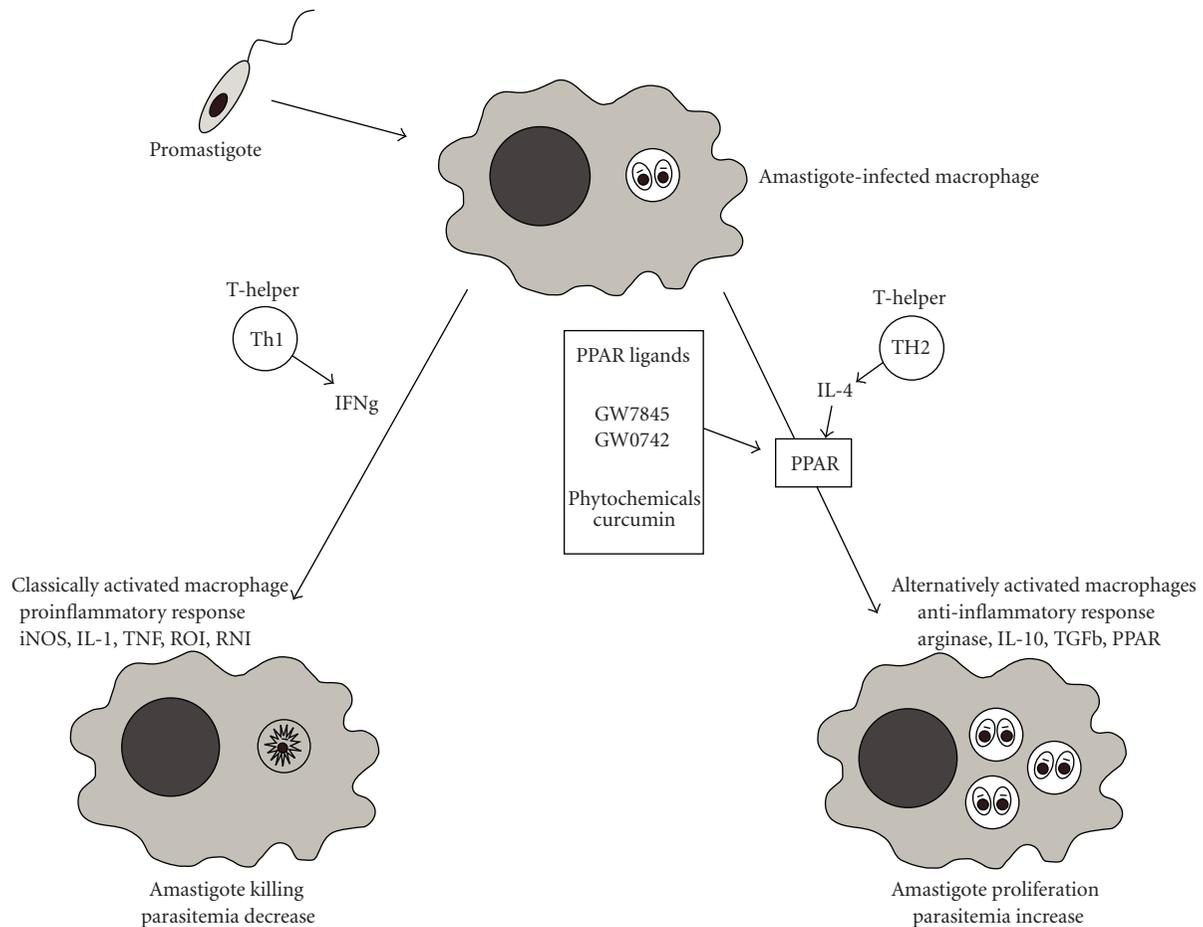


FIGURE 3: PPAR-mediated suppression of parasitocidal response in leishmaniasis. The sandfly vector carries leishmanial promastigotes to the mammalian host. The parasites then transform into amastigotes within phagolysosomes of host macrophages. Type 1 immunity, with T helper 1 (Th1) releasing interferon γ (IFN γ), leads to classically activated macrophages (caMac). CaMac produce nitric oxide from inducible nitric oxide synthase (iNOS), interleukin-1 (IL-1), tumor necrosis factor (TNF), and reactive oxygen and nitrogen intermediates (ROI, RNI) that act to eliminate the amastigotes. Type 2 immunity, with Th2 releasing IL-4, leads to activation of peroxisome proliferator-activated receptor (PPAR) and alternatively activated macrophages (aaMac). AaMac produce arginase, IL-10, transforming growth factor β (TGF β), and PPAR that allow amastigote propagation. PPAR agonists (GW7845 and GW0742) and some phytochemicals (curcumin) can activate PPAR and promote aaMac maturation. Activation of PPAR allows parasite survival as a chronic parasitic infection.

5.4. Interleukin-13. IL-13, another Th2 cytokine that shares a common receptor chain with IL-4, exerts similar effects on macrophages [13]. Functionally, it inhibits the activation of caMac and the production of Th1 cytokines and reactive nitrogen species. The effect of IL-13 is also mediated via the PPAR γ pathway. In *Toxoplasma*-infected macrophages, IL-13 activates PPAR by inducing the production of 15d-PG $_2$. Exogenous addition of PPAR γ agonists, rosiglitazone or 15d-PG $_2$, mimics IL-13 in that it induces CD36, the scavenger receptor whose transcription is turned on by PPAR γ [47]. This effect can be further extended to aaMac; IL-13 $^{-/-}$ mice are incapable of generating cells that express the aaMac phenotype (chitinase-like lectin YM1 or CD20) [48]. CD36 is classified as a class 2 scavenger receptor [49–51]. It is present on the cell surface of many cell types, including monocytes and macrophages. The molecule recognizes oxidized low density lipoprotein (LDL), oxidized phospholipids and lipoproteins, apoptotic cells, and microbial pathogens. With

respect to parasites, *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP-1), commonly found in the schizont stage, ring stage and early gametocytes (stage I and IIA), serves as a ligand for CD36. Binding to CD36 is necessary for phagocytosis of *P. falciparum*-parasitized erythrocytes by human and rodent monocytes/macrophages [52–57]. The function of PPAR γ in CD36-mediated phagocytosis is illustrated by the fact that IL-13-induced clearance of the parasitized erythrocytes is blocked by anti-CD36 and GW9662, a PPAR γ antagonist [47]. The activity, however, is enhanced by the agonists 15d-PG $_2$ and ciglitazone [52]. The role of PPAR γ in CD36-mediated phagocytosis is also confirmed by the observations that mice treated with rosiglitazone have reduced parasitemia of *Plasmodium chabaudi*, whereas this reduction is not seen in CD36 knockout animals [57]. CD36-mediated phagocytosis will facilitate removal of neutrophils and dead tissues to resolve inflammation as well as clearing of the parasite [52]. Consistent with this

TABLE 1: Effect of PPAR modulation on the outcome of parasitic infections.

Parasite	Animal model	Treatment	PPAR status	Outcome	Reference
Increase in parasite load					
<i>Leishmania major</i>	Murine cutaneous leishmaniasis	Gene knockout	PPAR γ ^{-/-} macrophages	Decreased footpad swelling	[41]
<i>Leishmania donovani</i>	Murine visceral leishmaniasis	Curcumin	PPAR γ \uparrow PPAR α \uparrow	Increased parasite load	[42]
Decrease in host tissue damage					
<i>Schistosoma japonicum</i>	Murine hepatic fibrosis	Rosiglitazone (Praziquantel)	PPAR γ \uparrow	Decreased fibrosis	[60]
<i>Plasmodium berghei</i>	Murine cerebral malaria	Rosiglitazone	Not stated	Increased survival	[57]
<i>Plasmodium chabaudi</i>	Murine malaria	Rosiglitazone	Not stated	Decreased parasitemia	[57]

concept that PPAR and aaMac resolve inflammation, Herbert et al. [58] reported that in the absence of IL-4/IL-13-induced aaMac, mice with their macrophage-specific IL-4 receptor knockout (LysMCreIL-4R^{-flox}) die of schistosomiasis. The mutant mice have increased Th1 cytokines and nitric oxide, hepatic and intestinal histopathology due to lack of aaMac to protect against tissue injury. Correspondingly, BALB/c mice with their T helper-1 responses and immunopathology down-modulated by aaMac survive *Schistosoma mansoni* infection better [59].

Henceforth, in sum, studies have revealed that PPAR γ suppresses immune reaction to parasitic infection; however, it also plays a role, perhaps through a CD36-dependent manner, in reducing parasitemia as well as resolving inflammation.

6. Effect of Pharmacological PPAR Ligands on Parasitic Infections

The anti-inflammatory actions of PPAR γ have received great attention because of the availability of synthetic PPAR γ activator molecules and their clinical use. The FDA has approved several synthetic PPAR ligands as therapeutic drugs [59, 61]. For example, clofibrate, an agonist of PPAR α , and its related compounds are used for the treatment of dyslipidemia. Rosiglitazone (drug name Avandia) and pioglitazone (drug name Actos), agonists of PPAR γ , are used in the management of type 2 diabetes. The drugs improve insulin sensitivity by controlling metabolic and endocrine functions of the adipose tissue. PPARs are attractive drug targets for energy homeostasis and control of lipid and glucose metabolism, as well as possible body weight control. Dual agonists of PPARs have been sought, even though troglitazone and muraglitazar (dual agonist for PPAR α/γ), originally approved by FDA were later withdrawn due to liver toxicity and increased cardiovascular risks, respectively [62, 63]. PPAR γ remains a promising target; safer therapeutics may still become a reality in the future.

Potentially, PPAR ligands may be used in parasitic diseases. To date, in experimental models PPAR ligands have

been shown to alleviate host tissue destruction by immune response, as seen in malaria and schistosomiasis. Recently, Lena Serghides and colleagues have shown that rosiglitazone, the PPAR γ agonist, is useful in alleviating cerebral malaria in a murine model [57]. Among the variety of symptoms of malaria, cerebral malaria is the deadliest complication that affects an estimated total of 785,000 children in Africa each year. Acutely manifested, the *Plasmodium falciparum* parasites induce fever, changes in mental status, and coma. The sequestration of parasitized red blood cells (PRBCs) and non-PRBCs in the cerebral capillaries and venules reduces microvascular blood flow and induces hypoxia in the brain. Furthermore, the parasites stimulate the host macrophages to release TNF α , IL-1, and uncontrolled production of nitric oxide, which diffuses through the blood-brain barrier and acts on synaptic function, leading to a state of reduced consciousness. Cotreatment of rosiglitazone (50 mg/kg of chow) with *Plasmodium berghei*-parasitized erythrocytes to susceptible mice leads to animal survival.

Similarly, PPAR ligands may be useful in human schistosomiasis, a chronic infection that can cause liver fibrosis. In a murine experimental model of hepatic fibrosis induced by *Schistosoma japonicum*, Chen and colleagues [60] reported that coadministration of rosiglitazone (4 mg/kg), daily for 6 weeks (at 4 weeks after infection) with the antischistosomal drug praziquantel (500 mg/kg) alleviates the symptoms of liver fibrosis. This was demonstrated by a decrease of fibrosis markers, type I and III collagen, and smooth muscle α actin. The decrease in fibrosis is accompanied by reduced binding activity of the transcription factor NF κ B and lowered levels of TNF α and IL-6 in the serum. Like cerebral malaria data, these findings also suggest that the PPAR γ ligand rosiglitazone may be clinically useful for treatment of liver fibrosis due to schistosomal infection.

7. Conclusion and Future Prospects

In conclusion, to date, the role of PPAR γ in parasitic diseases remains largely unexplored. Recent evidence suggests that PPAR γ activation may increase replication of parasites as well

as maintain survival of the host (Table 1). Perhaps it is a measure by which a chronic infection can be established. In a strong cell-mediated immunity, a typical type 1 immune response induces the production of TNF α , IFN γ , and nitric oxide which eliminates parasites. However, the host will severely suffer from tissue destruction during this process, as can be seen in cerebral malaria, when the children may be comatose, and in late stages of visceral leishmaniasis, when the patients may be cachexic and their liver and spleen are enlarged. A type 2 response with IL-4 and IL-13 which induces immunosuppressive PPARs would allow the parasites to infect, but spare the host of devastating tissue destruction. In leishmaniasis, for example, activation of PPAR γ by curcumin suppresses IFN γ and nitric oxide production and increases parasite burden. It may be of interest that curcumin is prevalently used for medicine, cosmetics, and cooking in India, where visceral leishmaniasis is endemic. Perhaps, through PPAR γ the host can resolve inflammation and repair tissues to strive for a balance between the host and the parasite that favors mutual survival.

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Review Article

Arginase in Parasitic Infections: Macrophage Activation, Immunosuppression, and Intracellular Signals

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A type 1 cytokine-dependent proinflammatory response inducing classically activated macrophages (CaM ϕ s) is crucial for parasite control during protozoan infections but can also contribute to the development of immunopathological disease symptoms. Type 2 cytokines such as IL-4 and IL-13 antagonize CaM ϕ s inducing alternatively activated macrophages (AaM ϕ s) that upregulate arginase-1 expression. During several infections, induction of arginase-1-macrophages was showed to have a detrimental role by limiting CaM ϕ -dependent parasite clearance and promoting parasite proliferation. Additionally, the role of arginase-1 in T cell suppression has been explored recently. Arginase-1 can also be induced by IL-10 and transforming growth factor- β (TGF- β) or even directly by parasites or parasite components. Therefore, generation of alternative activation states of macrophages could limit collateral tissue damage because of excessive type 1 inflammation. However, they affect disease outcome by promoting parasite survival and proliferation. Thus, modulation of macrophage activation may be instrumental in allowing parasite persistence and long-term host survival.

1. Macrophage Diversity

Macrophages are innate immune cells that play an indispensable role in the primary response to pathogens but also they play a role in the resolution of inflammation and tissue homeostasis. They can be polarized by the microenvironment to mount specific functions, as a consequence they represent a group of heterogeneous immune cells. The first indication of macrophage heterogeneity was showed by Gordon and colleagues during their studies of mannose receptor (MR) regulation in IL-4-treated M ϕ [1]. They demonstrated that macrophage treatment with IL-4 induced an alternative activation phenotype different to the classical macrophage activation (CaM ϕ), known to be dependent on interferon- γ (IFN- γ) and tumor-necrosis factor (TNF) [2–4]. Gordon later included the effects of IL-13 in the definition of alternative activation because IL-13 shares a common receptor chain with IL-4 and exerts similar effects on macrophages [5]. However, the name “alternative activated macrophages, (AaM ϕ)” may have led to some confusion

because it implicates that it is the only other way that macrophages can be activated. In fact, Mosser and colleagues showed later that this was the case. They demonstrated that macrophage treatment with classic stimulus, like LPS or IFN- γ , in the presence of immune complexes induces the generation of a cellular type different from the CaM ϕ . These cells release large amounts of IL-10 inhibiting the IL-12 synthesis. These M ϕ have been denominated “Type 2 M ϕ ” (T2a M ϕ) because they favor the development of type II adaptative immune response [6, 7].

In 1999 Goerdts and Orfanos proposed a classification of activation phenotypes based on grouping all activators other than IFN- γ and LPS/microbes into a common alternative activation group [8]. However, nowadays the classification has been extended. It has been proposed that a categorization in which M1 polarization included classical activated macrophages, while M2 polarization, mainly associated with antiparasitic and tissue repair programs, was subdivided in M2a or alternatively activated macrophages: M2b, corresponding to type II activated macrophages and M2c, which

includes heterogeneous macrophage deactivation stimuli such as glucocorticoids and TGF- β [9–11]. All of these types of macrophages can be found in a variety of immunological conditions, such as immune responses to different pathogens, tumors, and autoimmune diseases. Therefore, Mosser and colleagues propose a macrophage classification based on the fundamental macrophage functions such as host defense, wound healing, and immune regulation [12]. However, it is difficult to say which way is the best for classifying them. First, there are no specific biochemical markers to assign to each population. Second, it has been demonstrated that macrophages “sequentially” change their functional phenotype in response to microenvironmental changes. Therefore, macrophages can exhibit, at the same time, different kinds of markers associated with other types of macrophages [13, 14]. The plasticity of macrophages and high complexity of the in vivo environments result in the induction of various types of macrophages during protozoan infections and differentially affect the course of disease. Therefore, as a matter of simplicity, we will use the terms CaM ϕ and AaM ϕ . In this review we will focus on the relevance of arginase-1, an enzyme expressed in AaM ϕ , in parasitic infection and how the arginase-1 expressing macrophages can be exploited by parasites. In addition, we will discuss the T cell suppressive activity of these cells and some of the complex intracellular mechanisms involved in the induction of this enzyme.

2. Arginase-1 versus iNOS

CaM ϕ s have an enhanced ability to present antigens and eliminate intracellular pathogens. The antimicrobial actions have been attributed to the nitric oxide (NO) release and also to the synthesis of several products of NO reaction, such as peroxynitrite [15]. NO is produced from L-arginine by the inducible isoform of Nitric Oxide Synthase (iNOS) enzyme [16]. iNOS is induced in macrophages through the inflammatory cytokines such as TNF- α , IFN- γ , and IL-12 [17].

During infections with protozoan parasites such as *Leishmania*, *Plasmodium*, or *Trypanosoma*, an IFN- γ -dependent proinflammatory response triggering the development of CaM ϕ s is required to control parasitemia, especially during the acute phase of infection [18–21]. Therefore, CaM ϕ s are essential components of host defense, but their activation must be securely controlled because the cytokines and mediators that they produce can lead to host-tissue damage. On the other hand, depending on parasite virulence, host genotype and stage of infection, hosts can also produce type 2 anti-inflammatory immune responses which affect disease outcome [22, 23].

Type 2 cytokines such as IL-4 and IL-13 antagonize CaM ϕ s inducing arginase-1 expression in macrophages [24], a prototypic alternative activation marker in mouse macrophages [10]. The expression of arginase-1 induces a shift of arginine metabolism from NO production via iNOS toward production of L-ornithine, a precursor for polyamines and collagen, thereby contributing to the production of the extracellular matrix important for wound

healing [8, 25]. Although the term “alternatively activated” macrophages has been indicated for IL-4 and IL-13, stimulated macrophages [10] factors such as IL-10 or TGF- β [5] and phagocytosis of apoptotic cells [26] also antagonize CaM ϕ s and induce alternative, non-M1 activation states in macrophages.

AaM ϕ s in normal conditions, have been identified in tissues in which it is necessary to avoid the inflammation, such as lung and placenta [8, 27]. In addition, it was demonstrated an increase in arginase activity in peripheral blood of pregnant women and in term placenta [28]. Moreover, these macrophages have been identified during the phase of remission of acute and chronic inflammatory reactions like in psoriasis and rheumatoid arthritis [29, 30]. Therefore, generation of AaM ϕ s after inflammation would favor the maintenance of homeostasis. Nevertheless, several evidence obtained during different infections indicate that IL-4/IL-13-dependent AaM ϕ s exacerbate disease by limiting CaM ϕ s-dependent parasite clearance and promoting parasite proliferation [31, 32]. Therefore, the successful elimination of an infectious agent depends on the existence of temporary and space signals that regulate the type of macrophage activation.

3. Arginase-1 Expressing Macrophages in Helminth Infections

Helminths are worm parasites that represent a common infectious agent in developing countries. Protection against helminths depends on IL-4 and IL-13. However, when the infection progress to a chronic stage, Th2 cytokine results in AaM ϕ s recruitment, which leads to fibrosis and T cell suppression [33–36]. During *Brugia malayi* experimental infection, it has been observed that the IL-4-dependent macrophage recruitment actively suppress the proliferation of lymphocytes [37]. In addition, in a model of cysticercosis, *Taenia crassiceps* infected mice showed a Th1 response in early stages of the infection, which is replaced by a Th2 in the chronic stage [35]. In contrast to other helminth infections, CaM ϕ s are important for the clearance of *T. crassiceps* [35]. However, in chronic stages, the Th2 response results in AaM ϕ s, that exhibit arginase activity [33]. It has been reported that carbohydrates present in *T. crassiceps* can produce the detrimental change from Th1 response towards Th2 during this infection [38]. Besides, it was demonstrated that previous infection of mice with the *T. crassiceps* favors parasitemia and induces larger cutaneous lesions during both *Leishmania major* and *Leishmania mexicana* coinfections. These authors postulated that helminth infection may facilitate *Leishmania* installation by inducing arginase-1 expressing macrophages [39].

On the other hand, *Schistosoma mansoni* infection, as well as the other helminth infection mentioned above, leads to the development of a Th2 cell response. Using IL-4 and IL-13 knockout mice, Brunet and colleagues demonstrated that the Th2 immune response is critical for the survival during acute *S. mansoni* infection [40, 41]. They concluded that the severity of the infection in the knockout mice was due to an increase in the TNF- α and NO levels.

However, when mice are persistently infected, the chronic Th2 response becomes highly harmful contributing to the development of hepatic fibrosis and portal hypertension [42, 43]. In addition, the Th2 response was also associated with the accumulation of a large number of arginase-1 expressing macrophages in and around the granulomas [42] and arginase-1 expressing macrophages in peritoneal cavity and increased circulating polyamines [44]. The presence of arginase-1 expressing macrophages was originally hypothesized to be involved in promoting the collagen deposit and the fibrosis hepatic through proline synthesis mediated by arginase-1 [25]. However, Herbert and colleagues demonstrated later using mice deficient in AaM ϕ s (*Il4ra*^{-/flox};*LysMcre*) that the alternative macrophage activation is essential for survival during schistosomiasis and down modulates T helper 1 responses and immunopathology [45]. Moreover, the development of Th2-driven fibrosis in those mice *S. mansoni* infected appears normal [45]. Recently, it was demonstrated that macrophage-specific arginase-1 exhibits both anti-inflammatory and antifibrotic activities during Th2-driven inflammatory responses induced by *S. mansoni* [46]. The authors demonstrated that, instead of promoting Th2-disease, macrophage-specific arginase-1 contributes to the resolution of schistosomiasis by inhibiting CD4+ T cell effector function [46]. The data showed by Pesce and colleagues identify arginase-1-expressing macrophage as critical mediators of immune down-modulation in chronic schistosomiasis [46]. Additionally, it has been also recently demonstrated that the resistin-like molecule (RELM) α , which is another molecule expressed by AaM ϕ , is also involved in the Th2 cytokine-down modulation during the pulmonary inflammation observed in schistosomiasis [47].

On the other hand, infection of mice with the natural mouse gastrointestinal helminth parasite *Heligmosomoides polygyrus* triggers a highly polarized Th2 response. Memory CD4+ T cells rapidly accumulate at the host-parasite interface and secrete IL-4, inducing localized development of AaM ϕ s recruited to this site. These macrophages impair larval parasite health and mobility through an arginase-dependent pathway, contributing to eventual expulsion of adult worms [48]. It has been demonstrated that Th2 responses enhance gut contractility and luminal fluid secretion, leading to worm expulsion [49, 50]. Furthermore, specific inhibition of arginase activity during the infection with intestinal nematode parasite *Nippostrongylus brasiliensis* interfered with smooth muscle contractility and affected partially the protective immunity of the host [51]. Therefore, arginase-1 expressing macrophages are widely induced by helminths and they play different roles in those infections.

4. Arginase in Protozoan Infections

The type 1 pro-inflammatory response, crucial for parasite control during the acute phase of infection with protozoan parasites, should be carefully balanced by anti-inflammatory molecules like IL-10 and TGF- β to avoid excessive collateral damage to host tissues and cells. However, in many cases protozoan parasites take advantage of this anti-inflammatory

response to persist in the host. It has been reported that during *Trypanosoma brucei* infection, sustained CaM ϕ s could induce tissue damage, therefore mice that progress to a chronic stage, generate a Th2 response, able to favor the development of AaM ϕ [52]. In addition, it has been observed that *Leishmania major* has the ability to inhibit the production of IL-12 through the induction of IL-10 and TGF- β [53]. This imbalance underlies a shift from a Th1 to a Th2 immune response and reflects the susceptibility to the disease. This environment generates AaM ϕ facilitating the dissemination of the parasite in the host [54, 55]. Macrophage-IL-4 treatment previous to the infection with *Leishmania major* or *Leishmania infantum* biases the metabolism of L-arginine through arginase-1 towards the synthesis of polyamines, which promote the intracellular growth of the parasite [56]. Also, macrophage arginase-1-inhibition reduced the growth of the parasites due to an increase in NO production [56]. Additionally, in a mouse model of *L. major* infection, arginase-1 is induced in both susceptible and resistant mice during the development of the disease. The induction of the host arginase-1 in both strains is mediated by the balance between IL-4 and IL-12 and opposite to iNOS expression. Moreover, inhibition of arginase-1 reduces the number of parasites and delays disease outcome in susceptible mice, while treatment with L-ornithine increases the susceptibility of the resistant mice [54]. Furthermore, Kropf and colleagues also demonstrated that arginase-1 activity and production of polyamines are key factors in the regulation of leishmaniasis [55]. Moreover, another indication for the contribution of AaM ϕ to *Leishmania* susceptibility was demonstrated using macrophage/neutrophil-specific IL-4R α -deficient mice. Those mice showed a significantly delayed disease progression with normal Th2 and type 2 antibody responses but improved macrophage leishmanicidal effector functions and reduced arginase-1 activity [57]. Therefore, arginase-1 induction could be considered a marker of disease in leishmaniasis.

On the other hand, a Th1 immune response, involving IL-12, IL-18, and IFN- γ as well as NO-producing CaM ϕ , favors the control of the early *Trypanosoma cruzi* infection that causes Chagas disease [58, 59]. However, if uncontrolled, the Th1 immune response causes pathology. In this respect, IL-4 and IL-10 have been implicated in resistance to *T. cruzi* by balancing the early stage proinflammatory immune environment [60, 61]. Therefore, once again, the Th2 environment could induce AaM ϕ s and favor the installation of the parasite in the host. Arginase-1 activity, nevertheless, has not been necessarily induced by IL-4 during *T. cruzi* infection. We have demonstrated arginase-1 induction by the parasite component, cruzipain [62, 63]. We have showed that mice immunization with the immunodominant antigen cruzipain results in high Th2 cytokine secretion (IL-4, IL-5, IL-10), and marginal levels of IFN- γ and IL-12 [62]. In addition, cruzipain-stimulated macrophages exhibit increased arginase-1 activity and expression, and secrete IL-10 and TGF- β [63, 64]. Moreover, cruzipain-activated macrophages favor *T. cruzi* growth at the same extend as was observed in IL-4-treated macrophages [63] and arginase-1-inhibition leads to a drastic reduction in *T. cruzi* replication

in macrophages [64]. Additionally, we found an increase in arginase-1 activity during acute infection in BALB/c mice. Furthermore, arginase-1 inhibition leads to a reduction in the number of amastigotes in infected macrophages (Stempin, et al. unpublished data). Therefore, arginase-1 induction could favor *Trypanosoma cruzi* persistence in the host as was observed during *Leishmania* infection. In addition, it was demonstrated that peripheral blood monocytes of *T. cruzi* infected rats are not able to produce NO due to an increase in the arginase-1 activity [65]. The emergence of arginase-1-expressing macrophages is further promoted by the ingestion of apoptotic cells generated by NO during the early stage of *T. cruzi* infection, and the concomitant enhanced TGF- β secretion [26]. Therefore, this results in a decreased oxidative burst and persistent parasite growth in the infected macrophages because of the down regulation of iNOS activity. Moreover, TGF- β , by inducing ornithine decarboxylase activity, favors the synthesis of polyamines that are essential for the intracellular parasite replication [66]. Hence, *T. cruzi* might evade the host-protective Th1 immune response mediated by CaM ϕ s by instructing Th2 cytokine secretion, which stimulates AaM ϕ . Besides down regulating the inflammatory immune response, AaM ϕ s might favor the onset of a chronic infection during *Chagas* disease.

5. Alternative Activation Markers in Human and Murine Macrophages

AaM ϕ generation is associated with changes in macrophage function and distinctive gene signatures. Although the alternative activation markers for human and murine macrophages have long been considered analogous, there are differences between species. IL-4 and IL-13 induce genes conserved in both species such as MHC-II, MR, MGL1/2, and CD23 as well as divergent ones such as arginase-1, chitinase-like molecules (Ym1/2), and RELM α in mouse, and nucleotide G protein-coupled receptors (GPCRs) in humans [10, 67–69]

As mentioned before, mouse arginase-1 is a prototypic alternative activation marker and its induction is confined mainly to murine macrophages [70]. However, it was recently demonstrated that human mononuclear cells can adopt an alternative activation profile during the infection with the filarial nematode parasite *Wuchereria bancrofti*, the etiological agent of lymphatic filariasis [71]. The authors demonstrated that these cells express the arginase encoding genes and a member of the resistin family [71]. Under the appropriate environment, this outcome would indicate that human monocytes can undergo alternative activation, similar to that found for mouse macrophages [72].

In addition, it was demonstrated that arginase-1 is expressed constitutively in human granulocytes and would participate like a new effector mechanism against fungi, decreasing arginine availability in phagolysosome [73]. In contrast to murine arginase-1, human granulocyte arginase-1 expression is not influenced by proinflammatory or anti-inflammatory cytokines in vitro. On the other hand, its

location is not cytosolic but it is present in azurophilic granules and it was determined that human arginase-1 present in neutrophils has the capacity to suppress T cells [74].

Although the general functions and behaviors of murine and human AaM ϕ s are thought to be preserved, the genes required for such functions are to some extent different.

The divergences between species and their functional repercussions require further investigation [10].

6. Role of Arginase-1 in Immunosuppression

The ability of arginase-1-expressing macrophages to suppress T cell proliferation could explain their presence in placenta and lung of healthy individuals [8, 75]. However, the presence of suppressive arginase-1 expressing macrophages during infections as well as during cancer could be detrimental for the host [76].

As was mentioned above, during *Brugia malayi* experimental infection, it has been demonstrated that IL-4-dependent macrophages recruited in vivo actively suppress the proliferation of lymphocytes. These macrophages block proliferation by cell-to-cell contact, implicating a receptor-mediated mechanism. [37]. In addition, it was also recently demonstrated that arginase-1 plays an essential role in the T cell suppression observed at the site of pathology in non-healing leishmaniasis lesions [77]. Modollel and colleagues demonstrated that arginase-1 is highly expressed at the site of pathology in nonhealing lesions and causes local depletion of L-arginine which impairs the capacity of T cells in the lesion to proliferate and to produce IFN- γ , required for parasite killing. They clearly showed that arginase-1 is mediating T-cell immunosuppression since its inhibition or the injection of L-arginine reverses suppression and results in macrophage efficient control of parasite replication [77]. However, during schistosomiasis, macrophage-specific arginase-1 contributes to the resolution of the infection by inhibiting CD4+ T cell proliferation and cytokine production. The authors showed that arginine depletion by arginase-1 expressing macrophages was the primary suppressive mechanism while no significant contribution for IL-10 or TGF- β was observed in these studies [46]. Moreover, Terrazas and colleagues have showed that *Taenia crassiceps*-induced macrophages have suppressive T cell activity and this effect was IL-10, IFN- γ , NO independent, and cell-cell contact dependent [36]. *Taenia crassiceps*-induced macrophages showed an alternative activated profile with high expression of Fizz1, Ym1, and arginase-1 [36]. The authors demonstrated that the suppressive ability of the *Taenia crassiceps*-induced macrophages involves programmed death ligand molecules, PD-L1 and PD-L2, which were found to be upregulated in those macrophages [36]. These molecules bind to the receptor programmed death 1 (PD-1) which is expressed on activated T cells (B cells and myeloid cells also) and their interactions result in down-modulation of T cell responses [78, 79]. In addition, the selective upregulation of PD-L1 on macrophages by *Schistosoma mansoni* worms has shown to induce anergy of T cells during early acute stages of infection

before the subsequent emergence of egg-induced T cell suppression in the chronic stages of infection [80]. Studies in progress in our group demonstrated that the PD-1/PD-L pathway may be also involved in the immunosuppression observed during the acute phase of *T. cruzi* infection [81]. We observed change in the expression of these molecules during the acute phase of *T. cruzi* infection in CD3+, CD11c+, and F4/80+ peritoneal cells. In addition, *T. cruzi*-macrophages were able to suppress concanavalin A (ConA) T cell induced proliferation which is restored by blocking PD-1 pathway with antibodies or by inhibiting arginase-1 activity. In addition, our preliminary data suggest that PD-L2 could be involved in arginase-1 induction in *T. cruzi* infected macrophages (Dulgerian et al. unpublished data). Therefore, this may suggest that PD-1-PD-Ls interaction is important in arginase-1 modulation and in *T. cruzi* replication in macrophages.

In this context, it has been recently showed that the activity and expression of arginase-1 could be regulated by both PD-1 and CTLA-4 on the myeloid-derived suppressor cells (MDSCs) [82]. It has been extensively demonstrated that increased metabolism of L-arginine by myeloid cells can result in the impairment of lymphocyte responses to antigen during immune responses and tumor growth [76]. PD-1 molecules expressed on MDSCs can be involved in the regulation of MDSC function by regulating the activation and expression of arginase-1. The regulation of arginase-1 activity and expression by costimulatory or co-inhibitory molecules on the MDSCs might be important in inducing and maintaining of immunosuppression [76].

On the other hand, the role of human granulocyte arginase in immunosuppression was explored by Munder and colleagues [74]. They demonstrated that upon human polymorphonuclear granulocytes (PMN) cell death, arginase is liberated and very high activities of this enzyme accumulate extracellularly during purulent inflammatory reactions. PMN arginase induces a profound suppression of T cell proliferation and cytokine synthesis. This T cell suppression is due to arginase-mediated depletion of L-arginine in the T cell environment, which leads to CD3 ζ chain down-regulation [74]. Recently they showed that PMN arginase-mediated suppression reach also NK cells [83]. Granulocyte arginase severely impairs NK cell proliferation and IL-12/IL-18-induced secretion of IFN- γ [83].

7. Signals Involved in Arginase-1 Induction

One of the first works that investigated about the intracellular signals involved in arginase induction by LPS showed that protein kinase A (PKA) activation was required whereas the activation of the enzyme did not depend on PKC, neither the intracellular levels of Ca⁺⁺ [84]. Later, the signals involved in IL-13 arginase-1-induction were studied by Chang and colleagues [85]. They demonstrated that IL-13 down-regulates NO production through arginase induction via cAMP/PKA, tyrosine kinase (TK), and p38 MAPK signaling [85]. Recently findings correlate with the previous role of PKA in arginase induction in macrophages [86]. We

have previously shown that arginase-1 induction mediated by cruzipain involves the activation of multiple intracellular signals such as TK, PKA, and p38 MAPK [64, 87], similar to the signals triggered by IL-13 [85]. Furthermore, it was demonstrated that the transcription factor STAT-6 is required for arginase-1 induction in IL-4 and IL-13-treated macrophages [88, 89]. However, it was recently shown that some intracellular pathogens induced arginase-1 expression in mouse macrophages through the Toll Like Receptor (TLR) pathway [90]. In contrast to diseases dominated by Th2 responses in which arginase-1 expression is greatly increased by IL-4 and IL-13 signaling through the transcription factor STAT6, TLR-mediated arginase-1 induction was independent of the STAT6 pathway. Specific elimination of arginase-1 in macrophage favored host survival during *Toxoplasma gondii* infection and decreased lung bacterial load during tuberculosis infection. Therefore, TLR pathway could be also advantageous to certain pathogens [90].

8. Concluding Remarks

The diversity of macrophage activation states is probably as diverse as the number of environmental conditions to provide the appropriate immune response. It is influenced by the complexity of the stimulus, the sequence of exposure, and the status of macrophage differentiation at the time of exposure. In this review we have focused our discussion on the role of arginase-1-macrophages in parasitic infections. The outcome of protozoan infections is crucially dependent on the order, timing, and relative strength of type 1 versus type 2 responses and CaM ϕ versus AaM ϕ generation (Figure 1). During several infections induction of arginase-1-macrophages was showed to have a detrimental role by limiting CaM ϕ -dependent parasite clearance and promoting parasite proliferation. Additionally, the role of arginase in T-cell suppression has been explored recently. During several infections, arginase-1 T cell suppression contributes to failure to eliminate the pathogen. However, during schistosomiasis arginase-1-expressing macrophages suppress Th2-dependent inflammation and fibrosis, contributing to the resolution of the infection. Arginase suppressive activity has been mainly associated with L-arginine depletion leading to CD3 instability. In addition, PD-1 and their ligands have been also involved in arginase-1 suppression (Figure 2). On the other hand, although arginase-1 induction is confined mainly to murine macrophages, it was demonstrated that it is expressed constitutively in human granulocytes and it showed some similar function to the murine arginase (Figure 2). However, although the general functions and behaviors of murine and human AaM ϕ s are thought to be preserved, human alternative activated macrophages should be further investigated.

Additionally, recent findings have improved the understanding of signals involved in arginase-1 expression. In addition to the well-known role of STAT-6 in IL-4 and IL-13-arginase induction, during Th1 responses, a novel TLR dependent but STAT6-independent pathway of arginase-1 expression was identified.

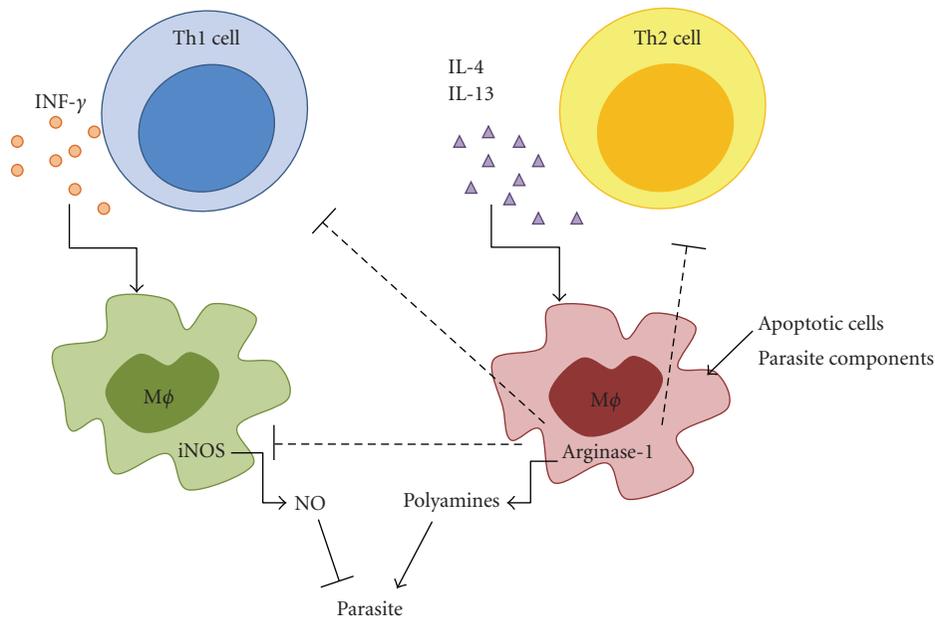


FIGURE 1: Role of macrophage activation in parasitic infections. A type 1 cytokine-dependent proinflammatory response inducing classically activated macrophages (CaMφs) leads to NO production and also to the synthesis of several products of NO reaction. CaMφs are crucial for parasite control during protozoan infections but can also contribute to the development of immunopathological disease symptoms. Type 2 cytokines such as IL-4 and IL-13 antagonize CaMφs inducing alternatively activated macrophages (AaMφs) that upregulate arginase-1 expression. Arginase-1 can also be induced during the infection by apoptotic cells or even directly by parasites or parasite components. Arginase-1 limits CaMφ-dependent parasite clearance promoting parasite proliferation. Additionally, arginase-1 suppresses T cell response. Therefore, generation of alternative activation states of macrophages could limit collateral tissue damage because of excessive type 1 inflammation. However, they affect disease outcome by promoting parasite survival and proliferation.

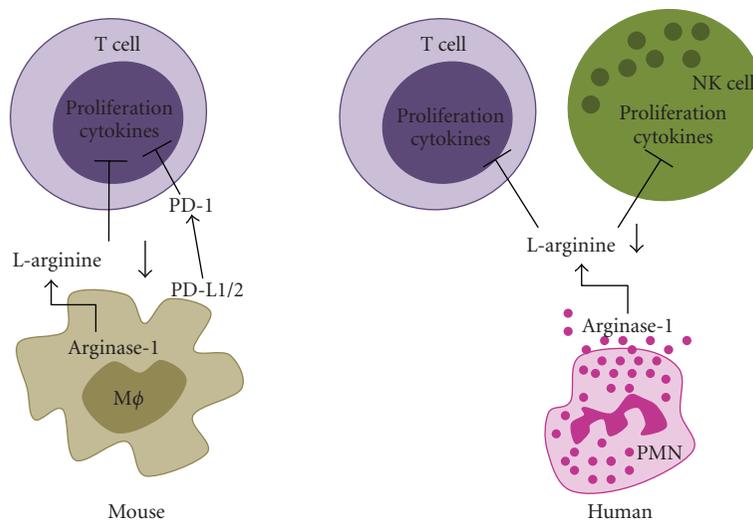


FIGURE 2: Role of arginase in immunosuppression. During parasitic infections, mouse macrophage-specific arginase-1 induces T cell suppression due to arginase-mediated depletion of L-arginine in the T cell environment, which leads to CD3 ζ chain down-regulation. Additionally, PD-1/PD-L pathway may be also involved in the T cell suppression. Furthermore, human granulocyte arginase can also induce immunosuppression by a similar mechanism. Upon human polymorphonuclear granulocytes (PMN) cell death, arginase is liberated and high activities of this enzyme accumulate extracellularly during purulent inflammatory reactions. PMN arginase induces a profound suppression of T cell and NK cell proliferation and cytokine synthesis.

Finally, our knowledge of how different types of macrophages affect the course of infectious diseases caused by protozoan parasites should provide therapeutic benefits in controlling these infections.

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Review Article

Parasitic Helminths: New Weapons against Immunological Disorders

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The prevalence of allergic and autoimmune diseases is increasing in developed countries, possibly due to reduced exposure to microorganisms in childhood (hygiene hypothesis). Epidemiological and experimental evidence in support of this hypothesis is accumulating. In this context, parasitic helminths are now important candidates for antiallergic/anti-inflammatory agents. Here we summarize antiallergic/anti-inflammatory effects of helminths together along with our own study of the effects of *Schistosoma mansoni* on Th17-dependent experimental arthritis. We also discuss possible mechanisms of helminth-induced suppression according to the recent advances of immunology.

1. Introduction: Autoimmunity, Allergy, and Helminth Infection

In 1989, the “hygiene hypothesis” was proposed by D.P. Strachan in an article that claimed an inverse relationship between the occurrence of hay fever and numbers of siblings [1]. According to the hypothesis, atopic disorders are due to reduced exposure to microorganisms in childhood. Nowadays, the concept is becoming more accepted with accumulating evidence not only in atopic diseases but also in autoimmune inflammatory diseases. For instance, the incidence of multiple sclerosis (MS) is higher in high latitude countries (= westernized developed countries) than in equatorial areas [2]. Not only residents of western countries but also immigrants from developing countries are at high risk of developing inflammatory bowel diseases (IBDs) and asthma [3]. In the case of type 1 diabetes (T1D), a similar geographical distribution to the diseases above and an inverse correlation to hygiene conditions are observed [4]. A population-based ecologic study in Canada showed that IBD, including ulcerative colitis (UC) and Crohn’s disease (CD), correlated with a high socioeconomic status, low rate of enteric infection, and high rate of MS [5].

Many studies have demonstrated that helminth infections lower the risk of autoimmunity or allergy. For instance,

an inverse correlation between autoimmune liver diseases and *Strongyloides stercoralis* infection was demonstrated in Okinawa, Japan [6]. Cross-sectional studies on the relationship between skin prick tests and helminth infections suggested a general protective effect on the atopic reaction [7]. The authors summarized effects of geohelminths on the risk of asthma according to previous studies; that is, hookworm lowered but *Ascaris* increased the risk of asthma and *Trichuris* had no effect. Collectively, it is concluded that at least some helminths seem to have anti-allergic or anti-inflammatory effects in humans.

Experimental studies have also shown protective effects of helminth infections in animal models of autoimmunity (e.g., colitis, arthritis, and diabetes) and allergy (e.g., airway hypersensitivity) [3, 8–10]. In this review, we discuss possible mechanisms of anti-allergic/anti-inflammatory effects of helminths in animal models including autoimmune arthritis. Possible clinical applications and future prospects are also discussed.

2. Helper T Cell Subset Dependence of Experimental Immunological Disorders

Based on T cell skewing patterns and their relative importance in the pathogenesis, disorders with excessive immune

responses had been briefly classified as “Th1 diseases” and “Th2 diseases” according to the Th1/Th2 paradigm. However, the recent discovery of a novel pathogenic T cell subset (Th17) [11] led investigators to the concept of “Th17 disease.” While most atopic immune disorders (e.g., hay fever and bronchial asthma) can be classified as Th2 diseases, the classification of autoimmune diseases is relatively difficult. Experimental autoimmune encephalomyelitis (EAE) as a model of MS was long thought to be a Th1 disease; however, the recent studies using IL-12/23 subunit (p35, p19, or p40) deficient mice revealed the progression of the disease to be dependent on the IL-23/IL-17 axis (= Th17 response) rather than IL-12/IFN γ axis (= Th1 response) [12]. The pathogenic role of IL-17 was shown directly by the finding that EAE development was significantly suppressed in IL-17-deficient mice [13]. The importance of the IL-23/IL-17 axis is supported also in human MS [12]. Regarding T1D, the diabetes observed in NOD mice (a model of T1D) has been classified as a Th1 disease despite the presence of some controversial study results [14–16]. Recent reports demonstrated that Th17 cells could also cause diabetes, but only after their conversion to Th1-type cells [17, 18]. This means that there is unknown plasticity of helper T subsets. Regarding IBD, the pathogenic roles of both Th1 and Th17 responses in TNBS-induced colitis (a model of IBD) are still controversial [19–23]. Collectively, some experimental autoimmune disorders cannot yet be distinctly classified as either Th1 or Th17 disease.

3. Protective Effects of Helminths against Immunological Disorders

The effects of schistosomes and other helminths on experimental autoimmunity/allergy are summarized in Table 1. Surprisingly, helminths have been shown to suppress all types (Th1, Th2, and Th17) of disease in the models described above [24–45]. Considering classical Th1/Th2 paradigm, it is reasonable to speculate that helminth-induced Th2-skewing with downregulation of Th1 immune responses results in an amelioration of Th1 diseases. As IL-4 is known to suppress Th17 development [46], Th17 response could also be suppressed as well as Th1 response in helminth-infected or helminth antigen-treated animals. In fact, STAT6-dependent IL-4/IL-13 signaling was shown to be essential in the suppression of TNBS-induced colitis [29] and EAE [25] by schistosome eggs, although the authors did not measure changes of IL-17. Given that the involvement of Th1 and Th17 in some forms of autoimmunity is still controversial, downregulation of both T helper responses may be beneficial for the amelioration of various kinds of autoimmunity. Along with other investigators, we recently found that schistosome-infected mice became resistant to experimental arthritis accompanying down-regulation of both Th1 and Th17 responses of splenocytes [47]. Likewise, Ruysers et al. reported suppression of TNBS-induced colitis by schistosome antigens, accompanying down-regulation of IL-17 gene expression in the colon and mesenteric lymph node (MLN) [28]. An intestinal nematode (*Heligmosomoides*

polygyrus) infection was also reported to suppress IL-17 production in MLN cells and lamina propria mononuclear cells [48]. The authors showed that the blocking of both IL-4 and IL-10 restored IL-17 production in vitro. Another study revealed that *Fasciola hepatica*-induced down-regulation of autoantigen-specific Th1 and Th17 responses (and protection from EAE) was dependent on TGF- β , not IL-10 [49]. Although the mechanisms of Th17's down-regulation by helminths are not yet established, some of the mechanisms might be common to those of Th1's down-regulation (e.g., through induction of IL-4 and IL-10, down-regulation of IL-12p40) and others might be distinct.

In our study on experimental arthritis in mice [47], we found no increase of Treg-related gene expression (Foxp3, TGF- β and IL-10) in the paws of *S. mansoni*-infected mice compared to the paws of uninfected control mice. As Treg cell population was known to expand in schistosome-infected or egg-treated mice [31, 32, 35, 50], the cells might participate in the regulation of the disease systemically rather than locally. To confirm the essential involvement of Treg cells in the antiarthritic effects of schistosome, further studies (e.g., persistent Treg depletion experiments) are necessary. In contrast to our result, in the case of diabetes in NOD mice, schistosome egg antigens induce infiltration of Treg cells at a local inflamed site (pancreas) [32]. Likewise, filarial nematode (*Litomosoides sigmodontis*) infection induced Treg cells and protected mice from the diabetes [39]. *H. polygyrus* also protected mice from the diabetes; however, the protection was not dependent on Treg cells [40]. In addition to schistosome and nematodes, tapeworm (*Taenia crassiceps*) infection also has anti-diabetic effects in multiple low-dose streptozotocin-induced diabetes (MLDS) in mice [45, in this issue]. In the study, alternatively activated macrophages (AAM ϕ) increased whereas Treg population did not increase. Taken together, these studies suggest that there may be various mechanisms in anti-diabetic effects of helminths.

We also found that schistosome-induced down-regulation of Th1 and Th17 occurred in the same period after the infection, corresponding to the beginning of egg-laying (unpublished observation). This result suggests that egg deposition is the major stimulus to lower Th17 responses (as well as Th1 response) in murine experimental schistosomiasis. Further studies using schistosome eggs are currently in progress in our laboratory.

Some epidemiological studies support that helminth infections are protective against atopic reactions and/or symptoms [51–53]. The helminth-induced suppression of Th2 diseases (atopic disorders etc.) is difficult to explain in terms of the Th1/Th2 paradigm. In the paradigm, theoretically, helminth infections are expected to cause IgE overproduction and hypereosinophilia, followed by exacerbation of allergic reactions. Indeed, persistent bronchoalveolar eosinophilia, airway hyperresponsiveness [54], and exacerbation of allergic airway inflammation [55] were observed in *Toxocara*-infected mice. One interesting explanation of anti-allergic effects of helminths is introduced in a review by Fallon and Mangan [56], in which Th2 responses are subdivided to “allergic” and “modified”, with helminth-induced Th2 responses corresponding to the latter

TABLE 1: Suppression of experimental immunological disorders by helminthes.

Animal models	Th types	Helminths	Treatment	Proposed suppressive mechanisms	Refs
Collagen-induced arthritis (CIA)	Th17	<i>Schistosoma mansoni</i>	Infection	IL-17 ↓, TNF-α ↓, IL-6 ↓, RANKL ↓, Anti-collagen IgG ↑	[47]
		<i>Ascaris suum</i>	Worm Ag		[37]
Experimental autoimmune encephalomyelitis (EAE)	Th17	<i>Acanthocheilonema viteae</i>	Purified Ag (ES-62)	IFN-γ ↓, TNF-α ↓, IL-6 ↓, Anti-collagen IgG ↓	[78]
		<i>Schistosoma mansoni</i>	Infection	IL-12p40 ↓, IFN-γ ↓, TNF-α ↓, IL-4 ↑	[24]
			Egg	IFN-γ ↓, IL-4 ↓, TGF-β ↑, IL-10 ↑	[25]
		<i>Schistosoma japonicum</i>	Egg Ag	IFN-γ ↓, IL-4 ↑	[26]
		<i>Fasciola hepatica</i>	Infection	IFN-γ ↓, IL-17 ↓, Dependent on TGFβ	[49]
		<i>Trichinella spiralis</i>	Infection		[27]
Type 1 diabetes in NOD mice	Th1?	<i>Schistosoma mansoni</i>	Infection, Eggs	Inhibition of Ab class switch (Anti-insulin IgG ↓)	[81]
			Egg Ag	Treg	[32]
Streptozotocin-induced diabetes	Th1?	<i>Litomosoides sigmodontis</i>	Infection, Worm Ag	IL-4 ↑, IL-5 ↑, Treg	[39]
		<i>Heligmosomoides polygyrus</i>	Infection	Independent of IL-10 and Treg	[40]
TNBS/DNBS-induced colitis	Th1? / Th17?	<i>Taenia crassiceps</i>	Infection	AAMφ	[45]
		<i>Schistosoma mansoni</i>	Infection	IL-2 ↑, IL-4 ↑	[30]
Piroxicam-induced colitis	Th17?		Eggs	IFN-γ ↓, IL-4 ↓	[29]
		<i>Schistosoma japonicum</i>	Worm Ag	IFN-γ ↓, IL-17 ↓, TGF-β ↑, IL-10 ↑	[28]
		<i>Hymenolepis diminuta</i>	Egg Ag	IFN-γ ↓, IL-4 ↓, IL-10 ↑, Treg	[31]
		<i>Heligmosomoides polygyrus</i>	Infection	IL-10 ↑	[41, 44]
DSS-induced colitis	Th17? / Th2?	<i>Schistosoma mansoni</i>	Infection (male worm)	IL-17 ↓, Independent of IL-10	[48]
Systemic anaphylaxis	Th2	<i>Schistosoma mansoni</i>	Infection	Macrophage infiltration	[64]
		<i>Schistosoma mansoni</i>	Infection	IL-10-producing Bcell	[34]
Asthma/Airway hypersensitivity or inflammation	Th2	<i>Schistosoma mansoni</i>	Infection (male worm)	IL-5 ↓, IL-10 ↑	[33]
		<i>Schistosoma japonicum</i>	Egg Ag, Eggs	Treg	[35]
		<i>Heligmosomoides polygyrus</i>	Infection	Treg	[36]
		<i>Ascaris suum</i>	Purified Ag (PAS-1)	IL-4 ↓, IL-5 ↓, Eotaxin ↓, RANTES ↓, IL-10 ↑	[38]
		<i>Litomosoides sigmodontis</i>	Worm Ag	IL-4 ↓, IL-5 ↓, Eotaxin ↓, IgE ↓	[42]
			Infection	TGFβ ↑, Treg	[43]

↓:downregulation, ↑:upregulation

type characterized by predominant Treg and IL-10 responses and a relatively weak IL-5 response. (The authors, however, describe that such modifications are localized in the lungs and different from systemic responses.) Along with this hypothesis, administration of *Ascaris* extract was shown to inhibit not only IL-5 production but also eosinophilic inflammation in a murine asthma model [42]. Filarial infection also suppressed airway hyperreactivity and pulmonary eosinophilia in a murine asthma model [43]. The suppressive effect of the filarial worms on hyperreactivity was dependent on Treg cells and TGF- β . As helminths seem to have both allergenic and immunomodulatory components [38] in their bodies, the balance of them may determine the outcomes (i.e., exacerbation or amelioration) of allergic disorders.

Helminthic infections often result in expansion and/or activation of Treg cells [31, 32, 35, 36, 50]. B cells are also suggested to be involved in disease suppression [34] through IL-10. However, the importance of IL-10 in helminth-induced suppression of atopic diseases is not fully established; that is, mesenteric lymph node cells from *Heligmosomoides*-infected IL-10-deficient mice could confer protection against allergic airway inflammation [36]. Likewise, in humans, anti-allergic effect of *Ascaris lumbricoides* is associated with Treg, but not with IL-10 [57]. In the case of mice, IL-35, a recently identified effector molecule of Treg cells [58], might be involved in the suppression. Other than the regulation of lymphocytes, helminth-derived products have potential anti-allergic effects on various kinds of cells. For instance, the filarial product ES-62 was shown to suppress the release of mediators from bone marrow-derived mast cells [59]. In addition, helminths generally induce AAM ϕ [60–63]. Such macrophages not only suppress the pathogenesis by the parasites themselves [61] but also may suppress pathological immune responses to autoantigens or allergens. The involvement of AAM ϕ in anti-diabetic effects was suggested in experimental tapeworm infection [45]. On the other hand, schistosome-modulated macrophages (but NOT AAM ϕ) were involved in suppression of dextran sodium sulfate-(DSS-) induced colitis [64]. These studies suggest the presence of various suppressive mechanisms by helminths. Immune cells and mediators possibly involved in the helminth-induced immunomodulation are illustrated in Figure 1.

4. Protective Effects of Helminths against Experimental Autoimmune Arthritis

Collagen-induced arthritis (CIA) is an autoimmune arthritis in mice and rats [65] widely used as a model of rheumatoid arthritis (RA). Although the role of the IL-12/IFN γ axis in CIA has been controversial [66, 67], recent findings suggest that IFN γ has ameliorating rather than exacerbating effects [68–71]. Instead, the IL-23/IL-17 axis was recently shown to be important in the pathogenesis of CIA [71–76] as well as RA in humans [77].

Regarding anti-arthritic effects of helminths, a filarial ES product ES-62 [78] and porcine roundworm *Ascaris suum* extract [37] were shown to suppress CIA in mice. As

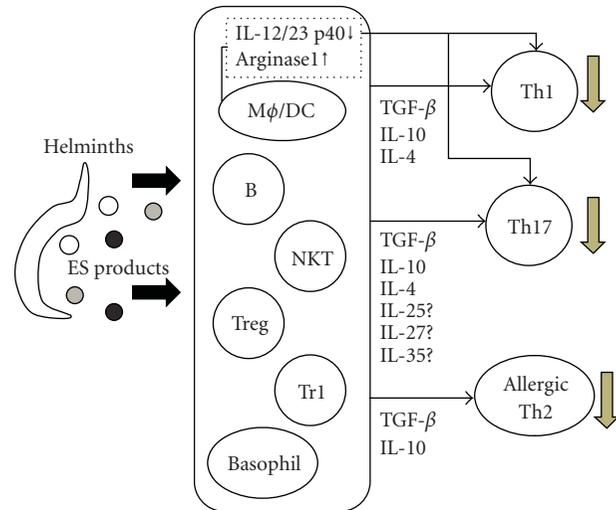


FIGURE 1: Possible involvement of immune cells and mediators in the helminth-induced immunomodulation on T helper subsets. Substances contained in the bodies of helminths or their excretory secretory (ES) products are recognized by innate immune cells via PAMPs receptors like TLRs. Thereafter, various changes occur in the immune cells, for example, down-modulation of IL-12/23p40 expression (DC), alternative activation (macrophages), proliferation and/or IL-10 production (Treg, Tr1, and B cells), and IL-4 production (basophils). We can observe suppression of immune disorders with down-regulation of pathogenic T helper subsets (Th1, Th17, and allergic Th2) as consequence of mixed effects of such immunological changes.

described, we examined effects of *S. mansoni* infection on CIA in mice. In humans, schistosomiasis has been reported as “arthritogenic” rather than anti-arthritic [79, 80]. In our experiments, however, *S. mansoni* infection lowered arthritis scores and numbers of arthritic paws [47]. Histopathological examination revealed that cell infiltration and bone/cartilage destruction were diminished in the infected mice.

In CIA and RA, the pathogenic roles of IL-1 and IL-6 are well established [82–84]. We observed that the marked augmentation of IL-1 β and IL-6 gene expression in the paws was almost completely abrogated by *S. mansoni* infection [47]. It was especially noteworthy that receptor activator of NF κ B ligand (RANKL) gene expression in the inflamed paws was also abrogated by *S. mansoni* infection. As RANKL expression is induced by proinflammatory cytokines including IL-17, TNF- α , and IL-1 β [85] and essential to osteoclast development followed by bone destruction [86–88], this result suggests that schistosome infection has anti-arthritic effects preventing bone destruction. Interestingly, we also found that intraperitoneally administered schistosome worm antigens (SWAP) or egg antigens (SEA) did not affect the progress of CIA (unpublished observation). In the antigen-administered mice, levels of IL-10, TNF α , and IL-17 produced by splenocytes were comparable to those in antigen nonadministered control mice. Thus, regarding schistosome, there is a considerable difference in immunomodulating effects between infection and antigen administration.

5. Future Clinical Applications of Helminths and Their Products

As described above, our experiments with schistosome showed that anti-arthritis effects were observed only in the viable worm infected mice. Likewise, Hunter et al. showed that anticolic effects of *Hymenolepis diminuta* were dependent on a viable infection [41], and surprisingly, Melon et al. showed that therapeutic efficacy of the viable tapeworm was superior to dexamethazone treatment [44, in this issue]. Because of these observations in experimental models, attenuated or non/lowly-pathogenic helminths are worth testing directly for therapeutic effects. In fact, porcine whipworm (*Trichuris suis*) eggs and *Necator americanus* infective larvae have been clinically tested for the treatment of chronic inflammatory or allergic diseases. Reddy and Fried [89] summarized the recent progress in clinical trials using these two intestinal nematodes. Summers et al. reported that the administration of *T. suis* eggs effectively ameliorated both UC and CD [90, 91] without adverse effects. *N. americanus* is also under clinical trials for asthmatic patients [92]. This worm is considered superior to porcine whipworm in that repeated administration is not needed.

Although it is not permissible to directly apply highly pathogenic helminths (e.g., schistosome) to clinical use, purified or synthetic immunomodulatory products from such worms can be considered for clinical purposes. Various immunomodulatory molecules (carbohydrates, proteins, and lipids) have been identified; for example, Lacto-*N*-fucopentaose III (LNFP III) contained in schistosome eggs is an oligosaccharide as the molecule affecting B cells (especially B-1 cells) to induce IL-10 production [93]. LNFP III was also reported to alternatively activate macrophages [94]. A chemokine-binding protein (CBP) from schistosome eggs inhibited the recruitment of neutrophils [95] to inflammatory foci. Peroxiredoxin (Prx) is an antioxidant protein found in various species including helminths [96, 97]. The molecules from *S. mansoni* and *F. hepatica* alternatively activate macrophages and are involved in the induction of a Th2-type immune response [97]. The IL-4-inducing principle of *S. mansoni* eggs (IPSE) is the molecule that induces "primary" IL-4 production from basophils [98]. Regarding anti-arthritis effects, a glycoprotein from *Spirometra erinaceieuropaei* was shown to suppress RANKL-induced osteoclastogenesis, suggesting that there may be more anti-arthritis substances in various helminths [99].

6. Concluding Remarks

Helminth-based immunotherapy for immunological disorders is still in its infancy. It should be pointed out that helminths do not always suppress autoimmune/allergic disorders. There are epidemiological and experimental reports that helminths exacerbate such disorders [10]. Apart from Th2-biasing abilities of helminths, allergens contained in the worms may partially explain the mechanisms of exacerbation. *Ascaris* extract has cross-reactivity with domestic mite allergens [100]. Moreover, *Ascaris* was shown to have an allergenic component (APAS-3) [38]. Mice infected

with *Toxocara canis* showed exacerbation of allergic airway inflammation [55] whereas hookworm (*Nippostrongylus brasiliensis*) infection persistently reduced airway responsiveness in mice [101], suggesting that there are considerable differences of outcomes even among lung-migratory nematodes. Thus, careful selection of "therapeutic" helminths and their target diseases is essential. Further studies on the mechanisms of immunomodulation are necessary for future human applications. The roles of Th1/Th17/Treg-related regulatory cytokines (IL-25, IL-27, IL-35, etc.) in the helminth-induced suppression of allergy/autoimmunity have not been sufficiently studied. It is well known that the roles of cytokines in human Th17 differentiation are very different from those in mice [12, 102]. For clinical applications in the future, we should ascertain changes in Th17-related cytokine patterns not only in animal models but also in patients with helminthiasis. It was reported that concurrent filarial infection suppressed both Th1 and Th17 responses to *Mycobacterium tuberculosis* [103]. This implies that helminths have down-regulating activity of both Th1 and Th17 in humans as well as in mice.

In human RA, the relative importance of Th1 and Th17 is still unclear, and therefore, suppression of both T helper responses is recommended [102]. Thus, at present we can conclude that regulatory effects on both Th1 and Th17 are promising characteristics of helminths as anti-inflammatory agents. However, the interpretation of experimental results of helminth-induced immunomodulation may change depending on changes in basic immunological knowledge. Indeed, it was recently reported that T-bet expression was more important in the pathogenesis of EAE than the Th1/Th17 balance [104]. Therefore, further studies of helminth-induced amelioration of immune disorders should strictly follow the studies of the diseases' pathogenesis.

As noted, in experimental animal studies, viable helminth infections seem to be superior to the administration of worm antigens or killed worms in the therapeutic effects. This might be due to that viable helminths can regulate secretion of immunomodulatory molecules in the most appropriate conditions for their survival. Taken together with successful treatment of UC and CD with viable porcine whipworms [90, 91], the optimal attenuation of human parasites by gene manipulation may be useful for the clinical application of parasitic helminths in the future.

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Research Article

Infection with *Hymenolepis diminuta* Is More Effective than Daily Corticosteroids in Blocking Chemically Induced Colitis in Mice

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Purpose. To compare infection with the tapeworm, *Hymenolepis diminuta*, with steroid (dexamethasone) administration in the inhibition of dinitrobenzene sulfonic acid- (DNBS-) induced colitis in mice. **Procedures.** Mice were treated with DNBS ± infected with *H. diminuta* or treated with daily dexamethasone (2 mg/Kg, ip.) and were assessed 72 hours post-DNBS by the calculation of disease activity and histological damage scores, and spleen cell cytokine production. **Results.** *H. diminuta*-infected mice showed increased IL-4 and IL-10 production by spleen cells compared to other groups and were protected from DNBS-induced colitis. In contrast, there was little benefit of dexamethasone in the treatment of colitis. Collagen deposition in the colon was not different between the groups. **Conclusions.** *H. diminuta* was superior to dexamethasone in the prevention of DNBS-induced colitis and did not result in additional side effects (i.e., collagen deposition). Comparisons with current therapeutics and long-term followup to studies are essential if “helminth therapy” is to become a viable treatment for specific inflammatory diseases in the gut or other tissues.

1. Introduction

During the last three decades there have been dramatic increases in autoimmune and inflammatory diseases, such as allergy/atopy, diabetes, and inflammatory bowel disease (IBD) that cannot be explained solely on the basis of genetics [1]. In the search for environmental triggers for these conditions, the hygiene hypothesis has arisen that suggests that reduced exposure to infectious agents (via increases in hygiene, sterile drinking water, and use of antibiotics) may result in the generation of greater numbers of autoreactive immune cells in humans, and hence the emergence of autoimmune and idiopathic inflammatory disease [2]. Compatible with this postulate is the geographical divergence in the occurrence of diseases such as IBD and areas of pandemic helminth infection [3]. Epidemiological data must be viewed cautiously when used in support of causation rather than association. They do nevertheless raise

the question: could infection with parasitic helminths protect against other concomitant disease?

Representatives of all classes of helminth parasite have been shown to modulate immunity in their hosts, both qualitatively and quantitatively [4–6]. Moreover, infection with helminth parasites evokes stereotypic immune responses in humans and mice that are dominated by T helper 2 (TH2) cytokines. Thus as putative modifiers of disease, the release of immunomodulatory or immunosuppressive molecules from helminths would be expected to impact on concurrent disorders in the host, and the stimulation of TH2 events has the potential to antagonize or inhibit diseases in which the immunopathology is driven by TH1 reactions. We have shown that infection with the rat tapeworm, *Hymenolepis diminuta*, protects mice from the colitic effects of direct instillation of dinitrobenzene sulfonic acid (DNBS) into the lumen of the colon [7]. A substantial amount of data has amassed showing that a variety of species of helminths

(e.g., *H. diminuta*, *Trichinella spiralis*, *Heligmosomoides polygyrus*, *Schistosoma mansoni*) reduce the severity of disease in rodent models of IBD, airways inflammation, and multiple sclerosis [8]. In the context of developing new treatments, two major possibilities arise from these proof-of-concept studies: (1) use of the immunological knowledge gleaned from these models to define new targets for pharmacological intervention; and (2) the prescription of a “therapeutic helminth(s)” to defined groups of patients that are refractory to other therapies. The latter is a provocative idea and, despite the spectre of iatrogenic disease [9], studies have been presented in which nematodes, namely, *Trichuris suis* and *Necator americanus*, have been used to treat IBD and as a putative forerunner to use in asthma, respectively [10, 11].

As the concept of a “therapeutic helminth” progresses it is important that appropriate comparisons with current therapies and potential side effects be rigorously assessed. Using the *H. diminuta*-DNBS murine model system, the current study addresses two questions. Will *H. diminuta* be as effective as steroids in the treatment of DNBS-induced colitis? And, is helminth therapy in this acute model associated with increased collagen deposition that could result in fibrosis and stricture formation? Using this acute and spontaneously resolving chemical model of colitis we present data showing that infection with *H. diminuta* is superior to daily steroid therapy and that infected mice, while receiving substantial anti-inflammatory benefit from infection, displayed no greater collagen deposition in their colons than mice receiving DNBS only.

2. Materials and Methods

2.1. Murine Model System. Male 6–8 Balb/c mice (Charles River Animal Supplies, Montreal, QB, Canada) were housed in filter-topped cages with free access to water and rodent chow and on a 12 hour : 12 hour light : dark cycle [7]. Colitis was induced in anesthetized mice via intrarectal (ir.) instillation of DNBS (3.0 or 2.5 mg in 100 μ L of 50% ethanol) delivered 3 cm into the colon via a polyethylene catheter [7]. Time-matched control mice received 50% ethanol only. A third group of mice were infected with five *H. diminuta* cystercoids via oral gavage in 100 μ L 0.9% NaCl, eight days prior to receiving DNBS [12]. The final group of animals was treated with the glucocorticoid, dexamethasone (DEX; Sigma Chemical Co., St. Louis, MO) and DNBS ($n = 3–8$ mice/group). Based on previous reports in which DEX was used to suppress inflammatory disease [13–15] mice was treated with either 1 mg or 2 mg/Kg administered by intraperitoneal (ip.) injection on three consecutive days beginning one hour after DNBS treatment (the effects of 1 and 2 mg/Kg DEX were not significantly different and so the data have been combined and are considered as a single group in the results section). All experiments adhered to the Canadian guidelines for animal welfare and complied with the specific ethical regulations of the University of Calgary.

2.2. Macroscopic Assessment of Colitis. Mice were examined daily, following treatment with DNBS, for signs of ill-health and intestinal dysfunction: weight loss, fur ruffling,

decreased activity, and wet or bloody anal area or feces. Upon autopsy, at 72 hours post-DNBS, the colon was excised and inspected for evidence of macroscopic damage. The appearance and length of the colon was recorded to give a Disease Activity Score based on the following criteria: >10% loss of body weight (0 or 1); wet anus, soft stool, or empty colon (0-1); anal bleeding (0 or 1); macroscopic ulcers present in the colon (0 or 1). If an animal deteriorated to a predetermined morbidity endpoint (e.g., rectal prolapse, obvious distress), it was humanely sacrificed and given a score of 5 [7].

Subsequently, the colon was divided based on length (contraction of the colon is characteristic of colitis in this model [7]) and segments preserved for further testing. The distal 20% was snap-frozen in liquid nitrogen for assay of eosinophils peroxidase (EPO) activity, the adjacent 10% segment was fixed in 10% formalin for histological analysis and the next 10% portion of colon was snap-frozen in liquid nitrogen and stored for measurement of collagen levels (the proximal colon was discarded).

2.3. Histological Assessment of Colitis. Formalin-fixed, paraffin-embedded segments of colon were sectioned (3 μ m), collected on coded slides, stained with haematoxylin and eosin and histological damage scored by an investigator using the following criteria (max score = 12): loss of architecture (0–3); inflammatory infiltrate (0–3); goblet cell depletion (0-1); ulceration (0-1); edema (0-1); muscle thickening (0–2); presence of crypt abscesses (0-1) [7]. Additional sections were stained with Mason’s trichrome stain, which identifies collagen as a blue reaction product.

2.4. Eosinophil Peroxidase Activity. Activity levels of EPO were determined as previously described [16]. Briefly the presence of MPO was assessed using a kinetic assay where H_2O_2 is broken down by the MPO released from the samples of colon. This assay was repeated on duplicate sample aliquots with the addition of 50 mM 3-amino-1,2,4-triazole (AMT; Sigma Chemical Co.) to inhibit EPO. EPO activity was calculated by subtracting the MPO + AMT value from MPO values.

2.5. Collagen Deposition. The amount of collagen in extracts of colon was measured via the Sircol colometric assay (Biocolor Ltd., N. Ireland, UK) following the manufactures instructions.

2.6. Cytokine Production. Spleen cells were isolated following a previously reported protocol [7], and 5×10^6 cells (in 1 mL of RPMI medium) were activated with the T cell mitogen, concanavalin A (ConA: at 2 μ g/mL). Culture media were collected 48 hours later and the levels of interleukin (IL)-10, IL-4, interferon (IFN)- γ , or tumour necrosis factor (TNF)- α were determined in duplicate serial dilutions using ELISA protocols stipulated by the manufacture (R&D Systems) [17]. ELISAs detection limits were 9 pg/mL. The bioactivity of the dexamethasone used in these experiments was assessed by its ability to suppress IL-4, IFN- γ , and TNF- α production by in vitro ConA-stimulated murine spleen cells.

2.7. Peripheral Blood Immune Cells. Blood smears were air dried and then stained using the Hema3 differential staining kit following the manufactures instructions (Fisher Scientific, Kalamazoo, MI). Mononuclear cells (T cells, B, cells, monocytes), neutrophils, and eosinophils were counted by a single investigator who was unaware of the treatment groups.

2.8. Statistical Analysis. Data are presented as the mean \pm the standard error of the mean (SEM), where n is the number of mice (3–8/group) examined. Statistical comparisons were by one way ANOVA followed by pairwise comparisons using the Student's t -test or Tukey's test for nonparametric data. A statistical difference was set at $P < .05$.

3. Results

In preliminary experiments we confirmed that the dexamethasone used in the in vivo analysis was bioactive. Treatment of spleen cells with ConA for 48 hours resulted in the production of 26 ± 2 pg/mL of IL-4, 508 ± 104 pg/mL of TNF α , and 4412 ± 870 pg/mL of IFN γ ($n = 6$): inclusion of DEX (1 μ g/mL) completely blocked ConA-induced production of these cytokines (levels of all 3 were undetectable in ELISA).

In two initial experiments, mice were treated with 3 mg DNBS ir; however, in these studies significant morbidity was observed with 66% (i.e., 6 of 9 (disease activity score = 4.6 ± 0.2)), 12.5% (1 of 8 (disease activity score = 2.6 ± 0.4)), and 25% (i.e., 2 of 8 (disease activity score = 3.8 ± 0.4)) of mice treated with DNBS, *H. diminuta* + DNBS and DNBS + DEX, respectively, reaching an endpoint that necessitated sacrifice prior to completion of the experiment. Despite the severity of the colitis, these data suggest that both infections with *H. diminuta* and daily DEX treatments reduce the effect of DNBS. Subsequent experiments (described below) were conducted with 2.5 mg of DNBS.

3.1. Increased Eosinophils Confirm Successful Infection with *H. diminuta*. Differential staining of peripheral blood revealed an increase in eosinophils ($4.0 \pm 0.6\%$ ($n = 3$)) in blood retrieved from *H. diminuta* + DNBS treated mice, confirming successful infection with the helminth. An effective infection was further substantiated by analysis of EPO activity in tissue homogenates that revealed significant increases in tissues excised from *H. diminuta*- + DNBS treated mice (5.7 ± 2.1 U/mg tissue) compared to control (2.1 ± 0.9), DNBS (1.4 ± 0.7), and DNBS + DEX treated mice (0.8 ± 0.2) ($n = 3$), indicating that the mice had received a viable infection of *H. diminuta*.

3.2. *H. diminuta* Is More Effective than Daily DEX in Inhibiting DNBS-Induced Colitis. As expected [7] mice treated with DNBS developed colitis as gauged by loss of body weight, shortening of the colon and disease activity and histology damage scores (Figure 1). Similarly, and corroborating our earlier report, mice previously infected with *H. diminuta* were protected against the procolitic effects of intrarectal DNBS treatment [7], and, in contrast, no anticolitic effects

were observed in the DEX treated group, using the dose and treatment regimen employed in these studies (Figures 1 and 2).

3.3. Infection with *H. diminuta* Induces IL-10 and IL-4 Production. Only spleen cells isolated from *H. diminuta* + DNBS treated mice ($n = 3$) produced substantial amounts of IL-10 (268 ± 122 pg/mL) and IL-4 (437 ± 192 pg/mL) in response to a 48-hour treatment with ConA. Spleen cells from control mice produced 0–24 pg/mL of IL-10 and IL-4; whereas neither cytokine was detected in culture medium from spleen cells isolated from DNBS- or DNBS + DEX-treated mice ($n = 3–6$). In contrast, lower amounts of IFN γ and TNF α were found in conditioned medium from ConA-stimulated spleen cells isolated from *H. diminuta* + DNBS and DNBS + DEX-treated mice compared to DNBS only treated mice (Figure 3). The latter shows that DEX was having an in vivo effect, despite the lack of inhibition of colitis using the indices of gut form employed here.

3.4. Collagen Deposition Is Not Apparent in this Acute Model of Colitis. Neither histochemical staining (Figure 2) nor biochemical analysis (Figure 4) revealed any significant increases in collagen deposition in the colon in any of the treatment groups (all examined 72 hours post-DNBS) compared to time-matched controls.

4. Discussion

Current medical management of many autoimmune and inflammatory diseases, including IBD, relies heavily on the use of steroids and broad-spectrum immunosuppressive drugs [18]. While effective in reducing disease symptoms, both classes of therapeutic are associated with a range of side effects. In the case of steroids, these include moon-face, fluid retention, insomnia and weight gain, with more serious effects of long-term use being osteoporosis and suppression of the hypothalamic-pituitary-adrenal axis. The use of biologicals (e.g., anti-TNF α antibody) is increasing as these drugs are proving very effective in treating some patients with aggressive disease that have failed other therapies [19]; however, their use comes at considerable financial cost. Thus, in the ongoing search for additional therapeutics, a number of investigators have revived an older notion that infection with helminth parasites can ameliorate concomitant disease [20]. The immunological basis of this postulate is that the immune response mobilized by the host to combat the parasitic helminth, whether it is TH2-immunity or a generalized state of immuno-regulation/suppression, is sufficient to antagonize or inhibit the immunopathological events underlying, for example, atopic disease or IBD.

Substantial proof-of-concept data have accumulated from analyses of rodent model systems to show that infection with helminth parasites either blocks the development of, or significantly reduces the severity of other diseases. With respect to intestinal inflammation, infection with nematodes (*H. polygyrus*, *T. spiralis*), trematodes (*S. mansoni*) and cestodes (*H. diminuta*) can reduce colitis evoked by chemical

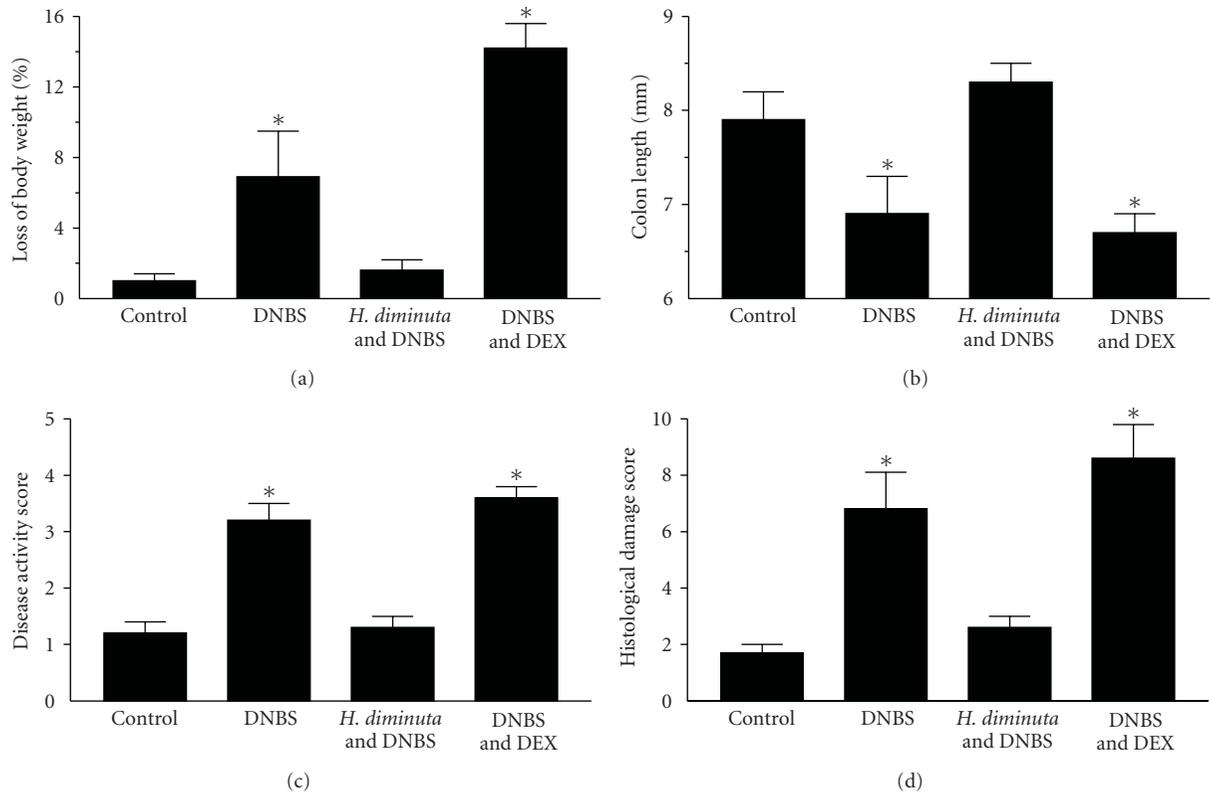


FIGURE 1: Bar graphs showing that infection with *H. diminuta* 8 days prior to DNBS (ir, 2.5 mg), in contrast to 3 doses of dexamethasone (DEX), reduces the severity of colitis as gauged by (a) body weight, (b) colon length, (c) disease activity scores, and (d) histological damage scores (mean \pm SEM; $n = 6-8$ mice from 2 experiments; * $P < .05$ compared to control).

haptizing agents, or that which spontaneously develops in the IL-10 deficient mouse (reviewed in [8]). The data from these investigations have been complemented by preliminary and provocative findings showing that “helminth therapy” could be a viable option for the treatment of IBD and possibly asthma [21]. The caveat here is that the introduction of a species into a new niche can have unforeseen consequences, and this needs to be borne in mind.

From the ability of infection with helminth parasites to block inflammatory disease two questions arise: (1) will immunological knowledge gleaned from the helminth-rodent model systems translate into new treatments for human disease? And, (2) are there side effects of “helminth therapy” (and by inference the application of immunological knowledge from the helminth-rodent model systems)? In relation to the former, analyses of infection with various parasitic helminths have implicated IL-10, transforming growth factor (TGF)- β , FoxP3⁺ regulatory T cells, and the inhibition of TH1 and TH17 events in the anti-colitic effect [4, 8, 22]. These cells and mediators have been implicated in the pathogenesis/pathophysiology of human inflammatory disease, confirming the value of the helminth-rodent models in the development of putative treatments for human disease.

The current study is, to our knowledge, the first to compare infection with parasitic helminths with another therapeutic modality in a model of colitis: dexamethasone (DEX) being selected as the comparator therapy. Consistent

with our earlier findings [12], mice infected with *H. diminuta* were significantly protected from the colitic effect of ir. instillation of DNBS. In contrast, and despite the DEX being bioactive (it blocked ConA-induced cytokine production from splenocytes in vitro), the severity of colitis-induced by DNBS was unaffected by three consecutive daily doses of DEX. This treatment regime (dose, route of administration, repeated treatment) was based on studies in which DEX blocked tri-nitrobenzene sulphonic acid- (TNBS-) induced colitis [13–15, 23]. However, only a single study has shown that DEX reduces DNBS-induced colitis in mice, and that study used a tenfold higher dose of steroid than used here [24]. Moreover, while our data are surprising, they are not unprecedented. Atug et al. reported that a 7-day DEX treatment did not alter TNBS-induced histopathology [25, 26] and others have shown that DEX inhibition of TNBS-induced inflammation was not accompanied by reduced local levels of IL-1 β and actually increased colonic levels of IFN γ [13]. Thus, while one can speculate on why DEX was ineffective in blocking DNBS-colitis in the present study (e.g., impact of microflora, suppression of a beneficial anti-colitic effect), and despite the reduction in the production in the synthesis of proinflammatory cytokines by spleen cells (e.g., TNF α and IFN γ), the important comparison remains that infection with *H. diminuta* was more effective in blocking DNBS-colitis than a steroid treatment regimen.

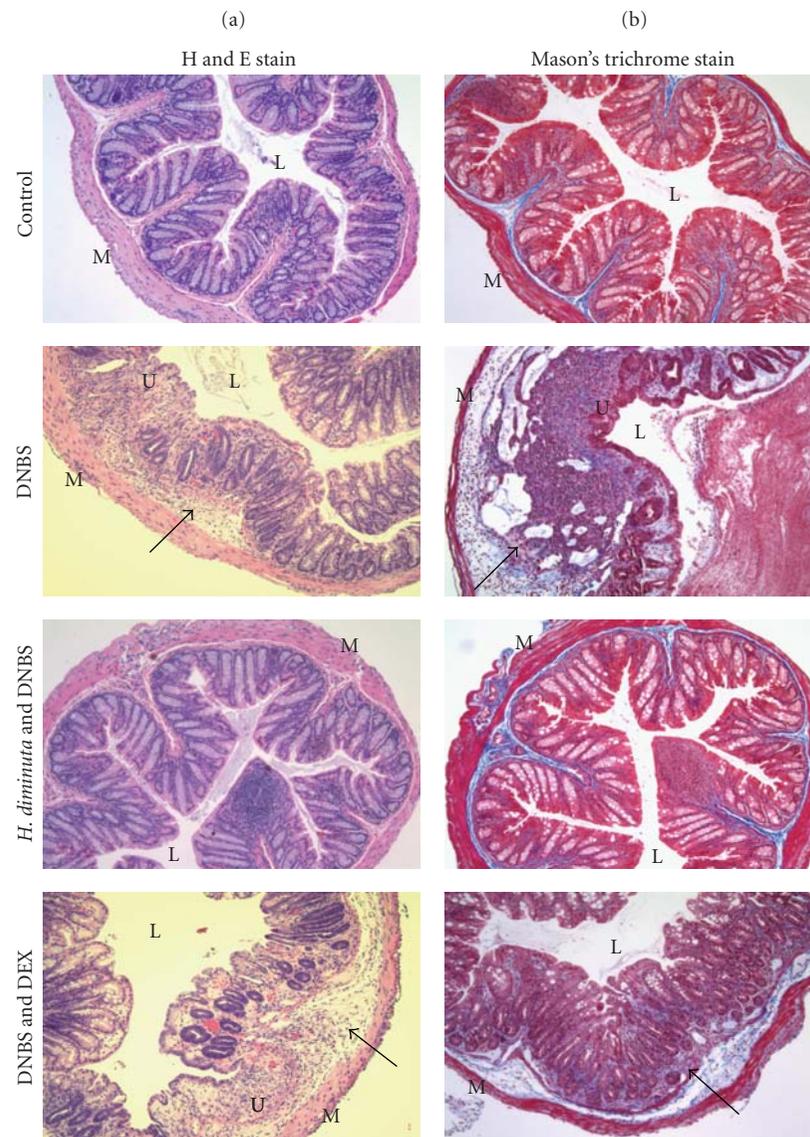


FIGURE 2: Representative images of gut morphology (hematoxylin and eosin (H&E stain); (a)) and collagen deposition ((b); collagen is stained blue) in colonic sections from the 4 treatment groups (M: muscle; L: gut lumen; U: ulcer; arrow, inflammatory infiltrate: original magnification = $\times 200$).

We have previously expressed the concern that “helminth therapy” would be of little value if it predisposed an individual to hypersensitivity/asthmatic conditions or promoted fibrotic disease (both of which can occur in TH2 dominated conditions) [16]. In the present study, we observed no evidence, neither histochemical nor biochemical, in support of additional deposition of collagen in the colon of mice cotreated with *H. diminuta* + DNBS. Furthermore, using the same experimental conditions, we found that the anticolitic effect of infection with *H. diminuta* was not associated with increased sensitivity to bystander protein antigens [7]. Thus, at least in this experimental paradigm with *H. diminuta* in the nonpermissive mouse host and an acute model of chemically induced colitis, significant adverse effects have not been detected in association with the anticolitic effect.

However, this does not preclude the possibility of long-term side effects, and studies to address this need to be conducted in this and other model systems.

In conclusion, significant momentum has been generated in the last few years in the assessment of the ability of infection with helminth parasites to block concomitant disease [8, 21]. These studies have the potential to identify one or more “therapeutic helminths” (the caveats of palatability and iatrogenic infection notwithstanding) and, perhaps more pertinently, can identify novel immunological molecules or signaling pathways that can serve as the basis of targets for new therapeutics. As this research area advances, we need to compare and contrast “helminth therapy” with other treatments and exhaustively assess potential side effects. In this context, we found that *H. diminuta* was more effective than

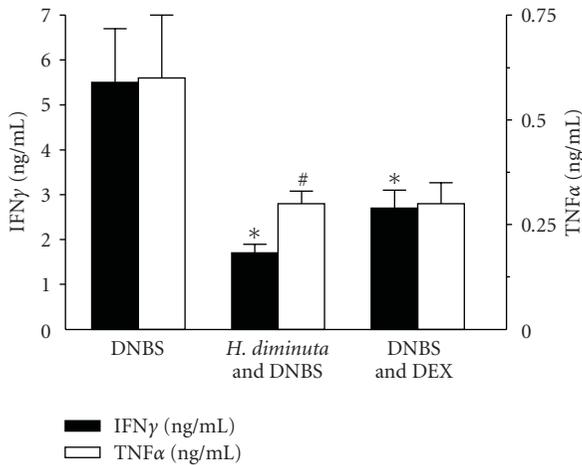


FIGURE 3: Bar graph showing concanavalin A ($2 \mu\text{g}/\text{mL}$)-stimulated cytokine production from spleen cells (5×10^6), excised from mice 72 hours post-DNBS treatment (mean \pm SEM; $n = 4$ -5 mice from 2 experiments; * $P < .05$ compared to DNBS only treated mice; # $P = .08$ compared to DNBS only; DEX: dexamethasone).

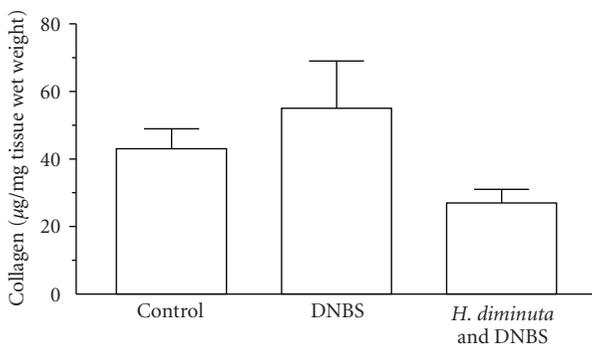


FIGURE 4: Bar graph showing the amount of collagen deposition in the colon of control, DNBS (ir, 2.5 mg) and *H. diminuta*-infected (5 cystercoids, 8 days prior to DNBS) + DNBS treated mice (mean \pm SEM; $n = 3$).

the steroid dexamethasone in preventing DNBS-induced colitis and that the benefit of infection with the helminth was not accompanied by increased deposition of collagen in the colon.

Acknowledgments

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Research Article

Taenia crassiceps Infection Attenuates Multiple Low-Dose Streptozotocin-Induced Diabetes

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Taenia crassiceps, like other helminths, can exert regulatory effects on the immune system of its host. This study investigates the effect of chronic *T. crassiceps* infection on the outcome of Multiple Low Dose Streptozotocin-Induced Diabetes (MLDS). Healthy or previously *T. crassiceps*-infected mice received MLDS and type 1 diabetes (T1D) symptoms were evaluated for 6 weeks following the induction of MLDS. *T. crassiceps*-infected mice displayed lower blood glucose levels throughout the study. A significantly lower percentage of *T. crassiceps*-infected mice (40%) developed T1D compared to the uninfected group (100%). Insulinitis was remarkably absent in *T. crassiceps*-infected mice, which had normal pancreatic insulin content, whereas uninfected mice showed a dramatic reduction in pancreatic insulin. Infected mice that received MLDS did not show an increase in their regulatory T cell population, however, they had a greater number of alternatively activated macrophages, higher levels of the cytokine IL-4, and lower levels of TNF- α . Therefore, infection with *T. crassiceps* causes an immunomodulation that modifies the incidence and development of MLDS-induced autoimmune diabetes.

1. Introduction

Parasitic helminths are a highly diverse group of organisms that display different morphologies, accessory structures, sexual and feeding behaviors and life cycle stages. Helminths distribute themselves across a variety of niches inside their hosts where they can cause a multiplicity of diseases. Helminth parasites also appear to follow varied and complicated oral and cutaneous routes of infection within host tissues. Surprisingly, despite these differences in features and behavior, helminths share a unique ability to exert profound regulatory effects on the immune systems of their hosts. One of the first observations made concerning helminth infection was the elicitation of a strong Th2-biased immune response [1, 2]. In the last few years, new regulatory has been identified to play a role in helminth infection [2, 3]. One of these mechanisms includes the induction of regulatory T cells (Tregs), which are now known to be involved in

pathogen susceptibility and the control of inflammation in helminth infections caused by *Litomosoides sigmodontis* [4], *Trichuris muris* [5], *Brugia malayi* [6], *Trichinella spiralis* [7], and *Heligmosomoides polygyrus* [8]. Dendritic cells (DCs) have also been reported to be affected by helminth-derived products [3, 9] and, more recently, a new population of macrophages called alternatively activated macrophages (AAMs ϕ) has been consistently observed in several worm infection models [10, 11].

There are a series of epidemiological and experimental studies supporting the idea that helminth infections can induce a protective effect against the development of both autoimmune and allergic diseases [12]. The “hygiene hypothesis” was the first to suggest that the increase in the prevalence of allergies and asthma in developed countries might be linked to the reduction in infections with parasitic and bacterial pathogens. Thus, parasitic infection might somehow “educate” the immune system to avoid exacerbated

inflammatory responses [13, 14]. Type 1 diabetes (T1D) is an autoimmune disease that has increased in prevalence over the last several years in developed countries and is caused by the selective destruction of insulin-producing β cells located in pancreatic Langerhans' islets by autoantigen-reactive inflammatory T cells. When the majority of β cells are destroyed, the pancreas' ability to secrete insulin in response to blood glucose levels is impaired, resulting in a disruption of glucose homeostasis. Previous studies have shown that the MLDS-induced diabetes model is a useful tool for understanding the basic mechanisms associated with the origin and modulation of induced T1D. T1D has been correlated predominantly with genetic background as well as proinflammatory cytokine profiles for TNF- α , IL-12 and IFN- γ [15, 16]. For example, C57BL/6 mice are more susceptible to developing MLDS-induced T1D than mice lacking STAT-4, a transcription factor that is essential for IL-12 signaling. STAT-4 deficient mice have a delayed onset of MLDS-induced T1D and show a milder form of the disease [17].

Taenia crassiceps is a cestode parasite that is useful in infection model systems for cysticercosis [18]. Infection of inbred mice with *T. crassiceps* induces a strong Th2-like immune response that is similar to the response elicited by infection with other helminths [19]. In addition to this Th2-like response, *T. crassiceps* infection is associated with a series of immunomodulatory events including the induction of AAM ϕ [20] and the inhibition of T cell proliferative responses to bystander and polyclonal stimuli [2]. We and others have found that infection with *T. crassiceps* alters the immune response to and susceptibility to concomitant pathogens such as *Trypanosoma cruzi* [21], vaccinia virus [22], or *Leishmania* [18], and also reduces the efficacy of vaccination [23]. Thus, it is clear that *T. crassiceps* infection is able to modify immune responses to concomitant pathogens.

The purpose of this study is to determine whether the immune modulation that is induced by *T. crassiceps* infection might affect the outcome of Multiple Low Dose Streptozotocin-Induced Diabetes (MLDS). To address this question, we compared the course of MLDS development in both healthy and *T. crassiceps*-infected mice. Our data suggest that *T. crassiceps* infection might modify the incidence and development of MLDS-induced autoimmune diabetes.

2. Materials and Methods

2.1. Mice. Six- to eight-week-old male BALB/cAnN mice and C57BL/6NHsd mice were purchased from Harlan Laboratories (México) and were maintained in a pathogen-free environment at the FES-Iztacala, U.N.A.M. animal facility in accordance with institutional and national guidelines.

2.2. Parasites and Infection Protocols. Metacystodes of *Taenia crassiceps* (ORF) were harvested in sterile conditions from the peritoneal cavity of female BALB/c mice after 2–4 months of infection. The cysticerci were washed four times in phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M

sodium phosphate buffer, pH 7.2) and used for mouse infection. Male BALB/c and C57BL/6 mice were infected with an intraperitoneal (i.p.) injection of 20 small, nonbudding cysticerci of *T. crassiceps* resuspended in 0.3 mL of PBS.

2.3. Multiple Low-Dose Streptozotocin-Induced Diabetes (MLDS). Mice infected with *T. crassiceps* for 6 weeks or uninfected controls received daily intraperitoneal injections of 40 mg/kg streptozotocin (Sigma-Aldrich; dissolved in 0.1 M sodium citrate, pH 4.5) for 5 consecutive days. Blood glucose was measured in animals that were fasted for 6 hours by an Accu-chek Advantage glucometer (Roche Diagnostics) once per week over a 6-week period. Untreated mice were included as controls. Animals were considered diabetic when fasting blood glucose was greater than means ± 2 SD on two consecutive tests.

2.4. Intraperitoneal Glucose Tolerance Test. Uninfected and *T. crassiceps*-infected mice were subjected to an intraperitoneal glucose tolerance test in order to establish the effects of the MLDS-induced diabetes model on glucose metabolism ($n = 6$ – 10 for each group). Uninfected and infected MLDS-treated mice were fasted for 6 hours prior to sample collection. A basal blood sample (0 min) was collected by tail-snip and plasma glucose was evaluated using an Accu-chek Advantage glucometer. Mice were then injected i.p. with filtered d-glucose (1.5 mg/kg). Glucose levels were evaluated again at 30-, 60-, and 120-minute time points.

2.5. Histology. Pancreata from C57BL/6 and BALB/c mice were collected 6 weeks after the induction of diabetes. Tissue was processed and embedded in paraffin, and 5- μ m sections were cut for histological analysis. Thin sections were stained with hematoxylin-eosin and evaluated microscopically for the presence of insulinitis using the following scoring system: grade 0, normal; grade 1, minor peri-islet cell infiltration; grade 2, moderate infiltration (<50% of islet area); grade 3, severe infiltration (>50% of islet area) with damage to islet architecture.

2.6. Immunohistochemistry. Immunoperoxidase staining was performed on 5- μ m paraffin sections using an avidin-biotin complex system, the Insulin Ab-6 (INS04 + INS05) mouse monoclonal antibody, and the commercial kit Dako EnVision + System-HRP (DAB). Sections were counterstained with hematoxylin. Images were captured using AxioVision Rel 4.6An and an AxioCam ICc3 connected to a Zeiss Microscope recorded the images.

2.7. Cytokine ELISAs. Peripheral blood was collected from tail snips once a week over a 6-week period. Serum IL-4 and TNF- α levels were measured by sandwich ELISA using commercial kits purchased from Peprotech (Rocky Hill, NJ, USA).

2.8. Isolation of Peritoneal Macrophages. BALB/c and C57BL/6 were sacrificed 6 weeks after induction diabetes. Peritoneal exudate cells (PECs) from mice STZ, STZ/Tc, and

Normal mice were obtained using 5 mL of ice-cold sterile PBS and the red blood cells were lysed by resuspending the cells in Boyle's solution. Following two washes, the viable cells were counted by trypan blue exclusion with a Neubauer hemocytometer. PECs were adjusted to 5×10^6 /mL in RPMI medium and then cultured in six-well plates (Costar, Cambridge, Mass). After 2 hours at 37°C and in 5% CO₂, nonadherent cells were removed by washing them with warm supplemented RPMI medium. Peritoneal macrophages were aseptically removed and processed for RNA extraction using the TRIzol reagent (Invitrogen, Carlsbad, CA).

2.9. Analysis of Cell Surface Markers in Macrophages. The Fc receptors on peritoneal macrophages were blocked with anti-mouse CD16/CD32 (Biolegend, CA, USA) and then stained with an APC-conjugated monoclonal antibody against F4/80 (Biolegend, CA, USA), PE-conjugated antibodies against PD-L1 and PD-L2, FITC-conjugated antibodies against CD23, or Alexafluor-conjugated antimannose receptor antibody (all obtained from Biolegend). The stained cells were analyzed on a FACsCalibur flow cytometer using Cell Quest software (Becton Dickinson).

2.10. T-Reg Cells Detection. Lymph nodes were macerated individually using frosted glass slides. The staining Treg cells were according to the manufacturer instruction (Mouse Treg Flow Kit, Biolegend).

2.11. Reverse Transcriptase-PCR. Total RNA was extracted from purified peritoneal macrophages obtained from BALB/c and C57BL/6. mRNA transcripts in peritoneal macrophages was determined by reverse transcription (RT)-PCR. The RNA was quantified and 3 mg of RNA were reverse transcribed using the Superscript II First Strand Synthesis Kit (Invitrogen) and an oligo dT primer, as recommended by the manufacturer. Once cDNA was obtained, conventional PCR was performed. The PCR reactions contained (in a 25 mL final volume) $5 \times$ PCR buffer blue, 10 mM dNTP, 40 nM each forward and reverse primer, 1 unit of Taq DNA polymerase (Sacace Biotechnologies, Italy), and 2 mL of the cDNA. The program used for the amplification of each gene was an initial denaturation at 95 °C for 5 minutes, 35 cycles of 95 °C for 40 seconds, the indicated melting temperature for 50 seconds and 72 °C for 40 seconds and a final extension step of 72 °C for 4 minutes. All reactions were carried out in a thermal cycler (Corbett Research, Australia). Finally, to observe the amplified products, a 1.5% agarose gel was prepared and samples were loaded with blue juice buffer containing SYBR Green (Invitrogen). The gels were visualized using a Fujifilm FLA 5000 scanner (Fuji, Japan) with FLA 5000 image reader V2.1 software to capture the shown images.

2.12. Statistical Analysis. One-tail Student *t*-test (glycemia, cytokine, and glucose tolerance test) was applied. $P < .05$ was considered statistically significant.

3. Results

3.1. *Taenia crassiceps* Infection Modulates Hyperglycemia and Diabetes Incidence by Multiple Low Dose Streptozotocin-Induced Diabetes. This study investigates whether *T. crassiceps* infection modifies the onset and development of MLDS-induced diabetes. Six weeks after infection with *T. crassiceps*, male BALB/c and C57BL/6 mice received MLDS (40 mg/kg) for 5 consecutive days. Blood glucose levels were analyzed each week for six weeks following the treatment with MLDS. Uninfected BALB/c mice became hyperglycemic one week post-MLDS injection (Figure 1(a)). By the second week following the induction of diabetes 100% of the uninfected mice were diabetic and remained diabetic until the end of the experimental period. In contrast, *T. crassiceps*-infected BALB/c mice displayed significantly lower blood glucose levels throughout the six-week period following MLDS injection compared to uninfected mice (Figure 1(a)). Interestingly, the onset of diabetes (as determined by glucose levels) was also different between uninfected and *T. crassiceps*-infected mice. The onset of diabetes in *T. crassiceps*-infected BALB/c mice occurred 2 weeks after MLDS induction; whereas the onset of diabetes occurred within the first week following MLDS-induction in uninfected mice. Additionally, only 40% of *T. crassiceps*-infected mice developed hyperglycemia compared to 100% in uninfected mice (Figure 1(b); $P < .05$). Interestingly, *T. crassiceps*-infected mice had normal glucose tolerance test values (Figure 1(c)). In contrast, uninfected mice could not down-modulate the hyperglycemia until 2 hours after the glucose tolerance test was administered.

A different trend was observed in C57BL/6 mice. C57BL/6 mice are known to be more susceptible to MLDS-induced diabetes [24] and are more resistant to *T. crassiceps* infection [25, 26]. C57BL/6 mice developed higher levels of hyperglycemia than BALB/c mice, but these high glucose levels were controlled after 4 weeks by *T. crassiceps* infection (Figure 2(a)). Shortly after MLDS-induction, 80% of *T. crassiceps*-infected C57BL/6 mice were diabetic (Figure 2(b)). However, by 4 weeks following MLDS-induction infected C57BL/6 mice began to recover from the hyperglycemia. Only 20% of *T. crassiceps*-infected C57BL/6 mice were diabetic at the end of this experiment. MLDS treatment caused significant elevations in glucose levels during an intraperitoneal glucose tolerance test in uninfected mice, but mice carrying *T. crassiceps* displayed normal glucose tolerance test values (Figure 2(c)).

3.2. Lack of Insulinitis in *T. crassiceps* Infected Mice Treated with MLDS. It is known that MLDS stimulates the recruitment of leukocytes to pancreatic islets, resulting in insulinitis [15]. Pancreata were harvested at the end of the 6-week experimental period for histopathological analysis. Figure 3 illustrates islet histopathology and the development of insulinitis in uninfected and *T. crassiceps*-infected BALB/c and C57BL/6 mice. A significant number of infiltrating leukocytes and the presence of insulinitis was observed in the pancreata from uninfected mice (Figures 3(a) and 3(b)), which was associated with loss of islet architecture in some cases. Conversely,

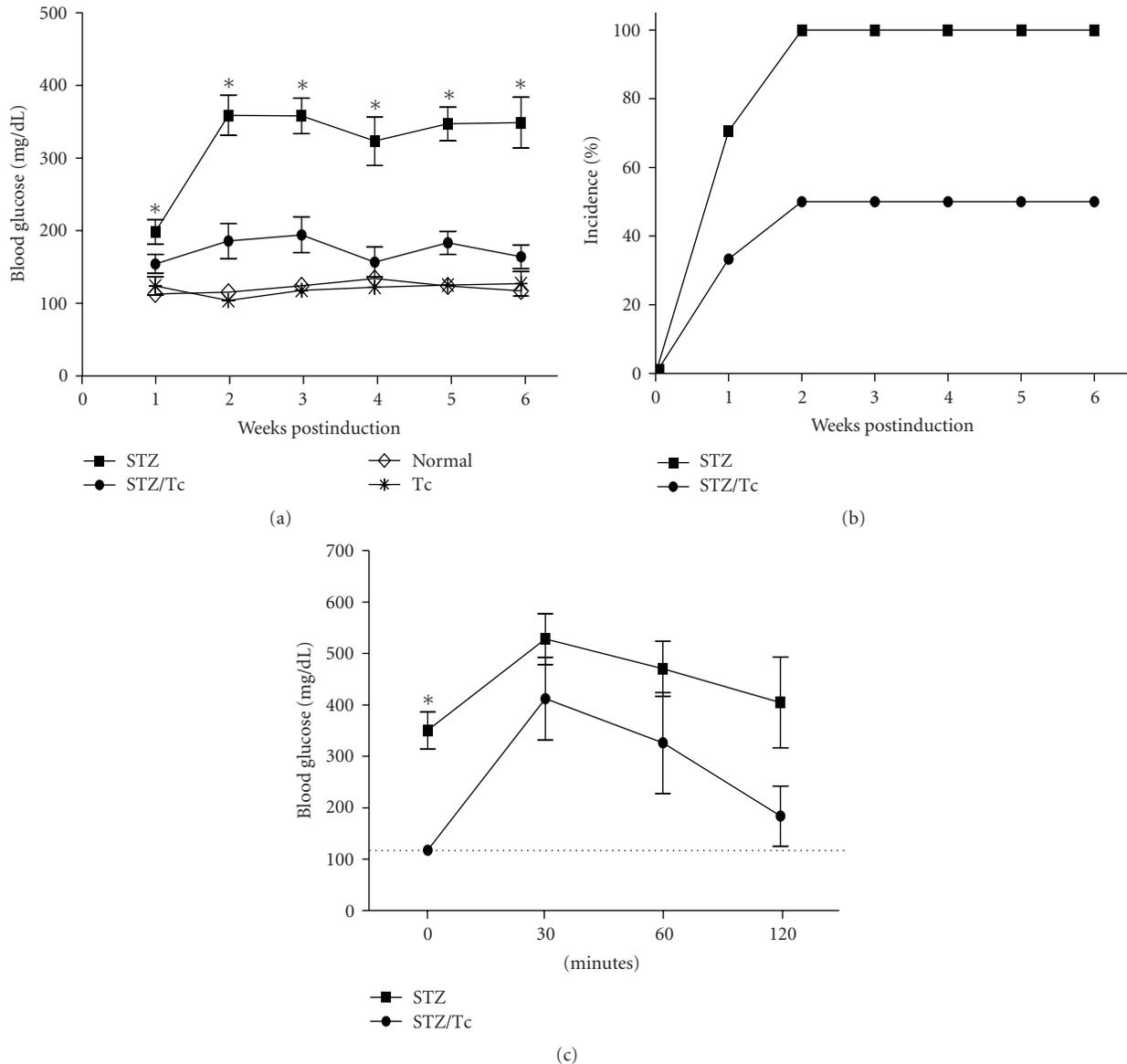


FIGURE 1: Hyperglycemia and diabetes incidence is modified by *T. crassiceps* infection in BALB/c mice. (a) Blood glucose levels throughout the MLDS protocol for uninfected BALB/c mice and BALB/c mice infected with *T. crassiceps* for 6 weeks. (b) Percent incidence of diabetes between uninfected and *T. crassiceps*-infected BALB/c mice. (c) Intraperitoneal glucose tolerance test for MLDS-treated uninfected and *T. crassiceps*-infected mice. Dotted line indicates normal glucose values. * $P < .05$, $n = 12$.

the islet histopathology from *T. crassiceps*-infected mice was devoid of both cellular infiltrates and insulinitis (Figures 3(a) and 3(b)). Similar results were observed in the pancreata from BALB/c and C57BL/6 mice.

3.3. Immunohistochemistry of Insulin in MLDS-Treated Mice. To determine whether islet cells from either uninfected or *T. crassiceps*-infected mice are able to produce insulin, we performed specific immunostaining of pancreatic tissue using an anti-insulin antibody. MLDS-treated *T. crassiceps* infected mice demonstrated strong insulin staining in their pancreatic islets as compared with normal (untreated and uninfected) mice (Figure 4(a)). In contrast, MLDS-treated uninfected

mice had weak insulin staining, suggesting a substantial loss of insulin granules in islet β -cells. A similar result was obtained from C57BL/6 mouse tissues (Figure 4(b)).

3.4. *T. crassiceps* Infection Increases IL-4 Levels in MLDS-Treated Mice. To analyze whether the inhibition of diabetes in helminth-infected MLDS-treated mice was due to an alteration in the Th1/Th2 balance, cytokine levels from the sera of uninfected and infected mice were measured. The Th1-associated cytokines IFN- γ and TNF- α have been reported to accompany the development of diabetes in both NOD and MLDS-induced mouse models [14, 27]. While BALB/c mice did not show significant changes in TNF- α levels in response

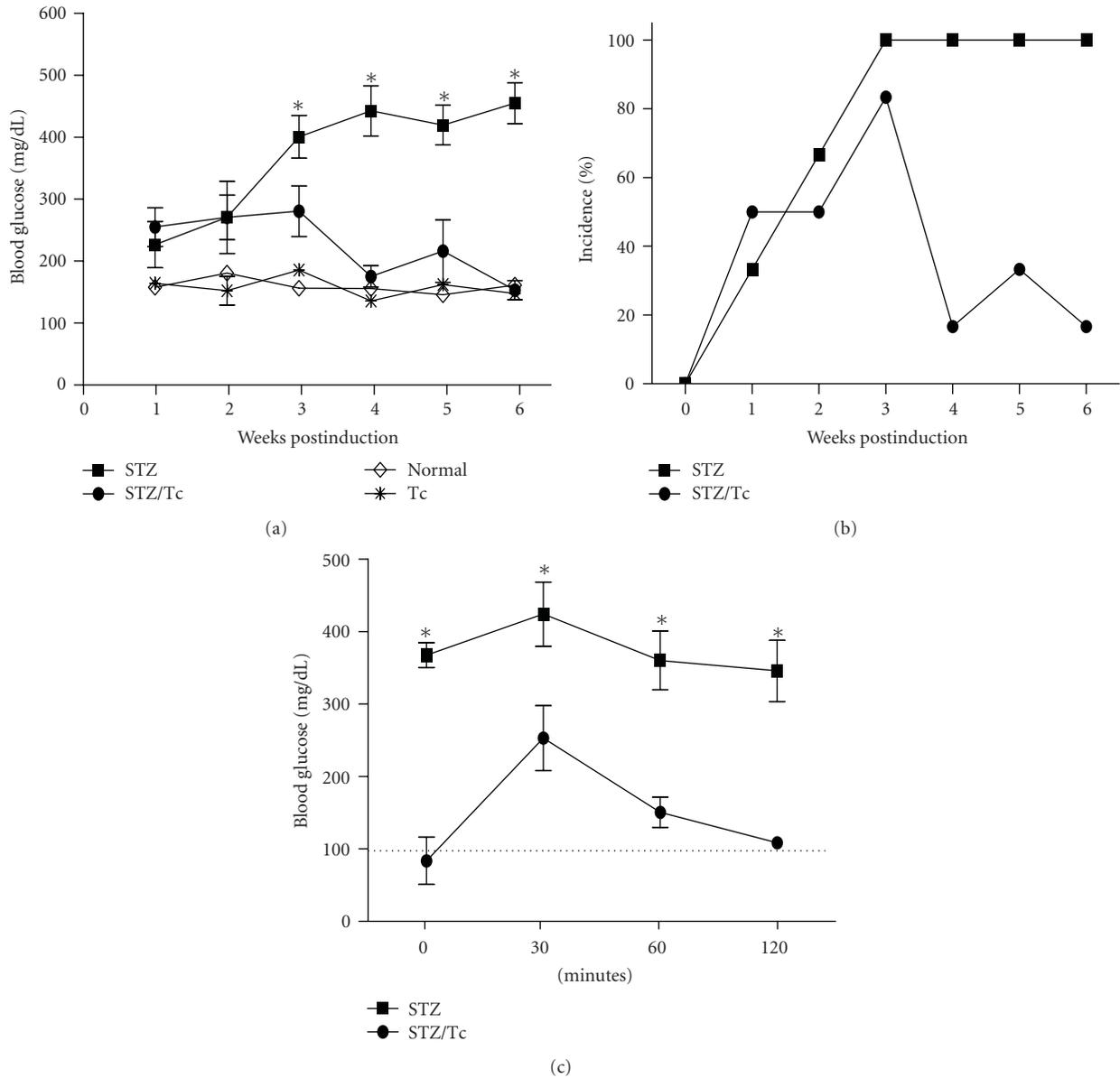


FIGURE 2: Hyperglycemia and diabetes incidence is modified by *T. crassiceps* infection in C57BL/6 mice. (a) Blood glucose levels throughout the MLDS protocol for uninfected C57BL/6 mice and mice infected with *T. crassiceps* for 6-weeks. (b) Percent incidence of diabetes between uninfected and *T. crassiceps*-infected C57BL/6 mice. (c) Intraperitoneal glucose tolerance test for MLDS-treated uninfected and *T. crassiceps*-infected mice. * $P < .05$, $n = 8$.

to MLDS treatment (Figure 5(a)), uninfected C57BL/6 mice had high levels of TNF- α and IFN- γ in their sera (Figure 5(c), and data not shown). Interestingly, C57BL/6 mice that were previously infected with *T. crassiceps* and then treated with MLDS displayed lower sera TNF- α levels but maintained elevated levels of IFN- γ , as compared to uninfected mice (data not shown). We also evaluated the effect of *T. crassiceps* infection on the presence of the Th2-associated cytokine IL-4. At some experimental time points, IL-4 levels were significantly enhanced in both *T. crassiceps*-infected BALB/c and C57BL/6 mice that received MLDS whereas uninfected mice with T1D had lower levels of serum IL-4 (Figures 5(c) and 5(d)).

3.5. Presence of AAM ϕ , but Not Tregs, in MLDS-Treated *T. crassiceps*-Infected Mice. In the last few years, new classes of regulatory cells that are induced by helminth infections have been identified. Tregs and AAM ϕ have been implicated in the control of immune pathology associated with helminth infection, but both cell types have also been associated with suppression of the immune response [28, 29]. In order to determine whether these cells play a role in *T. crassiceps*-mediated diabetes prevention, we performed FACS analysis of peritoneal cells and T cells from the mesenteric lymph node. Following MLDS treatment, neither uninfected nor *T.*

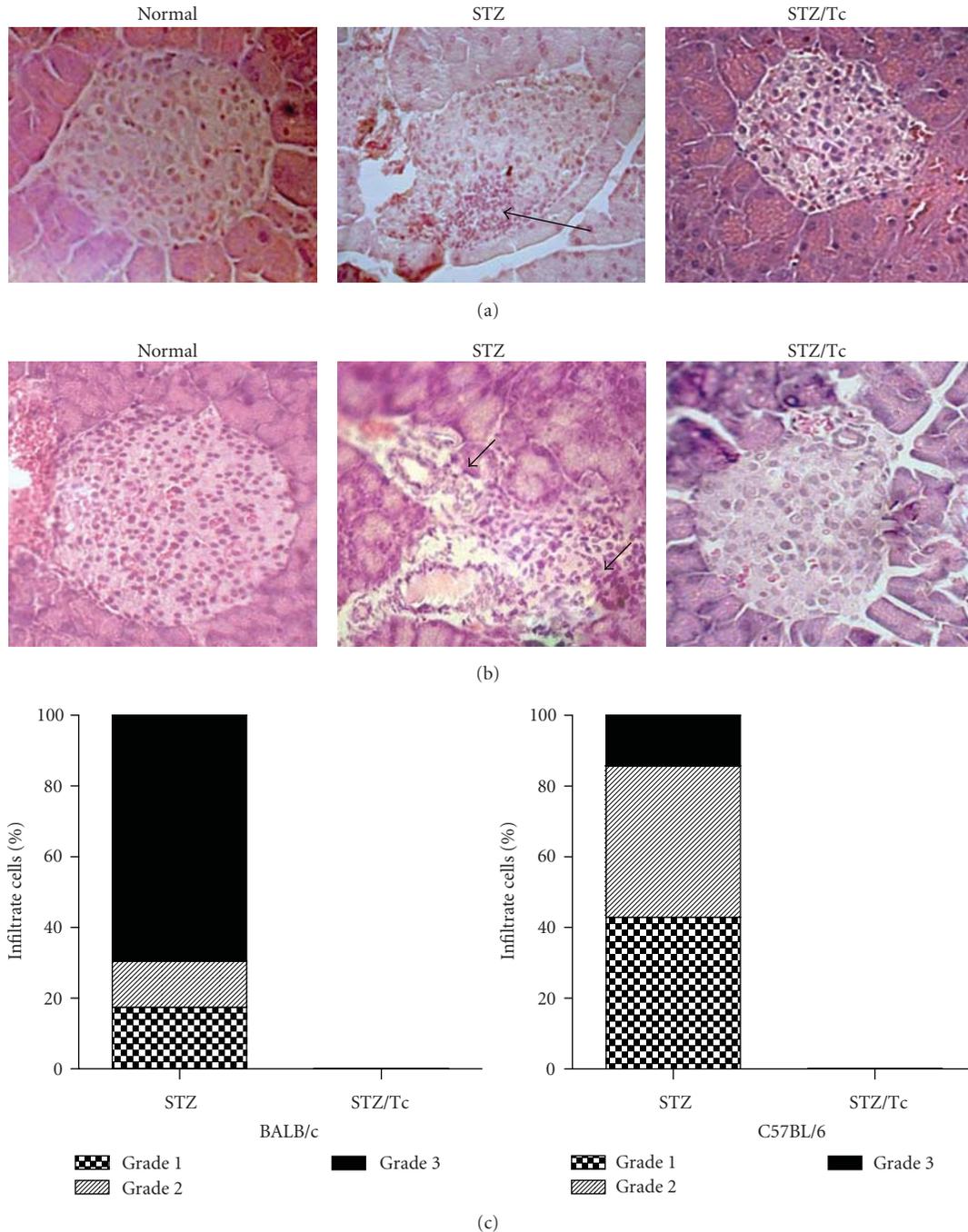


FIGURE 3: MLDS-mediated insulinitis in BALB/c mice. All mice received MLDS for 5 consecutive days and were sacrificed 6 weeks later to harvest tissues for histopathology. (a) H&E stained BALB/c mouse pancreas sections: normal islet, STZ (uninfected and MLDS-treated), STZ/Tc (*T. crassiceps*-infected and MLDS-treated). (b) H&E stained C57BL/6 mouse pancreas sections. (c) Score of cell infiltrates in BALB/c and C57BL/6 islets. Note the lack of leukocyte infiltrates in *T. crassiceps*-infected mice. Arrows indicate infiltration. Magnification $\times 400$.

crassiceps-infected mice displayed an increase in the population of Treg in the mesenteric lymph node (Figure 6(a)). Interestingly, *T. crassiceps*-infected mice displayed an increase in the percentage of AAM ϕ in the peritoneum, as determined by the expression of the mannose receptor, CD23, PDL1, and

MHCII (Figure 6(b)). To confirm the presence of AAM ϕ , RT-PCR was performed to detect mRNA transcripts for Fizz-1 and PDL-1. These transcripts were present in peritoneal cells from *T. crassiceps*-infected mice treated with MLDS, but not in uninfected, MLDS-treated mice (Figure 6(c)).

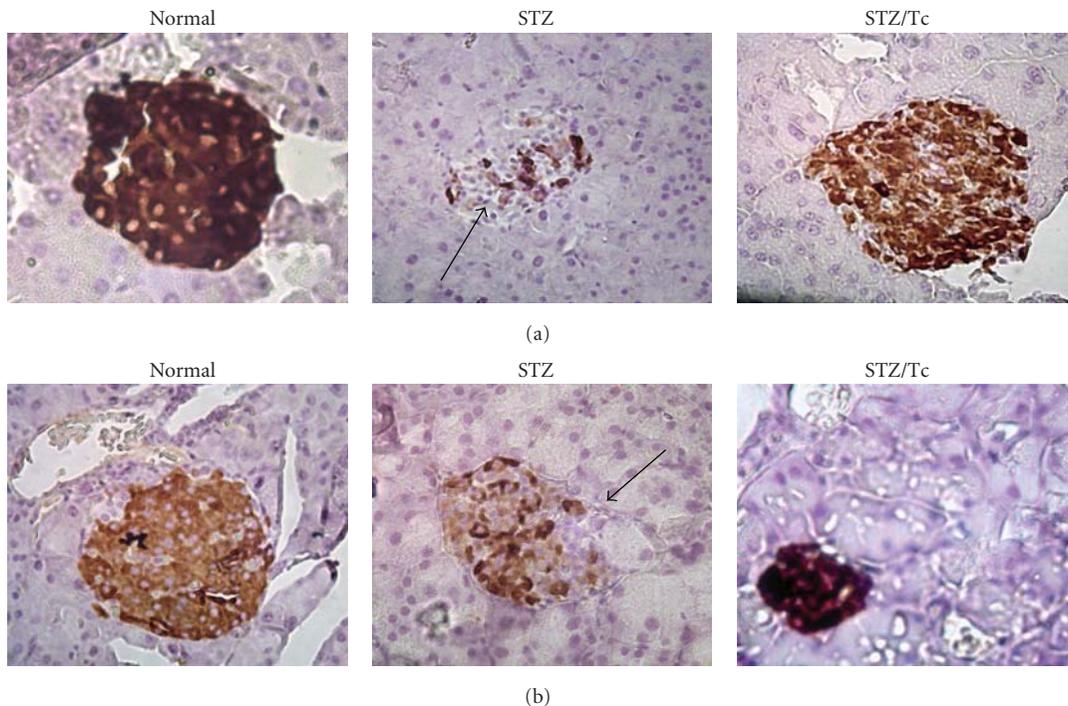


FIGURE 4: Insulin immunostaining of pancreatic islet cell from MLDS-treated mice. (a) Immunostaining of insulin in islets from BALB/c mice, insulin immunostaining in pancreatic islets of different uninfected (normal), uninfected and MLDS-treated mice (STZ), or *T. crassiceps*-infected MLDS-treated mice (STZ/Tc). Mice ($n = 4$) were sacrificed 6 weeks after the initial injection of MLDS and pancreata were processed for immunohistochemistry to specifically detect insulin. (b) Same as (a), just the sections belong to C57BL/6 mice. Note the lack of insulin staining in uninfected MLDS-treated mice. Magnification $\times 400$.

4. Discussion

T. crassiceps infection and its associated antigens can induce Th2-type responses in vivo [20] and can modulate the immune response to bystander antigens or live infections [2]. *T. crassiceps*, like other helminth parasites, has the ability to manipulate and down-modulate the immune responses of its hosts [10, 18]. It is widely accepted that the initiation and development of T1D is mainly caused by an autoimmune cell-mediated destruction of β cells in the pancreas [15]. Genetic, immunological, and environmental factors can also influence the onset and development of T1D [30]. In this study, we found that *T. crassiceps* infection could alter the development of MLDS-induced diabetes in mice. This study demonstrates that *T. crassiceps*-infected mice develop a mild, and sometimes transient, form of T1D after MLDS treatment. In contrast, uninfected mice exhibited an accelerated, more severe form of T1D.

There are likely several factors that contribute to the protection by *T. crassiceps* infection against MLDS-induced diabetes. First, the prominent Th2 environment that is induced by *T. crassiceps* infection [19, 31] might counteract the proinflammatory responses that are necessary to generate complete MLDS-induced diabetes. Second, *T. crassiceps* infection might alter T cell recruitment to the pancreas. Third, *T. crassiceps* infection might induce a regulatory cell response that dampens inflammatory processes during MLDS-induced diabetes.

The findings of this study demonstrate that infection with *T. crassiceps* maintains high levels of IL-4 and that there is a slight reduction in serum TNF- α levels after infected mice are treated with MLDS. TNF- α has been implicated as a critical player in leukocyte-mediated islet damage [32]. Thus, this type of immune regulation might be responsible for the decrease in pathology and lower incidence of T1D observed in *T. crassiceps*-infected mice. Consistent with a putative protective role for Th2 type cytokines (IL-4), NOD mice that express IL-4 in their pancreatic β cells are protected from insulinitis and autoimmune diabetes [33]. Other experimental models have demonstrated that helminth exposure (*Schistosoma mansoni*, *Trichinella spiralis*, *Hymenolepis diminuta* or *Heligmosomoides polygyrus*) to mice might drastically reduce the symptoms of autoimmune diseases such as Inflammatory Bowel Disease (IBD), Encephalomyelitis Autoimmune Experimental, and T1D, mainly by reducing IL-12 and IFN- γ production and enhancing IL-4 and IL-10 levels [34–45]. Female NOD mice that spontaneously develop diabetes due to Th1-mediated destruction of pancreatic β -cells are protected from developing diabetes when they are exposed to *S. mansoni* [34–36], and IL-4 has been shown to play an important role in this protection [46, 47]. It will be necessary to test our system in mice that either lack IL-4 or have been treated with a specific IL-4 inhibitor to fully understand the role that IL-4 plays in *T. crassiceps* protection against MLDS-induced diabetes.

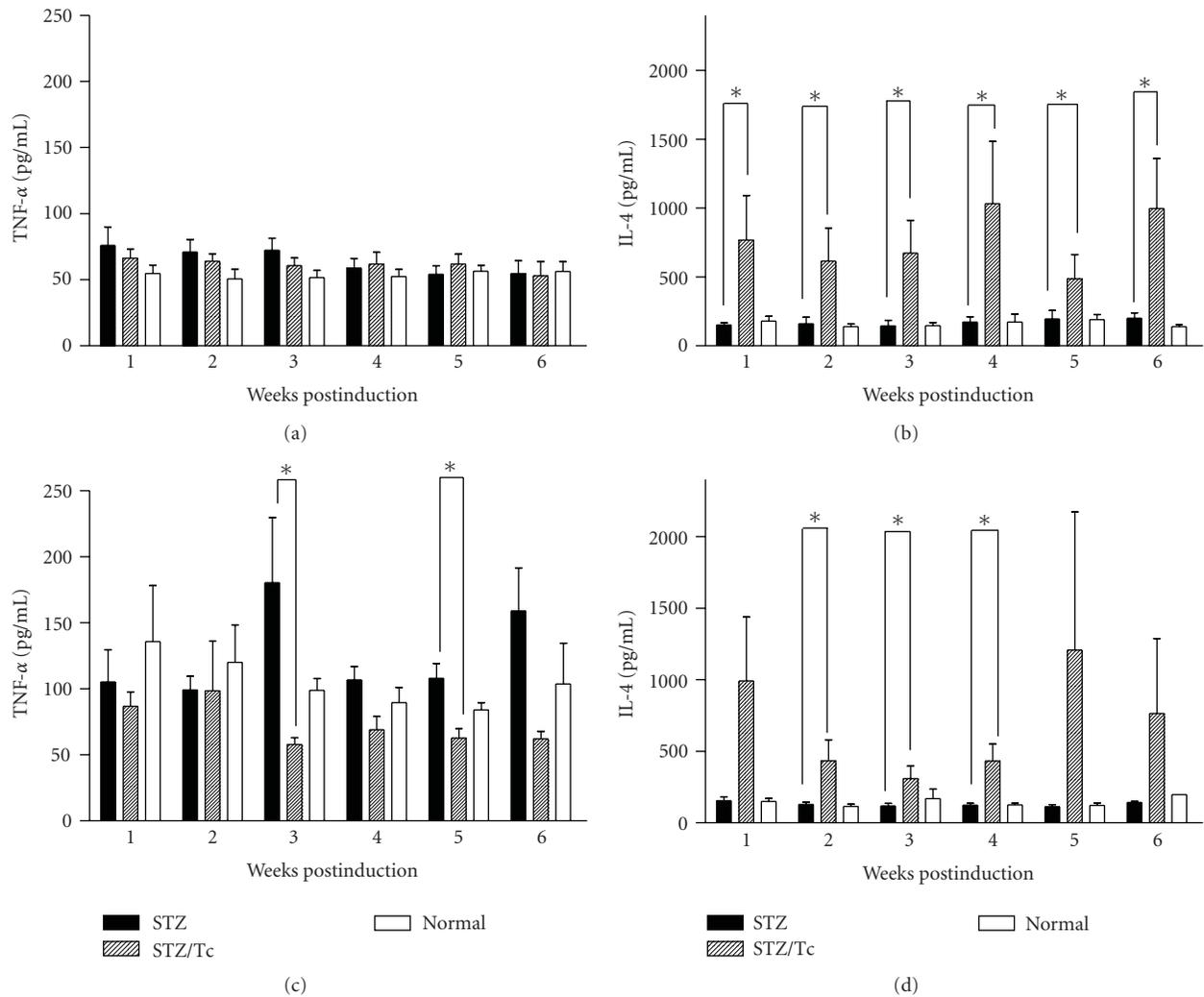


FIGURE 5: Cytokine profiles from the sera of mice treated with MLDS. BALB/c mice were bled at indicated time points and (a) TNF- α and (b) IL-4 were detected by ELISA. (c) TNF- α and (d) IL-4 for C57BL/6 mice. * $P < .05$, $n = 10$.

We found that *T. crassiceps* infection resulted in an increase in the population of F4/80⁺ macrophages that express CD23, PDL1, MR, Arg1, Ym1, but not iNOS, an expression profile that is now used to identify AAM ϕ . AAM ϕ might function as bystander suppressors of the immune response. Alternatively, they might inhibit cell infiltration in the pancreas, given their established suppressive activity, which is mediated through cell-contact, where PD-L1 and PD-L2 play a preponderant role, or by releasing soluble factors [20]. In contrast, Treg cells were not detected in *T. crassiceps*-infected mice. Mice that were infected with *T. crassiceps* were protected from both hyperglycemia and lymphocyte infiltration into the pancreatic islet despite the absence of regulatory T cells. This is interesting given the recent findings that demonstrate the importance of Foxp3⁺ regulatory T cell expansion in mice infected with helminths [4–8]. Tregs induced by *S. mansoni* and its associated antigens have been implicated in diabetes prevention in NOD mice [36]. This discrepancy might be explained by

differences in the classes of helminths used in these infection models, in the route of infection used, or by different suppressive mechanisms that are turned on by *T. crassiceps* versus *S. mansoni* [11, 19]. Only one published report demonstrates that macrophages are involved in preventing the pathology associated with IBD model [48]. Therefore, it is possible that AAM ϕ might actively participate in dampening the pathology associated with MLDS-induced diabetes, perhaps by the known ability of AAM ϕ to strongly suppress T cell responses.

Interestingly, MLDS-induced diabetes is associated with Th1-type responses [17] and C57BL/6 mice show a Th1-type response in the MLDS model [24]. We also show that *T. crassiceps*-infected C57BL/6 mice demonstrated a reversal of MLDS-induced diabetes even though they were more hyperglycemic at earlier time points following MLDS-treatment. Again, the specific regulatory cells and/or cytokines involved in these protective effects still need to be identified, however, according to our data, appears that AAM ϕ it may have an

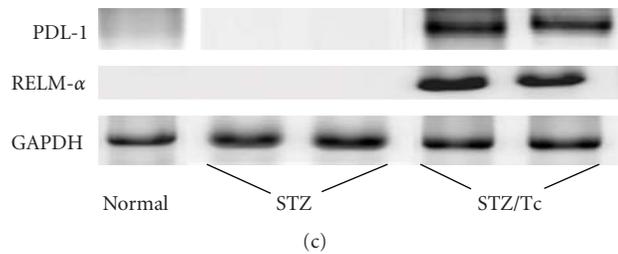
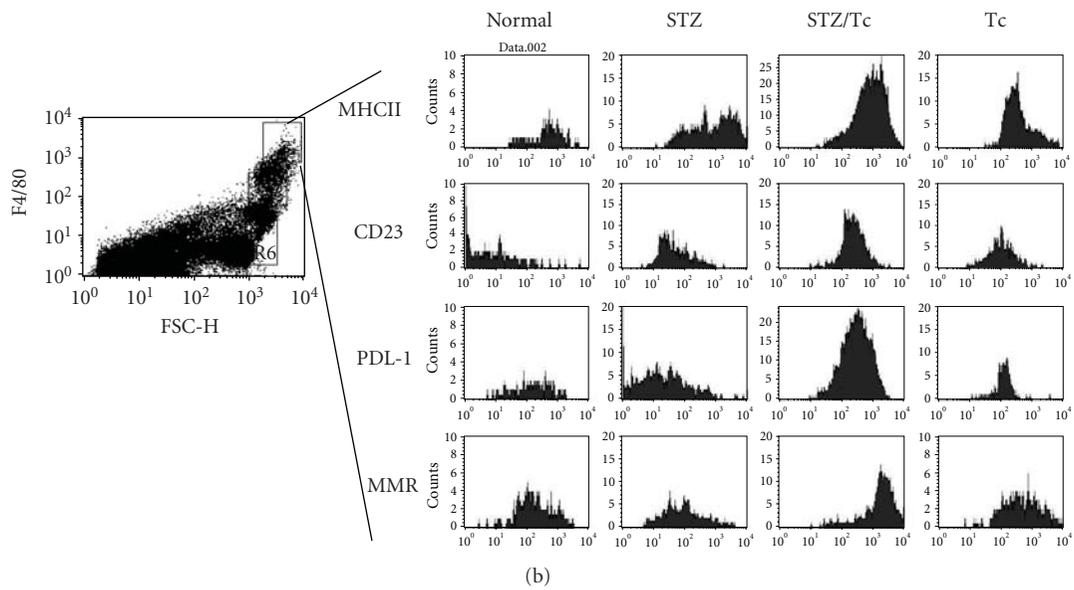
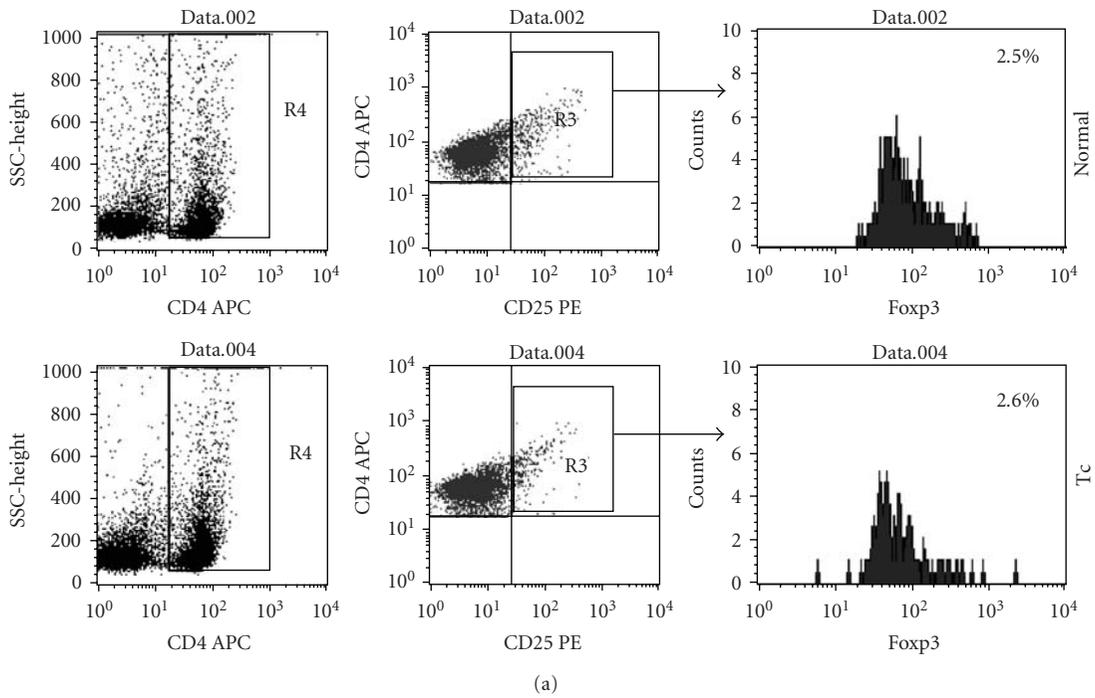


FIGURE 6: Flow cytometry analysis for the detection of regulatory T cells and alternatively activated macrophages. (a) Mesenteric lymph nodes from BALB/c mice were processed and cells were staining for Treg cell detection with anti-CD4, CD25, and FoXP3 (Tregs kit, Biolegend). (b) Peritoneal exudates cells stained with conjugated anti-F480, CD23, MR, PDL1, and MHCII and analyzed by flow cytometry. (c) RT-PCR analysis of PDL-1, RELM- α , and GAPDH in macrophages from uninfected and *T.crassiceps*-infected BALB/c mice.

important role instead T-regulatory cells that we could not detect in *T. crassiceps*-infected mice.

5. Conclusion

Our data support the notion that the protection from autoimmunity by helminth infection could be attributed to immunoregulatory mechanisms triggered by these parasites. Our study demonstrates for the first time that *T. crassiceps* infection protects against Multiple Low Dose Streptozotocin-Induced Diabetes, independently of the genetic background of the host. Furthermore, we present a possible protective role for AAM ϕ since we did not detect enhanced Treg cells. The analysis of *Taenia*-released products and repeating our experiments under conditions of macrophage depletion will be necessary to fully understand the mechanisms involved in this observed protection.

Acknowledgments

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Research Article

Immune Modulation by *Schistosoma mansoni* Antigens in NOD Mice: Effects on Both Innate and Adaptive Immune Systems

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We have shown that *Schistosoma mansoni* egg soluble antigen (SEA) prevents diabetes in the nonobese diabetic (NOD) mouse inducing functional changes in antigen presenting cells (APCs) and expanding T helper (Th) 2 and regulatory T cell (Treg) responses. A Th2 response to *S. mansoni* infection or its antigens is key to both the establishment of tolerance and successfully reproduction in the host. More recently we demonstrated that SEA treatment upregulates bioactive TGF β on T cells with consequent expansion of Foxp3⁺ Tregs, and these cells might be important in SEA-mediated diabetes prevention together with Th2 cells. In this study we profile further the phenotypic changes that SEA induces on APCs, with particular attention to cytokine expression and markers of macrophage alternative activation. Our studies suggest that TGF β from T cells is important not just for Treg expansion but also for the successful Th2 response to SEA, and therefore, for diabetes prevention in the NOD mouse.

1. Introduction

Helminths employ a range of immunomodulatory strategies to modulate the host immune response and utilize it to extend their longevity in the host and facilitate transmission. Characteristic of infection with helminths such as *Schistosoma mansoni* is the induction of a Th2 response. Two products released from eggs have recently been shown to play important roles in the immune deviation induced by *S. mansoni* infection [1, 2]. The NOD mouse provides a good animal model of the human autoimmune disease, Type 1 diabetes [3]. We have previously shown that *S. mansoni* infection or exposure either to the helminth eggs or soluble extracts of worms (SWA) or eggs (SEA) can prevent diabetes onset in this mouse [4–6].

As Type 1 diabetes is a Th1-mediated autoimmune disease [7], any skewing of the response towards Th2 would result in diabetes prevention. Other responses that have the potential to impact on diabetes would be the induction of immunoregulatory cytokines such as IL-10 and TGF β and regulatory T cells (Tregs) [8, 9]. We and others have shown that both SEA and SWA have profound effects on cells of the

innate immune system including dendritic cells (DCs) and NKT cells [5, 10, 11]. In the case of bone marrow derived DCs, it has been shown that exposure to *S. mansoni* antigens results in DC retention of a more immature phenotype while inducing them to mediate a Th2 response in vivo [5, 10]. Immature DCs have been associated with ability to induce a Th2 response as well as immune tolerance through induction of Tregs [12]. Although there was little evidence of phenotypic change in SEA exposed bone marrow derived murine DCs, recent studies showed increased expression of mannose receptor (MR), DEC-205, and DC-SIGN as well as induction of IL-10 [6]. SEA has, furthermore, been shown to induce Foxp3 expressing Tregs in a TGF β dependent manner [6]. Most studies in mice have been carried out using bone marrow derived APCs and it could be argued that it would be more relevant to examine the effect of *S. mansoni* antigens on isolated splenic DCs as well as in vivo on macrophage (M Φ) populations. In this manuscript we have addressed in detail the effect of SEA on functional and phenotypic changes in DCs and M Φ s and the impact that this might have on the development of different T cell subpopulations including Tregs.

2. Materials and Methods

2.1. Mice. Female NOD/Tac mice were housed and barrier bred in the Pathology Department, University of Cambridge animal facilities (Cambridge, UK) and used between 4–12 weeks of age. All work was conducted under UK Home Office project license regulations after approval by the Ethical Review Committee of the University of Cambridge.

2.2. Preparation of SEA and SWA. Preparation of SEA and SWA was described previously [13]. Briefly, for SEA, eggs were harvested from the livers of outbred infected mice, which were treated to prevent granuloma formation. Livers were homogenized through sieves and the eggs were collected, washed, and sonicated in PBS on ice, prior to centrifugation to separate the saline soluble fraction. SWA was prepared from adult worms recovered from the portal venous vasculature. Both antigens were sterile filtered and endotoxin removed to below <1 EU/mg using Polymixin B agarose beads (Sigma).

2.3. Cell Culture and Reagents. Cells were cultured in Iscove's modified Dulbecco's medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, 50 μ M 2-mercaptoethanol (All Sigma), and 5%–10% fetal calf serum (FCS) (Gibco). Anti-CD3 (α CD3) (2C11) and α CD28 (37.51) were obtained from BD Pharmingen. LPS from *Salmonella enterica* (Sigma) and the TLR1/2 agonist Pam₃CysK₄ (Invivogen) were used to stimulate DCs. Anti-TGF β (1D11.16) was precipitated from hybridoma supernatants and dialyzed against PBS before use.

2.4. TGF β Bioassay. The MLE/PAI cell line is derived from mink lung epithelial cells and contains firefly luciferase under PAI-1 promoter control. MLE cells were cultured with 50 μ L of tissue culture supernatants or a double-dilution standard curve derived from recombinant human TGF β 1 (R&D Systems) for 18 hours. Cells were then lysed and luciferase activity was measured using Promega's Luciferase Reporter System and a BetaLux scintillation counter (Wallac).

2.5. Flow Cytometry. Cells were stained with appropriate combinations of the following antibodies:

CD4 (RM4-5)-PCP-Cy5.5, CD25 (PC61)-PE, CD44 (IM7)-FITC, CD11b (M1/70)-PCP-Cy5.5, CD11c (HL3)-FITC or APC, galectin-3 (M3/38)-Alexa647, Foxp3 (FJK-16s)-Alexa647, CD205/DEC-205 (NLDC-145)-FITC, CD206/MR (MR5D3)-FITC, PD-L1 (M1H5)-PE, CD1d (1B1)-FITC, SIGN-R1 (22D1)-Alexa647, IL-4 (11B11)-PE, IL-17 (TC11-18H10)-PE, IL-10 (JES5-16A3)-APC, IL-13 (eBio13A)-Alexa647, and IFN γ (XMG1.2)-PE-Cy7. Appropriate isotype control antibodies were from the same manufacturer as for the specific antibody. Biotinylated polyclonal rat anti-mouse galectin-1 (R&D Systems) was used in conjunction with streptavidin-PE (BD Biosciences). Intracellular Foxp3 staining was according to the manufacturer's instructions (anti-mouse/rat Foxp3 staining set; eBioscience). For intracellular cytokine staining, cells were stimulated for 5 hours

with 0.5 μ g/mL PdBU, and ionomycin and cytokine secretion was blocked using 1 μ g/mL brefeldin A (all Sigma). Cells were fixed promptly in 1% paraformaldehyde PBS and permeabilized with 0.5% saponin (Sigma) in staining buffer. For live cell discrimination, 7-aminoactinomycin D (BD Biosciences) was used. For all staining, Fc receptor ligation was blocked using anti-Fc γ R 2.4G2 supernatant grown in house. Cells were acquired using a BD FACScalibur, a BD FACScan (BD Biosciences), or a CyAn-ADP (Beckman Coulter) and analyzed using FlowJo (TreeStar) software.

2.6. Cell Purification. Naïve T cells (>98% pure) were sorted from splenic cell suspensions on the basis of CD4⁺CD44^{low}CD25⁻ using a MoFlo (Beckman Coulter). Splenic dendritic cells (>90% pure) were purified by immunomagnetic selection on an autoMACS Pro (Miltenyi Biotec). Briefly, spleens were digested using Liberase CI (Roche) and passed through a 70 μ m strainer to obtain a single-cell suspension. Nonspecific binding was blocked using 2.4G2 anti-Fc γ R supernatant, and DCs were positively selected using pan-DC microbeads (Miltenyi Biotec). For T cell polarization, immature DCs were differentiated from bone marrow precursors using GM-CSF as previously described [5]. Peritoneal exudates cells, PECs (~50% CD11b^{hi} M Φ s), were flushed from the peritoneal cavity by lavage with cold PBS. Pancreatic and hepatic leukocytes populations were isolated from collagenase digested samples by centrifugation through a 33% Percoll (GE Healthcare) gradient as previously described (EJI).

2.7. RNA Isolation and Real-Time RT-PCR. Total RNA was extracted using an RNeasy Mini kit, converted to cDNA using a Reverse Transcription kit, and quantified in real time using SYBR green fast PCR (all Qiagen). cDNA was analyzed in duplicate reactions with amplification of target gene and housekeeping gene (hypoxanthine phosphoribosyl transferase 1, *Hprt1*) transcripts performed on the same reaction plate on a 7500 fast real-time PCR system (Applied Biosystems). Proprietary QuantiTect Primer Assays for all genes were purchased from Qiagen. Data are presented after normalization to the threshold cycle (CT) for *Hprt1*, either as $CT^{Gene1} - CT^{Hprt1}$ or as $Hprt - RE = 1000 \times 2^{(CT^{Gene1} - CT^{Hprt1})}$.

2.8. Statistics. Statistical analyses were performed using GraphPad Prism 4 software. Tests performed and calculated two-tailed *P* values are indicated in the individual figure legends. For nonparametric datasets, the Mann Whitney unpaired *t* test was employed.

3. Results

3.1. SEA Induces Functional and Phenotypic Changes on Dendritic Cells, Creating the Ideal Conditions for Th2 and Treg Cell Expansion. SEA triggers important changes in dendritic cell (DC) function, which are key in initiating modulation of the host immune system [14–19]. In the absence of additional TLR stimuli, we have shown that small consistent phenotypic changes occur when DCs are stimulated in vitro

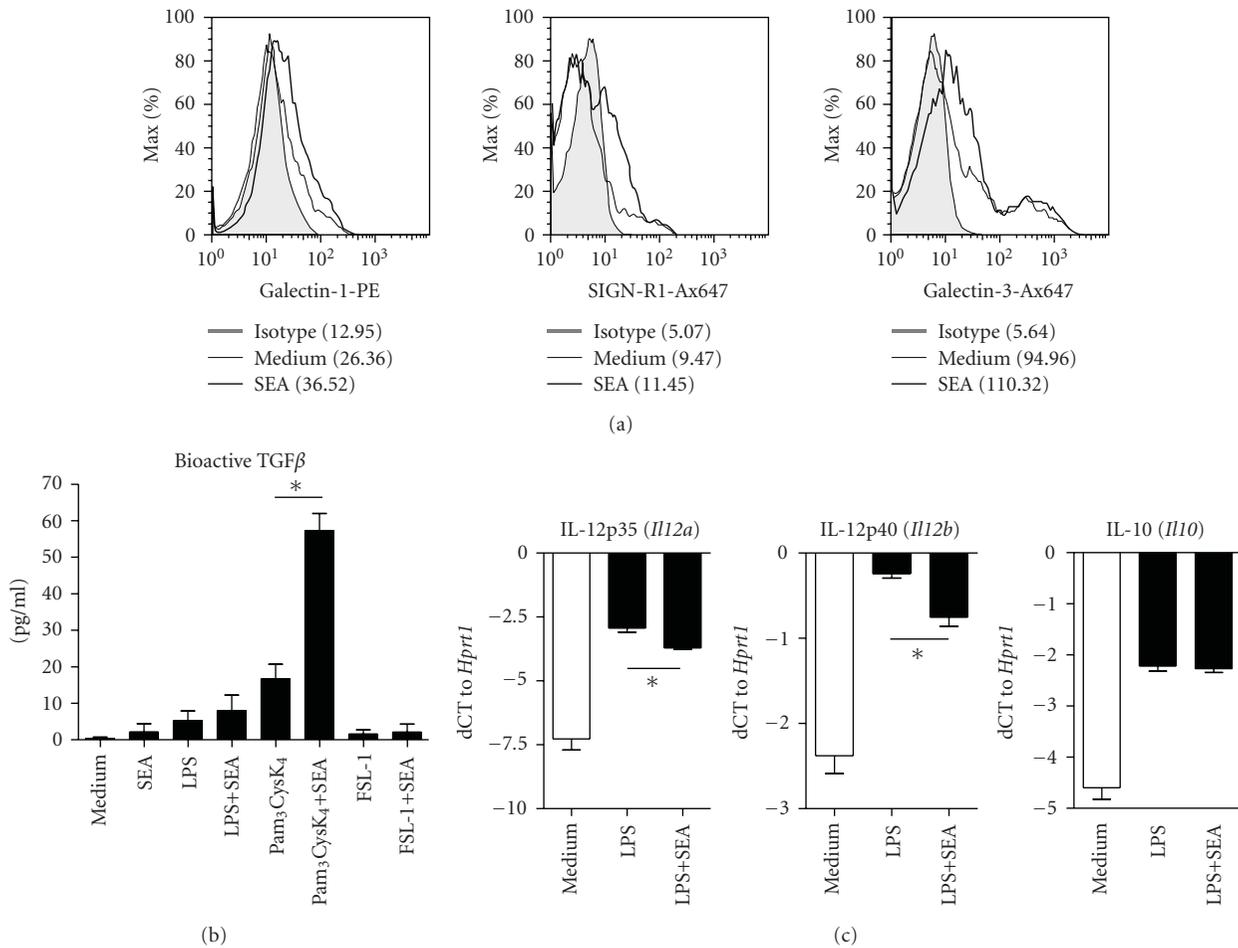


FIGURE 1: Schistosome soluble antigens change the phenotype of primary splenic DCs. (a) DCs, isolated by immunomagnetic selection, increase surface expression of CLR in response to SEA stimulation. DCs were cultured overnight with 25 $\mu\text{g/ml}$ SEA and were analyzed by flow cytometry, excluding nonviable (7-AAD⁺) cells. Data are representative of at least three independent experiments. (b) SEA enhances bioactive TGF β production by DCs stimulated with Pam₃CysK₄. DCs were isolated by immunomagnetic selection and cultured for 48 hours at 1×10^5 /well with 100 ng/ml LPS, 100 ng/ml Pam₃CysK₄, and/or 25 $\mu\text{g/ml}$ SEA. The supernatants were then cultured with a TGF β -responsive cell line and compared to a dose-response curve for recombinant human TGF β 1. Data shown are the means \pm SEM of triplicate wells. (c) SEA decreases LPS-induced IL-12p35 and p40 mRNA levels in splenic DCs. Cells (5×10^5) were stimulated for 4 hours in the presence of 100 ng/ml LPS and/or 25 $\mu\text{g/ml}$ SEA, and mRNA was analyzed in real time using qPCR. Data shown are the means \pm SEM of data from three experiments. * $P < .05$ by paired t test, two tailed.

with SEA [6]. This is particularly seen with C-type lectin receptors (CLRs). These receptors such as DEC-205, SIGN-R1, MR, and galectins 1 and 3, expressed on the surface of APCs, have been shown to recognize glycans present in schistosome antigens and are important for adaptive responses induced by the parasite [20–25]. Here we confirm that primary splenic DCs stimulated in vitro with SEA also upregulate the surface expression of galectins 1 and 3, and SIGN-R1, thus possibly increasing their ability to bind schistosome self-glycans (Figure 1(a)). Using bone marrow generated DCs, we have shown that the IL-10/IL-12 axis can be tilted by SEA [5, 6]. In Figures 1(b) and 1(c) we show that purified spleen CD11c⁺ DCs stimulated in vitro with SEA in the presence of TLR ligands upregulate the secretion of bioactive TGF β whereas IL-12 (p40 and p35) mRNA expression is downmodulated.

Interestingly, SEA in association of Pam₃CysK₄ (a TLR1/2 ligand) induces the secretion of bioactive TGF β , but this effect is not seen in combination with LPS (TLR4) or FSL-1 (TLR2/6, Figure 1(b)). No significant changes were found in IL-10 mRNA expression from purified splenic DCs (Figure 1(c)).

3.2. SEA Alternatively Activates M Φ s, which May Favor Treg and Th2 Cell Expansion In Vivo. We examined the effect of i.p. injected SEA on cellular phenotypes and function in the peritoneal cavity of NOD female mice. Examination of cytokine expression at the RNA level revealed that, while IL-4 mRNA was not detected in the control peritoneal cells (PECs), there was a substantial expression in PECs from SEA-treated mice, together with increased expression of IL-2, IL-6, IL-10, and TGF β (Figure 2(a)). Interestingly, IL-12p35

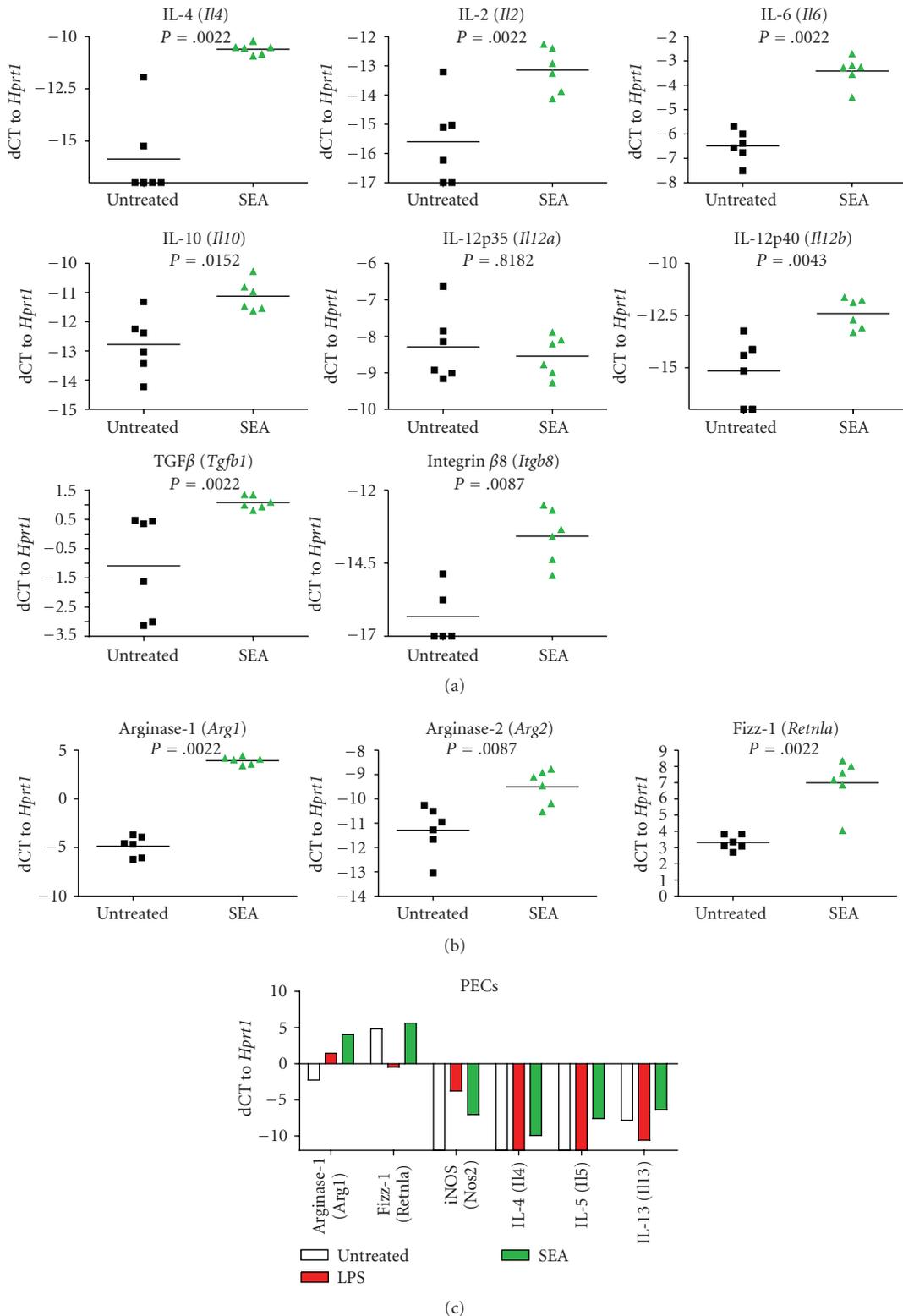


FIGURE 2: Schistosome antigens alternatively activate MΦs in vivo. (a) Increased expression of anti-inflammatory cytokines and mediators in the peritoneal cavity following SEA treatment of NOD mice are illustrated. (b) Induction of arginase and Fizz-1, markers of MΦ alternative activation in PECs is given. (c) Alternative activation in response to *S. mansoni* antigens shows a distinct pattern from that of the classical activation seen in response to LPS. Six-week-old female NOD mice were injected i.p. with 50 μ g SEA or 10 μ g LPS on day 0 and day 7, and PECs were collected for FACS and RT-qPCR analysis on day 10. Data shown are the responses of six mice per group for (a) and (b) or one mouse per condition for (c). Statistical analysis by Mann Whitney U test, two-tailed.

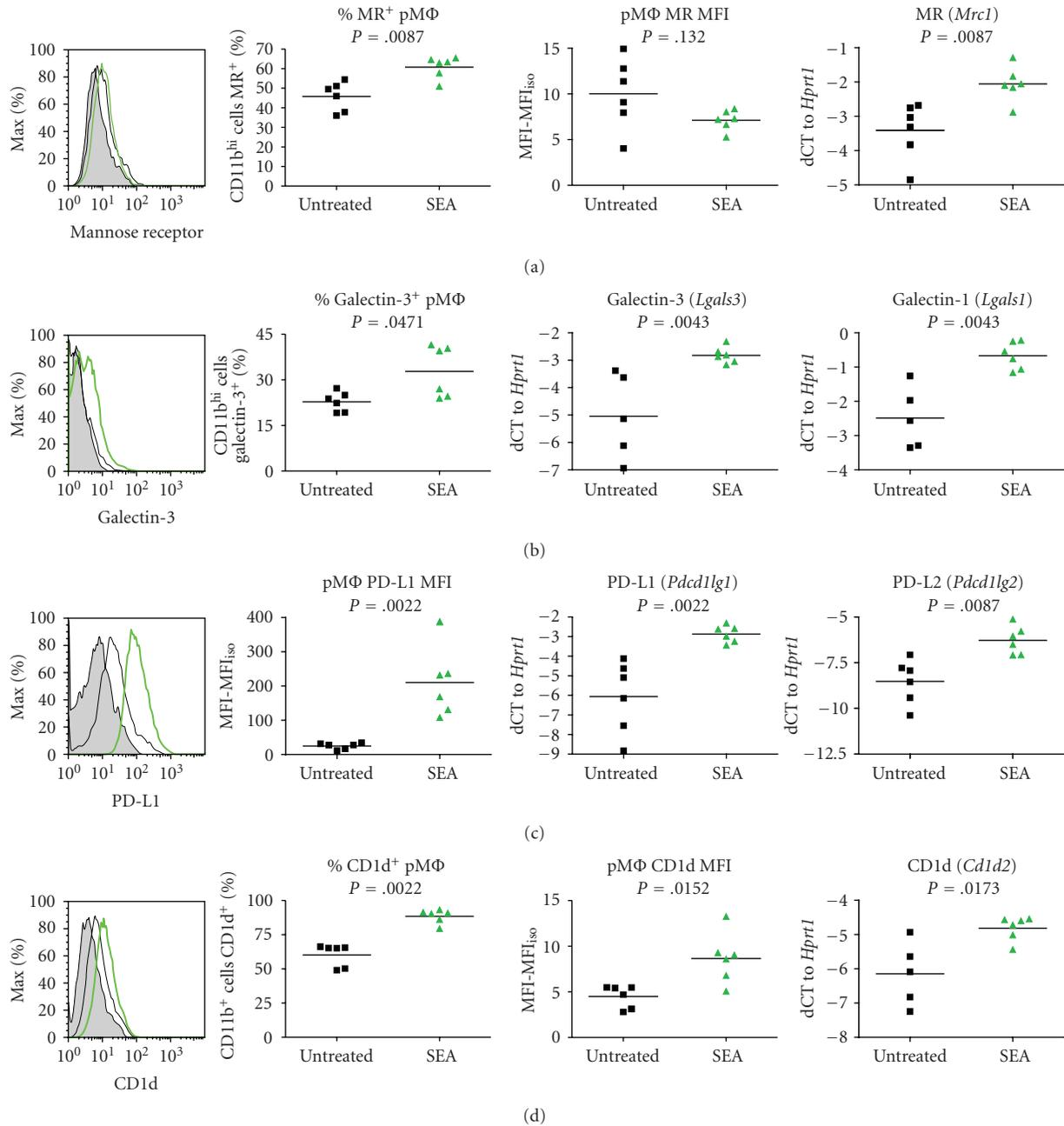


FIGURE 3: Schistosome antigens alternatively activate MΦs in vivo. CD11b⁺ peritoneal MΦs upregulate surface expression of (a) mannose receptor, (b) galectin-3, (c) PD-L1, and (d) CD1d, with accompanying increases in mRNA levels in whole PECs. Data from six mice per group treated and analyzed as in Figure 2 are shown. Statistical analysis by Mann Whitney U test, two-tailed.

expression was not affected by SEA while IL-12p40 was upregulated (Figure 2(a)). Expression of other subunits of the IL-12 family (EBI3, p28 for IL-27 and p19 for IL-23) was not affected by SEA treatment (data not shown). The significant upregulation of TGFβ suggests the presence of alternatively activated macrophages (aaMΦs) in PECs from SEA-treated mice and the increased expression of integrin β8 (Figure 2(a)) indicates an increased capacity to activate TGFβ at the point of the immunological synapse [26]. Further support for the presence of aaMΦs is the upregulated expression of arginase-1 and 2 as well as Fizz-1 (Figure 2(b)).

By comparison, treatment of NOD mice with LPS increased iNOS expression but, unlike with SEA, the full range of markers of alternative activation was not seen (Figure 2(c)).

Analysis of surface marker expression on PECs identified potentially relevant costimulatory molecules that might indicate the presence of aaMΦs as well as CLRs involved in the binding of SEA. After SEA injection there is an increased percentage of cells expressing MR on peritoneal MΦs (pMΦs) with no significant change in mean fluorescent intensity (MFI) (Figure 3(a)). This increased surface expression was mirrored by an augmented receptor mRNA

expression in whole PECs. The expression of SIGN-R1 and Dectin-1 was not altered by SEA when measured by FACS or by RT-PCR (data not shown).

Galectin-3 expression has been shown to be a feature of aaMΦs and its upregulation is IL-4 dependent [27]. Following SEA injection we find an increased percentage of galectin-3-expressing pMΦs and upregulation of both galectin 1 and 3 mRNA (Figure 3(b)). SEA increased PD-L1 expression on pMΦs, with an accompanying increase in message for PD-L1 and 2 in whole PECs (Figure 3(c)). At this time point SEA also increased the expression of MHC class II (data not shown) and the nonclassical class I molecule CD1d (Figure 3(d)). The negative costimulatory molecule programmed death ligand 1 (PD-L1) has been shown to be upregulated on aaMΦs [28].

Altogether our findings indicate that SEA alternatively activates MΦs in the peritoneal cavity and suggests that this anti-inflammatory signature might be tied to expansion of Tregs and Th2 cells.

3.3. SEA Induces IL-4, IL-10, and IFN- γ /IL-10 Double Producing T Cells In Vivo. Schistosome antigens are powerful inducers of Th2 responses, and this has been extensively explored using splenic and peripheral lymph node cells [29, 30]. In this study, we have extended our analysis to the pancreas in order to assess the potential impact of exposure to helminth antigens on diabetes and on the liver, the major site of immunopathology in schistosome infection. Kupffer cells, specialized liver MΦs, have also been shown to initiate Th2 responses to egg antigens, and we have previously observed changes in classical NKT cell populations in the liver following schistosome antigen treatment of NOD mice [5]. After a short course of treatment with SEA, the intracellular cytokine expression in CD4⁺ T cells is profoundly changed in the spleen, pancreas, and liver of prediabetic NOD mice (Figures 4(a), 4(b), and 4(c)). The systemic Th2/Treg response induced by SEA is accompanied by the signature of MΦ alternative activation in the pancreatic infiltrate of prediabetic NOD mice and is presumably beneficial for diabetes prevention (Figures 4(b) and 4(d)).

Together with the expected Th2 [5] and IL-10 Tr1 (CD4⁺ IL-10⁺) response, we have identified a population of CD4⁺ T cells expressing both IL-10 and IFN γ in response to SEA (Figures 4(b) and 4(c)). As IL-10-producing Th1 cells have been proposed as a self-regulating class of Th1 cells, the presence of this CD4⁺ T cell population in the pancreas might also mitigate Th1 driven diabetes pathology.

3.4. TGF β Is Also Important for SEA Th2-Induced Response In Vivo and In Vitro. We have previously demonstrated that TGF β is important for the generation and expansion of Foxp3⁺ Tregs in NOD mice [6]. Our preliminary experiments suggest that the Th2 response induced by SEA can also be regulated by TGF β . Figure 5(a) shows that the increased percentage of IL-4/IL-13 double-producing CD4⁺ T cells that we observe in SEA-treated NOD mice can be decreased by in vivo coadministration of SEA together with a TGF β

neutralizing antibody. This effect is particularly evident in the pancreas and not the spleen or PLN (Figure 5(a) and data not shown).

We have also confirmed our findings with an in vitro polarization assay. Using bone marrow generated DCs and naïve T cells from NOD mice, we have previously shown that SEA increases Foxp3⁺ Tregs in vitro and that TGF β neutralization reverts this effect [6]. Figure 5(b) shows that blocking TGF β in vitro reduces the generation of IL-10 and IL-4 single- and double-producing CD4⁺ T cells. Also in vitro we observe that SEA can induce the generation of IL-10/IFN γ double-expressing CD4⁺ T cells and that TGF β regulates this population. These preliminary data suggest that the role of TGF β in adaptive responses to schistosome antigens is quite complex and is not restricted to the generation/expansion of Foxp3⁺ Tregs.

3.5. Egg But Not Worm *S. mansoni* Soluble Antigens Induce Foxp3⁺ Tregs In Vivo and In Vitro. We have recently suggested that Foxp3⁺ Tregs have an important role to play in SEA immunomodulation and prevention of autoimmunity in the NOD mouse [6]. We found that in vitro only SEA, and not SWA, was able to induce Foxp3 in naïve T cells from NOD mice. To examine the effects of these helminth antigens on Tregs in vivo, we injected SEA and SWA into NOD mice.

Figure 6(a) shows that following a short in vivo treatment with *S. mansoni* antigens Foxp3⁺ Tregs increase only in SEA, but not SWA-treated mice. Further analysis of the liver of SEA-treated NOD mice revealed a highly significant increase in Foxp3⁺ Tregs, which is consistent with the fact that Tregs are found in the egg-induced granulomas in the liver and regulate their formation (Figure 6(b)) [31, 32].

These in vivo studies, therefore, support our in vitro studies and emphasize differences between the modes of activity of SEA and SWA.

4. Discussion

There has been considerable interest in defining the ways in which *S. mansoni* infection perturbs the immune system and induces a Th2 response. As we have previously shown that *S. mansoni* antigens are able to inhibit the Th1-mediated autoimmune disease Type 1 diabetes, we were particularly interested in clarifying this in NOD mice.

We focused our attention on DCs and MΦs as these cell types play a role in orchestrating the adaptive immune response. Much work has been carried out examining the effects of SEA on in vitro generated DCs, and while this has produced valuable insights, we felt that it is important to analyze the effects on populations of cells which would be exposed to *S. mansoni* antigens in vivo.

In our studies of splenic DCs we showed that following exposure to SEA in vitro these cells upregulated galectins 1 and 3, SIGN-R1, and DEC-205 following exposure to SEA in vitro. This is interesting because these C-type lectin receptors (CLRs) have been shown to recognize glycans present in schistosome antigens and are important for adaptive responses induced by the parasite. Galectins are

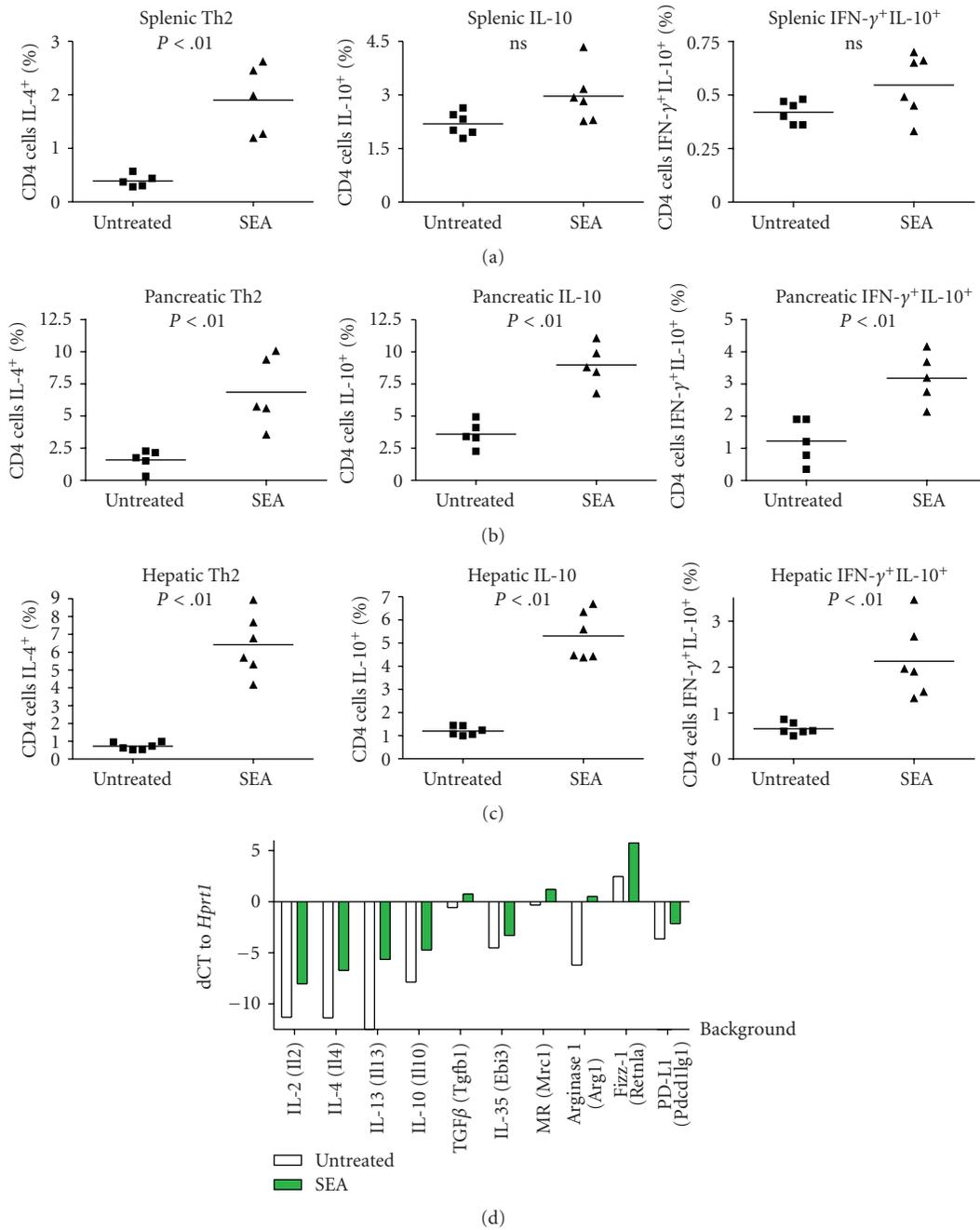


FIGURE 4: The systemic Th2 response to SEA is accompanied by local induction of IL-10 in the pancreas and liver. Increased proportions of CD4⁺ T cells expressing IL-4 were seen in the spleen (a), the pancreas (b), and the liver (c). In the pancreas and liver, SEA treatment triggered IL-10 production from Th1 cells. (d) Increased mRNA for Th2 and regulatory cytokines in the pancreas of mice treated with schistosome antigens are shown. Six-week-old female NOD mice were injected i.p. with 50 μ g SEA on day 0 and day 7, and lymphocytes from spleen, pancreas, and liver were collected for FACS and RT-qPCR analysis on day 10. Data shown are the responses of 5-6 mice per group for (a)–(c) or a pool of three mice per condition for (d). Statistical analysis by Mann Whitney U test, two-tailed.

of particular interest as they have the potential to alter sensitivity to cytokines and apoptosis [20, 33]. Exposure to SEA upregulates, therefore, the capacity of the DC to respond to further exposure to helminth glycans.

Some differences were observed in the response of DCs and M Φ s to SEA. This was seen in the effects of SEA on

IL-12. In vitro, we found that splenic DCs downmodulated IL-12p35 and p40 in response to SEA whereas the in vivo response of pM Φ s showed an upregulation of the IL-12p40 response with no effect on IL-12p35 (Figure 2(a)). This presents another interesting way in which the Th1 response could be modulated as it has been suggested that excess

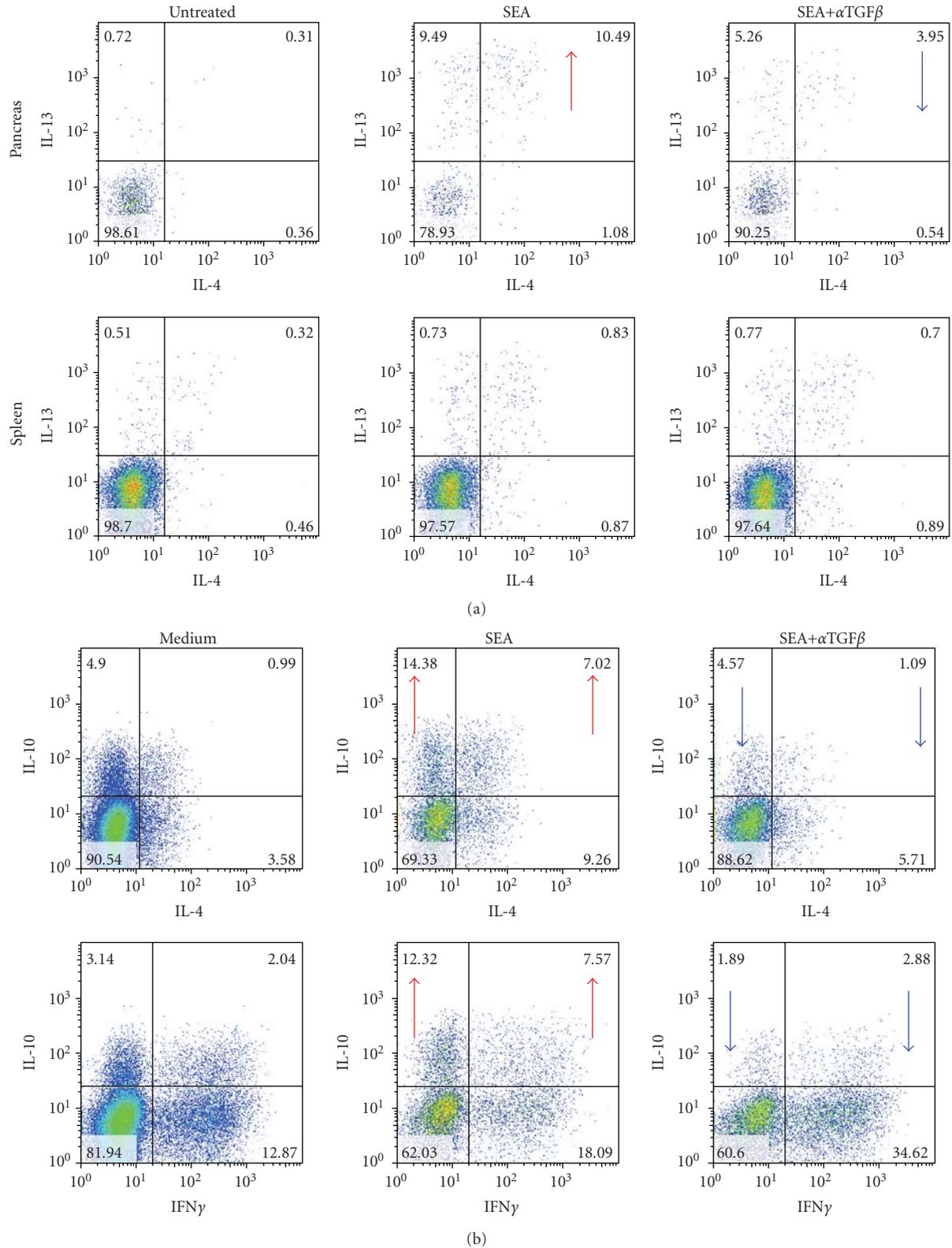


FIGURE 5: TGF β modulates the Th2 response to SEA in vitro and in vivo. (a) Treatment of NOD mice with anti-TGF β reduces the Th2 response to SEA in vivo. Female NOD mice (10-11 weeks of age) were injected i.p. with 50 μ g SEA on days 0 and 5, 5 mg anti-TGF β on days 0, 2, and 5, and cells were taken for analysis on day 10. Data shown are from a single experiment using anti-TGF β and SEA. (b) Antibody neutralization of TGF β in vitro diminishes the Th2 response to SEA. Naïve CD4⁺ T cells (5×10^5) were cultured for five days with bmDCs (10^5) and 0.5 μ g/ml α CD3 in the presence of SEA (10 μ g/ml) and/or anti-TGF β (20 μ g/ml). FACS plots from one of three independent polarization experiments are shown.

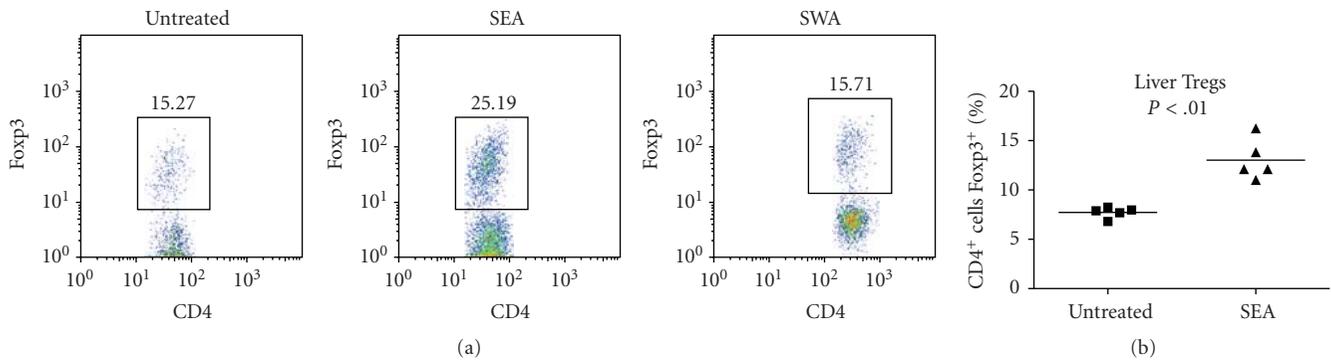


FIGURE 6: Egg, but not worm, antigens drive Foxp3⁺ Treg responses. SEA treatment increases the representation of Foxp3⁺ Tregs in the pancreas (a) and liver (b) of NOD mice. Female NOD mice were treated at eight weeks of age with 50 μ g SEA or SWA on day 0 and day 7. Pancreatic infiltrating cells were isolated and stained for intracellular Foxp3 expression on day 10. Statistical analysis by Mann Whitney U test, two-tailed.

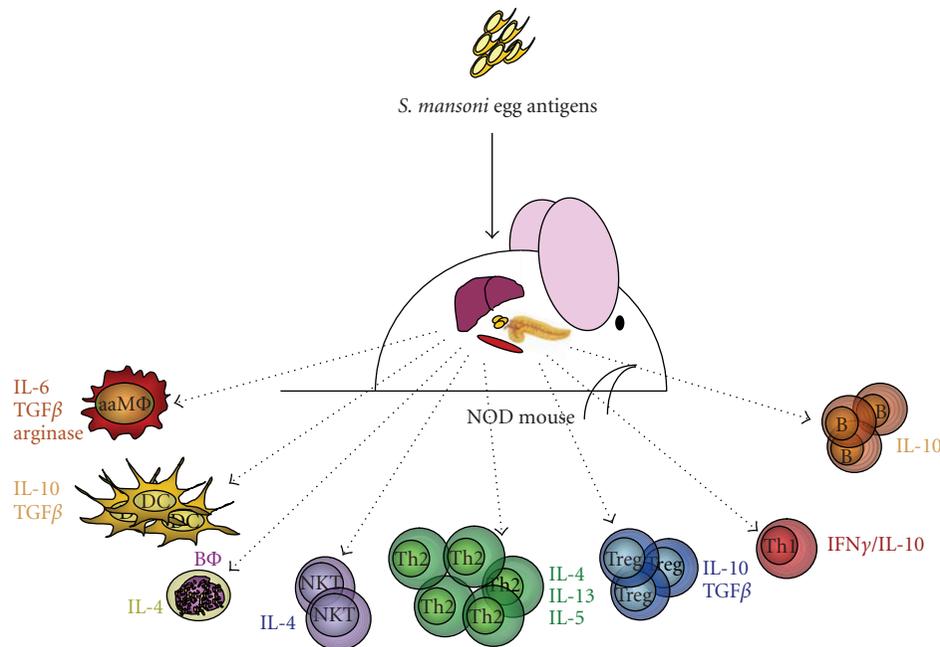


FIGURE 7: The cellular immune response to *S. mansoni* egg antigens in NOD mic: alternative activation of MΦs (aaMΦs), maintenance of dendritic cells (DCs) in an immature phenotype, IL-4 release from basophils (BΦs), expansion of classical natural killer T cells (NKT), expansion of T helper 2 (Th2) responses, expansion and activation of T regulatory cells (Tregs), as well as induction of IL-10 production in Th1 cells (Th1) and B cells (B).

IL-12p40 in both a monomeric (p40) and dimeric (p80) forms might antagonize the IL-12 receptor [34], providing a feedback mechanism to downmodulate Th1 responses.

With regard to effects of SEA administration *in vivo* on APC phenotype and function, several were noted that again had the potential to modify a diabetogenic Th1 response. SEA increased PD-L1 expression on pMΦs, with an accompanying increase in message for PD-L1 and 2 in whole PECs (Figure 3(c)). The negative costimulatory molecule programmed death ligand 1 (PD-L1) has been shown to be upregulated on aaMΦs [28], as well as playing a role in diabetes regulation in NOD mice [35]. Effects of *i.p.* injected SEA were noted in several sites, some of which were

particularly relevant to diabetes regulation. After a short course treatment of NOD mice with SEA, the intracellular cytokine expression in CD4⁺ T cells was profoundly changed in the spleen, pancreas, and liver of prediabetic NOD mice (Figures 4(a), 4(b), and 4(c)). The systemic Th2/Treg response induced by SEA was accompanied by the signature of MΦ alternative activation in the pancreatic infiltrate of prediabetic NOD mice and would be beneficial for diabetes prevention (Figures 4(b) and 4(d)). Together with the expected Th2 [5] and IL-10 Tr1 (CD4⁺ IL-10⁺) response, we have identified a population of CD4⁺ T cells expressing both IL-10 and IFNγ in response to SEA (Figures 4(b) and 4(c)). IL-10/IFNγ double-producing CD4⁺ T cells were first

identified in murine model of *Leishmania* infection and shown to mediate chronic pathology in the skin lesions with delayed/reduced clearance of infection [36, 37]. IL-10-producing Th1 cells have been proposed as a self-regulating class of Th1 cells, and therefore, this CD4⁺ T cell population in the pancreas might also mitigate Th1 driven diabetes pathology.

We have previously demonstrated that SEA has direct effects on DCs and T cells and suggest that the simultaneous interaction with both cell types is important for determining the host immune response. These in vitro studies had highlighted a role for TGFβ in the SEA-mediated induction of regulatory T cells; here we provide data suggesting that this immunomodulatory cytokine may also play a role in modulating the Th2 response in vivo as well as in vitro.

5. Conclusion

S. mansoni and its soluble antigens can prevent autoimmune diabetes in NOD mice. This paper summarizes and confirms our previous work demonstrating that SEA modulates the immune response through alteration of CLR expression, cytokine production, and Treg expansion in NOD mice. In particular we show how functional and phenotypic changes on DCs and MΦs, the expansion of different T helper cell subsets (Th2), and Treg cell types (IL-10/IFN-γ double producers and Foxp3⁺ Tregs) might contribute to diabetes prevention in NOD mice (Figure 7).

Abbreviations

SEA:	<i>S. mansoni</i> soluble egg antigens
NOD:	Nonobese diabetic
aa:	Alternatively activated
MΦ:	Macrophage
DC:	Dendritic cell
APC:	Antigen-presenting cell
Treg:	Regulatory T cell
Th:	T helper
CLR:	C-type lectin receptor
TLR:	Toll-like receptor
MR:	Mannose receptor
RT-PCR:	Reverse transcriptase polymerase chain reaction
Hprt-RE:	Expression relative to hypoxanthine phosphoribosyl transferase*1000
PLN:	Pancreatic lymph node
BΦ:	Basophil.

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