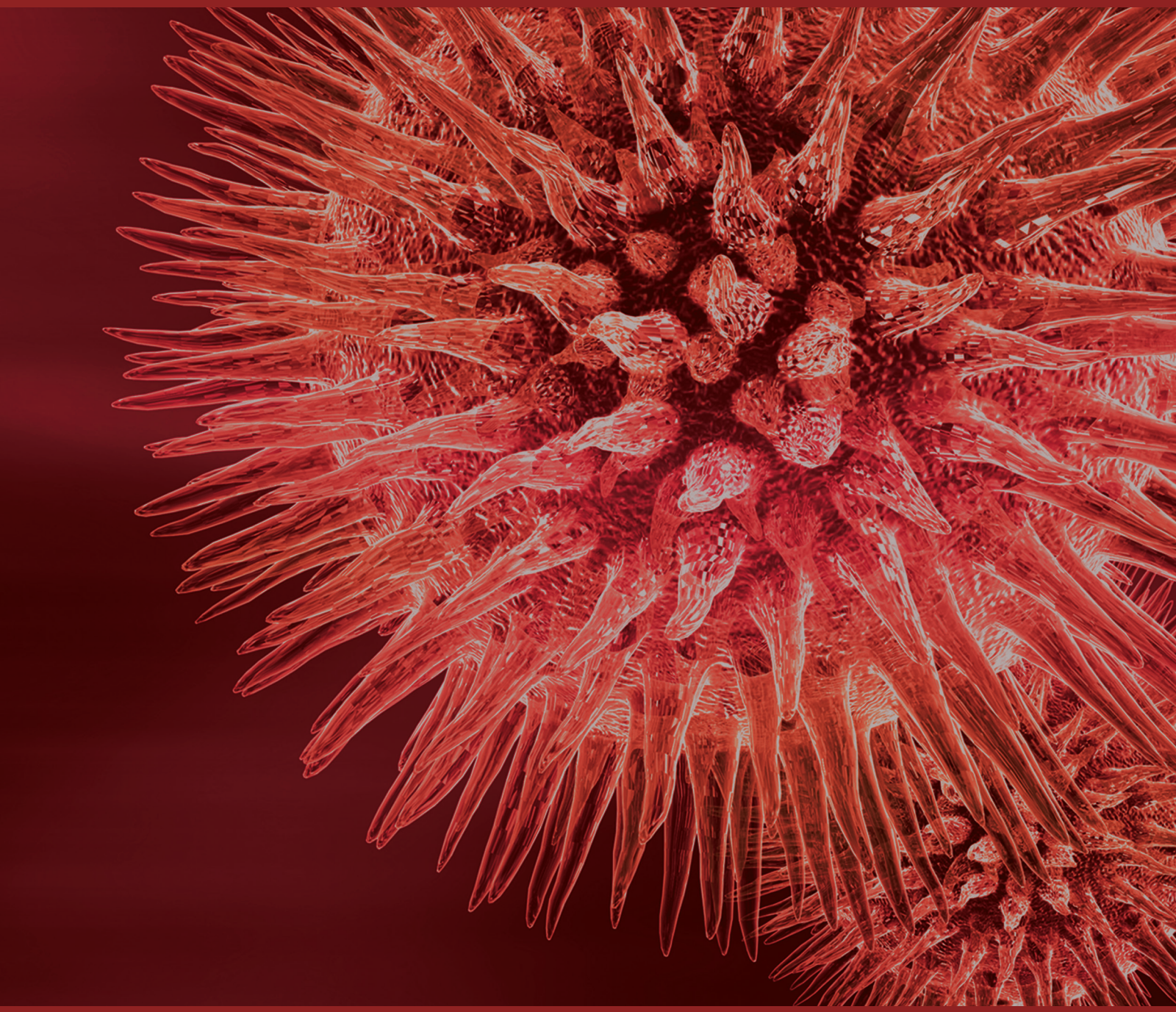
BioMed Research International

Immunology and Cell Biology of Parasitic Diseases 2014

Guest Editors: Luis I. Terrazas, Abhay R. Satoskar, Miriam Rodriguez-Sosa, and Abraham Landa-Piedra



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Editorial
Immunology and Cell Biology of Parasitic Diseases 2014

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Lack of clean water sources, starvation, insufficient hygiene, and poverty are some of the greatest barriers to health for the world's growing population. All these features are closely associated with parasitic infections. Approximately one third of the world's population has been infected with parasites at some point in their lives and these infections are often life-threatening. Since most parasitic diseases progress with few to no symptoms, patients do not obtain accurate diagnosis and treatment in a timely manner. Further, there are currently no accessible and effective antiparasitic vaccines despite enormous efforts and monetary investment into the development of vaccines, drugs, and treatments to combat these infections. Today parasitologists are looking for new alternatives for treatment such as immunotherapies, gene manipulation, or transfection in order to improve their fight against these "elusive" organisms. It is also clear that more studies on various parasites are necessary, even those which have low incidence, so that we are well-prepared during threats of reemerging parasitic infections.

In this special issue, we bring together several reviews as well as original reports that are intended to provide a summary of some of the current knowledge regarding the "immunology and cell biology of parasitic diseases." These papers include basic, clinical, and epidemiologic studies that we believe are interesting and very important in our field. The first section of this special issue is focused on helminthic diseases ranging from vaccine development to helminth therapy and includes some research on the basic mechanisms of susceptibility, modulation, and protection against these parasites.

The work by D. M. Lopes et al., "Dendritic Cell Profile Induced by *Schistosoma mansoni* Antigen in Cutaneous Leishmaniasis Patients," describes how helminth-derived molecules from *S. mansoni* can modulate dendritic cell activities during *Leishmania* infection. Additionally, V. H. Salazar-Castañon et al. have written an interesting review on how different helminth infections can improve or worsen the development of malaria in "Helminth Parasites Alter Protection against *Plasmodium* Infection." Data from their epidemiological and experimental studies indicates that helminth infections are a double-edged sword, in the context of malaria.

The next series of papers are focused on the biology of helminths. L. Jiménez et al. characterize the thioredoxin-1 gene and gene product from *T. solium* in their original paper "Characterization of a Thioredoxin-1 Gene from *Taenia solium* and its Encoding Product." Thioredoxin-1 is an essential component of the thioredoxin system and it performs functions such as antioxidative, protein-reducing, and signal-transducing ones for development, proliferation, migration, apoptosis, inflammation, and metabolism. It is secreted by *T. solium* and is able to modify the immune response by driving a Th2 biased response and allowing for the establishment of this parasite. Further, E.-V. Marcela et al. studied how the metacestodes of *T. crassiceps* "communicate" when they grow in vitro in a crowded manner. Their paper

is entitled "Crosstalk among *Taenia crassiceps* (ORF Strain) Cyst Regulates Their Rates of Budding by Ways of Soluble and Contact Signals Exchanged between Them."

Next, we put together a series of papers related to the development and regulation of immune responses to different helminths. K. E. Nava-Castro et al. demonstrated how early exposure to estrogens can imprint the immune response and have a positive or negative outcome during adulthood. Their results can be found in the paper "Diethylstilbestrol Exposure in Neonatal Mice Induces Changes in the Adulthood in the Immune Response to *Taenia crassiceps* without Modifications of Parasite Loads." Using the same model, M. Khumbatta et al. show the important role that somatostatin has on the immune response and susceptibility to experimental cysticercosis in their paper "Somatostatin Negatively Regulates Parasite Burden and Granulomatous Responses in Cysticercosis." A couple more papers focused on the immune response elicited by different helminth parasites. A. Prasad et al. report the first advances on immune response to the flat worm *Paramphistomum epiclitum* in their contribution "Evaluation of Antibody Response to Various Developmental Stage Specific Somatic Antigens of *Paramphistomum epiclitum* in Goats." Y. Gu et al. report a promising experimental vaccine against trichinellosis in their paper "Protective Effect of a Prime-Boost Strategy with the Ts87 Vaccine against *Trichinella spiralis* Infection in Mice." We have another paper on immunoregulation by helminths in the contribution of Y. Ledesma-Soto et al., "Extraintestinal Helminth Infection Limits Pathology and Proinflammatory Cytokine Expression during DSS-Induced Ulcerative Colitis: A Role for Alternatively Activated Macrophages and Prostaglandins." This paper suggests that helminth infections can modulate intense inflammatory processes such as colitis through different mechanisms and ameliorate signs of illness in mice.

Finally in this helminth section, B. Moguel et al. show in their review "Transfection of Platyhelminthes" how knowledge of the genome of helminths and genetic manipulation can be useful for designing new and more effective antihelminthic drugs. They also explain the molecular crosstalk that occurs between the host and parasite which has been partly inaccessible to experimentation.

The second section of this special issue deals with protozoan infections which are widely spread around the world. We started this section with an old "friend" from developing countries, the amoeba, which causes amebiasis and remains as a public health problem in this part of the world. A. Aceves-Cano et al. present an original research "Morphological Findings in Trophozoites during Amoebic Abscess Development in Misoprostol-Treated BALB/c Mice," where they show how trophozoites are altered with this kind of treatment. At human level A. K. Verma et al. contributed with the paper "The Trend in Distribution of Q223R Mutation of Leptin Receptor Gene in Amoebic Liver Abscess Patients from North India: A Prospective Study," they show that heterozygous mutant (QQ versus QR, $P = 0.049$) and homozygous mutant (QQ versus RR, $P = 0.004$) were significantly associated with amoebic liver abscess when compared with homozygous wild type (QQ).

D. M. Meneses-Ruiz et al. developed a new experimental vaccine focused on the expression of a lectin in their paper "Protection against Amoebic Liver Abscess in Hamster by Intramuscular Immunization with an *Autographa californica* Baculovirus Driving the Expression of the Gal-Lectin LC3 Fragment," where they found up to 75% protection using this novel vaccine compared to control animals. In the clinical field, we have a couple of papers related to amebiasis. The first paper is by L. R. Iyer et al. and is entitled "Differential Expression and Immunolocalization of Antioxidant Enzymes in *Entamoeba histolytica* isolates during Metronidazole Stress." Their work reports the behavior of the antioxidant enzymes during metronidazole stress on the strain HM1: IMSS versus clinical isolates of *Entamoeba histolytica* from India. Metronidazole is indiscriminately used in India. Their results revealed that the Indian isolate could tolerate higher concentrations of the drug compared to standard strains. Thus, the authors found an alarming resistance to metronidazole in the *Entamoeba* isolates from India. We have some more novel work with amoeba presented by Y. Toledano-Magaña et al., who used the highly phagocytic activity of *E. histolytica* as a tool to study the effects of new nanomaterials in their report "Effect of Clinoptilolite and Sepiolite Nanoclays on Human and Parasitic Highly Phagocytic Cells." They found that these nanomaterials were well tolerated by macrophages from human, mice, and the RAW 264.7 cell line as well as in *E. histolytica* trophozoite cultures. The final paper on amebiasis is an interesting comparative study between two species of amoebas: *Entamoeba histolytica* (pathogenic strain) and *E. dispar* (a noninvasive strain). Talamás-Lara et al. demonstrated a dramatic difference in the ability to phagocyte in these two species in their paper, "Erythrophagocytosis in *Entamoeba histolytica* and *Entamoeba dispar*: A Comparative Study." They discovered that *E. histolytica* displayed a superior erythrophagocytosis activity which possibly contributes to its more pathogenic nature.

Other protozoan parasites included in this special issue are *Trypanosoma*, *Leishmania*, and *Plasmodium*.

A. Y. Cervantes-Landín et al. contributed their work "High Molecular Weight Proteins of *Trypanosoma cruzi* Reduce Cross-Reaction with *Leishmania* spp. in Serological Diagnosis Tests." The aim of this study was to improve serological tests already standardized for Chagas disease diagnosis, by using a high molecular weight protein fraction from *T. cruzi* extracts. They developed an easier and cheaper assay which is much needed in poor countries that suffer from this debilitating disease. From the same group, A. Vizcaíno-Castillo et al. describe the processes of acute inflammation derived after experimental *T. cruzi* infection in their original paper "Exacerbated Skeletal Muscle Inflammation and Calcification in the Acute Phase of Infection by Mexican *Trypanosoma cruzi* DTUI Strain." In Leishmaniasis research, we have several interesting papers, such as "CK2 Secreted by *Leishmania braziliensis* Mediates Macrophage Association Invasion: A Comparative Study between Virulent and Avirulent Promastigotes" by A. M. B. Zylbersztejn et al., where the authors comparatively analyze the effect of the kinase enzyme CK2 of virulent and avirulent *L. braziliensis* strains on parasite growth and macrophage invasion. They show interesting

data that demonstrates that CK2 has a critical influence as a mechanism of invasion used by *L. braziliensis*. On the clinical side, N. Verma et al. show important cases where immunological changes were observed according to the treatment carried out by the patients, in their work "Clinicopathological and Immunological Changes in Indian Post Kala-Azar Dermal Leishmaniasis (PKDL) Cases in relation to Treatment: A Retrospective Study." In another review by S. A. G. Da-Silva et al. entitled "The Dialogue of the Host-Parasite Relationship: *Leishmania* spp. and *Trypanosoma cruzi* Infection," they have analyzed the host-parasite interaction in both *Leishmania* spp. and *Trypanosoma cruzi* infections. This review is comprehensive and is interesting since it indicates that there are some differences in host invasion strategies between these two major protozoan parasites. Three schematic figures summarizing the main escape mechanisms of *Trypanosoma* and *Leishmania* and modulation of host cells are included.

Another interesting original study related to malaria is presented by N. A. Mosqueda-Romo et al. in "Gonadal Steroids Negatively Modulate Oxidative Stress in CBA/Ca Female Mice Infected with *P. berghei* ANKA," where the authors highlight the important effect of sexual hormones on the development of *Plasmodium* infection.

Finally, a review of the basic cell biology of protozoan parasites and their virulence is discussed by C. Muñoz et al., in their latest paper, "Role of the Ubiquitin-Proteasome Systems in the Biology and Virulence of Protozoan Parasites."

We believe that this compilation of original research as well as the latest reviews written by authors from all around the world is a small sample about interesting yet complicated field of immunoparasitology. This research shows us that it is necessary to develop a deeper knowledge about the different mechanisms that parasites employ to invade the host and to avoid antiparasitic immune responses. We also require a better understanding of how they develop resistance to constant exposure to old drugs.

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Review Article

Role of the Ubiquitin-Proteasome Systems in the Biology and Virulence of Protozoan Parasites

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In eukaryotic cells, proteasomes perform crucial roles in many cellular pathways by degrading proteins to enforce quality control and regulate many cellular processes such as cell cycle progression, signal transduction, cell death, immune responses, metabolism, protein-quality control, and development. The catalytic heart of these complexes, the 20S proteasome, is highly conserved in bacteria, yeast, and humans. However, until a few years ago, the role of proteasomes in parasite biology was completely unknown. Here, we summarize findings about the role of proteasomes in protozoan parasites biology and virulence. Several reports have confirmed the role of proteasomes in parasite biological processes such as cell differentiation, cell cycle, proliferation, and encystation. Proliferation and cell differentiation are key steps in host colonization. Considering the importance of proteasomes in both processes in many different parasites such as *Trypanosoma, Leishmania, Toxoplasma,* and *Entamoeba*, parasite proteasomes might serve as virulence factors. Several pieces of evidence strongly suggest that the ubiquitin-proteasome pathway is also a viable parasitic therapeutic target. Research in recent years has shown that the proteasome is a valid drug target for sleeping sickness and malaria. Then, proteasomes are a key organelle in parasite biology and virulence and appear to be an attractive new chemotherapeutic target.

1. Introduction

In a paper published in 1978, Ciehanover et al. [1] reported the presence of a heat-stable polypeptide component of an ATP-dependent proteolytic system isolated from reticulocytes. A second paper from the same researchers reported that the ATP-dependent conjugation of reticulocyte proteins to the polypeptide was required for protein degradation [2]. Based on these findings, Hershko et al. [3] proposed in 1980 that the ligation of ubiquitin to proteins targets them for degradation by a protease that specifically acts on proteins with several ubiquitin molecules attached [3, 4]. A "protease," the 26S proteasome, was discovered by Hough and colleagues, who reported the identification and characterization of an ATP-dependent protease from rabbit reticulocyte lysates [5]. The real impact of this discovery would be dimensioned in the coming decades. In fact, Hershko's work on the ubiquitin enzymes was not only relevant but contributed to opening a new research field that was obscure and unexplored at that time. The 2004 Chemistry Nobel Prize award, conferred to Hershko, Ciechanover, and Rose "for the discovery of ubiquitin-mediated protein degradation," was not only a recognition of these researchers but a recognition of the importance of the ubiquitin-proteasome pathway to the life of the cell and to health, disease, infection, and immunity [6]. Many researchers have contributed to our current knowledge of this biological pathway. Many relevant reviews have been already published. In this context, the main goal of this review is to attract attention to a new role of proteasomes: the biology and virulence of protozoan parasites. General aspects of the ubiquitin-proteasome pathways and inhibitors will be only summarized.

2. The Ubiquitin-Proteasome System

The bulk of the turnover of intracellular proteins in eukaryotic cells is carried out by two self-contained proteolytic

systems, the lysosomes and proteasomes. Most proteins are degraded by the ubiquitin-proteasome system (UPS) [6].

Proteasomes are large complexes that perform crucial roles in many cellular pathways by degrading proteins in the cytosol and nucleus of eukaryotic cells to enforce quality control and regulate many basic cellular processes. Among these processes are progression through the cell cycle, signal transduction, cell death, immune responses, metabolism, protein-quality control, and development, in which proteasomes degrade short-lived regulatory or structurally aberrant proteins [6, 7]. The catalytic heart of these complexes, the 20S proteasome, is highly conserved in bacteria, yeast, and humans [8], with simpler versions also found in some *Archaea* and prokaryotes.

The 20S proteasome is a barrel-shaped assembly of 28 protein subunits. It forms a packed particle, a result of axial stacking of two outer α rings and two inner β rings made up of seven structurally related α and β subunits; the rings form an $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$ structure. The three subunits of each inner ring contain catalytically active threonine residues at their N termini and show N-terminal nucleophile hydrolase activity, indicating that the proteasome is a threonine protease [8]. The β1, β2, and β5 are associated with caspase-like, trypsin-like, and chymotrypsin-like activities, respectively, which confer the ability to cleave peptide bonds at the C-terminal side of acidic, basic, and hydrophobic amino acid residues, respectively. Those bonds that follow glycine and proline are less easily cleaved [9]. As revealed by structural studies performed by Huber and colleagues [10, 11], the potentially catastrophic elimination of inappropriate substrates is prevented by sequestration of active sites within the hollow structure of the 20S proteasome. Substrates access the central catalytic chamber through axial ports in the end rings of α subunits [12], although in the absence of activators, these channels are closed and proteasome activity is repressed. The 20S proteasome processively degrades client proteins, generating oligopeptides ranking in length from 3 to 15 amino acids. The resulting peptide products are subsequently hydrolyzed to amino acids by oligopeptidases and/or aminocarboxy peptidases [9].

Proteasomes are activated by protein complexes that bind to the end rings of α subunits. The best-known activator is PA700 [proteasome activator MW 700, also known as 19S or regulatory complex (RC)], which has been highly conserved from yeast to humans and binds to the 20S proteasome to form the 26S proteasome. PA700 is the only proteasome activator, that is, known to stimulate degradation of protein substrates. Thus, PA700 is thought to mediate most of the biological effects of the proteasome by facilitating substrate degradation [13, 14]. In contrast to PA700, two other evolutionarily conserved protein complexes that have been shown to bind specifically to and activate 20S proteasomes against model peptide substrates, PA28 (also known as 11S or REG) [7, 15] and PA200 [7, 16], do not recognize ubiquitinated proteins or use ATP. Proteasome activator PA200 enhances proteasome-mediated cleavage after acidic residues *in vitro*; however, in response to ionizing radiation, PA200 forms hybrid proteasomes with 19S caps and 20S core proteasomes that accumulate on chromatin, leading to an increase in proteolytic activity. A unique role for PA200 in genomic stability, that is, likely mediated through its ability to enhance post-glutamyl cleavage by proteasomes, has been reported [17]. Blm10/PA200 (*Saccharomyces cerevisiae*/human) does not utilize ATP and is generally believed to stimulate the hydrolysis of peptides but not proteins. Blm10/PA200 has been proposed to function in a surprisingly broad variety of processes [18], including 20S proteasome assembly [19], DNA repair [20], genomic stability [17], proteasome inhibition [21], spermatogenesis [22], and mitochondrial checkpoint regulation [23]. However, endogenous inhibitors like Hsp 90, P131, PR 39, and Tat have also been described. The biological role of 26S proteasomes and its activators and inhibitors have been reviewed extensively elsewhere [5, 7, 24, 25]. New regulatory mechanisms have emerged. The archaeal PAN ATPase complex is homologous to the eukaryotic 19S ATPases and contains a conserved C-terminal hydrophobic-tyrosine-X motif (HbYX), that is, essential for PAN to associate with the 20S proteasomes and open its gated channel for substrate entry [26]. Gate opening can be induced by C-terminal peptides from the 19S ATPase subunits, Rpt2, and Rpt5, but not by C-terminal peptides from PA28/26, which lack the HbYX motif and cause gate opening by distinct mechanisms. C-terminal residues in the 19S ATPases were also shown to be critical to the gating and stability of 26S proteasomes. Thus, the C termini of the proteasomal ATPases function like a "key in a lock" to induce gate opening and allow substrate entry [26]. Recently, it has been shown that binding of polyUb substrates to the 19S regulator stabilizes gate opening of the 20S proteasome and induces conformational changes in the 20S proteasome that facilitate channeling of substrates and their access to active sites. In consequence, polyUb substrates allosterically stimulate their own degradation, enhancing the peptidase activities of the 20S proteasome about two-fold in a process requiring ATP hydrolysis [27]. In addition, a recently published body of evidence suggests that many proteasome functions, such as substrate recognition, deubiquitylation, unfolding, and degradation, appear to be controlled allosterically [28, 29].

In this pathway, proteins are targeted for degradation by covalent ligation with ubiquitin. Ubiquitination tags the target protein with ubiquitin-like proteins (UBLs), such as ubiquitin, small ubiquitin-like modifier (SUMO), and NEDD8. Ubiquitination is a posttranslational modification of proteins in which the modifier is a polypeptide conjugated to the target proteins by an isopeptide bond between proteasome substrates: the C terminus of ubiquitin and one or more lysine side chains in the target proteins [30]. Protein modification by ubiquitin occurs in three successive steps that are mediated by three enzymes: the activating enzyme E1, the conjugating enzyme E2, and the ubiquitin ligase E3. This modification is reversible, and ubiquitinated proteins can be proteolytically deubiquitinated by specific deubiquitinating enzymes [30, 31]. Ubiquitin molecules can form polyubiquitin chains that are conjugated to target proteins, which are usually recognized and degraded by the proteasome [30, 32]; however, current knowledge of UPS strongly suggests that protein ubiquitination appears to be necessary but not essential. A recent paper reports

that proteasomes can degrade a significant proportion of cellular proteins independent of ubiquitination. Then, 26S proteasomes specifically recognize and cleave similar sites, independent of ubiquitination, suggesting that disordered regions likely constitute the universal structural signal for proteasome-substrate proteolysis by proteasomes. In the same way, the inactivation of ubiquitin-activating enzyme E1 does not prevent intrinsic proteasome substrates degradation [33].

The picture is completed by the deubiquitinating enzymes (DUBs) [30, 34]. They generate free Ub moieties from their initial translation products, recycle ubiquitin during breakdown of the poly-ubiquitin-protein conjugates, and/or reverse the effects of ubiquitination. All DUBs tested have remarkable specificity for ubiquitin. DUBs have been implicated in a variety of processes in animals and yeast, suggesting that individual DUBs are target-specific [34]. An intriguing possibility is that some DUBs can also regulate a protein's half-life by reversing ubiquitination. A large number of genes encode deubiquitinating enzymes, suggesting that many have highly specific and regulated functions. Interestingly, many of these enzymes are localized to subcellular structures or to molecular complexes. These localizations play important roles in determining functional specificity and can have major influences on their catalytic activities [34]. Indeed, recent findings strongly suggest that ubiquitination is regulated by both specific pathways of ubiquitination and deubiquitination. In summary, the protein substrates are first conjugated to multiple molecules of ubiquitin and then ubiquitin substrates are rapidly hydrolyzed by the 26S proteasome, an ATP-dependent complex comprising the core 20S proteasome enclosed by two proteasome activator (19S) regulatory complexes. Deubiquitination enzymes recycle the ubiquitin molecules and the pathway is modulated by protein activators and inhibitors. An overview of the ubiquitin-proteasome system is shown in Figure 1.

In summary, the ubiquitin-proteasome pathway is not only a degradation machine focused to destroy old or damaged proteins. This pathway is a major control point for regulating, among other things, short-lived proteins functioning as regulatory factors in a large array of cellular processes like cell-cycle progression [35], cell growth, stage-specific gene transcription [36], inflammatory response [37], and antigen processing [32]. Eukaryotic 20S proteasomes have several peptidase activities, as well as endoribonuclease, protein-chaperone, and DNA-helicase activities [38].

Until a few years ago, the role of proteasomes in parasite biology was completely unknown.

3. The Role of Proteasomes in Parasite Biology and Virulence

Parasitic protozoan are unicellular but complex cells that undergo multiple differentiation events to accommodate the various hosts and physical environments that they encounter in their life cycles.

Some proteases are involved in the differentiation of the infectious stages of a small number of protozoan parasites into their respective disease-causing stages [39–41].

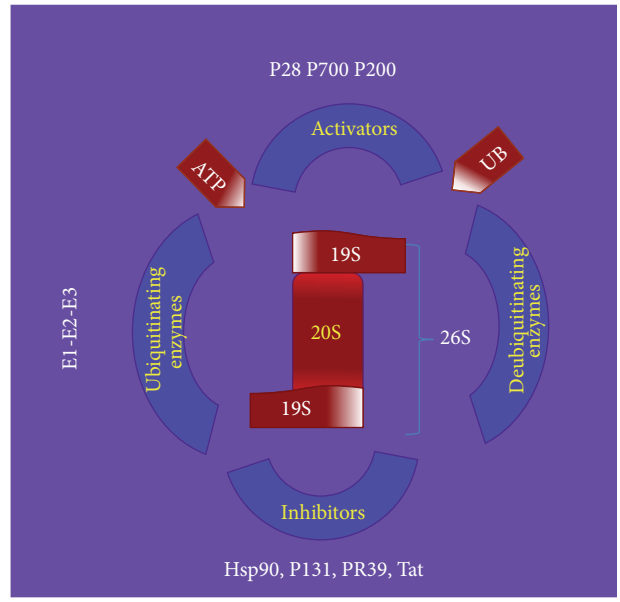

Figure 1: An overview of the main component of ubiquitin proteasome system.

The central role played by the proteolytic activities of the proteasome/ubiquitin system in regulating cell homeostasis has been demonstrated in a large number of fungi and higher eukaryotes and, more recently, in protozoan parasites [42].

A prominent feature of the life cycle of pathogenic parasites is the profound morphological changes they undergo during development in the vertebrate and invertebrate hosts. These developmental changes, during which shape, size, and cytoskeletal structures must adapt to the new stage, involve extensive and carefully controlled proteolysis. The intriguing question of what proteolytic system is involved in protein degradation in parasites led us to investigate the role of proteasomes in differentiation of protozoan parasites.

The protozoan parasites' 20S proteasomes are similar in morphology and size to the 20S proteasome isolated from archaebacteria, yeast, and mammals. Similarly, the composition of the protozoan proteasomes subunits is very similar to that of the eukaryotic proteasomes, with multiple α and β subunits instead of the single type of α and β subunit described in *Archaebacterias proteasomes*. Studies in different laboratories and with different models have found that inhibition of proteasomal function inhibits specific stages of morphological differentiation in *Trypanosoma*, *Plasmodium,* and *Entamoeba* and replication of *Plasmodium*, *Toxoplasma*, *Leishmania*, and *Trypanosoma*; however, invasion of the host cell is not inhibited in *Trypanosoma*, *Plasmodium*, *Leishmania,* or *Toxoplasma*. Differences between the proteasomes of mammals and parasites have been observed (*Trypanosoma*), as have differences in immunoreactive structures (*Trypanosoma* and *Toxoplasma*) and enzymatic activities (*Trypanosoma* and *Entamoeba*). These differences suggest that protozoan parasite proteasomes could be considered as a chemotherapeutic target, even though this organelle is also present in all host eukaryotic cells. In mammals cells,

the ubiquitin proteasome system is essential for all eukaryotic cells; any alteration to its components thus has potential pathological consequences [43]. Chemotherapy targeting parasite proteasomes could result in successful therapies.

The following are the main findings reported in the literature with respect to the role of protozoan parasite proteasomes.

3.1. Cryptosporidium. A DNA sequence composed of 1281 nucleotides (nt) consisting of a single open reading frame (ORF) encoding a putative 20S proteasome β1-type subunit was isolated from *Cryptosporidium parvum*. Southern-blot analysis suggested that the sequenced DNA exists in the *C. parvum* genome as a single copy. The predicted protein consists of 210 amino acids (aa), including characteristic amino acids common to all proteasomal subunits, and is more similar to the beta1-type subunit of yeast than to other types of beta subunits [44]. No studies have examined its biological role.

3.2. Giardia. The parasite has a single gene that encodes monoubiquitin; however, two-dimensional electrophoresis assays have shown that the *Giardia* 20S proteasome seems to be as complex as that of other eukaryotes [45]. A study of the seven genes that encode the α subunits of the *G. duodenalis* proteasome indicated that the α-proteasome gene family evolved quickly from a single gene in the Archaea to seven or more genes in Eukarya [46]. The *G. duodenalis* 20S proteasome appears to be similar to that described in eukaryotic cells, containing a divergent set of α subunits [47]. Proteomics approaches performed to discover novel proteins associated with the stage-specific, Golgi-like encystation-specific vesicles (ESV) identified cytoplasmic and luminal factors of the endoplasmic reticulum quality-control system, that is, several structural (α) and catalytic (β) proteasome subunits. In contrast, cytoplasmic proteasome complexes undergo a developmentally regulated relocalization to ESVs during encystation. In mammalian cells and in yeast, proteasome complexes localize at ER membranes in addition to the cytoplasm and the nucleoplasm. Confocal microscopy analysis demonstrated that the giardial 20S core complex and 19S cap structure were associated with ESV membranes during early encystation until at least 7 h after induction. As noted previously, the expression of proteasome subunits is not upregulated in encysting cells [47]. The confocal microscopy data indicated a relocalization from more peripheral sites in the cytoplasm to the vicinity of ESVs, indicating a high rate of retrotranslocation of organelle proteins destined for degradation. In light of these results, the authors proposed that proteasome recruitment during encystation is a consequence of quality control and cargo maturation processes in the ER and early ESVs (i.e., protein folding, heterooligomerization, and trimming) producing large amounts of material destined for degradation [48].

3.3. Entamoeba. The 20S activity in proteasomes was described based on the SDS-electrophoretic pattern and immunoblot analysis of a soluble *Entamoeba histolytica* extract fractionated by density-gradient centrifugation [49]. A study of the *E. histolytica* proteome confirmed the presence of ubiquitin-proteasome components [50]. On the other hand, the genes encoding the α proteasome subunits show a higher identity with mammalian proteasomes (60.1% homology with rat proteasomes and 60.5% with human proteasomes) than with proteasomes from *Thermoplasma acidophilum* and *Saccharomyces cerevisiae* (39.5% and 53.8%, resp.). In *E. histolytica* trophozoites, nuclear localization of the 20S complex was not evident even by high-resolution confocal microscopy [51]. Instead, fluorescent reactivity against the proteasome subunits EhotS and EhS2 was observed exclusively in the cytosol, exhibiting a homogeneous distribution with no apparent exclusion of compartments that resemble the ER and Golgi apparatus, as observed in other cell types [30, 51].

Recently, multiple *E. histolytica* ubiquitination components, including ubiquitin and its activating (E1), conjugating (E2), and ligating (E3) enzymes, have been cloned and characterized. EhUbiquitin is activated by and forms a thioester bond with EhUba1 (E1) *in vitro* in an ATP- and magnesium-dependent fashion. According to the authors, EhUba1 exhibits a greater maximal initial velocity of pyrophosphate-ATP exchange than its human homolog, suggesting that different kinetics of ubiquitin activation might exist in *E. histolytica* [52].

In a reptilian amoeba, *Entamoeba invadens*, encystation is inhibited by lactacystin, a specific and irreversible inhibitor of proteasomes [53]; however, lactacystin seems to have no effect on *E. invadens* excystation [54].

3.4. Leishmania. These parasites are protozoan parasites with an intracellular stage called the amastigote that replicates in mammalian macrophages and an extracellular stage called the promastigote that replicates in the intestine of hematophagous insect belonging to genus *Lutzomyia*.

Purified proteasomes from *L. mexicana* were studied using polyacrylamide-gel electrophoresis (SDS-PAGE), revealing 10 different bands with masses ranging between 22 and 32 kDa, suggesting a complexity similar to that of eukaryotic proteasomes [55]. Lactacystin affected *L. mexicana* replication only when used at concentrations higher than 5 μm, while MG132 blocked the same process at lower concentrations. These discrepancies might be due to the lower capacity of *L. mexicana* to incorporate these inhibitors. According to Christensen et al. [56], a new antigen that resembles an α subunit of the human 20S proteasome was identified in *Leishmania*. This antigen (LePa) is immunogenic in humans. Moreover, a DNA vaccine based on the LePa antigen induced an initial reduction in the size of lesions when mice were challenged with *Leishmania major*. The strong immunogenicity of the *Leishmania* proteasome was confirmed by Couvreur et al. [57], who reported that Antigen 24, an immunogenic complex isolated from *Leishmania infantum* used as reference antigen in the immunodiagnostic of human visceral leishmaniasis, was recognized by the serum of rabbits immunized with purified *L. mexicana* proteasomes. On the other hand, the *Leishmania chagasi* proteasome was

partially purified and showed sensitivity to lactacystin and clasto-lactacystin beta-lactone, which blocked the *in vitro* growth of the promastigote stage. Although pretreatment of the promastigotes with lactacystin did not inhibit cell invasion, proteasomal function seems to be essential for replication and intracellular survival of amastigotes in the host cell [58].

In synchronized *Leishmania* cultures, Dubessay et al. [59] reported the cell-cycle-dependent regulation of protein levels. A kinesin called LmjKIN13-1 is highly abundant in the G2 + M phase and present at very low levels after mitosis. This protein is degraded through ubiquitin-proteasome pathways, demonstrating that it has C-terminal redundant degradation signals. This observation suggests that in *Leishmania*, in which postranduccional regulation is rare or absent, the proteasome appears to be involved in the regulation of protein levels [59]. On the other hand, in *Leishmania donovani,* degradation of pteridine reductase 1 (PTR1) has been reported. In *Leishmania*, PTR1 is an essential enzyme in pterin and folate metabolism. Western-blot studies using *L. donovani* promastigotes transfected with PTR1-GFP showed that PTR1 was degraded in the stationary phase of growth, when parasites start metacyclogenesis. Similarly, a probable destruction box composed of nine amino acids (Q63ADLSNVAK71) and a lysine K156 residue (as a site of ubiquitin conjugation) were identified in *L. donovani* PTR1. This finding suggests that degradation of PTR1 during the stationary phase of growth is mediated by proteasomes, resulting in low levels of H4-biopterin, which promotes metacyclogenesis and subsequently results in highly infective parasite stages [60]. Two HIV-protease inhibitors, indinavir and saquinavir, have been shown to block proteasome functions; effects were observed on the growth of *L. major* and *Leishmania infantum*. After 24 h of treatment, both drugs exhibited dose-dependent antileishmanial activity, with lethal-dose values of 50% (LD50), 8.3 μM and 7 μm on *L. major*, and minor activity on *L. infantum*. These results suggest the potential use of these protease inhibitors against opportunistic infections in treated seropositive patients [61].

It has also been reported in *L. donovani* that the proteasome is involved in downregulation of methionine adenosyltransferase (MAT), an enzyme important for metabolic processes; its product, S-adenosylmethionine (AdoMet), plays a key role in trans-methylation, trans-sulfuration, and polyamine synthesis. The presence of proteasome inhibitors such as MG-132, MG-115, epoxomicin, and lactacystin in the culture medium prevented MAT degradation in both MAT-overexpressing and "mock-transfected" leishmanial strains. The role of the ubiquitin (Ub) pathway in MAT downregulation was also supported by immunoprecipitation experiments. Immunoprecipitated MAT cross-reacted with anti-Ub antibodies, providing evidence of a proteasome-mediated downregulation of the leishmanial MAT abundance [62].

3.5. Trypanosomes. The *Trypanosoma* proteasome is the most intensely studied of the parasite proteasomes. The proteasome of *Trypanosoma brucei* was the first to be purified and characterized; however, its role in the biology of the parasite was not described [63]. Subsequent work showed that proteasome activity appears to be essential for cell-cycle progression, although participation seems to differ between the blood and procyclic forms. The amount of lactacystin needed to inhibit proliferation of procyclic forms concentrations was 5–10 μm, five times higher than was needed to inhibit the same process in blood forms. According to the authors, this difference in sensitivity to inhibitors could be explained by differences in the cell permeability. DNA analysis by flow cytometry showed that in the procyclic forms, lactacystin inhibits the progression of the cell cycle in the G2 and M phases, while in blood forms, makes it in G1/S, G2, and M phases. According to the same authors, in *T. brucei,* lactacystin at 1 μM was unable to block the differentiation of blood forms to the procyclic stage [64]. These results suggest that in trypanosomes, proteasomes participate in the regulation of cyclin levels [65]. The 20S proteasome purified from procyclic and bloodstream forms has increased trypsin-like activity, unlike the eukaryotic proteasomes in which the chymotrypsin-like activity is higher. In addition, other differences between *T. brucei* and mammalian proteasomes have been found. (1) The 20S proteasome of trypanosomes has a molecular weight of 630 kDa, while that of mammals is 700 kDa; (2) the 2D gels from the trypanosome 20S proteasome have only 26 protein spots, fewer than observed in the 20S proteasomes isolated from rat livers [66]; (3) although the morphology and size of the *T. brucei* proteasome are similar to those described in mammalian proteasomes, the pore diameter of the *T. brucei* 20S proteasome is greater than that observed in the rat 20S proteasome; (4) polyclonal antibodies raised against the human 20S proteasome cross-reacted with the procyclic and bloodstreams forms of *T. brucei* 20S proteasome; however, they strongly recognized rat 20S proteasome. On the other hand, polyclonal antibodies obtained against the purified 20S proteasome isolated from blood forms of *T. brucei* 20S also reacted with the purified 20S proteasome isolated from procyclic forms of the parasite but not with the 20S proteasome from rat erythrocytes. The α5 subunit of *T. brucei* proteasome has only 50% sequence identity with that of the rat proteasome [67].

A 20S proteasome activator was also identified in procyclic and blood forms of *T. brucei*. *In vitro,* the 26 kDa PA26 spontaneously polymerizes with proteasome 20S to generate the activated 20S proteasome [68]. Its human counterpart, PA28α, was as effective as PA26 in associating with and stimulating the enzymatic activity of the rat 20S proteasome but was unable to activate the proteasome 20S of *T. brucei*. Moreover, unlike mammalian and yeast proteasomes, the *T. brucei* proteasome is unable to degrade the mammalian ornithine decarboxylase-antizyme (ODC) complex, which catalyzes the first step in polyamine biosynthesis. This inability is a significant difference between trypanosomes and mammalian proteasomes [69]. Moreover, the functional characterization of 11 non-ATPase subunits (regulatory particles not-ATPase (Rpn)) in the 19S regulatory complex showed that when Rpn10 was deficient, a complex without Rpn was formed, but cell growth stopped. This structural dispensability but functional indispensability of Rpn10 constitutes another unique

aspect of the *T. brucei* proteasome [70]. Similarly, proteomics and bioinformatic approaches have allowed the identification and mapping of *T. brucei* proteasomes [71].

Nine vinyl ester tripeptides selective for inhibition of mammalian proteasome trypsin-like activity have been tested for *in vitro* activity against *T. brucei*. Two showed trypanocidal activity in the low-micromolar range without displaying cytotoxicity against human cells; however, the compounds did not inhibit the trypsin-like activity of the trypanosome proteasome, although their effect correlates with inactivation of chymotrypsin-like activity. This finding suggests that the inhibitor sensitivities differ between mammalian and trypanosome proteasome. This difference may be exploited for rational antitrypanosomal drug development [72].

On the other hand, the role of the *T. cruzi* proteasomes in trypomastigote-to-amastigote differentiation has been clearly documented [73, 74]. Lactacystin significantly blocked the transformation of trypomastigotes to amastigotes in axenic medium at pH 5.0 [73]. The 20S proteasome was purified and characterized and shown to possess trypsin-like, chymotrypsin-like, and caspase-like activities. Treatment with lactacystin does not block cell invasion but strongly reduced discharge of the parasite. Similarly, leucine C^{14} metabolic labeling of trypomastigotes showed that proteolysis occurs during *T. cruzi* cell differentiation from trypomastigote to amastigote. This proteolytic pathway was blocked by proteasome inhibitors like lactacystin and vinyl sulphone, but not by serine or cysteinyl proteinase inhibitors, suggesting that the protein degradation that occurs during the parasite cell differentiation is proteasome-dependent [74]. Then, during parasite cell differentiation at acidic pH, an ATP-dependent proteolytic pathway was observed and 26S proteasomes were identified and characterized by first time in a protozoan parasite [74]. Similarly, these authors demonstrated that cytoskeleton proteins, especially the paraflagellar rod antigen, were degraded by a proteasome-dependent pathway. However, monoclonal antibodies raised against the *T. cruzi* 20S proteasome have been observed by electron microscopy and confocal studies, the presence of proteasomes in the nucleus, cytoplasm, and kinetoplasts. These findings were confirmed by detection of proteasome chymotrypsin-like activities in the kinetoplast, isolated by Percoll gradients [75]. In mammalian cells, the UPS has been found in the outer mitochondrial membrane associated degradation (OMMAD) quality controls proteins localized to the OMM [76]. Then, at the outer membrane, the UPS may play a role in recycling either membrane-embedded or imported proteins [77]. The role that proteasomes fulfill in *T. cruzi* kinetoplast is still unknown. However, we could speculate that proteasomes may be involved not only in quality control of proteins but also in kinetoplast morphology changes that occur when trypomastigotes differentiate to amastigotes or epimastigotes differentiate to metacyclic trypomastigotes.

According to Cardoso et al. [78], inhibition of the ubiquitin-proteasome pathway in *T. cruzi* epimastigotes does not block adhesion but does disrupt cell division. In the same way, *in vitro T. cruzi* metacyclogenesis was strongly inhibited (95%) by treatment with 5 μM of lactacystin.

Proteasomal proteolysis during the *in vitro* metacyclogenesis of *T. cruzi* has also been studied. Cardoso et al. [79] demonstrated that proteasome-dependent proteolysis occurs during metacyclogenesis. No peaks of ubiquitin-mediated degradation were observed, and the profile of ubiquitinated conjugates was similar at all stages of differentiation; however, an analysis of carbonylated proteins showed significant variation in the levels of oxidized protein at the various stages of differentiation, and proteasome inhibition also increased oxidized-protein levels. These observations suggest that different proteasome complexes coexist during metacyclogenesis. The 20S proteasome may be free or linked to regulatory particles (PA700, PA26, and PA200), at specific cell sites, and the coordinated action of these complexes would make it possible for proteolysis of ubiquitin-tagged proteins and oxidized proteins to cooccur in the cell. In addition, these findings strongly suggest that the coordinated series of biochemical adaptations occurring during *T. cruzi* metacyclogenesis may also be regulated by the activity of different proteasome complexes. These data also highlight the importance of ubiquitin-independent proteasomal degradation during metacyclogenesis [79]. The role of proteasomes in cell differentiation led us to propose this organelle as a trypanosome virulence factor [80].

Two genes encoding the α1 and α6 subunits of the *T. cruzi* proteasome have been cloned and characterized [81]. Considering that the most part structural studies have been performed in trypanosomes [67, 69, 74, 75], a subunits composition of human, yeast, and trypanosomes is shown in Table 1.

3.6. Plasmodium. The presence of the *Plasmodium* proteasome was first shown using inhibitors. Lactacystin inhibits the *in vitro* development of exoerythrocytic forms of *Plasmodium berghei* but does not inhibit sporozoite invasion of the host cell. The inhibitory effect of lactacystin is stage-specific, and although no infected rat survived, lactacystin reduced the parasitemia of the infected animals. The authors thus suggested the proteasome as a promising chemotherapeutic target [82]. On the other hand, lactacystin inhibited the growth of three different lines of *Plasmodium falciparum* at similar molar concentrations and was more effective against chloroquine-resistant parasites [83]. The genes encoding the β subunits of *P. falciparum* 20S proteasome have been already cloned [84].

Phosphoethanolamine methyltransferase (fepm), an enzyme of central importance in the serine decarboxylase phosphoethanolamine methyltransferase (SDPM) pathway, is negatively transcriptionally regulated and degraded by the proteasome in the presence of choline. Immunoblotting, pulse-chase, and chromatin immunoprecipitation experiments have shown that Pfpmt degradation occurred not only in wild-type cells but also in transgenic parasites that express Pfpmt constitutively. The proteasome inhibitor bortezomib blocked choline-mediated Pfpmt degradation. These data were the first evidence that a metabolite can mediate transcriptional regulation and proteasome degradation in *Plasmodium* [85].

Table 1: Proteasome subunits composition in diferent species of eukaryotic cells.

Protein complex	Subunits	Systematic nomenclature	Miscellaneous nomenclature		
			Human	Yeast	Trypanosomes
20S	α Type	α1	Iota	SCL1, YC7	Tbα1
		α2	C3	PRE8, Y7	Tbα2
		α3	C9	PRE9, Y13	Tbα3
		α4	C6	PRE6	Tbα4
		α5	zeta	PUP2, DOA5	Tcα5, TbPSA5
		α6	C2	PRE5	Tcpr29α6, Tbα6
		α7	C8	PRE10, YC1	Tbα7
	β Type	β1	Y, delta	PRE3	Tbβ1
		β2	Z	PUP1	Tbβ2
		β3	C10	PUP3	Tbβ3
		β4	C7	PRE1	Tbβ4
		β5	X, MB1, epsilon	PRE2, DOA3	Tbβ5
		β6	C5	PRE7	Tbβ6
		β7	N3, beta	PRE4	Tbβ7
		β1i	LMP2, RING12		
		β3i	LMP10, MECL1		
		β5i	LMP7, RING10		
19S	ATPase	RPT1	57, MSS1	YTA3, CIM5	TcS7
		RPT2	54, P56	YTA5, mts2	TcS4
		RPT3	56, Tbp7, P48	YTA2	TcS6
		RPT4	S10b, p42	SUG2, CRL3, PLS1	TcS10b
		RPT5	S6′ , Tbp1	YTA1	TcYTA-1
		RPT6	58, p45, Trip1	SUG1, CRL3, CIM3/let1	TcS8
	Non ATPase	Rpn1	S2, p97	HRD2, NAS1/mts4	TcRpn1, TbRpn1
		Rpn2	S1, p112	SEN3	TbRpn2
		Rpn3	S3, p58	SUN2	TbRpn3
		Rpn5	p55	NA55	TbRpn5
		Rpn6	S9, p44.5	NAS4	TbRpn6
		Rpn7	S10a, P44		TbRpn7
		Rpn8	S12, p40, MOV 34	NAS3	TbRpn8
		Rpn9	S11, p40.5	NAS7, mts1	TbRpn9
		Rpn10	S5a, MBP1	SUN1, MCB1, pus1	TbRpn10
		Rpn11	S13, Poh1	MPR1, pad1, mts5	TbRpn11
		Rpn12	S14, p31	NIN1/MTS3	
		Rpn13	ADRM1	DAQ1	
		Rpn15	DSS1, SHFM1	SEM1	

Gliotoxin (GTX), a metabolite of fungal origin, may have an *in vitro* antimalaria effect. GTX showed activity against chloroquine-sensitive and -resistant strains of *P. falciparum*. GTX cytotoxicity was significantly lower against normal liver cell lines [86]. According to the same researchers, GTX blocked chymotrypsin-like activity in the *P. falciparum* proteasome. In the same way, MLN-273, a proteasome inhibitor belonging to the peptidyl boronic acid family, has shown to inhibit the early intraerythrocytic stages of *P. falciparum,* as well as the exoerythrocytic stagesof *P. berghei*. The inhibitor did not affect the erythrocytes or the liver cells but caused a significant reduction in parasite protein degradation. According to these authors, the use of proteasome inhibitors as antineoplastic drugs suggests the possibility of malaria chemotherapy based on proteasome inhibitors [87]. From this perspective, proteasome inhibitors like bortezomib (Velcade: [(1R)-3-methyl-1-[[(2S)-1-oxo-3-phenyl-2-[(pyrazinylcarbonyl) amino] propyl] amino] butyl] boronic acid), which has been approved for the treatment of patients with myeloma, and a similar boronate called Z-Leu-Leu-Leu-B (OH) 2 (ZL3B), were assessed against four strains of *P. falciparum* (3D7, HB3, W2, and Dd2) which had different levels of sensitivity to antimalaria drugs like pyrimethamine and chloroquine. Both drugs were equally effective against susceptible and resistant parasites, blocking intraerythrocytic parasite development. These data strongly suggest that these

drugs could be used alone or in association with malaria chemotherapy [88].

A comprehensive study of proteasome inhibitors active against *P. falciparum* laboratory strains and field isolates from Gabon showed that epoxomicin was highly active against *P. falciparum* and showed no signs of cross-resistance with similar drugs or any other proteasome inhibitor in an area with high-grade chloroquine resistance [89].

Although the *Plasmodium* proteasome has been suggested as potential antimalarial drug target, the toxicity of inhibitors has prevented validation of this enzyme *in vivo*. A screen of a library of 670 analogs of the recent US Food and Drug Administration-approved inhibitor, carfilzomib, was performed to identify compounds that selectively kill parasites. One of them, PR3, displayed significant parasite-killing activity *in vitro* but dramatically reduced toxicity in host cells. According to the authors, this parasite-specific toxicity was not due to selective targeting of the *Plasmodium* proteasome over the host proteasome but due to a lack of activity against one of the human-proteasome subunits. Subsequently, they used PR3 to significantly reduce parasite load in *P. berghei* infected mice without host toxicity, thus validating the proteasome as a viable antimalarial-drug target [90].

3.7. Toxoplasma. The *Toxoplasma gondii* proteasome has been examined in terms of its intracellular localization and enzymatic activity. Studies of immunofluorescence with antibodies against proteasome have shown that, unlike eukaryotic cells (in which the proteasome is located both in the nucleus and cytosol), in *Toxoplasma,* proteasomes are restricted to the cytosol. Chymotrypsin-like activity was detected, with *Km* values close to those observed in eukaryotic cells [91]. The pretreatment of free tachyzoites with proteasome inhibitors (10 μm lactacystin) or 5 μm gliotoxin [92] did not block the entry of the parasite or the formation of the parasitophorous vacuole but did block intracellular parasite growth and DNA synthesis. However, Shaw et al. [93] showed that lactacystin (2 μm) did not block parasite entry or the establishment of the parasitophorous vacuole but did inhibit parasite growth and daughter-cell budding, as well as DNA synthesis. Pretreatment of host cells with lactacystin did not block parasite entry or development. These results highlight the possible role of *Toxoplasma* proteasome activity in intracellular development and regulation of parasite replication. In the same way, parasite penetration of host cells was not modified by a high gliotoxin concentration (1 μm), but replication was markedly decreased (approximately 50% inhibition by 0.5 μm gliotoxin). Gliotoxin reduced the chymotrypsin-like activity of the *Toxoplasma* proteasome with five times lower potency than in HeLa cells [92]. The major findings about the role of proteasomes in protozoan parasites are summarized in Figure 2.

4. Concluding Remarks

Protease activity is essential to many biological systems and processes. In parasites, proteases are essential for host-tissue degradation, immune evasion, and nutrition acquisition [94]. Until less than twenty years ago, the presence and biological role of proteasomes in parasites was not known.

Since the first report of proteasomes in protozoa [63], the first description of its biological function [73], and the first description of the existence of the 26S proteasome in protozoa [74], several reports have confirmed the role of proteasomes in parasite biological processes such as differentiation, the cell cycle, proliferation, and encystation. Proliferation and differentiation are key steps in host colonization. Considering the importance of proteasomes in both processes in many different parasites such as *Trypanosoma*, *Leishmania*, *Toxoplasma,* and *Entamoeba*, parasite proteasomes might serve as virulence factors.

Despite the many parasitic biological phenomena in which the proteasome participates, information relating to how proteasome participates in such phenomena is not known. The majority of the parasite proteins that are degraded by proteasomes have not been identified. Proteasome targets and biological pathways involving proteasomes in key biological process remain to be clarified.

Proteasome inhibitors have been valuable research tools in cellular biology through the elucidation of important biological processes associated with the ubiquitin-proteasome protein-degradation pathway. The ubiquitin-proteasome system is a privileged pharmacological target for drug development due to the tremendous potential for intervention in multiple pathologies including cancer, neurodegenerative diseases, immune diseases, and infections. The pharmacological potential of the UPS was revealed after the unpredicted success of proteasome inhibitors for the treatment of some hematological malignancies.

Moreover, after US Food and Drug Administration approved bortezomib (Velcade) for the treatment of relapsed multiple myeloma, the proteasome has emerged as a new therapeutic target for diverse pathologies. Drug-discovery programs in academia and the pharmaceutical industry have developed a range of low-nanomolar natural and synthetic 20S-proteasome inhibitors and entered them in human clinical trials as significant anticancer and anti-inflammatory leads. The landscape of proteasome inhibitor-based therapeutics is quickly evolving, with promise beyond clinical oncology, and represents an exciting example of translational medicine.

Several pieces of evidence strongly suggest that the ubiquitin-proteasome pathway is also a viable parasitic therapeutic target. Research in recent years has shown that the proteasome is a valid drug target for sleeping sickness [72, 95, 96]. Although the structure of the trypanosome proteasome resembles that of its mammalian counterpart, the enzyme complexes differ from each other with respect to peptidase activity, substrate specificity, and inhibitor sensitivity. In addition, enzymatic analyses have demonstrated that the trypanosome and mammalian proteasomal functions are particularly sensitive to inhibition of the trypsin-like and chymotrypsin-like activities, respectively [97, 98]. Thus, compounds specifically targeting the trypsin-like activity of the trypanosome proteasome may constitute a basis for

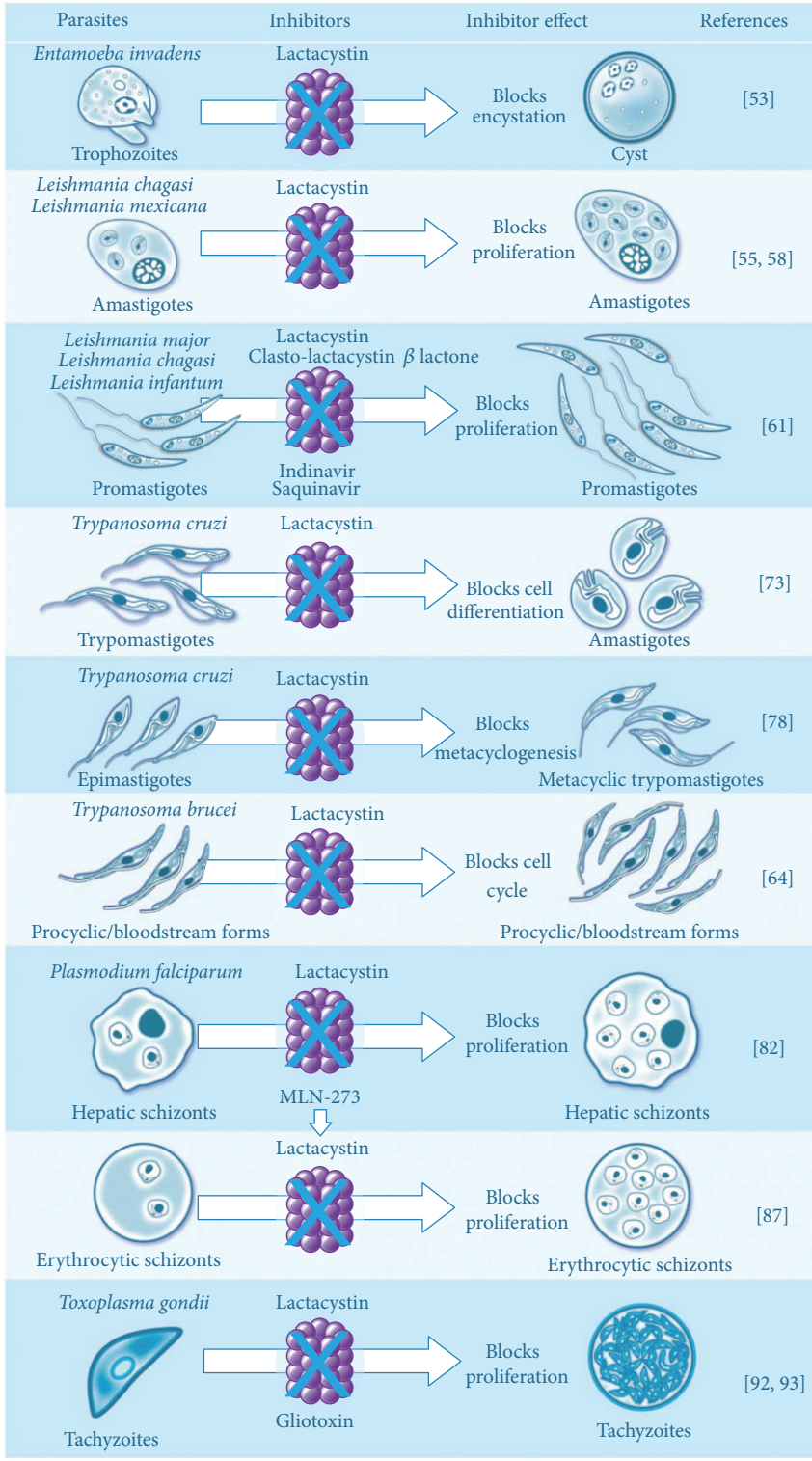

Figure 2: Role of the ubiquitin proteasome system in biology of protozoan parasites and effect of different proteasome inhibitors on proliferation and cell differentiation.

rational antitrypanosomal drug development. However, the emergence and spread of *P. falciparum* resistance to existing antimalarials necessitates the search for novel drug targets and chemotherapeutic compounds. Inhibition of the proteasome is a promising strategy to develop novel antimalarial drugs.

Diseases caused by parasites affect hundreds of millions of people worldwide, with devastating health and economic effects; however, parasites have been largely neglected in terms of drug development because they affect poor people in poor regions of the world. Most of the drugs currently used to treat these diseases are decades old and have many limitations, including drug resistance. Proteasomes are a key organelle in parasite biology and virulence and appear to be an attractive new chemotherapeutic target.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effect of Clinoptilolite and Sepiolite Nanoclays on Human and Parasitic Highly Phagocytic Cells

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Nanoclays have potential applications in biomedicine raising the need to evaluate their toxicity in *in vitro* models as a first approach to its biocompatibility. In this study, *in vitro* toxicity of clinoptilolite and sepiolite nanoclays (NC) was analyzed in highly phagocytic cultures of amoebas and human and mice macrophages. While amebic viability was significantly affected only by sepiolite NC at concentrations higher than 0.1 mg/mL, the effect on macrophage cultures was dependent on the origin of the cells. Macrophages derived from human peripheral blood monocytes were less affected in viability (25% decrease at 48 h), followed by the RAW 264.7 cell line (40%), and finally, macrophages derived from mice bone marrow monocytes (98%). Moreover, the cell line and mice macrophages die mainly by necrosis, whereas human macrophages exhibit increased apoptosis. Cytokine expression analysis in media of sepiolite NC treated cultures showed a proinflammatory profile (INFγ, IL-1α, IL-8, and IL-6), in contrast with clinoptilolite NC that induced lees cytokines with concomitant production of IL-10. The results show that sepiolite NC is more toxic to amoebas and macrophages than clinoptilolite NC, mostly in a time and dose-dependent manner. However, the effect of sepiolite NC was comparable with talc powder suggesting that both NC have low cytotoxicity *in vitro*.

1. Introduction

Clinoptilolite and sepiolite clay are zeolites that belong to a complex group of aluminosilicates used for nanocomposites applications [1, 2]. They are used as metal oxides supports, antimicrobials [3], enzyme stabilizers [4], for absorption of heavy metals [5–7], and additives for the development of nanocomposites [8]. Because of this, in the last decade they have attracted increasing interest in biomedicine, mainly, nanoclays (NC) dispersed into polymeric matrices which have been proposed as good candidates for drug delivery systems [9–14], dental adhesives [15], bone tissue engineering [16], and immunosensors [17]. However, their use in humans has been hampered by the insufficient information regarding their safety, and toxicological assessment on *in vitro* and *in vivo* models is absolutely necessary [18–20]. These evaluations are needed because the properties of nanomaterials, such as the surface area, zeta potential, and size, can modify their biological interactions compared to microsized materials [21]. Moreover, it has been reported that toxicity of nanomaterials also depends on the model used [22], emphasizing the need for appropriate methodologies and a unified evaluation [23].

According to the International Agency for Research on Cancer, there are few reports regarding *in vitro* and *in vivo* clinoptilolite and sepiolite NC biocompatibility [24]. In this sense, macrophages can be an appropriate model for *in vitro* cytotoxic studies [25, 26] due to the NC applications as nanovehicles which can reach the bloodstream and other tissues. Macrophages are pivotal cells of the innate immune response, specialized in the scavenging of foreign bodies in mammals and widely used in toxicity assays [27–29]; besides, macrophages are considered one of the most phagocytic cells

in mammals. On the other hand, the amoeba *Entamoeba histolytica*, the protozoan parasite causing human amoebiasis, is among the most active phagocytic and proteolytic cells in nature, and it has been used as a model to evaluate toxicity of carbon nanotubes [30]. Even though the two systems are quite far apart (mammal and protozoan, for defense and feeding, resp.), they share the characteristic of being the most active highly phagocytic cells in nature, favoring the uptake of the material and the study of toxicity thereof even at low concentrations. In this work, we evaluated the cytotoxicity of clinoptilolite and sepiolite NC *in vitro* by determining their effect on the viability of macrophages from human, mice, and the RAW 264.7 cell line as well as in *E. histolytica* trophozoite cultures, the type of cell death induced (apoptosis or necrosis), and the cytokine profiles released by treated macrophages, all of them as a first approach to determining clinoptilolite and sepiolite NC biocompatibility.

2. Materials and Methods

2.1. Characterization of Clinoptilolite and Sepiolite NC. Characterization analysis was performed in the USAI, Facultad de Química, UNAM. To determine the chemical structure of NC, X-ray powder diffraction was obtained in a Bruker diffractometer model D8 Advance, with a copper anode as X-ray source ($K_{\alpha 1}$ = 0.154060 nm); chemical composition was determined using a ICP-ms, Bruker Aurora M90 following the percentage of the enlisted elements Si, Al, Fe, Ca, Mg, Ti, P, Mn, Na, K, and S. Finally, samples after the suspension procedure in culture media (below) were observed in Low Vacuum Transmission Electron Microscopy (TEM) and Scanning Electron Microscopy (SEM). Qualitative and semiquantitative microanalysis was performed to determine the size and shape of nanoclays.

2.2. Nanoclays and Talc Suspension. Suspensions of clinoptilolite NC (Valfor-100, Silicatos y derivados S.A. de C.V. Mexico), sepiolite NC (sepiolite powder, Sigma-Aldrich, USA) and asbestos-free talc powder (Talc, tested according to Ph. Eur; Sigma-Aldrich, USA) were obtained by sonication of each NC in culture media four times during 15 s each with amplitude of 50% and a frequency of 130 kHz using a sonicator tip Branson Sonifier, USA. TYIS-33 media supplemented with 10% of adult bovine serum and high glucose DMEM media supplemented with 10% of fetal bovine serum were used for amoeba and macrophages, respectively. This treatment was efficient preventing NC aggregates without affecting particle size (data not shown). Stock suspensions of each NC containing 1000 μg/mL in each media were prepared as mentioned, stored at 4°C, and used for the following experiments.

2.3. Parasite Culture and Treatment. *E. histolytica* HM1-IMSS trophozoites were axenically grown at 37°C in TYI-S33 medium supplemented with 10% bovine serum, 3% vitamins (Diamond Vitamin Tween 80 Solution 40x, Sigma Aldrich, USA), and 0.1% antibiotic (Penicillin-Streptomycin 10,000 U/mL, GIBCO, USA). Amoebas (1×10^5/mL) were placed in tubes with supplemented TYI-S33 and added with clinoptilolite or sepiolite NC so that the final volume was 3 mL and the concentrations of clinoptilolite and sepiolite NC were as follows: 10, 100, 500, and 1000 μg/mL.

2.4. Amoebic Viability. The viability and morphology of amoebic trophozoites were assessed at 24, 48, and 72 h of coincubation with the NC employing two different methods: (1) the vital marker trypan blue to evaluate viability and (2) the carboxyfluorescein diacetate (CFDA Vibrant kit, Invitrogen, USA) plus propidium iodide to evaluate morphology. In brief, amoebic culture tubes were incubated on ice for 5 min in order to detach the parasites, and 10 μL of Trypan blue 0.4% or 1 μL CFDA 5 μM plus 1 μL propidium iodide 1.5 μM was added to aliquots of 100 μL and incubated at room temperature for 15 min. Viable and dead cells were counted in a fluorescence microscope Olympus BX51 using a haemocytometer. Of six independent experiments each one by triplicate was done for each NC assayed.

2.5. Macrophage Culture. Macrophages from three different sources were used: macrophages derived from human peripheral blood monocytes (HMDM), macrophages derived from CD1 mice bone marrow monocytes (MMDM), and the RAW 264.7 cell line. HMDM were derived from monocytes isolated from blood samples of 10 healthy individuals at the Hospital Arnau de Vilanova (Lleida, Spain) with written consent. Monocytes were isolated in a Ficoll gradient and then placed in Petri dishes with supplemented RPMI 1640 medium at 37°C under 5% CO_2 for 5 days, changing the medium every 48 h for the monocytes differentiation into macrophages.

MMDM were obtained from four-week-age CD1 mice. Once euthanized, the femur and tibia were carefully removed and kept in PBS. After treatment with absolute ethanol for 3 min, the femur and tibia were washed with RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% gentamicin, and 1% of 2-mercaptoethanol. Bone marrow cells were removed from the bones using scissors, washed twice with nonsupplemented RPMI 1640 medium, and followed by erythrocytes lysis. Adherent white blood cells were washed with PBS and suspended in supplemented RMPI 1640 medium. Cells were harvested in Petri dishes with 10 mL of medium and incubated at 37°C and 5% CO_2, changing the medium every 48 h during 5 days for monocytes differentiation into macrophages. The RAW 264.7 cell line was cultured in RPMI 1640 medium supplemented as described above and maintained at 37°C under a 5% CO_2. Macrophages were sorted by flow cytometry using a specific F4/80 antibody.

2.6. Macrophages Treatment with the NC and Viability Assays. Effect of clinoptilolite and sepiolite NC was determined for the three types of macrophages. For each experiment, 1×10^5 macrophages per well were placed in 96-well plate with 100 μL of supplemented RPMI 1640 and enough NC suspension to reach concentrations of 10, 100, 500, and 1000 μg/mL in each well. RAW 264.7 and MMDM culture treated were incubated for 60 h, whereas HMDM were

incubated only during 48 h, taking an aliquot every 12 h for determining viability and death as described below. Of three independent experiments each one by triplicate was done for each NC assayed. There are no reports of the concentrations of clinoptilolite and sepiolite NC to which humans could be exposed; however, the reports where NC toxicity has been analyzed reported concentrations between 1 and 1000 μg/mL.

2.7. Transmission Electron Microscopy. Amoebic and macrophage culture treated with each NC were centrifuged at 1800 rpm for 5 min and washed 3 times with phosphate buffer solution (PBS, pH 7.4). Cells were then fixed in 4% formaldehyde and 1% glutaraldehyde in PBS by mixing equal volume of fixative and cell suspension. After centrifugation at 1800 rpm for 10 min, the pellet was kept in fresh fixative overnight. Then, the cells were treated 3 times for 15 min with 8% (0.2 M) sucrose in PBS after fixation with 1% OsO4 in PBS for 1 h and rinsed with PBS for 30 min. For dehydration process, ethanol solutions (50, 70, and 95%) were added to the pellets for 15 min each one, absolute ethanol for 15 min twice, and 100% of propylene oxide for 30 min. Infiltration was done with LR white resin (Ted Pella Inc., USA), first adding 1 : 1 LR White: Propylene Oxide for 2 h to the pellets and then stored overnight in 2 : 1 LR White: Propylene Oxide. Samples were embedded in gelatin capsules and baked in 60°C oven for 48 h. Ultrathin sections of 0.5 μm were collected on Formvar/Carbon 200 mesh and Nickel grids and stained with uranyl acetate for 15 min and lead citrate for 3 min.

2.8. Viability, Apoptosis and Necrosis. The viability, apoptosis, and necrosis of macrophages treated with NC were determined every 12 h using the Annexin V-FITC Apoptosis detection kit I (BD Pharmingen, USA), according to the protocol of BD Pharmingen. Briefly, for each time point, the culture medium of each well was placed in an Eppendorf Tube and stored at −20°C until use for cytokine analysis, and the wells were refilled with 100 μL of PBS. Annexin V and propidium iodide markers were added and the cells further incubated for 15 min. After the incubation time, the cells were fixed with 50 μL of a stock solution of p-formaldehyde 3.7%, and the samples were read in a FACS Canto flow cytometer (Becton Dickinson, USA).

2.9. Th1, Th2, and Treg Cytokines Determination. The cytokine secretion pattern of macrophages incubated with clinoptilolite or sepiolite NC was determined in the supernatant of cultures at 24, 36, and 48 h after coincubation. The expression of GM-CSF, IFN-γ, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17, and TNF-α was measured using the mouse and human Th1/Th2 10plex FlowCytomix Multiplex kit (eBioscience, USA) according to the provider protocol. Briefly, marked beads were added to the supernatant, and after incubation for 15 min the cytokines were measured in a FACS Canto flow cytometer.

2.10. Statistical Analysis. Data were analyzed with a Two-way ANOVA ($P < 0.05$) followed by a Tuckey post hoc test ($P < 0.05$) (Microsoft Excel, 2010).

3. Results

3.1. Characterization of Clinoptilolite and Sepiolite NC. X-ray powder diffraction pattern of nanoclays showed a typical distribution of diffraction planes associated with the sepiolite and clinoptilolite zeolites. Sepiolite NC pattern showed a perfect match with the pattern obtained from PDF-2 database of ICCD (International Center for Diffraction Data) and the clinoptilolite NC pattern corresponded to a Nickel Ammonium Aluminium Silicon Hydroxide Oxide Hydrate (see Figure S1 in the Supplementary Material available online at http://dx.doi.org/10.1155/2015/164980). The chemical composition of nanoclays was determined by atomic absorption spectroscopy resulting in clinoptilolite nanoclays: 21.7% Na_2O, 0.0078% MgO, 57.3% SiO_2, 9.2% Al_2O_3, 1.4% CaO, 1% TiO_2, 0.01% MnO, and for sepiolite nanoclays: 2.16% Na_2O, 27.79% MgO, 37.15% SiO_2, 12.01% Al_2O_3, 3.72% K_2O, 3.92% CaO, 3.60% TiO_2, and 7.22% MnO. In addition, the microanalysis of metallic elements and carbon was evaluated in the SEM micrographs (Figure S2). Results showed that 80% of clinoptilolite nanoparticles had a size down to 30 nm, with an average size of 17.5 nm, and 70% of sepiolite nanoparticles had a diameter size down to 20 nm. As this nanoclay is a fiber, fibers over 500 nm were also found (Figure 1).

3.2. Effect of Clinoptilolite and Sepiolite Nanoclays on E. histolytica Cultures. The viability and growth of *E. histolytica* trophozoites were affected to different extents by the nanoclays. When treated with clinoptilolite NC, viability and growth were not significantly affected by incubation with any concentration at any time evaluated (Figure 2(a)). The integrity of trophozoites treated with clinoptilolite NC was confirmed on CFDA plus PI stained cells under fluorescence microscopy. In contrast, treatment with sepiolite NC, decreases the viability of trophozoites around 13 to 21% at 72 h with concentrations higher than 100 μg/mL in a dose-dependent manner ($P < 0.05$ with respect to the untreated cultures). A slight recovery of amoebic viability was observed at 48 h for cultures treated with 100 and 500 μg/mL; this recovery was apparent for cultures treated with 1000 μg/mL until 72 h posttreatment (Figure 2(b)). The staining with CFDA plus PI showed that viability of amoebas seems to diminish over the time in a dose dependent manner, shifting from green to yellowish fluorescence, including red nuclei of death cells (Figure 2(c)).

In order to demonstrate the uptake and cellular location of NC on treated cells, transmission electron microscopy (TEM) of *E. histolytica* trophozoites and human peripheral blood macrophages treated for 24 h with 100 μg/mL of each NC was performed. Clinoptilolite NC were found dispersed in the cytosol and as aggregates inside the amoebic vacuoles (Figure 3(b)); in contrast, sepiolite NC were mainly found as aggregates inside large vacuoles and, in some cases, seem to cause the rupture of the vacuolar membrane (Figure 3(c), red arrow). In HMDM, clinoptilolite NC were observed inside phagocytic vacuoles larger than those observed in the amoebic cultures (Figure 2(e), green arrow); in the case of sepiolite NC, the distribution and size of the phagocytic

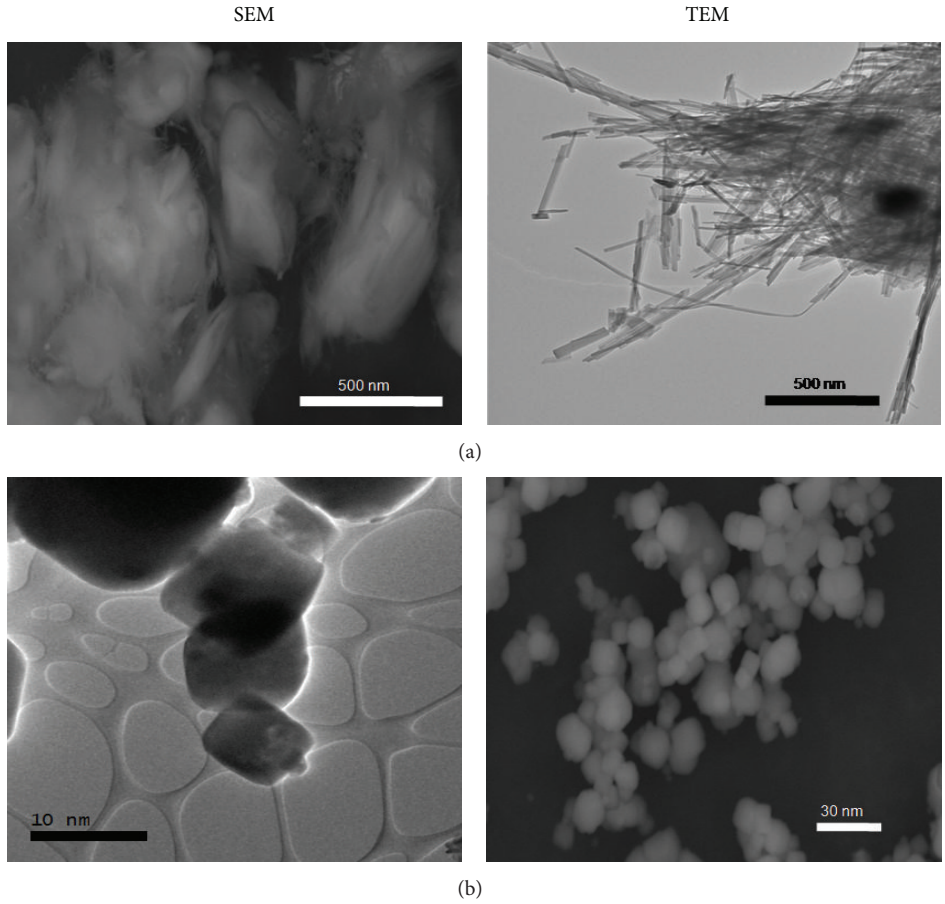

Figure 1: Transmission and scanning electron microscopy of sepiolite and clinoptilolite nanoclays prepared in the DMEM culture media. Sepiolite nanoclays are fibers with a diameter size down to 20 nm (a), whereas clinoptilolite nanoclays are deformed octahedrons with a size down to 30 nm (b).

vacuoles were similar to those of amoebas (Figure 3(f), pink arrow).

3.3. Effect of Clinoptilolite and Sepiolite NC on Macrophage Cultures. A dose and time dependent effect was observed on the viability of macrophages when treated with NC and talc, used here for comparing with the nanoparticle due to his long and proven history of safe use [31] as well as *in vitro* modest effect on peritoneal mouse macrophages [32]. Viability of RAW 264.7 macrophages decreased 15% at 24 h, reaching 20% at 60 h when treated with clinoptilolite NC, whereas sepiolite NC and talc affected the viability by 25% at 24 h to around 40% at 60 h (Figure 4; upper panels). HMDM showed a similar pattern but were less affected; in this case, clinoptilolite and sepiolite NC decreased the viability in around 25% and talc 14% at 60 h. However, a dramatic effect on the viability was rapidly observed in MMDM cultures, decreasing it to 65%, 73%, and 82% when treated with clinoptilolite NC, sepiolite NC, and talc at 24 h, respectively. The viability drops to 80%, 98%, and 88% at 60 h, respectively (Figure 4, upper panels). Cell death determined in the treated macrophage cultures using the Annexin V/propidium iodide kit showed that most RAW 264.7 and MMDM died by necrosis at all assay time, with no clear distinction between the different treatments (Figure 4, lower panels). In contrast, HMDM cultures, which were the least affected in viability, showed that about two thirds of the cells die by apoptosis at all times tested, suggesting that the cell processes that activate in macrophages the clinoptilolite NC are different than those activated by sepiolite NC and talc, leading to different outcomes (Figure 4, middle panels).

3.4. Cytokine Secretion Pattern from HMDM and MMDM Treated with NC. The cytokines secreted by HMDM and MMDM (not RAW 264.7) in the presence of both NC and talc were determined in the culture media at 24, 36, and 48 h post-treatment by flow cytometry. Cytokines were undetectable at 12 h (data not shown) and were not determined at 60 h. In general terms, proinflammatory cytokines increased over the time in the two types of macrophages, but at different levels depending on treatments (Figure 5). A proinflammatory profile was clearer with sepiolite NC and the talc treatments, compared with clinoptilolite NC treatment. Thus, in HMDM treated with sepiolite NC or talc, some amounts of IL-1α and IL-6 were detected that slightly increased over the time. In contrast, release of IL-6 and INFγ was not detected

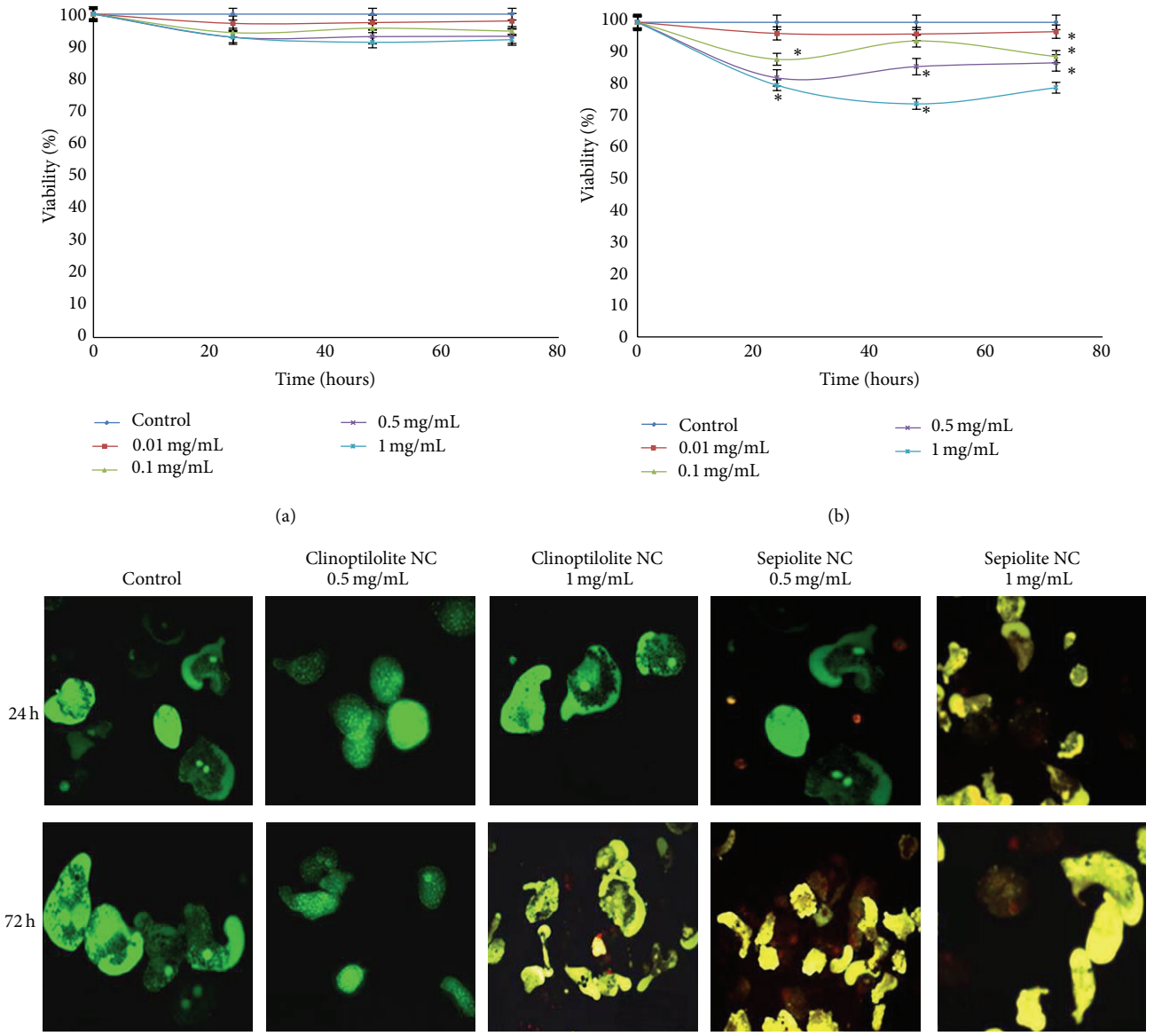

Figure 2: Viability of *E. histolytica* after the treatment with nanoclays. Amoebic cultures were treated with clinoptilolite (a) or sepiolite (b) NC for the period of time indicated and the viability measured by the Trypan blue method. Bottom pictures show CFDA/PI stained trophozoites from the treated cultures at the concentrations and time indicated. Controls are amoebas from a culture without treatment. The shifting of green to yellowish fluorescent indicates decrease of viability. Red nuclei come from dead cells.

in neither MMDM nor HMDM treated with clinoptilolite at any time evaluated (Figures 5(a) and 5(b), resp.). The cytokine/chemokine IL-8 was released without a clear pattern by any treatment, but its production was considerably higher in MMDM treated with sepiolite NC or talc, compared with clinoptilolite NC ($P < 0.05$) (Figure 5(a)). Few levels of this cytokine were detected in medium from HMDM treated. The other cytokine highly expressed was IL-17, which appeared under any treatment at different times, with its production being higher in MMDM and HMDM treated with talc. INFγ was increasingly induced only by talc in MMDM and by sepiolite NC and talc in HMDM, but not by clinoptilolite NC. GM-CSF and Th2 cytokines IL-4 and IL-5 were not detected under any condition tested (not shown). Low levels of the regulatory cytokine IL-10 were detected in media from treated MMDM (Figure 5(a)) in contrast with high levels detected in HMDM treated with clinoptilolite NC with respect to sepiolite NC and talc ($P < 0.05$) (Figure 5(b)).

4. Discussion

Clinoptilolite and sepiolite have been proposed for pharmaceutical applications including tablet manufacture [33], slow release systems [34], in combination with drugs for cancer therapy [35], being as adjuvants [36], being as adsorbent trapping lead in children with ADHD by intravenous administration [5], and, in general terms, for diverse therapy in humans [37]. In this sense, the development of nanoparticles as drug

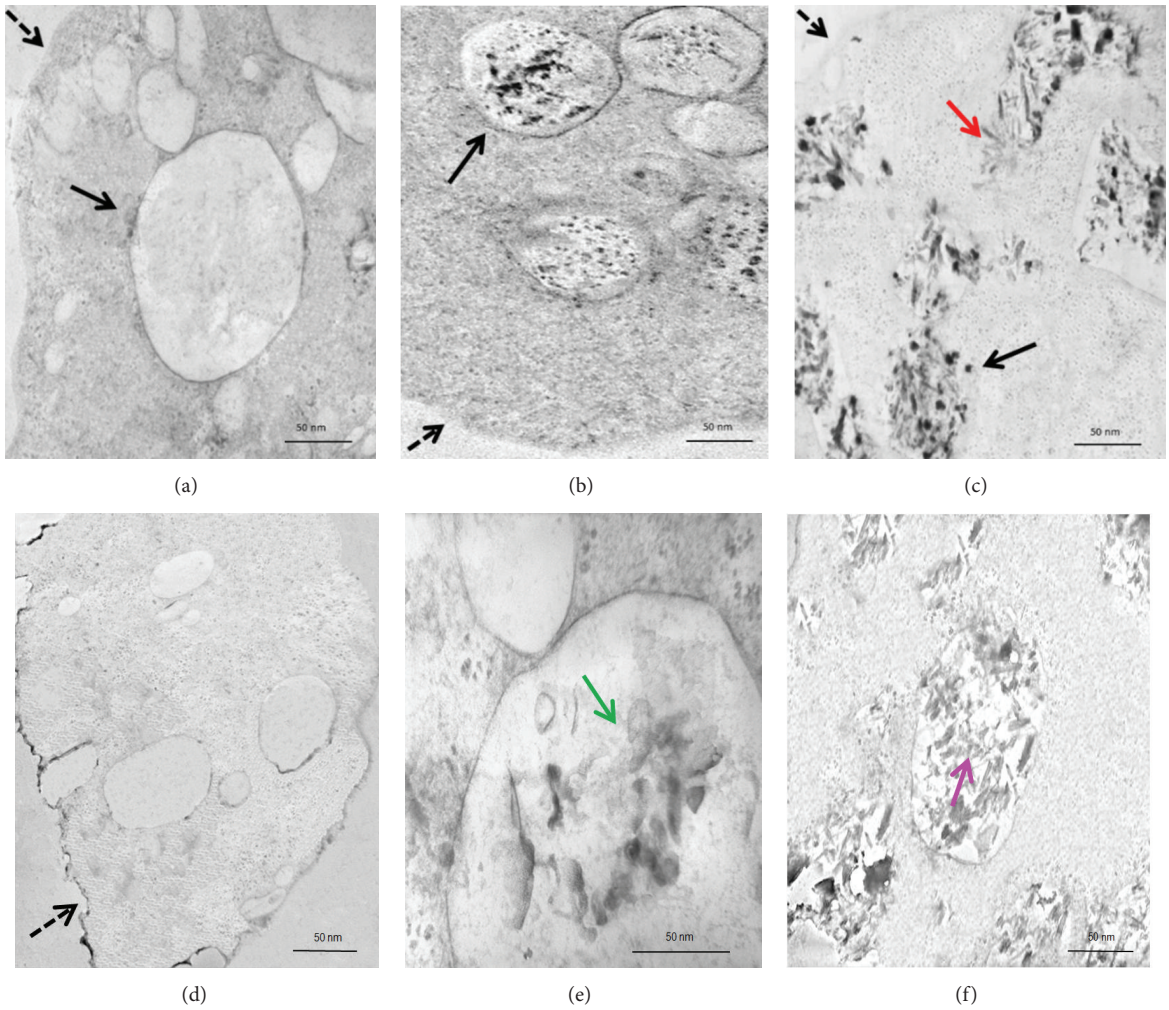

Figure 3: Uptake of nanoclays by amoebas and macrophages. TEM of 5 μm thickness cross section of amoebas (a–c) and human macrophages (d–f) nontreated (a) and (d) or treated with clinoptilolite NC (b) and (e) or sepiolite NC (c) and (f). Dashed arrows are showing the cytosolic membrane of amoebas and macrophages. Black arrows are showing the vacuolar membrane, many of them containing high accumulation of clinoptilolite and sepiolite NC. Red arrow shows a possible vacuolar membrane rupture for sepiolite NC accumulation. The green and pink arrows show the accumulation of clinoptilolite and sepiolite NC in macrophages, respectively.

delivery systems increased the interest in nanoclays [3–8] due to their physical and chemical properties in comparison to the natural clays. However, even if the toxicity of microsized clays has been evaluated [38, 39], the International Agency for Research on Cancer reports that there are few reports regarding *in vitro* and *in vivo* clinoptilolite and sepiolite NC biocompatibility [24]. Thereby, in this work we evaluated the cytotoxic effect of clinoptilolite and sepiolite NC in two of the most highly phagocytic cells reported [27–29], as a first approach to determining nanoclays biocompatibility. One of the precautions that need to be taken into account for *in vitro* tests with clinoptilolite is that this nanoclay could modify the ion composition of the culture media and thereof mask the toxicity of this nanoclay [40]. In order to prevent this, nanoclay suspensions stocks were prepared in supplemented TYI-S-33 and DMEM culture medium, exposing the nanoclays to serum and glucose prior to its addition to the experimental cultures decreasing the probability of affecting the cellular cultures by the ion-exchange features of NC. In addition, the nanoclays suspensions were sonicated in order to reduce any possible unspecific effect of the NC aggregation. With this treatments we assumed that ion composition of the medium and dispersion ratio of NC was nearly constant during the experiments, so neither of the two is the principal cause of cellular death in our cultures.

Our results showed that even when both NC were highly phaghocytosed by amoebas, clinoptilolite NC was not toxic to *E. histolytica* trophozoites, in contrast to sepiolite NC that showed significant cytotoxic effect, suggesting that clinoptilolite is less toxic than sepiolite NC, at least against the parasite. As the sepiolite NC-treated trophozoites did not show evidence of lysis or significant morphological changes, we think that amoebas probably die by an apoptotic process, a type of cellular death known that occurs in amoeba [41] (Figure 2). However, apoptosis of amoeba was not analyzed in this work and should be conducted in further studies.

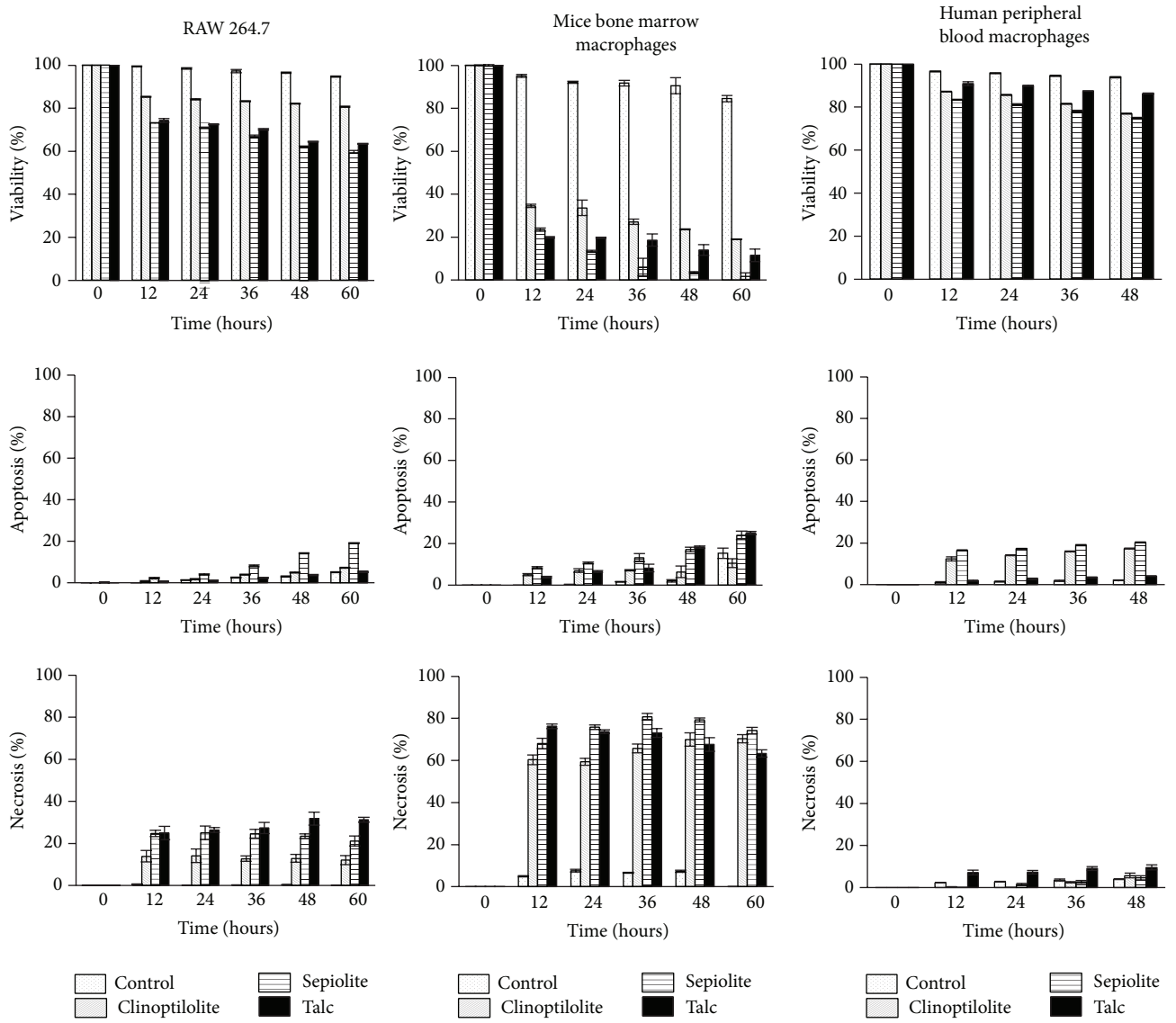

Figure 4: Viability, apoptosis, and necrosis of macrophages culture after treatment with nanoclays. Macrophages of RAW 264.7 cell line, from mice bone marrow monocytes and from human peripheral blood monocytes stained with Annexin V and propidium iodide to evaluate viability, apoptosis, and necrosis. Treatments with nanoclays and talc at 0, 12, 24, 36, 48 and 60 h are shown.

On the other hand, viability of macrophages cultures was affected at different extents, but in a dose and time dependent manner by the NC. Even when the viability of the macrophages was affected in a larger extent than the *E. histolytica* trophozoites, in agreement with the results obtained with amoebas, clinoptilolite NC were less toxic than sepiolite NC in all the macrophage cultures tested, supporting clinoptilolite NC as more biocompatible. However, sepiolite NC treatment showed a comparable effect with asbestos-free talc powder used as nanoparticle compound with a proven long history of safe use [31], suggesting that even when sepiolite NC is more cytotoxic than clinoptilolite NC, both are relatively harmless. Sohaebuddin et al. [22] have reported that a different type of cells has a different cytotoxic response against nanoparticles. Remarkably, we observed in this study that the cytotoxic effect also depends on the cell origin, with the MMDM cultures being the most affected (more than 80% of viability reduction at 60 h) followed by RAW 264.7 and finally HMDM (Figure 4). The RAW 264.7 cell line has widely been used for analyzing cytotoxicity of silica, polymers, metal oxides, silver, and gold nanoparticles [42–45], including two reports on hydroxyapatite and boehmite NC [46, 47], showing higher susceptibility than the results obtained here with the NC. Thus, our results suggest that not only the type but also the origin of the cell should be taken into account when testing cytotoxicity of NC, which could be extensive to any nanoparticle. However, we cannot rule out that other factors can influence the results and therefore, such proposal needs to be confirmed using a wide variety of cells from different sources.

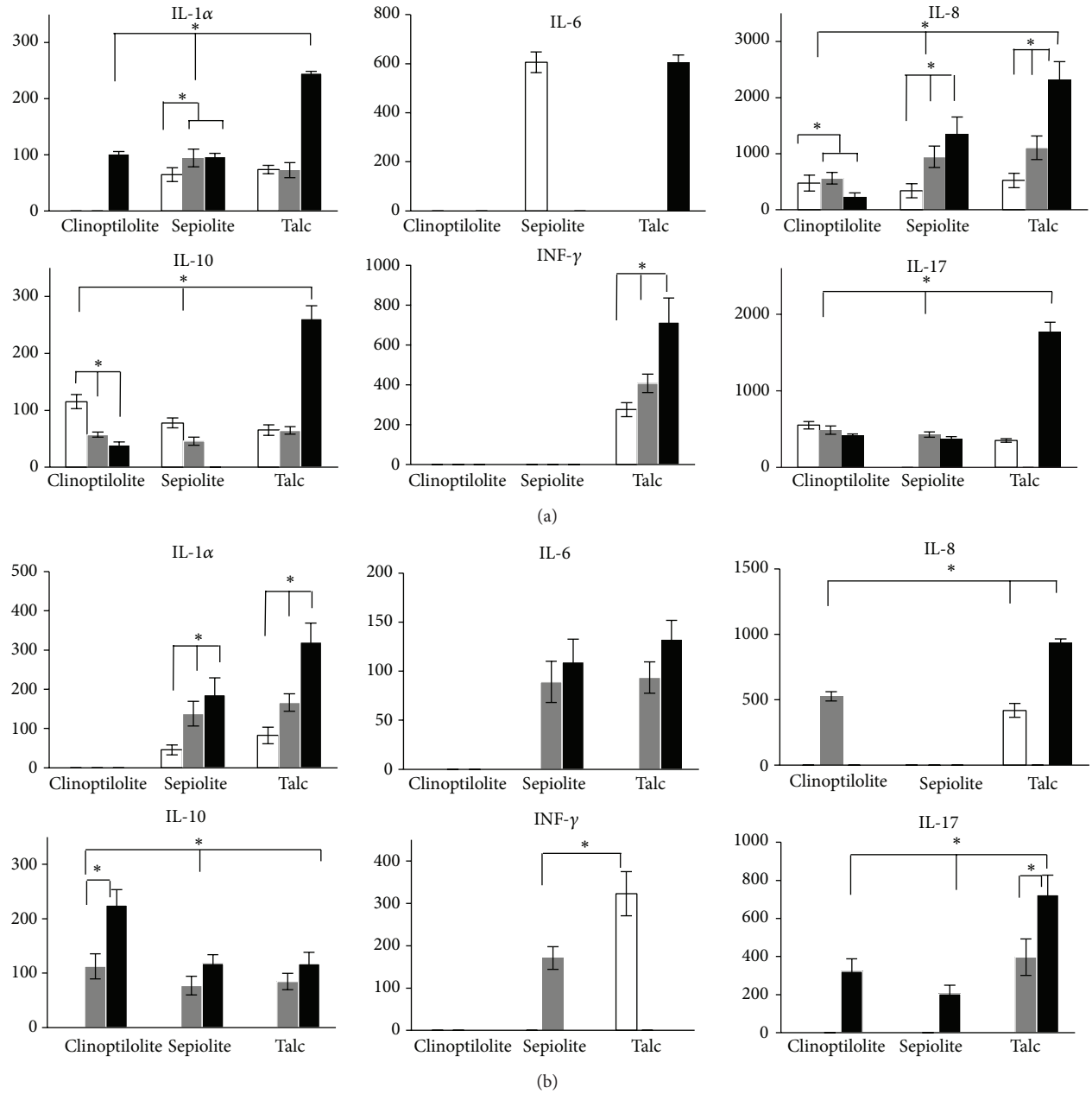

Figure 5: Cytokine secretion pattern from NC treated macrophages cultures. A panel of Th1 and Th2 cytokines was determined in the culture media of mice bone marrow macrophages (a) and human peripheral blood macrophages (b) at 24 h (open bars), 36 h (gray bars), and 48 h (black bars) posttreatment with NC and talc. All cytokine levels are shown in pg/mL $^{*}P < 0.05$.

In this work, the results obtained in the RAW 264.7 cell line macrophages were similar to the effect reported for montmorillonite NC on the human hepatic cell line HepG2, where cell viability was reduced in more than 20% with the same dose of 1 mg/mL [48]. Even when different cell lines were used, clinoptilolite NC and montmorillonite NC showed a similar effect on viability probably due to their structure [49]. However, we cannot rule out the possibility of differences associated with the particular characteristics of each cell line. Remarkably, HMDM cultures were the less affected by the treatments with NC or talc, never showing more than 25% of viability reduction at the time and doses tested (Figure 4). This is particularly interesting if we consider that potential biomedical applications in humans of NC will lead in any point to the encounter of the nanoparticles with the highly phagocytic scavenger macrophage, and its use is highly recommended by the international standard ISO 10993-5 for the biological evaluation of medical devices. In this sense, HMDM has been used to evaluate the toxicity of many particles present in dust, polluted air, polymers, and others [50–52]. However, in our knowledge, HMDM cultures have only been used to assess the cytotoxicity of MWCNTs

and nanoparticles of titanium and zinc metal oxide, but not nanoclays [53, 54].

Interestingly, uptakes of both NC by amoebas and HMDM appear to be similar, due to their location inside large phagocytic vesicles or lysosomes, suggesting a phagocytic or macropinocytic process (Figure 3). In this sense, the greater aggregation of sepiolite NC inside larger vesicles could affect the vacuolar membrane compared to the less aggregation of clinoptilolite NC, which could be also related with the higher cytotoxic effect of sepiolite NC on the cultures. As the toxicity of nanoparticles depends on the size and composition of the material [22] and the aggregation state is associated with the NC structure, the nanofiber structure of sepiolite NC could aggregate and puncture the vesicle membrane more easily than the deformed octahedron clinoptilolite NC, explaining the higher toxic effect of sepiolite NC.

Regarding the type of cell death induced by the NC, noteworthy, the predominant cell death was different depending on the macrophage culture studied. Thus, RAW 264.7 and MMDM cultures mainly died by necrosis (two- and threefold over the number of apoptotic cells, resp.), whereas HMDM preferably died by an apoptotic pathway (twofold over the number of necrotic cells). HMDM death could be comparable with amoebic cultures, where the morphology of the death trophozoites suggests an apoptotic process. The mechanisms triggering the macrophage death by the NC and talc as well as the underlying signaling events leading to apoptosis or necrosis are unknown, but they could involve surface scavenger receptors and activation of mitochondrial caspase 9 as described for the toxic effect of zinc oxide nanoparticles [55].

In terms of cytotoxicity, apoptosis death could be more preferable than necrosis, due to the potential of necrotic cellular debris to promote a proinflammatory response that is associated with tissue damage [56]. The proinflammatory response involves the release by the macrophages and other innate cells of cytokines and chemokines that promote recruitment of new cells to the site of infection or damage. Therefore, the cytokine secretion pattern in the supernatant of primary culture macrophages (MMDM and HMDM) treated with the NC and talc was analyzed by flow cytometry. As expected, a correlation between the cytokine patterns released by the NC exposed macrophages and the observed cellular death pathways was found. Thus, the MMDM cultures, which were the most affected by the treatments and mainly dying by necrosis, secreted higher levels of proinflammatory cytokines Il-1α, IL-8, and IL6, in comparison with HMDM cultures. In agreement with our results, it has been reported that human macrophages/monocytes stimulated with single-walled carbon nanotubes (CNT) or silica induced the release of IL-1α, IL-6, and IL-8 associated with a proinflammatory outcome [53]; also, the application of natural clinoptilolite in mice food for 28 days produced an increased serum LSA concentration which could be related with the release of TNF-α and IL-1 by macrophages [57]. As mentioned before, MMDM cultured with NC released higher amount of proinflammatory cytokines than HMDM, mainly IL-8 and IL-6 (Figure 5). In addition, the release of IL-8 in MMDM was higher with sepiolite NC than with clinoptilolite NC. The induction of some of these proinflammatory cytokines by the NC could be related to their agglomeration state [58] and to the particle size, the bigger the particle the higher release [59]. Therefore, the higher IL-8 release from MMDM in the presence of sepiolite NC could be related to the bigger size of sepiolite NC in comparison with clinoptilolite NC.

This was also observed for INFγ, another important proinflammatory cytokine, which was only detected in two time points of HMDM treatment with sepiolite NC and talc (36 h and 24 h postexposure, resp.), but not with clinoptilolite NC (Figure 5). On the other hand, TNFα was undetectable in the macrophage cultures with any NC or talc (not shown), suggesting low toxicity as the most biocompatible materials have been shown to induce low TNF-α levels that tend to drop to zero over the time [60]. Moreover, the expression of cytokine IL-17 at late time (48 h) suggests that NC and talc have the potential to induce an allergic response, as it has been described in alveolar macrophages of animals orally treated with PLGA NPs coated with chitosan and PEG [61]. In addition to the proinflammatory pattern of cytokines, HMDM cultures release higher amount of IL-10 than MMDM cultures in the presence of NC, a regulatory cytokine probably produced to counterbalance the proinflammatory profile. Interestingly, the expression of IL-10 has been associated with a greater biocompatibility contributing to the inhibition or resolution of the inflammation associated with nanocomposites [59], which could be related with the lower toxicity of NC on HMDM.

The results of this work showed that clinoptilolite and sepiolite NC are well tolerated when tested in highly phagocytic cell cultures, showing results comparable with asbestos-free talc powder suggesting that both could be highly biocompatible. However, when compared, clinoptilolite NC appears to be less toxic than sepiolite NC, which is very important taking into account the potential biomedical application of clinoptilolite in humans. These cytotoxic assays could contribute to the necessary knowledge for future application of nanoclays; however, additional studies regarding the cellular physiology alterations of cells from different lineages as well as *in vivo* studies at short and long term exposure to confirm the safety of clinoptilolite and sepiolite nanoclays are necessary before thinking in their use for biomedical applications.

Conflict of Interests

The authors declare that there is no conflict of interests regarding to the publication of this paper.

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Research Article

Protection against Amoebic Liver Abscess in Hamster by Intramuscular Immunization with an *Autographa californica* Baculovirus Driving the Expression of the Gal-Lectin LC3 Fragment

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In a previous study, we demonstrated that oral immunization using *Autographa californica* baculovirus driving the expression of the Gal-lectin LC3 fragment (AcNPV-LC3) of *Entamoeba histolytica* conferred protection against ALA development in hamsters. In this study, we determined the ability of AcNPV-LC3 to protect against ALA by the intramuscular route as well as the liver immune response associated with protection. Results showed that 55% of hamsters IM immunized with AcNPV-LC3 showed sterile protection against ALA, whereas other 20% showed reduction in the size and extent of abscesses, resulting in some protection in 75% of animals compared to the sham control group. Levels of protection showed a linear correlation with the development and intensity of specific antiamoeba cellular and humoral responses, evaluated in serum and spleen of hamsters, respectively. Evaluation of the Th1/Th2 cytokine patterns expressed in the liver of hamsters showed that sterile protection was associated with the production of high levels of IFNγ and IL-4. These results suggest that the baculovirus system is equally efficient by the intramuscular as well as the oral routes for ALA protection and that the Gal-lectin LC3 fragment is a highly protective antigen against hepatic amoebiasis through the local induction of IFNγ and IL-4.

1. Introduction

Entamoeba histolytica is the protozoan parasite that causes amoebiasis in humans. This disease is widely prevalent in population of developing countries with poor living conditions and hygiene. The parasite has been estimated to infect 40 million people around the world, although the real number of *E. histolytica* cases is unknown due to the inclusion in this estimation of cases with the morphologically identical *E. dispar* and *E. moshkovskii* species. However, since *E. histolytica* is the unique specie considered as pathogen for humans, it appears to be responsible for 10 million cases of amoebic dysentery/amoebic liver abscesses and about 100,000 deaths every year [1]. In Mexico, amoebiasis was ranked as the sixth highest cause of morbidity with an incidence of 498 cases per 100,000 habitants in 2008 [2].

Amoebiasis treatment relies on the use of imidazole derivatives such as metronidazole, which is highly effective but has the drawback of inducing side effects, is mutagenic at high concentrations, and induces the development of cellular resistance [3]. Thus, there are reports of *in vitro* induction of resistant cultures to high concentrations of metronidazole by continuous exposure to increasing concentrations of the drug as well as the description of patients with amoebic

liver abscesses reluctant to the treatment [4]. Another option that has been shuffled for controlling amoebiasis is the development of a vaccine. In this regard, there have been many trials of immunization in experimental animals using different amoeba antigens in combination with adjuvant [5].

The galactose-binding lectin is among the antigens most commonly used for protection assays. This is a protein complex of three subunits that are preferably located at the surface of the parasite and whose main component, the heavy subunit of 170 kDa, is also one of the most immunogenic *E. histolytica* molecules [6]. Along with other proteins, such as the family of serine-rich proteins [7] and the 29 kDa cysteine-rich Alkyl hydroperoxide reductase [8], the Gal-lectin is considered as one of the main targets for an effective vaccine against amoebiasis. The gal-lectin, with its cysteine-rich portion of the 170 kDa lectin subunit, is the target for serum of 95% of patients with amoebic liver abscess [9] as well as IgG and IgA anti-Gal-lectin antibodies recovered from serum and feces of patients with intestinal amoebiasis, respectively [10, 11]. Oral or nasal immunization of mice, gerbils, and nonhuman primates with the cystein-rich section of galactose-inhibitable lectin LC3 and cholera toxin as adjuvant induced high level of specific serum IgG and fecal IgA [12, 13] antibodies that inhibit *in vitro E. histolytica* adherence to CHO cells [14]. Moreover, intraperitoneal immunization of gerbils with the LC3 fragment with Titermax adjuvant elicited IgG antibodies that conferred 71% of protection against ALA [15]. Recently, it was demonstrated that LC3 is one of the main targets of antibodies elicited by natural infection of female baboons with *E. histolytica* [16]. Thus, 73% and 46% of such animals showed serum anti-LC3 IgG and IgA antibodies, respectively, and 49% exhibited fecal anti-LC3 secretory IgA antibodies. Noteworthy, the specificity of recognition of epitopes in LC3 and the native Gal-lectin by the infected baboons was similar to the specificity of recognition of human asymptomatic subjects and ALA patients [16].

Although promising results have been obtained in protection assays against amoebiasis using various experimental models such as mice, hamsters, and gerbils, the use of these strategies to protect humans in the future is hampered by the use of adjuvants that are potentially toxic and proinflammatory to mammals, such as bacterial toxins or oil-based adjuvants. In a previous report, we proposed the use of viral vectors such as the baculovirus as a strategy for the delivery of amoebic antigens in studies of protection [17]. Baculoviruses are insect viruses capable of infecting mammalian cells, but not of replicating in them. The most promising is *Autographa californica*, an envelope and double-stranded DNA nucleopolyhedrovirus that can drive the expression of foreign genes in mammalian cells without causing cytotoxic effects [18]. This virus has the ability to transduce a wide range of mammalian cells, including liver and kidney cells, and therefore it has been proposed as a delivery vector for human gene therapy, mainly as oncolytic agent. In this sense, *A. californica* has been proposed as a tool for targeting and transferring therapeutic genes into carcinoma cells from patients with prostate [19], colorectal [20], and lung [21] cancer as well as ameliorating the collateral ischaemia in organ transplantation [22]. Noteworthy, from 27 nonhuman virus species that are in preclinical studies, *A. californica* was classified in the category of "negligible" regarding the relative environmental risk [18], suggesting that its use in human for any medical purpose, including gene therapy and vaccines, is safe. As vaccine delivery strategy, the baculovirus *A. californica* has the advantage of priming the immune response by itself, innate and adaptive as well as humoral and cellular [23], preventing the need for adjuvants with potential adverse effects such as bacterial toxins and oil-based formula. Thus, baculovirus displaying target antigens on their surface and/or driving the expression of them under the control of a cytomegalovirus promoter has been highly efficient in inducing immune responses that confer protection against viral and parasitic infections in experimental models [24–35].

In a previous report, we described the potential of an *A. californica* nucleopolyhedrovirus (AcNPV) system driving the expression of *E. histolytica* Gal-lectin LC3 fragment of conferring sterile or partial protection against ALA in 79% of orally and 21% of nasally immunized hamsters. Although the protection was associated with the development of an anti-amoebic cellular immune response measured in spleen, the underlying cytokines and cell populations responsible were not identified. Herein, we complement the previous study by evaluating the potential of an intramuscular immunization with the AcNPV/CMV/Gal-lectin LC3 system for conferring protection against ALA in hamsters, determining whether this route of immunization confer a greater level protection than the mucosal delivery and evaluating in the liver the locally produced cytokines and cell populations responsible for protection.

2. Materials and Methods

2.1. Cells and Cultures. Axenic HM1: IMSS trophozoites were maintained in TYI-S-33 medium supplemented with 15% of adult bovine serum (Biofluids International Inc., MD, USA) and 3% of Diamond's vitamin mix (JRH Biosciences, Kansas, USA). Trophozoites virulence was maintained through successive passages into hamster's liver.

sf9 insect cells (*Spodoptera frugiperda*) (Invitrogen, San Diego, CA) were cultivated as a suspension in Grace's medium (Invitrogen, USA) supplemented with 10% heat inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 1% penicillin/streptomycin (100 U/mL), and 0.1% pluronic F-68 (Invitrogen, USA). AcNPV-LC3 baculovirus production was described in [17]. Recombinant AcNPV-LC3 baculovirus was amplified by infecting sf9 insect cells at a multiplicity of infection (MOI) of 0.1 and purifying them from culture's supernatants 6 days after and concentrated by ultracentrifugation, resuspended in PBS and titered by using a plaque assay following the manufacturer's instructions (Invitrogen, USA).

2.2. Immunization and Challenge Protocols. Male Syrian golden hamsters (*Mesocricetus auratus*) 4 to 6 weeks of age were maintained free of pathogens with water and food *ad libitum*. Following a protocol approved by the Institutional Animal Care Committee, animals were divided in 2 groups of 20 hamsters (AcNPV-WT and AcNPV-LC3 groups) and

1 group of 10 hamsters (sham group). Hamsters of groups AcNPV-WT and AcNPV-LC3 were intramuscularly immunized with three doses at two-week intervals with 1×10^8 PFU of wild-type or LC3 recombinant baculovirus prepared in 20 μL PBS, pH 7.4, respectively. Sham group received 20 μL of PBS by intramuscular route following the same protocol. Two weeks after the last immunization, all animal were infected by intraportal route with virulent *E. histolytica* trophozoites. In brief, hamsters were anesthetized with sodium pentobarbital (50 mg/kg; Anestesal, Pfizer), a laparatomy practiced in aseptic conditions, and 10^6 trophozoites in 100 μL PBS were directly injected in the portal vein. The site of injection was immediately obtruded by applying a gel foam pad, intestine carefully returned to the abdominal cavity, and the abdominal layers sutured with surgical staples (Reflex 9, USA). Blood samples were collected from all animals before treatment and after immunization prior to challenge. Sera were obtained by centrifugation and stored at −70°C until use.

2.3. ELISA. 96 well plates were coated overnight with 500 ng/well of trophozoite's total extract in carbonate buffer, pH 9.6 at 4°C. After blocking with 1% BSA-Tween 20, hamster's sera diluted in 1% BSA (1 : 50) were added to the wells and incubated 1 h at 37°C. After extensive washings with PBS-Tween 20, a HRP-conjugated anti-hamster IgG antibody (Becton Dickinson, USA) was added to 1 : 1000 dilution and incubated for 1 h at 37°C. The wells were washed and the antigen-antibody complexes were developed with OPD and read at 490 nm in a spectrophotometer.

2.4. Cytokine Determination. Cytokine determination was performed in soluble liver extract from hamsters with commercially available mouse IFNγ, IL-12 p70, IL-4, and IL-10 ELISA MAX Standard kits (BioLegend) according to manufacturer's instruction (capture ELISA). Each cytokine assay was performed in triplicate each time. In brief, liver extracts were obtained by sonication (three times, 30 sec each on ice) of tissue fragments, containing or not ALA, in the diluent solution provided with the kit in the presence of complete protease inhibitor cocktail (Roche). Extracts were centrifuged at 5000 rpm for 10 min at 4°C and the soluble fraction, once determining protein concentration by the Lowry method, stored at −20°C until use. For cytokine determination, each well was added with 5 μg of extract in 100 μL of diluent solution and incubated for 2 h at room temperature. Secondary antibodies were used 1 : 200 dilution in diluent solution for 1 h at room temperature and the immune complex developed using Avidine-HRP/TMB substrate and reading at 650 nm in a spectrophotometer.

2.5. Spleen Cells Proliferation by Flow Cytometry. After sacrifice at 7 days after challenge, hamster's spleens were removed and its cellular fraction obtained by perfusion in 2 mL supplemented RPMI medium. The cells were incubated with hemolysis solution and washed by centrifugation with RPMI media. After the erythrocytes lysis, 1×10^6 lymphocytes were gating with 1 mL of PBS/0.1% BSA solution and incubated with 5 mM/mL of CFSE (CellTrace CFSE Cell Proliferation Kit C34554, INVITROGEN) during 10 min at 37°C. After the incubation, the cells were washed in RPMI media and cultivated in 96-well plates at 37°C with 5% CO_2 and 95% humidity for 72 h in RPMI medium alone or stimulated with concanavalin A (1 μg/well), or amebic total extract (50 μg/well). Finally, the lymphocytes were harvested and fixed with 3.7% of formaldehyde solution. All the treatments were carried out by triplicate for each animal. Samples were analyzed on a FACSCanto II flow cytometer (BD) on a minimum of 20,000 events using the DIVA software. The computational analysis was carried out in FACSDiva Software Version 6. The statistical analysis was performed with Batch Analysis Report. Proliferation data from each experimental group was obtained by pooling the means of the triplicate for each animal and is presented as mean ± SD.

2.6. Assay for Liver Function. Blood samples were obtained from hamsters at day of sacrifice. Serum was separated from the blood samples by centrifugation at 5000 rpm for 10 min at room temperature. The serum marker of liver function alanine amino transferase (ALT) was determined by the method EAGLE-UV at Department of Pathology, Faculty of Veterinary Medicine, UNAM.

2.7. Statistical Analysis. The comparison of the infection rates was done using Fisher's exact test. The Kruskal-Wallis test was used to compare antibody and proliferation of lymphocytes between groups. A P value < 0.05 was considered statistically significant in these analyses. All statistical analyses were performed using SPSS statistical software version 19.0.

3. Results

3.1. Intramuscular Immunization with AcNPV-LC3 Inhibits the Development of ALA in Hamsters. In order to evaluate the potential of recombinant baculovirus driving the expression of the amoebic Gal-lectin LC3 fragment of protecting against ALA, hamsters were intramuscularly immunized with the viral particles and intraportally challenged with virulent *E. histolytica* trophozoites. Description on how the recombinant baculoviruses were obtained and evaluated for driving the expression of the Gal-lectin LC3 fragment was previously reported [17].

A comparative macroscopic description of livers showed variable grades of ALA development (Figure 1). Massive development of ALA extended throughout the liver was observed in 8 out of 10 (80%) nonimmunized but challenged hamsters (Sham group; Figures 1(e) and 1(f) and Table 1). By contrast, the development of ALA was variable in immunized and challenged animals. Thus, 11 out of 20 hamsters immunized with the recombinant baculovirus AcNPV-LC3 and 5 out of 20 immunized with the wild-type baculovirus showed no gross (Figures 1(a) and 1(b)) or microscopic evidence (not shown), of ALA development, suggesting that intramuscular immunization with the baculovirus *per se* is capable of giving up to 25% sterile protection, which is increased to more than twice (55%) by immunizing with the recombinant baculovirus of LC3 (Table 1). Moreover,

Table 1: ALT levels in hamsters immunized with AcNPV-LC3 and challenged with *E. histolytica*.

Parameter	Infected	IM AcNPV-LC3 Total protection	IM AcNPV-LC3 Partially protection
Weight (g)	15 ± 3.9	6 ± 2.3	6.3 ± 3.2
ALT (U/L) <87	258.8 ± 163.2	32.2 ± 10.1	169.4 ± 112.3

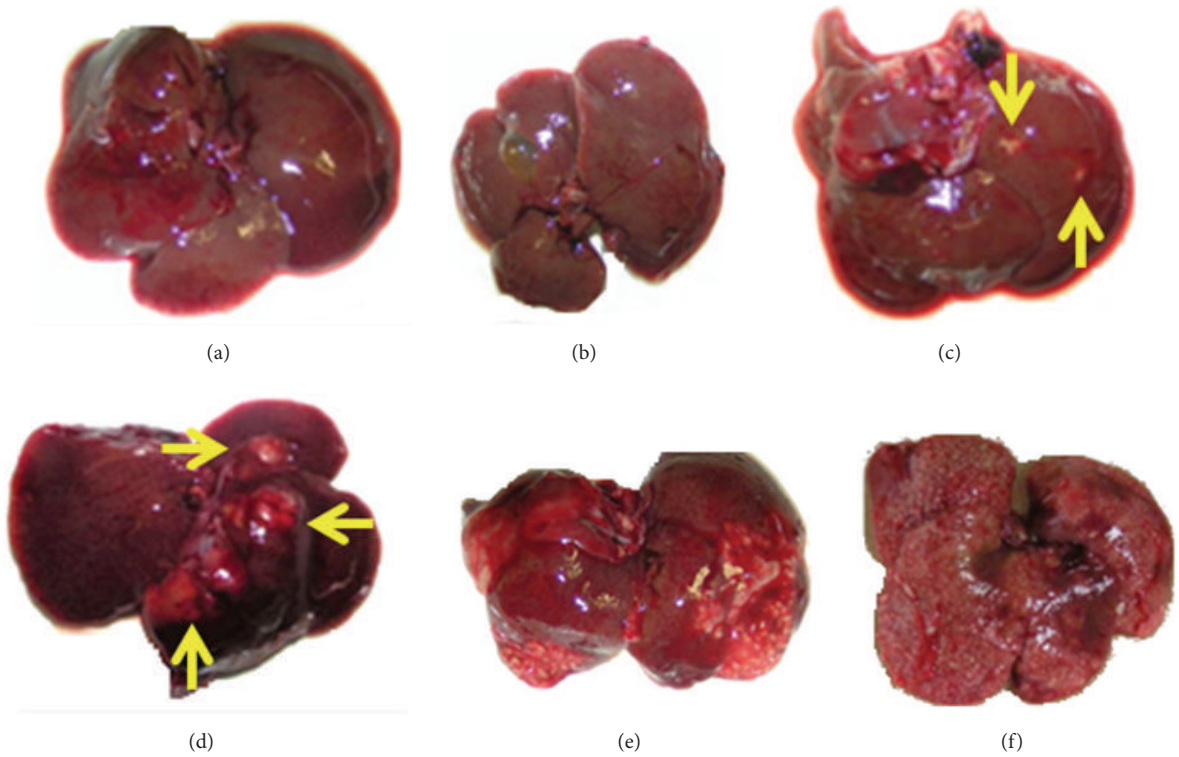

Figure 1: Macroscopical evaluation of protection from ALA challenge in hamsters intramuscularly immunized with Ac-NPV-LC3. Hamsters were IM immunized with baculovirus or PBS and then challenged with virulent *E. histolytica* trophozoites by intraportal route. After sacrifice, livers were exscinded showing different grades of ALA. Sterile protection ((a) and (b)) and partial protection showing few abscesses (c) or no more than 5 large abscesses usually located in one lobe (d) (yellow arrows) were observed in AcNPV-LC3 and AcNPV-WT immunized animals. Massive abscesses development ((e) and (f)) was mainly observed in hamsters nonimmunized but infected.

within the immunized animals that developed ALA, 1 out of 5 animals immunized with the wild virus and 4 out of 9 immunized with AcNPV-LC3 showed partial protection (Table 1), some showing few millimetric abscesses scattered throughout the liver (Figure 1(c)) or no more than 5 large abscesses usually located in one lobe (Figure 1(d)).

The protection against the development of ALA was confirmed by analyzing the hepatomegaly associated and by testing liver function, in particular the determination of alanine aminotransferase (ALT), a pyridoxal cytoplasmic phosphate-dependent enzyme involved in cellular nitrogen metabolism, amino metabolism acid, and liver gluconeogenesis. ALT levels are often low in blood but increased in the case of liver diseases or events that include damage of that tissue [36]. The results showed high levels of ALT, about three times above the reference value, in the serum of nonimmunized and infected animals (sham group), which correlates with an increase of more than twice the weight of the liver as a result of abscesses (Table 1). By contrast, the liver weight and ALT levels in the serum of immunized animals correlated with the degree of protection, being these normal values in hamsters fully protected (ALT < 87 U/L and liver around 6 g) and intermediate in animals showing partial protection (Table 1).

Overall, it can be considered that 75% of hamsters immunized with the recombinant baculovirus ($P < 0.002$ versus WT group by Fisher's test) and 30% of hamsters immunized with the wild baculovirus ($P < 0.02$ versus AcNPV-LC3 group by Fisher's test) showed any evidence of protection against the development of ALA (Table 2).

3.2. Intramuscular Immunization with the Recombinant Baculovirus AcNPV-LC3 Induces High Levels of Serum Anti-Amoebic Antibodies in Hamsters. Analysis of sera from hamsters by ELISA against whole amoeba antigen showed the development of high levels of specific IgG antibodies

Table 2: Infection rate in hamsters intramuscularly immunized with AcNPV-LC3 and intraportally infected with virulent *E. histolytica* trophozoites.

Groups	Infection rate (Infected/Total) %	Total protection %	Partially protection %	Protection efficacy %	*P* value (Fisher's Test)
Infected	8/10 (80%)	0	0	0	0
IM AcNPV-WT	15/20 (75%)	25	5	30	0.02 (vs IM AcNPV-LC3)
IM AcNPV-LC3	9/20 (45%)	55	20	75	0.002 (vs IM sham)

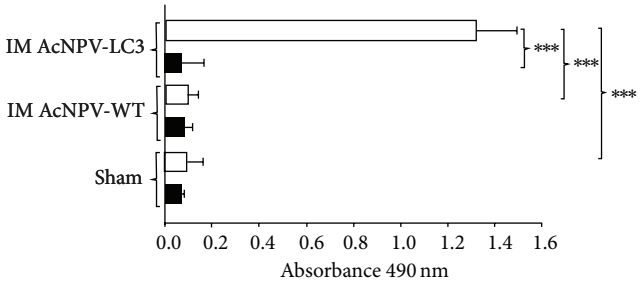

Figure 2: Humoral anti-*E. histolytica* immune responses in AcNPV-LC3 intramuscular immunized and challenged hamsters. IgG antibodies in sera against total extract of *E. histolytica* trophozoites were measured by ELISA at day 0 (preimmune sera, black bars) and day 35, once finalizing the immunization protocol (postimmune sera, 7 days prior to intraportal challenge with trophozoites; white bars). Bars represent mean optical densities ($^{***}P < 0.001$).

after intramuscular immunization with the recombinant baculoviruses AcNPV-LC3 (Figure 2; white bars; $P < 0.001$ versus control groups). Since the sera of animals immunized with wild baculovirus, as well as sham control animals, did not show reaction against the antigen of amoeba, antibodies from hyperimmune sera of immunized hamsters were assumed to be directed against the LC3 fragment Gal-lectin (Figure 2).

3.3. Intramuscular Immunization with the Recombinant Baculovirus AcNPV-LC3 Induces a Cell-Mediated Antiamoebic Response in Hamsters. Antigen specific proliferation of lymphocytes was determined in sensitized spleen cells from hamsters, by incubating splenocytes in the presence of whole amoeba antigen. The results showed that the level of proliferation correlated with the degree of protection obtained with the recombinant baculovirus AcNPV-LC3 (Figure 3), being greater in the immunized animals protected entirely (rate 20) followed by those which were partially protected (rate 13; Figure 3(c), $P < 0.05$ between them; $P < 0.001$ versus the other groups). Proliferation in the AcNPV-LC3 immunized but not protected hamsters was twice of the animals from control groups, but not statistically different. By contrast, animals not protected and even those totally or partially protected by immunization with the wild baculovirus did not show any degree of splenocyte's proliferation against whole amoeba antigen (Figure 3).

3.4. Protection against ALA Correlated with the Local Production of IFNγ and IL-4. In order to identify soluble elements of the local cellular immune response associated with the protection obtained against ALA, the Th1 (IFNγ and IL-12) and Th2 (IL-4 and IL-10) associated cytokines in extracts of livers from protected hamsters were evaluated. The results animals immunized with AcNPV-LC3 and completely protected had on average 9-fold more IFNγ than infected animals (450 pg/mL versus 50 pg/mL, $P < 0.01$), and almost twice the levels in partially protected hamsters, but with no statistical significance (Figure 4(a)). Similarly, there was no statistical difference between the IFNγ levels from liver of the partially protected and the nonprotected animals. In the same way, this trend was observed in AcNPV-WT group, where totally protected animals have 6 times average of IFNγ more than infected animals (396.5 pg/mL versus 65.2 pg/mL, $P < 0.05$, Figure 4(a)) and twice with respect to animals that showed partial protection. Finally, no difference between groups was observed for the other Th1 cytokine IL-12 (data not shown).

With respect to the Th2 cytokine profile, differences were only observed in the levels of IL-4, but not IL-10 (data not shown). As with IFNγ, IL-4 was on average 8-fold higher in the livers of animals fully protected compared with immunized and infected animals (80 pg/mL versus 10 pg/mL, $P < 0.01$, Figure 4(b)). In the same way, the partially protected hamsters showed a tendency to have higher concentrations of IL-4 than the infected, but the difference was not statistically significant. With regard to AcNPV-WT immunized group, the totally protected animals showed IL-4 six times higher more than infected animals.

4. Discussion

In a previous study, we demonstrated the potential of *A. californica* nucleopolyhedrovirus (AcNPV) driving the expression of the *E. histolytica* Gal-lectin LC3 fragment of conferring sterile or partial protection against ALA in 79% of orally and 21% of nasally immunized hamsters, but the underlying cytokines and cell populations responsible were not identified [17]. In this report we aimed to determine whether the intramuscular immunization of hamsters with the same AcNPV-LC3 recombinant baculovirus was able to confer a greater level of protection than the mucosal vaccination against the development of ALA following an intraportal challenge with virulent amoebic trophozoites and whether the protection was correlated with a particular type of local immune response.

The results showed that intramuscular immunization of hamsters with the AcNPV-LC3 recombinant baculovirus

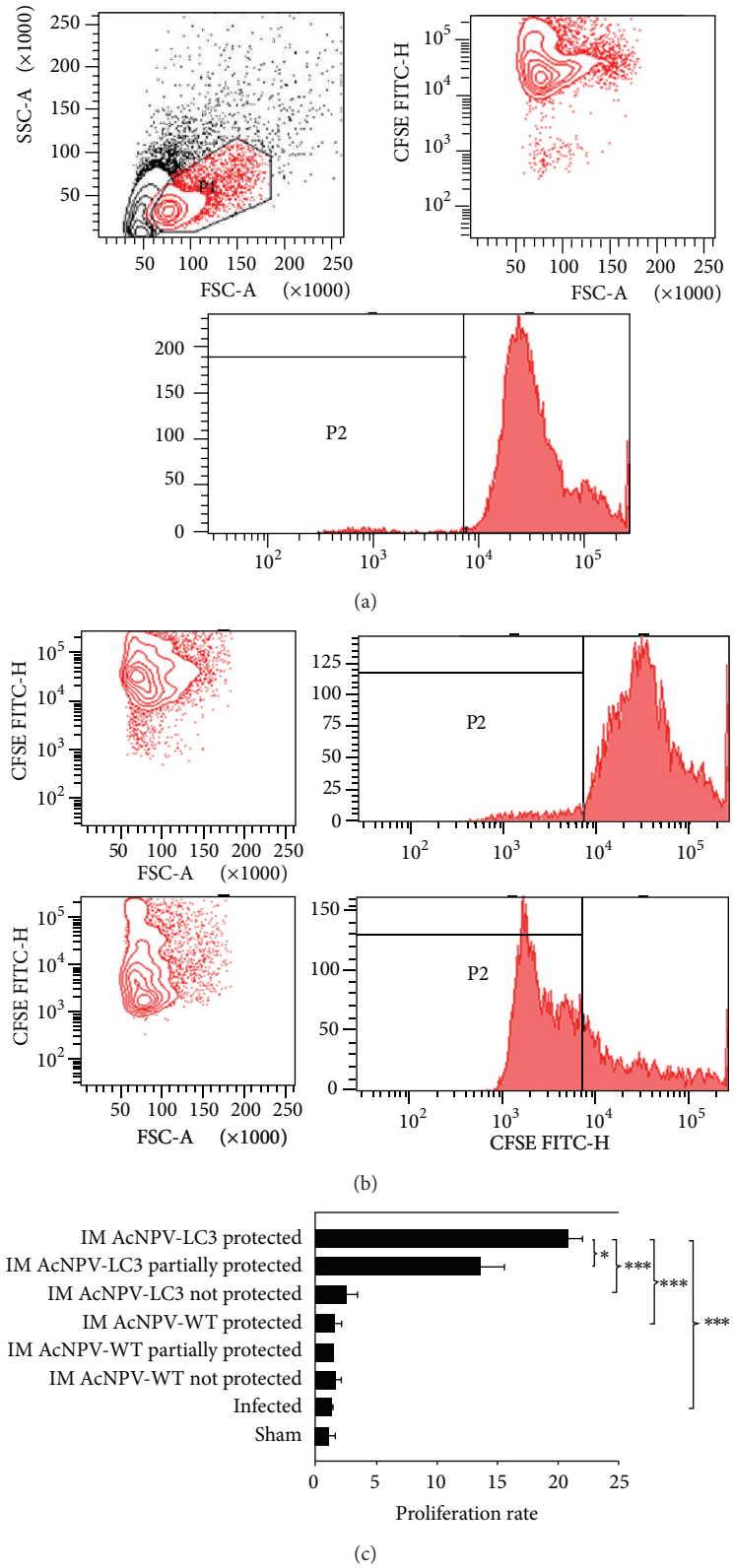

FIGURE 3: Cellular anti-*E. histolytica* responses in AcNPV-LC3 intramuscular immunized and challenged hamsters. Cellular immune response was evaluated in splenocytes after immunization and challenge (day 49) by staining of the harvested cells with CFSE and stimulation with *E. histolytica* total extract during 72 h followed by flow cytometry analysis. In (a), the first panel shows dot plot of splenocytes stained with CFSE without antigenic stimulation. The cell population selected according to their size and granularity (P1 region) and its corresponding dot plot and histogram are shown (a). (b) shows a representative dot plot and histogram of IM AcNPC-LC3 splenocytes from nonprotected (upper) and sterile protected (bottom) hamsters. (c) shows the proliferation rate of all experimental groups classified by protection level. Bars represent mean of percentage of proliferation ($^{*}P < 0.05$; $^{***}P < 0.001$).

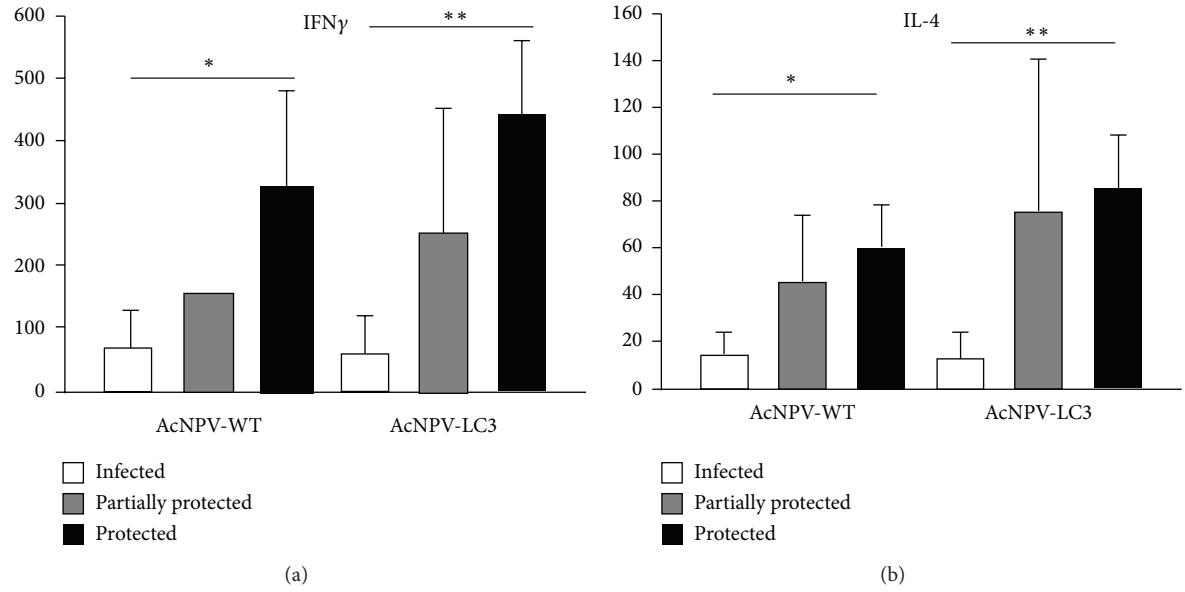

Figure 4: IFNγ and IL-4 local productions in liver parenchyma of hamsters intramuscularly immunized with AcNPV-WT, AcNPV-LC3 and challenged. Cytokines in soluble livers extracts from hamsters of AcMPV-WT and AcMPV-LC3 groups were evaluated by ELISA. Concentrations in extracts from immunized and nonprotected hamsters (white bars), partially protected hamsters (grey bars), and hamsters with sterile protection (black bars) are shown. The results show a direct relationship between IL4 (a) and IFNγ (b) expression and protection degree ($^{*}P < 0.05$, $^{**}P < 0.01$).

confers an overall level of protection similar to that previously obtained with the oral route (75% versus 79%, resp.). Moreover, the sterile protection levels obtained by both routes were also similar (55% oral versus 58% intramuscular). This result was unexpected because being ALA a systemic type infection, it was more probably that the intramuscular route of immunization had higher efficiency to induce a systemic protective response than the oral route. Therefore, this supports the proposal by our group and others that the oral route may be the appropriate to administer a vaccine against amoebiasis in general, because, in addition to the classic advantages of this route as its manageability, noninvasion, and no needs for specialized personnel, oral route triggers local and systemic immune responses that in addition to conferring protection against intestinal amoebiasis, may also protect against the development of liver abscesses if trophozoites manage to pass from the intestine to the liver. This is noteworthy as extraintestinal amoebiasis, and in particular, ALA is considered the main cause of human death by *E. histolytica* [37].

The effectiveness of the Gal-lectin as vaccine against amoebiasis has been extensively studied [5]. Recently, a new model of intestinal infection with *E. histolytica* in baboons (Papio sp) and the efficacy of nasal vaccination against amoebic colitis using a Gal-lectin synthetic peptide was reported [38]. Notably, a vaccination schedule of four doses of 1600 μg peptide/nostril in combination with cholera toxin as adjuvant once a week was able to eradicate amoebic infection in all baboons by the 51st day after intracecal challenge with trophozoites. Moreover, serum IgG and IgA antibodies induced by natural infection of baboons with *E. histolytica* are directed to epitopes present in the LC3 fragment of the Gal-lectin, and this immunodominance is shared with humans carrying asymptomatic *E. histolytica* infection or recently cured of ALA [14]. These results, together with the obtained by our group, previously and reported here, using recombinant baculoviruses driving the expression of the LC3 fragment [17] as well as other protection assays using LC3 as immunogen [15], highly support that Gal-lectin, in particular its LC3 fragment, is the target of protective antibodies and cellular immune responses resolving intra- and extraintestinal amoebiasis, and, therefore, it should be the basic component of an effective vaccine against human amoebiasis.

The immune elements responsible for protection against intestinal amebiasis or ALA are not entirely known. In this study, we found an association of sterile protection against ALA with the local production of high levels of IFNγ, a cytokine of Th1 profile. This result is in agreement with previous studies where protection against ALA in hamsters correlated with the production of the same cytokine in protective assays using the EhCPADH surface complex as immunogen [39]. In addition, relevance of IFNγ as playing a role in the innate immunity against ALA came from studies in SCID mice with targeted disruption of the IFNγ receptor gene [40]. Moreover, T cell derived IFNγ and IL-17 were also essential for protection against intestinal amoebiasis in a murine model vaccinated with recombinant LecA fragment of the Gal-lectin, determined by adoptive T cell transfer and IFNγ/Il-17 neutralizing assays [41, 42]. The source of the protective IFNγ in our assays is unknown, but it could be from T cells responding specifically to the amoeba antigen

as observed in the cell proliferation assays, in which the best protected animals showed higher response to the amoeba extract. However, NKTs cells as source of the early production of IFNγ in the liver during the ALA are also well known [43]. In this regard, interestingly, the wild-type baculovirus was able to confer up to 25% protection by itself, which was also associated with local production of high levels of IFNγ, suggesting that activation of the innate immune response by the baculovirus, probably IFNγ from local NKT cells, is partly responsible for the observed protection. Thus, it has been demonstrated that baculovirus recognition by TL9 leads to the production of various cytokines that triggers innate immunity, including IFNγ [23], which in turn has the potential to confer high levels of protection in several experimental models, such as influenza where intranasal immunization with a wild-type baculovirus protected 100% of mice from a lethal challenge [44].

In this report, a correlation of partial and sterile protection against ALA with the local production of high levels of IL-4 was also observed (Figure 4(b)). Although the production of IL-4 has been associated with susceptibility to intestinal infection by amoeba [45], a previous study by our group using lactoferrin as an oral therapy for the treatment of intestinal amebiasis in C3H/HeJ mice showed production of this cytokine in tissue of ceca from animal cured [46]. As we suggest in such paper, it is likely that the production of IL-4 is the result of a feedback mechanism for controlling excessive inflammation (a typical feature of the intestinal mouse and liver hamster amoebiasis), restoring the physiological anti-inflammatory state that predominates in both intestine and liver. IL-4 also augments fibroblast growth and collagen production during granulomas formation, a characteristic mark of the chronic development of ALA [47], a reaction trying to contain the infection. Thus, it is possible that the role of IL-4 depends on whether liver abscesses develop or not, so contributing to the formation of granulomas at later stages if ALA develops or decreasing excessive inflammation at earlier stages.

Protection against ALA in hamsters was also associated with the induction of serum anti-amoebic antibodies, particularly IgG (Figure 2). This is consistent with a variety of previous studies demonstrating that antibodies are important in protection against amoeba, particularly secretory IgA against intestinal amebiasis, and serum IgG and IgA against hepatic amoebiasis [48, 49]. It is possible that the high levels of IFNγ observed in protected hamsters have also contributed to the increased production of IgG antibodies mentioned, mainly IgG2a isotype (Th1 response), linking the two arms of the immune response in the liver infection by *E. histolytica*. Taken together, the results suggest that both responses, cellular and humoral anti-LC3 fragment of the Gal-lectin, are important for the development of resistance in hamsters against ALA.

Immunization in humans has always faced with the difficulty of identifying safe adjuvants that do not generate unwanted side effects. Most protection assays against intestinal and/or liver amoebiasis described above used toxins derived from bacteria or mineral or vegetable oils suspensions, which although inducing a good immune response also usually generate local or systemic inflammatory responses that can have implications for the health of the vaccinated subject [50]. In this work, we reinforce the probability of using recombinant baculovirus system as an alternative approach to the use of toxic adjuvants for the safe release of amoeba antigens into mammals, due to its *per se* capacity of stimulating the innate immune response, and, thus, conditioning the generation of a specific antiamoeba adaptive immune response. In this sense, many studies demonstrating the phenotypic and functional maturation of dendritic cells and macrophages as well as direct activation of B cells induced by AcNPV, resulting in antigen presentation and Th1 responses, have been reported [51, 52]. Noteworthy, the acceptability for using baculovirus in humans has been recently increased with ongoing trials of gene therapy based on the use of such viruses as delivery system [53], which lays the foundation for safe and extended use of recombinant baculovirus systems in other areas such as vaccines [54].

Finally, further studies of T cell adoptive transfer and neutralization of cytokines, IFNγ and probably IL-17, are needed for determining the mechanisms underlying protection against ALA in baculovirus-immunized hamsters, as well as immunization studies using AcNPV-LC3 in an animal model closer to humans such as primates, in order to determine the safety and effectiveness of this system with view to its use as a strategy of vaccination against amoebiasis in humans.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Characterization of a Thioredoxin-1 Gene from *Taenia solium* and Its Encoding Product

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Taenia solium thioredoxin-1 gene (*TsTrx-1*) has a length of 771 bp with three exons and two introns. The core promoter gene presents two putative stress transcription factor binding sites, one putative TATA box, and a transcription start site (TSS). TsTrx-1 mRNA is expressed higher in larvae than in adult. This gene encodes a protein of 107 amino acids that presents the Trx active site (CGPC), the classical secondary structure of the thioredoxin fold, and the highest degree of identity with the *Echinococcus granulosus* Trx. A recombinant TsTrx-1 (rTsTrx-1) was produced in *Escherichia coli* with redox activity. Optimal activity for rTsTrx-1 was at pH 6.5 in the range of 15 to 25°C. The enzyme conserved activity for 3 h and lost it in 24 h at 37°C. rTsTrx-1 lost 50% activity after 1 h and lost activity completely in 24 h at temperatures higher than 55°C. Best storage temperature for rTsTrx-1 was at −70°C. It was inhibited by high concentrations of H_2O_2 and methylglyoxal (MG), but it was inhibited neither by NaCl nor by anti-rTsTrx-1 rabbit antibodies that strongly recognized a ~12 kDa band in extracts from several parasites. These TsTrx-1 properties open the opportunity to study its role in relationship *T. solium*-hosts.

1. Introduction

Thioredoxin (Trx) is a small (~12 kDa) enzyme that belongs to the reductase family. Trx reduces disulfides in several proteins using its conserved dithiol active site. It is ubiquitous and multifunctional; it is involved in processes such as maintenance of cellular homeostasis, cell proliferation, detoxification of peroxides (H_2O_2, hydroperoxides), DNA synthesis, signaling, and inhibition of apoptosis. Likewise it reduces diverse molecules of low molecular weights, such as glutathione disulfide, as well antioxidants dehydroascorbate, lipoic acid, and lipoamine [1–4]. All these events oxidize Trx, and it is reduced by thioredoxin glutathione reductase (TGR) and NADPH + H^+; these components form the thioredoxin system in platyhelminths [5].

Trx has been classified into cytosolic (Trx-1) and mitochondrial (Trx-2); the latter is synthesized with an additional N-terminal extension that targets the mitochondrial protein, where it is cleaved to yield the ~12 kDa form [3]. All Trx enzymes have a similar structure, the Trx fold that is formed by a central domain with five-stranded β-sheet, surrounded by four α-helices, and the active site (CGPC), located between β strand 2 and α-helix 2 [3].

In Cestoda, Trx and TGR have been reported in *Echinococcus granulosus* and *Taenia crassiceps*. On the other hand, these organisms and *Taenia solium* possess a typical 2-Cys peroxiredoxin, which reduces H_2O_2 and hydroperoxides to water and its corresponding alcohol using the thioredoxin system. This shows that these organisms are able to regulate hydroperoxides levels and repair enzymes inactivated by oxidative stress [5–9].

Neurocysticercosis is the most common parasitic brain disease worldwide; moreover the high relationship between epilepsy and neurocysticercosis is considered now as a "biological marker" of the social and economic development of a community [10]. No commercial vaccine exists to prevent this parasitic disease and the treatment relies on two drugs, albendazole and praziquantel, to which *T. solium* has started to develop resistance [11, 12]. Therefore, the identification and biochemical characterization of new targets are important tools for development of vaccines or therapeutic drugs.

In this study, we describe the cloning and characterization of a gene that encodes a thioredoxin-1 from *Taenia solium* (*TsTrx-1*) and present a partial biochemical characterization of its encoding product.

2. Material and Methods

2.1. Taenia solium Trx Gene and cDNA Isolation. A Trx probe was generated by RT-PCR using the SuperScript One Step RT-PCR Kit (Invitrogen, Carlsbad, CA) with 1 μg of *T. solium* larval total RNA prepared by TRIzol (Invitrogen, Carlsbad, CA) and two degenerated primers called TRX-1 and TRX-2 designed from the well conserved regions (TWCGPCK and MPTLFVFK) in Trx enzymes. The RT-PCR program for cDNA synthesis was 1 cycle at 50°C for 30 min, 30 cycles at 94°C for 1 min, 54°C for 30 sec, and 72°C for 1 min, and a final extension cycle at 72°C for 15 min. The fragment (probe) obtained was cloned into pCRII vector (Invitrogen), sequenced on an automated DNA sequencer ABI Prism model 373 (Perkin-Elmer, Applied Biosystem, Foster City, CA), and the nucleotide translation to amino acids sequence was analyzed with the PCGENE program. Screenings for *T. solium* cysticerci cDNA and genomic DNA libraries were carried out using 45,000 and 120,000 λZAPII phages, respectively. Both libraries were hybridized with the aforementioned probe, as previously described [8, 9]. Phage positive clones obtained after three screening rounds of each library were converted to Bluescript plasmids using ExAssist helper phage (Stratagene, La Jolla, CA). Plasmids were sequenced and analyzed as before. Intron detection was carried out with the PCGENE and analyses of amino acid sequences were performed through BLAST (National Center for Biotechnology Information NCBI (http://www.ncbi.nlm.nih.gov/BLAST/)). Alignment of the multiple amino acid sequences was performed by Clustal X (http://www.clustal.org/). The proximal promoter analysis for detecting putative transcription binding sites was carried out with the TRANSFAC program (http://www.gene-regulation.com/pub/databases.html).

2.2. Transcription Start Site Determination. Taenia solium larval total RNA (200 ng) was used as template for the transcription start site (TSS) determination using the Smart RACE cDNA Amplification Kit (Clontech Mountain View, CA). RACE fragments were amplified by PCR using reverse primer TRXRE-1 designed from the region DEMAKENAN (5′-GTTAGCATTCTCCTTTGCCATTTCGTC-3′) and forward primer SMARTII from kit (5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′) following manufacturer's directions. The resulting bands were cloned into pCRII (Invitrogen), sequenced, and compared with the results obtained with the neural network analysis tool (http://www.fruitfly.org/) to confirm transcription start site (TSS) found by the 5′-RACE method.

2.3. Transcripts Relative Expression. For the real time-PCR, 3 μg of total RNA from *T. solium* larval and adult stages was reverse-transcribed to cDNA using SMARTScribe Reverse Transcriptase and 5′-CDS primer A (Clontech) according to manufacturer's instructions. cDNA 200 ng was used for each reaction in a volume of 10 μL using the primers TRX-X1 and TRX-X2 designed from the regions (MSVEAVV) and (IQANV-) of *TsTrx-1*. Primers SOZ-2 and SOZ-6 were designed on the regions (KHGFHVH) and (GNAGGR-) of *T. solium* Cu/Zn superoxide dismutase (TsCu/ZnSOD) [13]. The reactions were performed with LightCycler 480 SYBR Green I Master in the LightCycler 480 System (Roche, Germany). The real time-PCR program used was 95°C for 10 min and then 40 cycles at 95°C for 15 sec and 52°C for 1 min and 72°C for 30 sec. The mRNA levels of *TsTrx-1* were normalized using the *TsCu/ZnSOD* as a housekeeping gene, and relative amounts of mRNA were calculated using the comparative CT method.

2.4. Purification of Recombinant TsTrx (rTsTrx-1). Plasmid pRSET containing the cDNA coding region from TsTrx-1 was expressed on BL21(DE3) bacteria with 1 mM IPTG during 4 h. Bacteria were centrifuged at 10,000 ×g and the pellet was disrupted by sonication in a TrisED buffer (10 mM Tris, 1 mM EDTA, and 1 mM DTT, pH 7.5) plus 4 M urea. The supernatant was applied onto a Ni+ sepharose column (His Trap HP GE Healthcare) and eluted with TrisED plus urea using a linear gradient of imidazole (0, 50, 100, 200, 300, and 400 mM). Fractions containing high Trx activity were dialyzed in TrisED buffer and reloaded in the Ni+ sepharose column for a second purification process without urea. The Trx obtained was concentrated and proteins concentration was determined by the Lowry method. Purification process of rTsTrx-1 was visualized by 15% SDS-PAGE staining with Coomassie Brilliant Blue.

2.5. Production of Antibodies and Western Blot. A 10-week-old New Zealand rabbit was immunized subcutaneously with 100 μg of the purified recombinant enzyme plus 10 μg of saponin as adjuvant. Immunizations were conducted on days 1, 15, and 30. Antisera were obtained one week after the third immunization.

For western blot analysis, 1 μg/mm of rTsTrx-1, rTrx-*E. coli*, and human T-cell recombinant Trx (rTrx-human, Sigma-Aldrich, St. Louis. MO) and likewise 5 μg/mm of parasites crude extract and *T. solium* cysticerci excretion-secretion antigens (E/S Ag), prepared as described in [14], were separated by 15% SDS-PAGE and transferred onto a nitrocellulose membrane (Amersham Biosciences, Sweden). The membrane was blocked with 1% BSA in PBS containing 0.05% Tween 20 buffer and incubated for 2 h at room temperature with the anti-rTsTrx-1 serum (dilution 1 : 100). After 3 washes of 5 min with PBS containing 0.05% Tween, the membrane was incubated for 1 h with horseradish peroxidase- (HRP-) conjugated goat anti-rabbit antibody (1 : 2000). Bands recognized by the anti-rTsTrx-1 serum were visualized with 3,3′-diaminobenzidine (DAB) and H_2O_2 as substrate; normal rabbit serum was used as negative control at the same dilution.

2.6. Effect of pH, Temperature, Methylglyoxal (MG), H_2O_2, NaCl, and Anti-TsTrx-1 Antibodies on rTsTrx-1 Activity. The oxidoreductase activity of rTsTrx-1 was determined by the

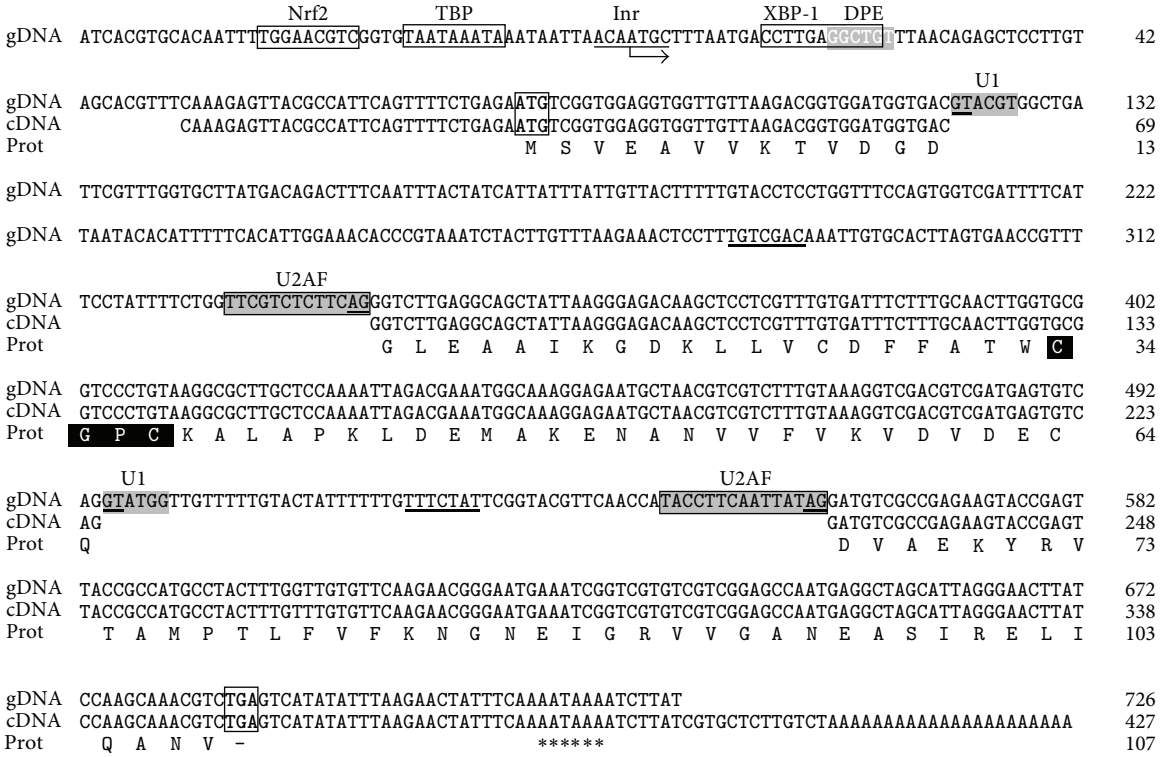


Figure 1: Genomic (gDNA) and complementary DNA (cDNA) nucleotides sequences and deduced protein from *Taenia solium* thioredoxin-1. Putative transcription factors sites (Nrf2, TBP, and XBP-1) are placed inside a box; DPE is in a white letter inside the grey box; TSS inside the Inr is underlined and signalized by an arrow. Start (ATG) and stop (TGA) codons are inside the box; donor (GT) and acceptor (AG) introns sequences are underlined. Putative branch point is underlined by a black bar; putative U1 and U2AF splicing binding sites are in grey inside a box; polyadenylation sites are indicated by asterisks (∗). Thioredoxin residues from the active site (CGPC) are highlighted in white on a black background.

dithiothreitol (DTT)/insulin reduction method described by Holmgren [15]. Briefly thioredoxin reactions are coupled to DTT using insulin as the protein substrate. rTsTrx-1 or rTrx-*E. coli* at 1, 10, and 20 μg were added to 160 μM of insulin in PE buffer (100 mM potassium phosphate, pH 6.5, containing 2 mM EDTA and 1 mM DTT). Insulin reduction was monitored by measuring turbidity at 650 nm for 30 min in a spectrophotometer Ultrospec 3100 Pro (Amersham Biosciences). A sample without rTsTrx-1 was used as reference control.

Assays to determine the pH enzymatic stability were carried out with rTsTrx-1 dialyzed for 8 h in citrate buffer at pH of 3, 5, and 6, in PE buffer at pH of 6.5, 7.5, and in Tris buffer at pH of 8, 9, and 10. The thermal stability of the enzyme was assayed incubating rTsTrx-1 at temperatures between 15 and 100°C during 1, 3, and 24 h. In both assays the Trx residual activity was measured. In addition, the optimal storage temperature was analyzed by incubating the enzyme at temperatures ranging from 25° to −70°C during 1 to 28 days.

To determine whether concentrations of 0 to 8 mM of MG, 0 to 2 M of NaCl, 0 to 200 mM H_2O_2, and 1, 10, and 20 μg of IgG fraction, coming from sera of rabbit immunized with rTsTrx-1 and normal rabbit serum (control), affected the TsTrx-1 activity, the enzyme was incubated in each one for 30 min at 37°C and the activity was measured as before. For all these assays 20 μg of enzyme was used.

3. Results

3.1. Isolation of cDNA and Gene Encoding TsTrx-1 and Its Characterization. Through RT-PCR, using total RNA from larval *T. solium* and primers designed on two conserved regions from several Trxs, we obtained a ~153 bp DNA fragment that evidenced high homology with *Trx* genes. This was used as probe to isolate the transcript and the coding *TsTrx-1* gene by screening a genomic DNA and cDNA λZAPII libraries, respectively. Figure 1 shows the isolate genomic DNA sequence; it spans 771 bp and codes for the same Trx-1 as the cDNA. The 5′-RACE experiments on the proximal promoter of the *TsTrx-1* gene showed that the transcription start site (TSS) corresponds to an adenine (A) located within the initiator (Inr, AC<u>A</u>ATGC) sequence and mapped at 81 bp upstream of the translation start codon (ATG). Moreover, we identified a putative TATA box located at −19 pb and a GGCTGT motif (downstream promoter element, DPE) at +22 pb, both from the TSS. Additionally, putative binding sites for Nrf2 and XBP1 transcription factors were found at −32 bp and +14 bp, respectively (GenBank accession for *TsTrx-1* gene is KM401604).

Table 1: Comparison of the structural coding regions of *Taenia solium Trx-1* gene (*TsTrx-1*) with other *Trx-1* genes from *Homo sapiens*, *Mus musculus*, *Schistosoma mansoni*, and *Echinococcus granulosus*.

Organism	Size of structural coding region of gene (kbp)	Number of introns, size (kbp), and position	Number and size of exons (bp)	Size encoding product (amino acids)
Taenia solium	0.605	I: 0.218 (DGD^{13}–^{14}GLE) II: 0.065 (ECQ^{65}–^{66}DVA)	1: 39 2: 156 3: 129	107
Echinococcus granulosus	0.609	I: 0.220 (DGD^{13}–^{14}ALE) II: 0.065 (ECQ^{65}–^{66}DVA)	1: 39 2: 156 3: 129	107
Schistosoma mansoni	0.685	I: 0.237 (KQD^{10}–^{11}GDL) II: 0.126 (KLE^{64}–^{65}ETA)	1: 30 2: 162 3: 129	106
Mus musculus	~13	I: 4.2 (ESK^{8}–^{9}EAF) II: 0.897 (FFH^{43}–^{44}SLC) III: 5.6 (DCQ^{63}–^{64}DVA) IV: 1.1 (GQK^{85}–^{86}VGE)	1: 24 2: 105 3: 60 4: 66 5: 63	105
Homo sapiens	~17	I: 5.0 (ESK^{8}–^{9}TAF) II: 0.478 (FFH^{43}–^{44}SLS) III: 5.9 (DCQ^{63}–^{64}DVA) IV: 0.558 (GQK^{85}–^{86}VGE)	1: 24 2: 105 3: 60 4: 66 5: 63	105

The structural coding region for the *TsTrx-1* gene spans over 770 pb. It has three exons split by two introns (intron I: 218 bp length; intron II: 65 bp length) that possess the donor-acceptor sites (NGT-AGN). Moreover, the putative binding sites for the splicing machinery U1 (intron I: ^{121}**GT**ACGT^{126}; intron II: ^{495}**GT**ATGG^{500}) and U2AF (intron I: ^{326}TTCGTCTCTTC**AG**^{338}; intron II: ^{547}CCTTCAATTAT**AG**^{459}) were identified. Additionally, the putative branching point in each intron (intron I: ^{281}TGTCGAC^{287}; intron II: ^{522}TTTCTAT^{528}) was identified too. Figure 1 also shows the isolated cDNA with 427 bp with an open reading frame (ORF) from 31 (ATG) to 354 (TGA) bp that encodes for a protein with 107 amino acids with a theoretical molecular weight of 11,579 Da and pI of 4.39. It presents the motif (CGPC) that corresponds to the catalytic active site of Trx enzymes. Furthermore, a putative classic polyadenylation (AATAAA) site was located between 380 and 385 bp downstream of the stop codon (GenBank accession for TsTrx-1 cDNA is KM401605).

Table 1 depicts a comparison of the coding structural region of the *TsTrx-1* gene with *Trx-1* genes from *E. granulosus*, *Schistosoma mansoni*, human, and mouse [16–19]. It shows that the *Trx-1* genes of *T. solium*, *E. granulosus*, and *S. mansoni* have a similar size, in contrast to mammalian *Trx-1* genes that are bigger. It also depicts that *T. solium* and *E. granulosus* present an identical structural coding region to that of the *Trx-1* gene composed by three exons and two introns. Similarly, *S. mansoni Trx-1* gene also presents three exons and two introns, which are slightly larger than *T. solium* and *E. granulosus Trx-1* genes. In contrast, human and mouse genes present 5 exons and 4 introns with similar sizes between them, but introns are larger than those of cestodes. It is noteworthy that the second introns of *TsTrx-1* and *E. granulosus* genes coincide with the second intron of *S. mansoni* and third intron of human and mouse *Trx-1* genes. Figure 2 shows that TsTrx-1 mRNA was expressed higher in larvae than in adult, as determined by real time-PCR assays.

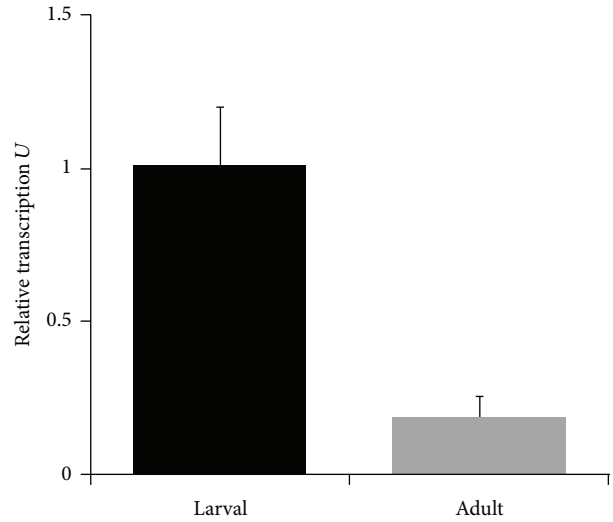
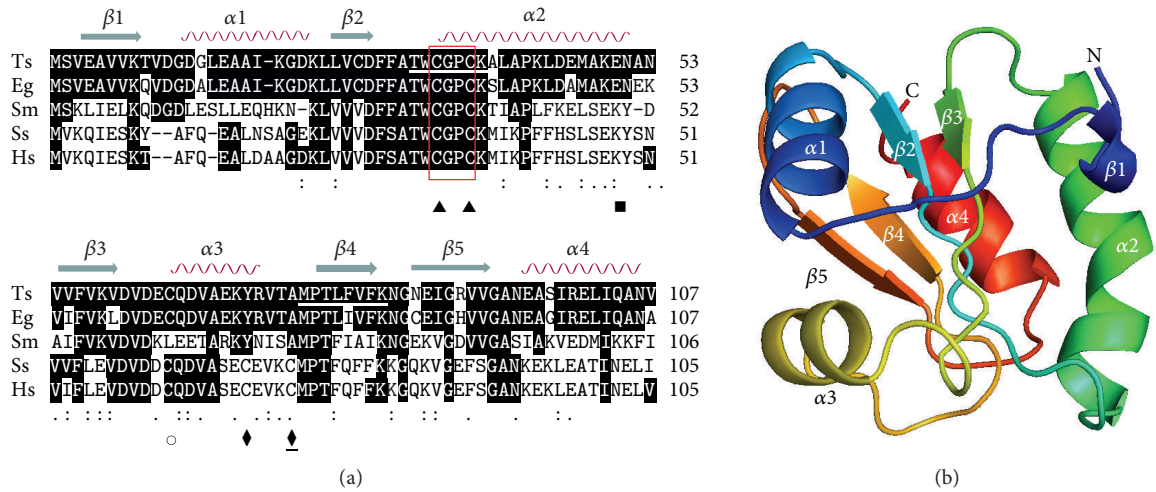


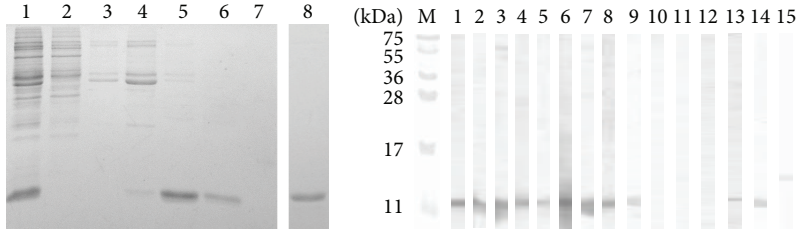
Figure 2: Relative transcription of *T. solium* thioredoxin-1 (*TsTrx-1*) gene from larvae and adult stages of *T. solium* was done by real time-PCR using TRX-X1 and TRX-X2 primers.

The comparison of the deduced amino acid sequence of TsTrx-1 with *E. granulosus* Trx revealed (Figure 3(a)) an 87.85% identity, followed by a 46.72% with *S. mansoni*. In contrast, a low identity, between 41.12 and 43.92%, was found with pig and human Trx-1, respectively. TsTrx-1 has the



(a) (b)

FIGURE 3: (a) Alignment of Trx-1 from *Taenia solium* (Ts. GenBank accession KM401605) with other thioredoxins from *Echinococcus granulosus* (Eg. GenBank: AF034637.1), *Schistosoma mansoni* (Sm. GenBank: AAL79841.1), *Sus scrofa* (Ss. GenBank NM_214313.2), and *Homo sapiens* (Hs. GenBank AF085844.1). Identical residues are highlighted in white on a black background. The symbols in the residues indicate (-) absence and (:) homology. Tyrosine 49 where nitration occurs (■) in mammalian. Cysteines: (▲) from active site, (○) conserved cysteine in mammalian and helminths, and (⧫) only present in mammalians where S-nitrosylation occurs; likewise (⧫) it is involved in glutathionylation. In a box is the active site and underlined are the residues used for primer design to produce the Trx-1 probe. Secondary structure elements are shown above the alignment. (b) Structure model of TsTrx-1. It shows the Trx fold formed by a central domain with five-stranded β-sheet, surrounded by four α-helices. The model was drawn with the Swiss Model program (http://swissmodel.expasy.org/).



(a) (b)

FIGURE 4: Purification process of the recombinant *T. solium* thioredoxin-1 (rTsTrx-1) and specificity of rabbit anti-TsTrx-1 serum. (a) 15% SDS-PAGE showing the crude extract of *Escherichia coli* produced with 4 M urea induced with IPTG, 1: after and 2: before. Crude extract was run through the nickel chelator column; 3: wash column. Eluted fractions with imidazole at 4: 50 mM, 5: 100 mM, 6: 200 mM, and 7: 400 mM. Eluted fractions from 100 and 200 mM were mixed and dialyzed and run through the same column to obtain 8: a pure rTsTrx-1. (b) Western blot showing the reaction from anti-TsTrx-1 serum with 1: pure rTsTrx-1 and crude extracts from 2: *Taenia solium* larvae, 3: *T. solium* adult, 4: *T. saginata* adult, 5: *T. taeniaeformis* adult, 6: *T. crassiceps* larvae, 7: *Hymenolepis diminuta* adult, 8: *Fasciola hepatica* adult, 9: *Entamoeba histolytica*, 10: *E. coli*, and 11: *Homo sapiens* recombinant Trxs. 12: a preimmune serum was incubated with a crude extract of *T. solium* larvae as a negative control. 13: *T. solium* cysticerci E/S Ag. Strips 14 and 15 show the rTrx-*E. coli* and Trx-*Homo sapiens* stained with Ponceau red. Molecular mass standards are indicated in the middle of both figures.

two conserved cysteines (Cys34 and Cys37) in its active site and cysteine 64, which is conserved in cestodes and mammalian, but it is not presented in *S. mansoni* Trx-1. Cysteine 27 is shared only by *T. solium* and *E. granulosus* Trx-1; unfortunately, its function is still unknown. *T. solium* and *E. granulosus* Trx-1 as well as *S. mansoni* lack tyrosine 49 and cysteines 69 and 73, present in mammalians, which are involved in nitration, glutathionylation, and S-nitrosylation and dimer formation [20]. Figure 3(b) shows a model constituted by a central domain with five-stranded β-sheet, surrounded by four α-helices, showing the classical Trx fold and the active site (CGPC) located between β strand 2 and α-helix 2 [3].

3.2. Production and Characterization of rTsTrx-1. *Escherichia coli* containing the expression vector pRSETB with the coding region for TsTrx-1 were induced with IPTG for 4 h; bacteria were centrifuged and the pellet was disrupted with 4 M urea. Because rTsTrx-1 was produced with six histidines in the amino terminal, the supernatant was through to a nickel affinity chromatography. Figure 4(a) shows the expression levels of the recombinant enzyme in *E. coli* and the purification steps were run on a 15% reduced SDS-PAGE. Lane 1 shows all the soluble proteins from *E. coli* induced with IPTG (a large band ~12 kDa is highlighted in the sample), whereas lane 2 presents the soluble proteins from *E. coli* before induction with IPTG. Lane 3 shows the wash fraction

before the elution step. Imidazole fractions were showed at lanes 4 to 7 (50, 100, 200, and 400 mM); rTsTrx-1 was eluted in the 100 to 200 mM imidazole. These fractions were pooled and loaded again on the same nickel affinity chromatography, following the same procedure without urea. A single band with an apparent *Mr* of 12 kDa (rTsTrx-1) was obtained in fractions with 100 to 200 mM of imidazole (lane 8), pooled fractions were dialyzed in TrisED buffer, and protein concentration was determined. The entire process yielded 10 mg/L of culture medium. Figure 4(b) shows the strong recognition of a band of ~12 kDa by the specific anti-TsTrx-1 antibodies in the western blot membranes containing the purified rTsTrx-1 (lane 1), crude extracts from larvae (lane 2), and adult (lane 3) *T. solium* stages and crude extracts from adult *T. saginata* (lane 4), adult *T. taeniaeformis* (lane 5), larval *T. crassiceps* (lane 6), adult *Hymenolepis diminuta* (lane 7), adult *Fasciola hepatica* (lane 8), and a weakly recognition for *Entamoeba histolytica* (lane 9). Anti-TsTrx-1 antibodies were not recognized in *E. coli* (lane 10) and *Homo sapiens* (lane 11) Trxs. The preimmune serum obtained from rabbit before immunization (lane 12) also did not recognize any band. Additionally, anti-TsTrx-1 antibodies strongly recognized a ~12 kDa band in cysticerci *T. solium* E/S Ag. Strips 14 and 15 are *E. coli* and *Homo sapiens* Trxs stained with Ponceau red.

3.3. Enzyme Activity and Optimal pH. Figure 5(a) shows the insulin reduction activity performed with concentrations of 1 to 20 μg of rTsTrx-1 at room temperature; all showed detectable insulin precipitation, but velocity and quantity of insulin reduction were dependent on rTsTrx-1 concentration. The maximal rate of precipitation was obtained with 20 μg (1.5 μM); however, there are no significant differences between 10 and 20 μg; even 5 μg reduced by half the amount of insulin precipitated. Therefore, the 20 μg concentration was used for the characterization assays. Similar results were obtained with rTrx-*E. coli* (control assay) which showed a better activity at 20 μg. A control reaction without rTsTrx-1 enzyme was carried out in parallel. Figure 5(b) depicts the enzyme exhibiting a triangle-shaped curve showing high activity (more than 84%) over a broad range of pH, between 6 and 7.5, but the optimal pH was 6.5 in PE buffer. The same figure shows that the activity was maintained around 58% at pH of 3, 5, 8, and 9 and was lost completely at pH 10.

3.4. Temperature Effect. Figure 6(a) shows the residual activity after exposing the rTsTrx-1 to different temperatures (15 to 100°C) at times (1, 3, and 24 h). The activity/temperature plots show a descending pattern. The enzyme maintained 100% activity for 3 h and lost it in 24 h at 37°C; at 55°C, 100% activity was maintained for 1 h; ~50% activity was lost at 3 h and lost activity completetly in 24 h. Finally at 70 and 100°C, the enzyme lost ~50%, ~75%, and 100% of activity at 1, 3, and 24 h, respectively. As negatives controls, we used a similar reaction without the Trx enzyme at all times; as expected, there was no activity. Noteworthy is that the loss of activity was not reversible in any condition.

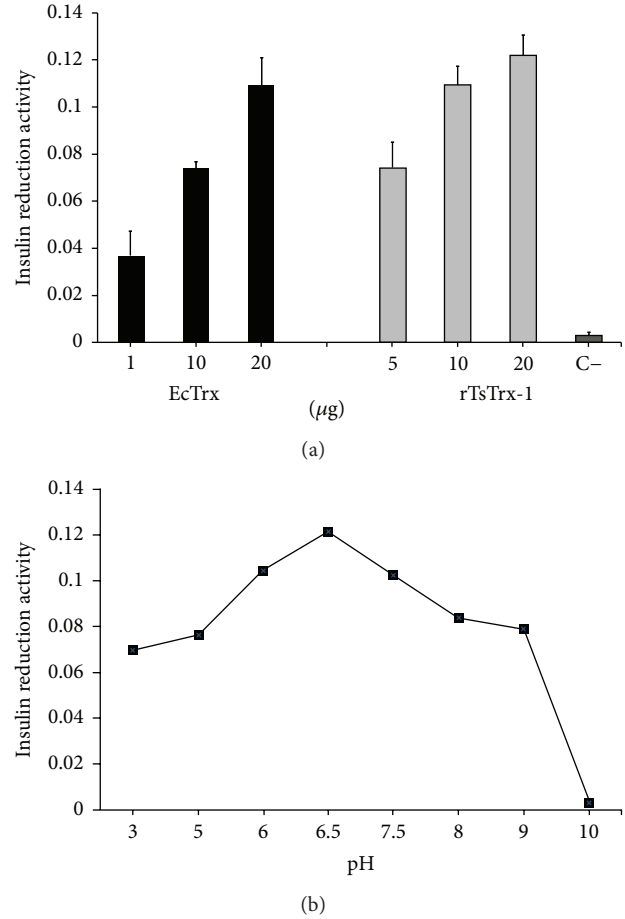


Figure 5: (a) Thioredoxin-catalyzed reduction of insulin. The increase in turbidity at 650 nm is plotted against the 1, 10, and 20 μg of *E. coli* rTrx (dark bars) and 5, 10, and 20 μg of *Taenia solium* thioredoxin-1 (rTsTrx-1, grey bars). (C–) Control lacking rTsTrx-1. (b) The pH enzymatic stability was determined incubating 20 μg of rTsTrx-1 at different pH between 3 and 10. The residual activity was measured as before.

To determine the best temperature to store the rTsTrx-1, assays such as those showed in Figure 6(b) were done. It shows that the enzyme stored at 25°C gradually lost 10%, 30%, and 60% of activity at 3, 7, and 14 days, respectively; at this same temperature, the enzyme lost the activity completely at 28 days. Decrease in activity at 15°C was of 10, 33, 42, and 70% at the tested times. Storage at 4°C induced a ~10% activity loss at 14 days and activity loss of 50% at day 28, whereas storage at 20°C induced a gradual activity loss of 33%, 50%, 85%, and almost 100% between days 3 and 28; at −70°C, the enzyme presented the best stability; it lost only 9% activity at 3 days and ~25% of activity between days 7 and 28.

3.5. Effect of NaCl, MG, H_2O_2, and Anti-TsTrx-1 Antibodies on rTsTrx-1. Exposure of rTsTrx-1 at 37°C for 30 min to different NaCl concentrations showed that concentrations of 250, 500, 1000, 1500, and 2000 mM decreased its activity ~ 9, 17, 17, 40, and 50%, respectively (Figure 7(a)). Moreover, exposure of rTsTrx-1 at 3 mM MG decreased 25% of its

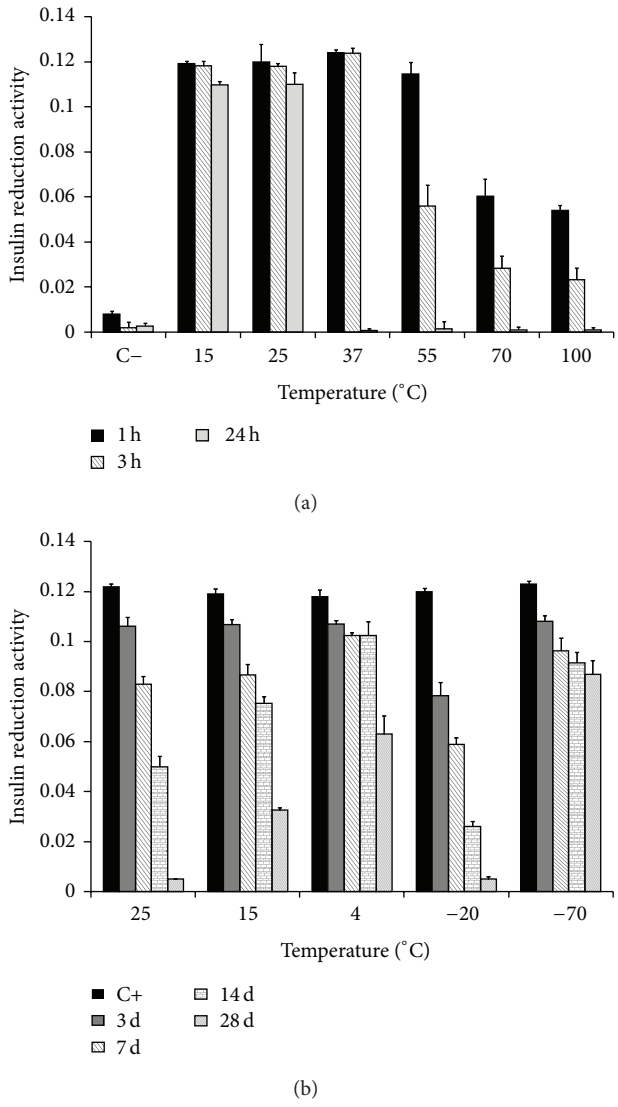


Figure 6: Effect of temperature on *T. solium* thioredoxin-1 (rTsTrx-1) activity. (a) rTsTrx-1 was incubated for 1, 3, and 24 hours at 15°C, 25°C, 37°C, 55°C, 70°C, and 100°C. (C−) Control lacking rTsTrx-1. (b) rTsTrx-1 was incubated during 3 to 28 days at 25°C, 15°C, 4°C, −20°C, and −70°C. (C+) Control of enzymatic activity was performed with a freshly made rTsTrx-1. Residual activity was determined by reduction of insulin assay.

activity, and higher concentrations inactivated rTsTrx-1 activity completely (Figure 7(b)). Concentration until 1 mM of H_2O_2 did not affect the enzyme activity of rTsTrx-1, whereas concentrations between 10 mM and 100 mM of H_2O_2 reduced the enzymatic activity (17, 25, 50, and 60%); concentration of 200 mM completely disrupted the activity (Figure 7(c)). A reaction without enzyme (negative control, C−) and a reaction with 1.5 μM rTsTrx-1 without treatment (positive control, C+) were used as controls. As expected, no activity was detected in C−; in contrast 100% activity was obtained in C+. Finally, anti-TsTrx-1 antibodies were incapable of inhibiting enzymatic activity (Figure 7(d)) and, as expected, normal IgG did not affect the TsTrx-1 activity.

4. Discussion

The analysis of the 5′-flanking region of the *TsTrx-1* gene reveals putative sites for Nrf2 and XBP1 transcription factors; these sites are presented in promoters for typical 2-Cys-peroxiredoxin of *T. solium* and *Trx-1* from human genes and both factors are positive regulators of antioxidant genes under stress condition [9, 16, 21–23]. This suggests *Trx-1* gene in cestodes could be regulated in this way. On the other hand, a putative TATA box was found at −19 bp and a DPE putative site at +22 bp, both related to TSS. It is known that the TATA box usually appears at −30 bp and DPE at +28 to +32 bp, even in Taeniidae family genes [9, 24, 25], and, for these reasons, neither TATA box nor DPE in the *TsTrx-1* gene is at a classical distance to be functional [26]. However, these assertions must be corroborated with functional studies. The comparison analysis of Inr sequences from the *TsTrx-1* gene with other Inr sequences from genes of the Taeniidae family suggests conservation in this motif with similarities to the mammalian consensus Inr sequence YYANWYY [9, 13, 24–26].

Comparison analyses of the coding region for the *Trx-1* gene show that *T. solium* (0.605 kb) and *E. granulosus* (0.609 kb) were identical. Even *S. mansoni Trx-1* gene was showed to have similar structure with three exons and two small introns; the composition of nucleotides and amino acids sequences is different. In contrast, they are different from the structure of human (~17 kb) and mouse (13 kb) genes, which have five exons and four big introns. These differences between number and sequence of introns could be used to design specific primers for the diagnosis of cysticercosis/taeniasis caused by *T. solium* using PCR with cerebrospinal fluid (CSF) and human feces [27]. This point is important for epidemiological studies, because it would allow identifying active infection of carriers of this parasite, which will be easy to treat with antihelminthic drugs. Moreover, the difference in introns and the presence of an intron in the same position in different *Trx-1* genes of different organisms suggest that it was present in the ancestor; however more detailed studies should be done to use intron position as marker of evolution [28].

The confrontation of cysticerci with the host immune response (inflammation) and oxidative stress in tissues with high oxygen, such as brain and muscle, in contrast to the adult stage that lives in the small intestine, where it is exposed less to these factors, could be the reason why RNA expression of the *TsTrx-1* gene is higher in cysticerci than in adults.

The primary sequence shows a typical catalytic site (CGPC), which executes the oxidoreductase activity, and the typical thioredoxin fold; even more, it shows higher identity with Trx from *E. granulosus*, a cestode, less identity with *S. mansoni*, a trematode, and poor identity with pigs and humans (intermediate and accidental hosts of *T. solium*). Polyclonal antibodies produced against rTsTrx-1 strongly recognized a ~12 kDa band in various *Taenia* species and *F. hepatica* and weakly in *E. histolytica* but did not recognize *E. coli* and human Trxs. These points suggest that specific regions of TsTrx-1 could be used in vaccination assays against *T. solium*.

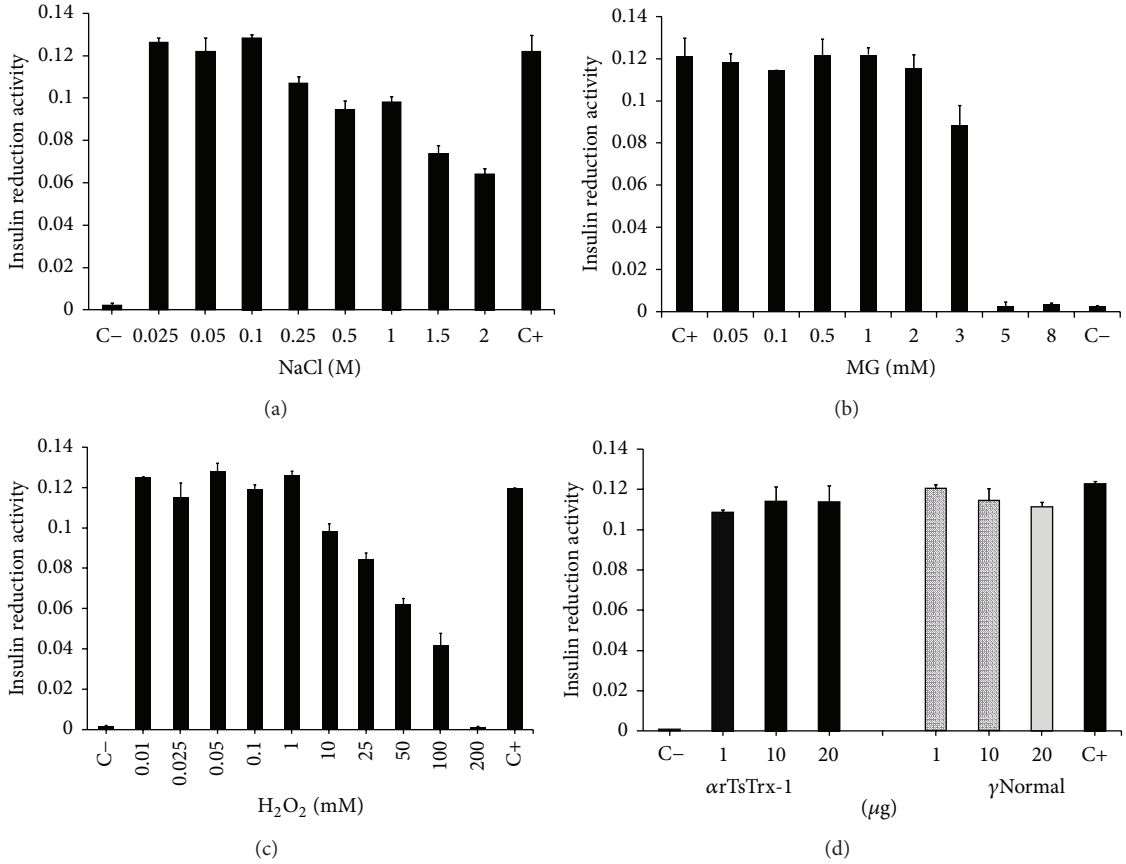


Figure 7: Effect on *T. solium* thioredoxin-1 (TsTrx-1) after incubation with different concentrations of (a) NaCl (25–2 M), (b) methylglyoxal (MG, 0.05–8 mM), (c) H_2O_2 (10 μM–200 mM), and (d) IgG rabbit anti-*T. solium* Trx-1 and IgG from preimmune serum (1, 10, and 20 μg). (C−) Control lacking rTsTrx-1 and (C+) control of enzymatic activity was performed with a freshly made rTsTrx-1.

Noteworthy, TsTrx-1, *E. granulosus*, and *S. mansoni* Trx-1 have a cysteine at position 27 close to the cysteines of the active site; moreover they lack cysteines 69 and 73, which regulate the activity and biological functions of the mammalian Trx-1. These findings give rise to the following questions. Does cysteine 27 have a role in the catalytical activity? Are the helminths Trx-1 not regulated by posttranscriptional modifications, and which is the biological consequence of this? [20].

The rTsTrx-1 enzyme exerted its optimal reductase activity in a range of pH and temperature of 6.5 to 7.5 and 4 to 37°C, respectively. Likewise, it presented 100% activity in a buffer with 100 mM NaCl and lost it gradually in a buffer with concentrations higher than 250 mM NaCl, losing up to 50% activity at 2 mM NaCl. On the other hand, the best way to store this enzyme to not lose its activity was at −70°C for 28 days and at 4°C for 14 days. The known biochemical properties of the enzyme, such as pH, temperature, buffers, cofactors, salt concentration, additives as glycerol, and oxidants and inhibitors, let us preserve its activity, which will help to determine enzymatic mechanisms with inhibitors or observe its effect on cells or organisms *in vitro* and *in vivo*.

The presence of MG and ROS especially H_2O_2 triggers oxidative stress, DNA damage, and apoptosis in cells. MG is a reactive carbonyl compound that causes glycation of proteins and is formed principally by glucose metabolism; it induces oxidative stress by inactivating antioxidant enzymes, such as Cu/Zn superoxide dismutase, glutathione peroxidase, and Trx reductase and decreases Trx protein level [29]. Activity of rTsTrx-1 was not affected by 2 mM of MG. On the other hand, ROS are continuously produced by the host's inflammation caused by the immune response. It is known that helminths lack catalase and present low activity of glutathione peroxidase; cysticerci of *T. crassiceps* (cestode) resist concentration of 2.5 mM of H_2O_2 *in vitro* for 2.5 h. We observed that rTsTrx-1 enzyme resists 1 mM H_2O_2. Both findings indicate that rTsTrx-1 is highly resistant to oxidant molecules and, together with the 2-Cys-peroxiredoxins of *T. solium*, could constitute the hydroperoxides-regulating system in this parasite [8, 9].

Trx is an essential component of the thioredoxin system, where it performs functions such as antioxidative, protein-reducing, and signal-transducing ones. In mammalian and helminths, the antioxidative activity is the most studied. However, there is evidence that Trx participates in signaling pathways, interacting with different proteins, to control processes such as development, proliferation, migration, apoptosis, inflammation, and metabolism [30]. No signal sequence was found on the *TsTrx-1* gene; however Trx-1 was found in the cysticerci E/S Ag and several reports have observed

that mammalian cells stimulated with lipopolysaccharide and viral infections are able to secrete Trx-1 [31, 32]. In addition, now it is known that helminths constitutively secrete Trx-1 and molecules that are able to modify the immune response by altering the normal signaling of host immune cells, letting them drive Th2 immune response, which allows for their long term establishment, such as the cases of 2-Cys-peroxiredoxins from *S. mansoni* and *F. hepatica* [33, 34].

In conclusion, the Trx-1 tools presented here could help to perform studies to know the role that Trx-1 plays in the host-parasite relationship; likewise its antioxidant and biochemical properties could be used to inactivate TsTrx-1 by drug or by vaccine. Furthermore, its importance in immune signaling pathways could let us think of it as a therapeutical molecule to other diseases.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors' Contribution

Lucía Jiménez and Oscar Rodríguez-Lima contributed equally to this work.

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Research Article

Clinicopathological and Immunological Changes in Indian Post Kala-Azar Dermal Leishmaniasis (PKDL) Cases in relation to Treatment: A Retrospective Study

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Post-kala-azar dermal leishmaniasis (PKDL) is an important factor in kala-azar transmission; hence its early detection and assessment of effective treatment is very important for disease control. In present study on 60 PKDL cases presented with macular, mixed papulonodular, or erythematous lesions, *Leishmania parasites* were demonstrated microscopically in 91% of papulonodular and 40% of macular lesions. Cellular infiltrates in skin biopsy imprint smears from lesions were mononuclear cells, 25–300/OIF (oil immersion field), predominantly histiocytes with vacuolation, many lymphocytes, some plasma cells, and *Leishmania* amastigotes 0–20/OIF. Cases with no demonstrable parasites were diagnosed on the basis of past history of VL, lesion's distribution, cytopathological changes, and positive DAT (86.83%). Following antileishmanial treatment with SAG, papulonodular forms of PKDL lesions disappeared clinically but microscopically the mononuclear cells (20–200/OIF) persisted in the dermal lesions. Response observed in macular PKDL lesions was poor which persisted both clinically and cytopathologically. Follow-up of PKDL will assess the effectivity of treatment as either disappearance of lesions or any relapse. Studies on involvement of immunological factors, that is, certain cytokines (IL-10, TGF-β, etc.) and chemokines (macrophage inflammatory protein, MIP 1-α, etc.) in PKDL, may provide insight for any role in the treatment response.

1. Introduction

Leishmania, a protozoan parasite, is the causative agent of various forms of leishmaniasis, like visceral form (VL), mucocutaneous form (MCL), and cutaneous form (CL), of which VL is fatal. Postkala-azar leishmaniasis (PKDL) is a cutaneous form of leishmaniasis and usually occurs one to several years after apparent cure of VL caused by *Leishmania donovani* [1]. In some cases, previous history of symptomatic VL may be absent [2] indicating the possibility of subclinical infection. PKDL is common in India where it occurs in 6–20% of kala-azar (VL) cases following its attack [3]. It is characterized by hypopigmented macules (discrete or confluent) all over body sparing palm, soles, scalp, and axillae and erythematous papules or nodules predominantly over face.

PKDL has long been suspected as a potential reservoir of kala-azar infection. It is considered an important factor in disease transmission between kala-azar outbreaks in India [4]. Since PKDL cases harbour leishmania parasites superficially in the skin lesions, it is easy for the vector sandfly to pick up the parasites from the lesions and therefore it is considered an important source for transmission of the disease. Hence, for effective control of VL and to interrupt the transmission of kala-azar infection, its reservoir host PKDL needs to be detected early and treated adequately. The present study was undertaken in PKDL cases from VL endemic areas of Bihar, India, with objective to detect the different forms of PKDL cases and to correlate the cytopathological changes and parasite load in their dermal lesions with immunological changes and clinical response after full course of treatment with sodium antimony gluconate (SAG).

2. Materials & Methods

PKDL cases ($n = 60$) from endemic zones of Bihar were included in this study for parasite load, pathological changes in the skin, and immune response before and after treatment with sodium antimony gluconate (SAG). All cases were examined for any past history of kala-azar and details of its treatment, pattern of distribution, type, duration, site, size, coalescence, and sensation of skin lesions present. The study was approved by Institutional Ethical Committee and written informed consent was obtained from all the subjects.

Skin biopsies were collected aseptically from edges of the dermal lesions. One part was put in the biphasic culture media and from other part, biopsy imprint smears were prepared on clean glass slides and stained with Leishman/Giemsa stain. The remaining part was fixed in 10% buffered formalin for histological sections.

Venous blood was collected for haematological (TC, DC of WBC, Hb%, and total platelet counts) and the immunological tests, that is, direct agglutination test (DAT, [5]) and migration inhibition factor (MIF, [6]).

2.1. Skin Biopsy/Smear Collection from PKDL Patients and Microscopy. Skin biopsies/smears were collected from the dermal lesions of PKDL patient aseptically who had given his/her consent before screening for the study.

Two clean glass slides were labelled by diamond pencil with laboratory number, which was also noted on patient's request form for skin biopsy/smear filled up and sent by the clinician to the Pathology laboratory. Selected site of skin lesion was sterilized with 70% alcohol swab and allowed to dry. Effort was taken to blanch the area by pressing with forceps on the base of the site. Using sterile scalpel with surgical blade and smooth end forceps, a tiny piece of skin tissue was cut superficially to avoid scarring. Imprint smears of skin tissue were prepared on the grease-free glass slides labelled with diamond pencil. After 5 minutes of soft pressure, the cut area of skin was covered with medicated dressing (handyplast) which is ventilated and containing antiseptic 0.2% Nitrofurazone.

For collection of skin smear, the skin is grasped between the thumb and forefinger of the hand until the site is blanched. Approximately 5 mm long and 1-2 mm deep incision was made with scalpel blade. The site of the incision is scraped inside with scalpel to obtain tissue fluid and pulp. Smears are made on the grease-free clean glass slides and prenumbered with diamond pencil.

After collection, skin smear slides were immediately fixed with methyl alcohol and allowed to dry at room temperature. Slides were stained with diluted (1 : 9) Giemsa stain for 25 minutes. Smears are examined under the oil immersion objective of the microscope for demonstration of *Leishmania* parasites [7]. Gradation of the parasite load in skin smear was done as per the WHO criteria [8].

2.2. Preparation of DAT Antigen [9]. Antigen prepared from crude *Leishmania donovani* promastigotes (96 h stationary-phase cultured promastigotes of a reference strain of *L. donovani* (WHOM/IN80/DD8), in a monophasic medium with 20% foetal calf serum) was used in the DAT study to observe the antibody titre [10]. Following trypsin treatment, the harvested promastigotes were washed twice with Locke's solution (154 mM NaCl, 2 mM $CaCl_2$, and 2 mM $NaHCO_3$, with 0.25% glucose) and then fixed, for 18–20 h, in 2% (w/v) formaldehyde in Locke's solution. After washing in citrate saline (0.5 M NaCl and 0.05 M sodium citrate at pH 7.4), the parasites were stained with 0.02% Coomassie Brilliant Blue, washed again with citrate saline, resuspended, at a concentration of 7.5×10^7/mL, in citrate saline containing 0.43% (w/v) formaldehyde, and then stored at 4°C until used.

2.3. Optimizing and Evaluating the Performance of the DAT. To determine a suitable threshold titre for seropositivity in the DAT and then to explore the sensitivity and specificity of the test, samples of the fingerprick blood were collected, onto 1-mm-thick filter paper. These subjects were parasitologically confirmed, active and untreated cases of VL ($N = 108$), the cases of tuberculosis ($N = 10$), malaria ($N = 10$), leprosy ($N = 10$), and filariasis ($N = 10$), and apparently healthy controls who lived either in the nonendemic districts ($N = 452$) or, adjacent to an endemic area, in Muzaffarpur district ($N = 189$). Bone marrow and/or splenic aspirates from each of the VL cases were investigated. Amasigotes had been found in the aspirates, and when the blood samples had been collected, each of these cases had splenomegaly or recent history of fever.

The blood spots were allowed to dry before 0.5 cm-diameter circles (each covered in dried blood) were cut from the dried filter paper sheets. Each of the circles was then immersed in 165 μL saline (0.9% NaCl, pH 7.4) to give a solution of serum diluted 1 : 50 in saline [11].

The dilution sera were then investigated in DAT [11], which were performed in microtitre plate (Nunc, Roskilde, Denmark) that each had 96, V-shaped wells. In these tests, saline supplemented with 0.2% (w/v) gelatin and 0.78% (v/v) β-mercaptoethanol was dispensed into the wells of the plate (at 50 μL/well), so that a 50 μL sample of each test serum (already diluted 1 : 50 during its elution from a filter-paper circle of dried blood) could be further diluted, in twofold series. Some wells (the first in each row) were left serum-free, as controls. Reference sera, known to be positive or negative for antileishmanial antibodies, were also included, as controls. The suspension of stained parasites was then dispensed into the wells, at 50 μL/well, before the plates were covered and the contents of each well were mixed (by gentle swirling each plate across the bench top for 30 s). After incubation at 18–22°C for 18 h, each plate was read by eye, the end-point titre for each test serum being the highest dilution giving visible agglutination (i.e., blue mats or blue dots, with or without fuzzy edges that were larger than those seen in the negative-control wells).

The sensitivity of the DAT was evaluated by comparing the results of the VL/PKDL cases with those of the apparently healthy controls. The specificity of the test was evaluated, however, by comparing the results of the VL cases with those of the patients who had nonleishmanial infections. The way in

which the titre used as the threshold for positivity influenced the sensitivity and specificity of the DAT and the relationship between sensitivity (true positivity) and specificity (false negativity) were used to identify the optimal threshold titre. The DAT was then used, with this optimal titre, to see how useful it would be in detecting subclinical infections.

Migration inhibition factor (MIF) assay was evaluated in PKDL and active kala-azar (as control) cases to measure the cell mediated immunity (CMI) status [12]. In brief, lymphocytes were separated from heparinized blood and cells were washed three times with RPMI-1640. Finally, the aliquots were distributed in 4 cells cut in a preparation of agarose in a petri dish (15 × 90 mm). Two wells were filled with soluble or LD antigen while the rest were filled with the medium (control wells). The petri dish was incubated overnight at 37°C in humidified chamber with 5% CO_2. Next day, the cells that had migrated under the agarose were fixed and stained. The diameter of the migration areas was measured to calculate the MI [13].

All these tests were done before and after treatment with SAG (10 mg/kg/day for 90 days).

Biopsy imprint smears were examined under microscope (Oil immersion and high power fields) for detection of leishmania parasites and cytological changes in skin lesions of postkala-azar dermal leishmaniasis during different phases of collections.

3. Results

Out of 60 PKDL cases, 39 were males and 21 females aged between 7 and 57 years. Only 54 cases had past history of VL of duration ranging between 3 months and 10 years, and the remaining 6 cases had no past history of VL. The duration of dermal lesions in these cases was in range of 2 months to 20 years. Twenty-five (41.67%) PKDL cases had only hypopigmented macular lesions, 15 (25%) cases had papuloerythematous lesion and 20 (33.33%) cases had mixed lesions (with all macular, erythematous, and papulonodular type) (Table 1).

Microscopically leishmania parasites were detected in 95% of mixed papulonodular lesions whereas only in 40% of cases with macular lesions. PKDL cases, where no parasites were demonstrated microscopically, were diagnosed on basis of clinical presentation, past history of kala-azar and its treatment, histopathological changes and cytological findings in the imprint smears of biopsy from skin lesions, and DAT results. The leishmania parasite density in the positive cases was 0–20/OIF of the biopsy imprint smears. They were found mostly (88%) extracellular. Most of the parasites (73%) were of proper morphology with healthy looking but few (27%) were with faint cytoplasmic border.

3.1. Cytopathological Changes. The dermal infiltrates observed in the biopsy imprint smears consisted of mononuclear cells (25–300/OIF), a mixture of histiocytes, lymphocytes, and occasional plasma cells. In papulonodular lesions, the histiocytes were predominant cells with many activated macrophages having vacuolated appearance and scattered epitheloid cells and plasma cells. In hypopigmented macular lesions, the predominant cells were lymphocytes with some histiocytes and scarce plasma cells. DAT was positive in 86.83% of PKDL cases with titre ranging between 1 : 800 and 1 : 25600 and MIF was positive (>20%) in 70% of PKDL cases studied, with result range of 17–38% (mean 27.9%). All the ten active kala-azar cases were negative for migration inhibition factor (<20%) (Table 2).

Pathologically, after schedule treatment with SAG, papulonodular form of all PKDL cases was negative for leishmania parasites except one. Mononuclear cells in imprint smears came down to 20–200 cells/OIF with many histiocytes, increase of lymphocytes, and occasional plasma cells. Although the lesions improved clinically, the cytological changes in the dermal lesions persisted even after treatment. In case of macular lesions, there were no significant changes in the lesions both clinically and pathologically after treatment. The number of mononuclear cells was in the same range (15–50/OIF). Thus, it was very difficult to decide the complete cure in these forms. After treatment, the DAT titre was in the range of 1 : 800 to 1 : 6400 whereas MIF was positive in all the ten PKDL cases with value of range 33–65% (mean 46.8%). In 3 PKDL cases, the lesions persisted cytopathologically even after one year of treatment. In all PKDL cases, no significant haematological changes were observed in either group except increase of eosinophils (ranged 10–30%) of total leucocytes.

4. Discussion

Persistence of Kala-azar in endemic form in Bihar and other affected areas and the absence of zoonotic reservoir of kala-azar infection as observed in several studies [14, 15] raised a possibility of existence of human reservoir in the form of PKDL or asymptomatic carriers in the community, as they harbour leishmania parasite in the skin or blood. They may help in persistence of transmission of infection and spread of the disease.

In our study, diagnosis of PKDL was confirmed pathologically by microscopic demonstration of parasites in the skin lesions of patients presented with clinical features of PKDL. Clinical diagnosis was based on characteristic skin lesions in the patients treated for kala-azar. Diagnosis may be problematic in cases with long clinical interval with up to 30 years, being reported between completed treatment of Kala-azar and the appearance of skin lesions [16–18].

A definitive diagnosis is demonstration of parasites in the skin lesions, but parasites are often scanty and require a prolong research. In our study, thin skin biopsies were collected superficially from suspected PKDL lesions to prepare imprint smears for microscopic demonstration of leishmania parasites resulting in high positivity of 95% in mixed papulonodular lesions (Table 1). This shows that leishmania parasites are most abundant just beneath the epidermis and it should be taken into consideration when taking biopsy and making smears [2]. Culture is often difficult due to risk of bacterial and fungal contamination in the skin tissues [19]. Taking biopsy and smears is often resented in young

Table 1: Showing age, sex, duration of past history of Kala-azar and leishmania positivity in various types of PKDL cases.

Type of lesion	Hypopigmented macular	Papuloerythematous	Mixed macular Papulonodular lesion	Total
Number of PKDL Cases	25	15	20	60
With past history of Kala-azar	21	14	19	54
No past history of Kala-azar	04	01	01	06
Mean age range (years)	14.15 (7–30)	21.78 (10–36)	26.86 (17–57)	20.45 (7–57)
Sex (male/female)	15/8	6/9	18/4	39/21
Duration of past History of Kala-azar in mean (range)	5 years (4 months–10 years)	8.95 years (1–22 years)	8.75 years (3–18 years)	7.5 years (4 months–22 years)
Leishmania parasite positivity number (%)	10 (40%)	13 (86.67%)	19 (95%)	40 (66.67%)
DAT positivity (%) cases titre	83.4% (1 : 800–1 : 6400)	83.33% (1 : 1600–1 : 6400)	93.75% (1 : 12800)	86.83% (1 : 800–1 : 12800)

Table 2: Clinicopathological changes in PKDL lesions in relation to treatment and immune response.

Parameters	Before treatment	After treatment
Clinical changes	Nodulo-papular	Absent or low papular lesion
	Erythematous over whole face	Over chin only
	Macular over all body	Present but slightly fainter
Microscopic findings		
Leishmania parasite Positivity with density	Positive in 91% of papulonodular cases and 40% of macular cases. Mostly extracellular, only few intracellular. (0–20/OIF)	Negative in all except one.
Cytological changes	Mononuclear cells (+++)	Number decreased (++)
	25–300/OIF, mostly clustered	20–200/OIF, Scattered.
	Histiocytes predominant (15–250/OIF)	Many Histiocytes (10–140/OIF)
	Lymphocytes: 10–40/OIF	Lymphocytes: 10–60/OIF
	Plasma cells, epitheloid cells occasional	Plasma cells scarce
	Many activated macrophages with vacuolated appearance	Some activated macrophages with vacuolation present
Immunological response	DAT—75% positive	DAT—66% positive
	MIF +ve 70%, value 17–38%	MIF +ve all, value 33–65%

children since face is the most common site of the lesion. Other techniques such as PCR are promising but yet to be assessed for their application in PKDL diagnosis since limited studies have been conducted in these cases [19, 20]. DAT is useful in differential diagnosis, for example, with leprosy [21], although a negative result does not exclude kala-azar or PKDL, as found in our present study also.

Studies of the immune responses showed that unlike kala-azar patients, in most (70%) of the PKDL cases, T-cells reacted in response to leishmania antigen as observed in MIF assay and there was no suppression of CMI response in them. In contrast to patients with kala-azar who show no response to leishmania antigen, patients with PKDL develop some degree of immunological competence to the parasite, but the response is insufficient to bring about its elimination from the skin [22].

In the present study, we have observed that, after scheduled course of treatment with SAG in PKDL cases, although skin lesions disappeared clinically in papulonodular and erythematous cases with no parasite in most of the cases, the dermal pathology with cellular infiltration persisted as observed microscopically in the imprint smear cytology of skin biopsy collected from the same site. But the density and number of the mononuclear cells per oil immersion field (OIF) of the microscope were comparatively less (20–200/OIF) than before initiation of the treatment (25–300/OIF) (Table 2). This indicates that the cases might have responded clinically, but the cytological response within the dermal lesion is gradual. Hence, microscopic examination for imprint smear cytology along with leishmania parasite detection in thin skin biopsy from PKDL lesion site should be followed at the interval of 3–6 months to ensure the complete treatment both clinically and pathologically. In macular cases, no conspicuous difference was observed in the lesions both clinically and microscopically in the imprint smear cytology and in DAT results. Hence, it is very difficult to decide the complete cure in these forms of PKDL.

Further studies on the involvement of certain immunological factors such as some cytokines (IL-10, TGF-β, IFN-γ, etc.) and chemokines (macrophage inflammatory protein,

MIP 1-α, Rantes, etc.) in the dermal lesions of PKDL cases (before and after chemotherapy) may provide insight for any role in the disappearance of lesion and treatment response.

Some of the PKDL cases required prolonged treatment and these cases are being followed up for two years, cytopathologically with parasite detection and correlating with clinical and immunological changes to observe any redevelopment of dermal lesions or complete recovery. There is no satisfactory test of cure in macular PKDL cases as parasites are often difficult to find in smears or biopsies from these skin lesions and there are no conclusive immunological findings in relation to treatment in these cases that ensure the response of the specific chemotherapy. The PCR may prove an important tool in this respect but is yet to be evaluated [23].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Transfection of Platyhelminthes

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Flatworms are one of the most diverse groups within Lophotrochozoa with more than 20,000 known species, distributed worldwide in different ecosystems, from the free-living organisms in the seas and lakes to highly specialized parasites living in a variety of hosts, including humans. Several infections caused by flatworms are considered major neglected diseases affecting countries in the Americas, Asia, and Africa. For several decades, a particular interest on free-living flatworms was due to their ability to regenerate considerable portions of the body, implying the presence of germ cells that could be important for medicine. The relevance of reverse genetics for this group is clear; understanding the phenotypic characteristics of specific genes will shed light on developmental traits of free-living and parasite worms. The genetic manipulation of flatworms will allow learning more about the mechanisms for tissue regeneration, designing new and more effective anthelmintic drugs, and explaining the host-parasite molecular crosstalk so far partially inaccessible for experimentation. In this review, availability of transfection techniques is analyzed across flatworms, from the initial transient achievements to the stable manipulations now developed for free-living and parasite species.

1. Platyhelminth Transfection Studies

The phylum Platyhelminthes or flatworms represent one of the most diverse groups within Lophotrochozoa with about 20,000 species distributed worldwide including free-living and parasitic organism classified into 17 major groups [1, 2]. All these acoelomate worms have bilateral symmetry; they are hermaphrodite with some exceptions and have a simple centralized nervous system and a mesodermal germ layer [3, 4]. Flatworms are characterized by a high degree of morphological diversity and reproduction modes (Table 1). The phenomenon of asexual reproduction that is uncommon in the animal kingdom occurs in all major groups of flatworms. This supports the presence of a population of totipotent stem cells called "neoblasts" in free-living worms and "germ or germinal cells" on flukes and tapeworms [4]. Several human infections caused by flatworms are considered major neglected tropical diseases (NTDs) by the World Health Organization: cysticercosis, schistosomiasis, fascioliasis, paragonimiasis, and echinococcosis [5].

Developing techniques to manipulate flatworms is a growing topic in contemporary research as judged by the number of reports published during the last decade [6]. Maintenance of parasite species under laboratory conditions has been challenging and genetic manipulation is still difficult [7]. However, since the 90s, attempts have been made to identify and characterize the regions controlling the expression of genes in several species of flatworms [8]. Due to the lack of a good expression system for heterologous genes in these organisms, several mammalian cell lines have been employed as transfection targets to identify functional promoters in flatworms [9, 10]. In this regard, the recently described genomes for several of these organisms, including the free-living planarian *Schmidtea Mediterranean* [11], and the parasites *Schistosoma mansoni*, *S. japonicum* [12, 13], *Taenia solium* [14, 15], *Echinococcus granulosus,* and *E. multilocularis* [15] represent a considerable advantage. Those genome projects allowed us to identify orthologous genes of each species and group and their functional promoters as well as to carry out *in silico* metagenomic studies. Transfection studies for each of the three groups of Platyhelminthes done so far are described in this short review.

1.1. Tricladida. Planarians have the capacity of regenerating complete worms from a small fragment of their bodies

Table 1: Main characteristics of the groups where genetic transfection has been achieved*.

Group	Biologic interactions	Adult body	Life cycle	Example of genus
Tricladida	Mostly free-living	Nonsegmented	Simple	*Dugesia, Schmidtea*
Trematoda	Endoparasites of invertebrates and vertebrates	Nonsegmented	Complex	*Fasciola, Schistosoma*
Cestoda	Endoparasites of vertebrates	Segmented	Complex	*Taenia, Echinococcus*

[16]. In 1981, Baguñà described a group of cells conferring these regenerative properties as "neoblasts" [16–18]. In order to understand the basis of tissue regeneration in these flatworms, several studies were conducted [18], which could represent a valuable contribution to human regenerative medicine [16] as well as to the establishment of stable germ cell lines useful in transfection studies [19]. However, it was not until the advent of the molecular biology and genetic tools that further investigation in this phenomenon was possible. Thus, in 1999 the Dglvs gene (*Dugesia* VASA-like) was reported as the first gene expressed in neoblasts [20] and, almost simultaneously, a successful application of RNA interference (RNAi) was reported [21]. Since then, several related neoblast genes have been described and strategies for transient transfections have been developed for Tricladida [16]. The most used method for introducing exogenous genes in the different stages of these organisms is microinjection, which is also frequently used for silencing genes such as Djpum, nanos, ß-catenin, ndk, DjFGFR1, and DjFGFR2 [16, 18, 22, 23]. This method, although highly efficient in adult flatworms, was very invasive for early developmental stages. More recently, a novel method for introducing exogenous materials into developing planarian embryos by nanosecond exposure of eggs to pulsed laser has been reported. This represents the first report of planarian embryos being genetically modified without compromising their normal development [17]. However, availability of suitable vectors for stable transfections is required to allow incorporation of exogenous genetic material into the genome of these organisms. For example, in the case of planarians three mobile elements (mariner, Hermes, and PiggyBac) have been introduced using the green fluorescent protein (EGFP: enhanced green fluorescent protein) as reporter gene, using microinjection followed by electroporation to transfect the parenchymal cells of adult flatworms [19]. Until now, the three transposons have shown good efficiency of integration into the genome in neoblast cells. PiggyBac and Hermes appear to be quite stable showing a good expression after eight months of transfection [19].

2. Digenean Trematodes

Trematode infections reach high prevalence in developing countries [5, 24]. The helminth infection with the largest global prevalence is schistosomiasis with 207 million cases worldwide, mainly caused by three species of blood flukes: *S. haematobium*, *S. mansoni,* and *S. japonicum*. In the case of trematodes, extensive studies on vaccines, drug development, and diagnostic methods are available [24]. Moreover, the complete genomes of *S. mansoni* and *S. japonicum* have been elucidated [12, 13]. Attempts of identifying genes and introducing heterologous genetic material have been carried out for more than a decade. New technologies have enabled success to identify, to silence, and to carry out transient transfections of several genes. Stable transfections have been achieved, allowing the approach to questions about the involvement of specific genes in disease pathogenesis or the identification of new target candidates for drug treatment [24]. Several reviews are available where the genomic history of schistosomes, including advances on transfection, is well organized [8, 10, 25–29]. Table 2 summarizes the progress in the transfection of *S. mansoni* and *S. japonicum*.

Other trematodes causing infections of high global prevalence (>40 million cases) [24], such as *Clonorchis sinensis* (liver fluke), *Opisthorchis viverrini* (liver fluke), *Paragonimus spp* (lung fluke), *Fasciolopsis buski* (intestinal fluke), and *Fasciola hepatica* (intestinal fluke), have not been successfully transfected; successful methodologies developed for *S. mansoni* could be adapted for these trematodes [27]. However, the promoter region of cathepsin 1 from *F. hepatica* has been characterized through transient transfection of mammalian Vero cells [30]. Another case is the *Paragonimus westermani* retrotransposon sequences belonging to three LTR (long terminal retrotransposons) retrotransposon families [31]. Two of these retrotransposon sequences appeared to maintain their mobile activities as suggested by the presence of mRNA transcripts [31]. The ability to integrate into the flatworm genome makes transposons and retrotransposons excellent candidates to develop stable transfections [32].

Three methods have been exploited for nucleic acid delivery into schistosomes [28]: biolistic (particle bombardment/gene gun), electroporation, and infectious retroviral vectors (Table 2). Electroporation has been considered as the most efficient method for transfection of sporocysts and schistosomules. However, biolistic has also been successfully used on miracidia and adults [33]. The choice of a delivery method depends on the organism and the life cycle stage under study. Moreover, experiences in schistosomes can also help to choose and adapt one transfection method on related organisms.

An application of transient transfection methodologies is the silencing of specific genes through RNAi, involving studies on worm viability, development, tegument physiology, egg development, signaling pathways, and drug discovery. Efficacy of RNAi can be influenced by the method of delivery: the more often used in schistosomes are soaking and electroporation [8] and the most frequently used RNAi in schistosomes is dsRNA (long double stranded), followed by siRNA (small interfering) [29]. The properties of each RNAi have been important to define their use; for example, it has been suggested that siRNA accumulates faster in certain tissues [50], whereas dsRNA is more stable to RNAse digestion [51].

Table 2: Transfection of heterologous genes in Schistosomes.

Species	Agent and method	Promoter	Reporter gene	Life stage transfected	Transfection type	References
S. mansoni	RNA and plasmid by particle bombardment	Spliced Leader	Luciferase	Adult worm	Transient	[34]
	Plasmid by particle bombardment	Hsp70	GFP	Adult worm and sporocysts	Transient	[35]
	Plasmid by particle bombardment	ER60	GFP	Female miracidia with sporocysts	Transient	[36]
	Plasmid by particle bombardment	SmCNA	GFP	Adult worm	Transient	[37]
	Plasmid by particle bombardment	ER60	GFP	Adult worm	Transient	[38]
	Plasmid by particle bombardment	Hsp70	EGFP	Miracidia	Transient	[39]
	RNA by electroporation	—	Luciferase	Schistosomula	Transient	[40]
	RNA by particle bombardment and electroporation	—	Luciferase	Sporocysts, miracidia, and adult worm	Transient	[41]
	VSVG-pseudo MMLV plasmid by cation polybrene	SL and hsp70	EGFP and Luciferase	Schistosomula	Transient	[42]
	Electroporation	SmACT1.1	Luciferase	Schistosomula	Transient	[43]
	PiggyBac by electroporation	Actin and HSP70	Luciferase	Schistosomula	Stable	[44]
	VSVG-pseudo MMLV plasmid by lipofectamine	Sma-Zinc	Luciferase	Adult worm and schistosomula	Stable	[45]
	RNA and VSVG-pseudo MMLV by electroporation	—	CY3 and luciferase	Eggs	Stable	[46]
	MLV pseudotyped plasmid by lipofectamine or polyethylenimine	MLV 5′, Pol II, *vasa-like,* Actin, Pol III U6	Luciferase and EGFP	Schistosomula, eggs, and adult worms	Stable	[47]
S. japonicum	Plasmid by electroporation	CMV	EGFP and luciferase	Schistosomula and adult worm	Transient	[48]
	VSVG-pseudo pantropic retrovirus plasmid by cation polybrene	LTR	hTERT	Schistosomula	Stable	[49]

SL: splice leader, hsp70: heat-shock protein 70, ER60: endoplasmic reticulum 60, SmCNA: *Schistosoma mansoni* calcineurin 1, CMV: cytomegalovirus, SmAct 1: *Schistosoma mansoni* actin 1, Sma-Zinc: *Schistosoma mansoni* Zinc finger protein, hTERT: human telomerase reverse transcriptase, VSVG: vesicular stomatitis virus glycoprotein, MMLV: Moloney murine leukemia retroviral, and LTR: retrovirus long terminal repeat.

In addition, dsRNA experiments are cheaper than the siRNAs counterpart [29, 51]; however, siRNAs can be more efficient inhibitors when multiple sequence oligonucleotides are used against the same target [8, 28, 29, 51, 52]. Developments of RNA silencing in schistosomes and other trematodes have accumulated during the last decade (Table 3).

Table 3 shows that although the most widely used RNAi is dsRNA gene silencing also can be efficiently achieved with siRNA [29]. The RNAi agent and the delivery method can be defined after the gene target and the stage of the parasites are selected. It is worth mentioning that initial attempts towards knocking down the expression of *S. mansoni* essential genes through *in vivo* administration of siRNA on infected hosts have produced encouraging results [53]. This strategy, that takes advantage of the low mRNA levels of the homologue gene in the host's tissues (hypoxanthine-guanine phosphoribosyl transferase: HGPRTase), is restricted in the case of other essential genes [54].

3. Cestodes

Among cestodes the most important infections in public health are cysticercosis and hydatosis or echinococcosis, with high global prevalence in endemic countries [5, 85]. In the case of these parasites, extensive studies on immunodiagnosis, drug and vaccine development, and so forth have been carried out [85–87]. However, transfection studies on cestodes have been scarce. An important development in the manipulation of these parasites is the isolation of germinal

Table 3: RNA silencing in trematode parasites.

Species	Rnai	Target gene	Life stage target	Silencing efficacy	References
S. mansoni	dsRNA	SGTP1 and GAPDH	Miracidia and sporocyst	70–80% (t); 40% (p)	[55]
	dsRNA	SmCB1	Schistosomula	10-fold (t)	[56]
	dsRNA	SmCB1 and SmCB31	Cercariae and adult worms	80% (t)	[57]
	dsRNA and siRNA	SmAP	Cercariae and adult worms	>90% (t); >70% (p)	[58]
	siRNA	SmRPNII/POH1	Schistosomula	80% (t)	[59]
	dsRNA	Cathepsin D	Schistosomula	100% (t)	[60]
	siRNA	HGPRTase	Cercariae	↓ 27% parasite load, 65% (t)	[53]
	dsRNA	SmLAP 1 and SmLAP2	Eggs	↓ 80% hatching	[61]
	dsRNA	32 genes (antioxidants, transcription factors, cellular signaling, and metabolic enzymes)	Miracidia	Mobility, growth, and viability affected	[62]
	siRNA	SmAP	Adult worms	80% (t)	[63]
	dsRNA	SmTK4	Adult worms	17–63% (p)	[64]
	dsRNA	SmAQP	Adult worms	90–95% (t)	[65]
	dsRNA	SmPAL	Adult worms	Inconsistent results	[66]
	dsRNA	SmGTP-1 and SmGTP-4	Adult worms	*In vivo*: SmGTP-1 55% (t), SmGTP-4 85% (t); *In vitro*: SmGTP-1, 70% (t), SmGTP-4, 90% (t)	[67]
	dsRNA	11 genes	Schistosomula	40–75% (t)	[68]
	dsRNA	SmCa1 and SmCa2	Miracidia	35% (p)	[69]
	sh-RNA	Luciferase	Schistosomula	47.5% (p)	[70]
	dsRNA	SmAP	Adult worms	95% (t)	[71]
	dsRNA	Sm-NPP-1	Schistosomula and adult worms	55% (t)	[72]
	siRNA	SmCD59	Schistosomula	60% (t)	[73]
	dsRNA	$SmCaMK_2$, SmJNK, SmERK1, SmERK2, and SmRas	Schistosomula	SmERK1 92% (t), SmERK2 56% (t), SmRas 42% (t)	[74]
	dsRNA	SmACC-1 and SmACC-2	Schistosomula	SmACC-1 60% (t), SmACC-2 90% (t)	[75]
	siRNA	SmAP, SmNPP-5, and SmATPDase1	Schistosomula and Adult worms	SmAP 90% (t), SmNPP-5 >90% (t), SmATPDase1 80% (t)	[54]
	siRNA	Sm5HTR	Schistosomula and adult worms	Larvae: 100% (t) and ↓ 80% motility; adult male and female: 90% (t) and ↓ 60% motility, 80% (t) and ↓ 50% motility, respectively	[76]
S. japonicum	dsRNA	SjGCP	Adult worms	75% (t)	[77]
	siRNA	Mago Nashi	Schistosomula	66–81% (t)	[78]
	dsRNA	Prxs 1 and Prxs 2	Schistosomula and adult worms	~20% (t)	[79]
	dsRNA	(SHSP) Sjp40	Adult worms	80% (t)	[80]
	siRNA	SjAR (SiRNA1 and SiRNA2)	Schistosomula	48% (t) and 73% (t)	[81]

Table 3: Continued.

Species	Rnai	Target gene	Life stage target	Silencing efficacy	References
S. haematobium	siRNA and dsRNA	Luciferase and Sh-tsp-2	Eggs, schistosomula, and adult worms	>75% (p) for both	[82]
F. hepatica	dsRNA	FheCL and FheCB	Metacercariae	FheCL1: 80% (t)	[83]
	dsRNA	FhLAP	Young larvae	>90% (p)	[84]

SGTP: facilitated diffusion glucose transporter, GAPDH: glyceraldehyde-3-phosphato-dehydrogenase, SmCB: *Schistosoma mansoni* cathepsin B, SmAP: *Schistosoma mansoni* alkaline phosphatase, SmRPNII/POH1: *Schistosoma mansoni* proteasome subunit, HGPRTase: hypoxanthine-guanine phosphoribosyl transferase, SmLAP: *Schistosoma mansoni* leucine aminopeptidase, SmTK4: *Schistosoma mansoni* SYK kinase, SmAQP: *Schistosoma mansoni* aquaporin gene, SmPAL: *Schistosoma mansoni* peptidylglycine alpha-amidating lyase, SmGTP: *Schistosoma mansoni* glucose transporter, SmCa: *Schistosoma mansoni* calmodulin sensing, Sm-NPP-1: *Schistosoma mansoni* neuropeptide precursor 1, SmCaMK: *Schistosoma mansoni* calmodulin-binding kinase, SmJNK: *Schistosoma mansoni* C-JUN-N-terminal kinase, SmERK: *Schistosoma mansoni* extracellular signal-regulated kinase, SmRAS: small GTPase superfamily, SmACC: *Schistosoma mansoni* acetylcholine-gated chloride channels, SmHTR: *Schistosoma mansoni* serotonin-activated G protein-coupled R, SjGCP: *Schistosoma japonicum* gynecophoral canal, Prxs: peroxiredoxin, Sjp40: *Schistosoma japonicum* short heat-shock protein, SjAR: *Schistosoma japonicum* aldose reductase, FheCL and FheCB: *Fasciola hepatica* cathepsin L and B, FhLAP: *Fasciola hepatica* leucine aminopeptidase, and sh-tsp-2: transcription of tetraspanin 2. (↓): knockdown; (t): transcript; (p): protein.

cells lines. For *T. crassiceps* it was possible to regenerate complete cysticerci from cellular clusters [88]; for *E. multilocularis* new metacestodes were regenerated from the germinal layer [89]; in the case of *E. granulosus,* the isolation and *in vitro* maintenance and propagation of germinal cells have been reported [90, 91]. The most significant development in the transfection of cestode parasites was achieved on *E. multilocularis* using axenic cultures of metacestodes. After some time in coculture with rat hepatocytes, the germinal cells formed a laminar layer and then clustered until the regeneration of the metacestode vesicles [92]. The first attempts of a transient transfection were done by lipofection of germinal cells with a cyanofluorescent gene as a reporter under the control of *elp* (encoding the ezrin-radixin-moesin- (ERM-) like protein), an *E. multilocularis* gene promoter [92]. Transient expression of the fluorescent protein was detected. Furthermore, these cells of *E. multilocularis* were infected with the intracellular bacterium *Listeria monocytogenes*, demonstrating a good nucleic acid carrier system [92]. The use of an attenuated, self-destructive bacteria is exciting, as it can reach the cytosol of the host cells and induce the expression of a heterologous gene under the control of the P_{acta} promoter [93]. This approach could be useful for other Platyhelminthes where cell lines can be isolated and maintained *in vitro*. In the case of gene silencing, an experiment in which *elp* and 14-3-3 were used as target genes, employing soaking and electroporation to deliver the siRNA, showed that the protein expression of 14-3-3 and *elp* decreases ~22% and ~72%, respectively, on day fifteen, after transfection of the protoscoleces of *E. multilocularis* [94]. Another silencing experiment was performed in the cestode of ruminants *Moniezia expansa*; the aim was to silence the transcription of actin (Me-act-1) gene using dsRNA. The reduction of actin expression was detected by immunohistochemistry and western blot techniques, in addition to severe damage in the morphology of tegument [95]. These studies demonstrated that the transfection and gene silencing techniques can be successfully used in cestodes. In fact, we have achieved successful transient transfection of *T. crassiceps* cysticerci *in vitro* by microinjection using a cytomegalovirus promoter and GFP as a reporter (submitted for publication). In addition, we are conducting assays to achieve stable transfection using PiggyBac transposon, as well as developing strategies for the introduction of dsRNA to silence target genes in *T. crassiceps*.

Thus, the genetic manipulation of cestode parasites is currently under examination with the goal of developing reliable methodologies for stable transfection and *in vitro* maintenance of cell lines.

4. Conclusion

The new technologies for genetic manipulation and transgenesis have been used in trematode parasites, specifically in *S. mansoni* [29], which is a starting point for other flatworms. However, the progress in helminths and especially in cestodes has been limited by the inability to produce stable cell lines, although the recent advances in *Echinococcus* are encouraging. It is important to remark that the advance in Platyhelminth parasites is still limited in comparison with the protozoan parasitic organisms, where highly reproducible transfection methods, including stable transfections [9], have been available for some time. Transposons, bacteria, viruses, and constructs with sequences that allow integration of exogenous sequences into the flatworms genome have been already used, but successful experiments have been only reported for *Schistosoma* [10, 28, 29]. It is expected that similar gains can be achieved in other flatworms. If so, molecular helminthology will be transformed from descriptive to more functional investigations. The need to develop methods for the production and *in vitro* cultivation of germ cell lines for genetic manipulation is emphasized [89].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

CK2 Secreted by *Leishmania braziliensis* Mediates Macrophage Association Invasion: A Comparative Study between Virulent and Avirulent Promastigotes

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CK2 is a protein kinase distributed in different compartments of *Leishmania braziliensis:* an externally oriented ecto-CK2, an intracellular CK2, and a secreted CK2. This latter form is constitutively secreted from the parasite (CsCK2), but such secretion may be highly enhanced by the association of specific molecules, including enzyme substrates, which lead to a higher enzymatic activity, called inductively secreted CK2 (IsCK2). Here, we examined the influence of secreted CK2 (sCK2) activity on the infectivity of a virulent *L. braziliensis* strain. The virulent strain presented 121-fold higher total CK2 activity than those found in an avirulent strain. The use of specific CK2 inhibitors (TBB, DRB, or heparin) inhibited virulent parasite growth, whereas no effect was observed in the avirulent parasites. When these inhibitors were added to the interaction assays between the virulent *L. braziliensis* strain and macrophages, association index was drastically inhibited. Polyamines enhanced sCK2 activity and increased the association index between parasites and macrophages. Finally, sCK2 and the supernatant of the virulent strain increased the association index between the avirulent strain and macrophages, which was inhibited by TBB. Thus, the kinase enzyme CK2 seems to be important to invasion mechanisms of *L. braziliensis*.

1. Introduction

Leishmania braziliensis is an etiological agent of leishmaniasis in the New World [1] that can differentiate from avirulent to virulent promastigotes in the sandfly midgut and from promastigotes to amastigotes in mammalian macrophages. The relationship between the parasite and host cells commonly involves signal transduction pathways that are triggered upon

the interaction between the surfaces of both parasites and macrophages [2].

The invasion of host immune cells by pathogens is the first step of a complex series of events that ultimately allow parasite proliferation and infection of a whole organism. The presence of enzyme activities on the parasite surface can promote the subversion of host cell membrane proteins that regulate access to the intracellular environment. Once inside, the parasite must ensure their replication and the suppression of host immunity. This process involve complex signal transduction pathways that use the reversible phosphorylation of proteins promoted by protein phosphatases and kinases, which continuously modulate the host-pathogen interaction [3]. In this manner, active enzyme secretion following invasion is likely associated with host cell machinery subversion. Evidence for the existence of such a mechanism is available in several models of host and pathogen interaction [4].

Protein kinases are involved in many cellular processes [5]. This family has hundreds of members capable of phosphorylating specific amino acid residues, such as serine, threonine, or tyrosine, as its target proteins [6–8].

Two families of protein casein kinase (CK), CK1 and CK2, whose name is due to the fact that casein is a suitable substrate, are commonly used for *in vitro* activity testing. Unlike CK2, CK1 protein kinase is used as the only phosphate ATP donor [9].

L. braziliensis genome analysis by TriTrypDB has shown that the ck2 gene has a single copy, located on chromosome 34.

CK2 protein kinase frequently appears as a quaternary structure, with a molecular weight of approximately 130 kDa, composed of two types of catalytic (α) and regulatory (β) subunits, and a general structure of $\alpha\alpha'\beta 2$ or $\alpha 2\beta 2$ [8–10]. The α and α' subunits are catalytically active and have molecular weights between 42–44 kDa or 38 kDa, respectively. They are structurally similar but encoded by different genes [9, 11, 12] and have distinct functions [8]. A third subunit isoform, designated as α'', also occurs [13, 14]. The β regulatory subunit (approximately 26 kDa in animal cells) is naturally inactive but enhances the catalytic activity of α5-10-fold [9] and can interact with other proteins [15]. The association between the α and β subunits determines the sphere protein structure. The complex is stabilized by the C-terminal domain, which is involved in the strong interaction between the two subunits through four electrostatic interactions between the positively charged region of the catalytic site (α) and a highly negative region in the regulatory subunit (β). This closed configuration is responsible for the catalytic site of an obstruction, which allows restricted access to protein substrates [16]. In this manner, positively charged molecules are able to modulate CK2 activity to promote the exposure of the active enzyme site. Thus, negative molecules (such as heparin) inhibit this activity, whereas positive molecules (such as polyamines) increase this activity [8]. In the absence of negative charges, CK2 maintains a stable and closed conformation. In contrast, in the presence of positive compounds, the β subunit is dislocated allowing the enzyme to assume the open conformation less stable, which facilitates substrate access [16].

This protein is highly conserved in nature [17] and regulates cell development and differentiation and the cell cycle [18]. This enzyme is inhibited by heparin [9] and by cell-permeable drugs, such as DRB (5,6-dichlorobenzimidazone1-β-D-ribofuranoside) [19] and TBB (4,5,6,7-tetrabromobenzotriazole) [20]. CK2 can be found in the nucleus, in the cytoplasm, and on the cellular surface of mammalian cells [18] and has been previously described on the surface of intact cells [21]. Its secretion may be induced by its potential phosphorylatable substrates, such as casein and phosvitin [22, 23]. The presence of CK2 activity has been previously described in trypanosomatids [23–26]. Moreover, when these organisms were incubated in the dephosphorylated casein presence, increased CK2 secretion was observed [23, 26].

Some papers have described CK2 activity in various protozoan parasites species, such as *L. tropica* [23], *L. major* [25], *L. amazonensis* [24], *L. donovani* [27], *Herpetomonas muscarum muscarum* [26], *Trypanosoma cruzi* [28], *T. brucei* [29], and *Toxoplasma gondii* [30]. Furthermore, constitutive and inducible CK1 and CK2 activities are present on the surface of cells and in enzymatic secretions from *L. major* promastigotes; therefore, these proteins appear to be involved in *L. major* and *L. amazonensis* promastigote cell growth, morphology, and infectivity in *in vitro* mice peritoneal macrophages and *in vivo* BALB/c mice [25]. The participation of these enzymes in the parasite-host interaction may be crucial for successful infection.

In the present study, we identified and characterised secreted CK2 (sCK2) enzyme activity in supernatants of highly virulent *L. braziliensis* promastigotes. In addition, we evaluated the contribution of this enzyme in parasite survival and infectivity in host cells.

2. Material and Methods

2.1. Microorganisms and Growth Conditions. We used promastigotes of *L. braziliensis* virulent strain (MHOM/BR/2002/EMM IOC-L2538) cultured for 60 passages until it became avirulent. This strain was kindly provided by Dr. Léa Cysne. The promastigotes were grown in Schneider's medium supplemented with 10% foetal calf serum at 28°C. The amastigotes were isolated from the lymph nodes of hamsters infected 8 to 10 weeks earlier. The parasites were maintained in culture (*in vitro*) for no more than 8 passages to maintain infectivity. Six days after culture *in vitro*, the parasites were harvested by centrifugation, washed twice with 0.9% saline, and washed once with a buffer containing 20 mM Tris-HCl, pH 7.5, 10 mM NaF, 1 mM sodium orthovanadate, 150 mM NaCl, 1 mM glucose, and 1 mM $MgCl_2$. Then, the parasites were maintained in the same buffer until the protein kinase activity assays were performed. Cell viability was assessed before and after incubation by motility and cell dye exclusion (Trypan blue method) [31]. The viability of the parasites was not affected by our laboratory conditions or procedures. For the experiments on cellular interaction, the parasites were washed in RPMI 1640 medium and maintained in this medium until the experiments were performed.

2.2. Growth Course. The parasites (virulent and avirulent promastigotes) were grown in Schneider's medium supplemented with 10% foetal calf serum at 28°C in the absence or presence of heparin (10 μg/mL), TBB (1 μM), DRB (6 μM), and casein (1 mg/mL) for 6 days. The number of cells was evaluated after each 24 h period using Neubauer's chamber.

2.3. Induction of sCK2 Secretion. Intact promastigotes were incubated in a buffer containing 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM NaF, 1 mM sodium orthovanadate, 1 mM glucose, and 1 mM $MgCl_2$ in the absence or presence of dephosphorylated casein (1 mg/mL), albumin (1 mg/mL), foetal bovine serum (1 mg/mL), inactivated human serum (1 mg/mL), and fixed BALB/c mice peritoneal macrophages for 30 minutes at 37°C. The parasites were centrifuged, and the supernatant was analysed to determine CK2 activity as follows. To assess the molecular mass of sCK2, intact promastigotes were incubated in the presence of dephosphorylated casein (1 mg/mL) for 30 min at 37°C. Next, the cells were centrifuged to obtain the supernatant, which was applied to a gel filtration superose 6H/R column coupled to a Shimadzu HPLC system as previously described [26]. All harvested fractions were assayed for CK2 activity [32] using TBB (1 μM) and heparin (10 μg/mL) as inhibitors to identify the fraction of enzyme activity corresponding to CK2.

2.4. Protein Kinase Activity. Protein kinase activities from intact promastigotes (ecto-CK2, eCK2), cytoplasmic contents (iCK2), and the supernatants of parasites medium of incubation (sCK2) were measured as previously described [32]. iCK2 was obtained by three cycles of parasite freeze and thaw, followed by centrifugation. Each of the above mentioned CK2s was assayed as follows. Briefly, either dephosphorylated casein (5 mg/mL) or the CK2-specific peptide RRRADDSDDDDD (50 μM) [23, 27] was used as substrate in the presence of 100 μM ATP-[γ-^{32}P] (1,000 cpm/pmol) in the following reaction mixture: 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM NaF, and 0.02 g sodium azide for 30 min at 37°C. The fraction of enzyme activity inhibited by heparin (1 μg/mL) was considered CK2 activity (this value was evaluated as the difference between the kinase activity measured in the absence of heparin and that measured in the presence of heparin). Whenever indicated, sCK2 activity was measured in the absence or presence of 500 μM putrescine, 500 μM spermidine, and 500 μM spermine. Protein concentration was determined according to the literature using bovine serum albumin (BSA) as a standard [33].

2.5. Western Blotting Analyses. CK2 obtained from HPLC purification (1 mg/mL) was submitted to 7.5% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) (26°C/150 V/60 mA), transferred to a PVDF membrane (4°C/100 V/300 mA), and blocked in a buffer containing 150 mM NaCl, 0.05% Tween 20, 5% BSA, and 10 mM Tris (TBS-Tween-BSA), pH 7.6. CK2 was then incubated in TBS-Tween-BSA, pH 7.6, containing primary anti-human αCK2 (goat polyclonal IgG, dilution 1 : 7,500). The sample was then washed with TBS-Tween-BSA, pH 7.6, incubated in the same buffer containing secondary antibody (donkey anti-goat IgG conjugated to peroxidase, dilution 1 : 25,000), and analysed using a SuperSignal West Pico kit (Pierce) and Kodak Diagnostic Film (T-Mat S).

2.6. Endogenous Phosphorylation Assays. Promastigotes were washed 3 times with PBS and incubated in a buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM NaF, 1 mM sodium orthovanadate, 150 mM NaCl, 1 mM glucose, and 1 mM $MgCl_2$ in the absence (constitutively secreted CK2 [CsCK2]) or presence (inducible secreted activity [IsCK2]) of dephosphorylated casein (1 mg/mL) for 30 minutes. After incubation, the parasites were harvested by centrifugation, and the cell-free supernatant (1 mg/mL) was assayed for endogenous phosphorylation activity [31] in the presence of 100 μM ATP-[γ-P32] (1000 cpm/pmol) and a reaction mixture containing 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, and 0.02 g sodium azide. Where indicated, the PKC inhibitor bisindolylmaleimide (BIS; 0.4 μM) and the CK2 inhibitors heparin (10 μg/mL) and TBB (1 μM) were used. The reaction was conducted at 37°C for 30 minutes and stopped by increasing the temperature to 100°C for 2 minutes. The samples were analysed by 7.5% SDS-PAGE and stained by Coomassie Brilliant Blue R250. The phosphoproteins were revealed by exposure to Phosphorimager.

2.7. Interaction between Murine Macrophages and L. braziliensis Parasites. Peritoneal macrophages were extracted from BALB/c and maintained in RPMI 1640 medium at 37°C in a 4% CO_2 atmosphere for 4 hours. The parasites were harvested by centrifugation, washed twice with 0.9% saline, and then washed once with RPMI 1640 medium. The promastigotes were maintained in contact with the macrophages for 2 hours at a ratio of 10 parasites: 1 macrophage. Then, the supernatant with the unbound parasites was removed. The macrophages were washed with saline solution and incubated for 2 more hours. The macrophages were then fixed, stained with Giemsa, and observed by optical microscopy. Virulent parasites or macrophages were preincubated in the absence or presence of DMSO (1%, drugs diluents), TBB (1 μM, CK2 inhibitor), DRB (6 μM, CK2 inhibitor), putrescine, spermine, and spermidine (500 μM, CK2 stimulators). These drugs were also added to the medium during the interaction experiment. The supernatant of the incubation medium of *L. braziliensis* virulent parasites (1 mg/mL) and the secreted CK2 purified from this supernatant (1 mg/mL) were added to the medium during the interaction experiment between avirulent promastigotes and macrophages or between latex beads and macrophages in the absence or presence of TBB (1 μM). Latex beads (100 nm) were used in the macrophage interaction assay at a dilution 1 : 20. The assay conditions were the same used for the promastigote-macrophage interaction assay.

Table 1: Effect of casein on the secreted CK2 activities of *Leishmania braziliensis* avirulent and virulent samples.

Systems	Virulent strain pmol·Pi mg^{-1}·min^{-1} ± E.P.	Avirulent strain pmol·Pi mg^{-1}·min^{-1} ± E.P.	Virulent activity/avirulent activity
Constitutive ecto-CK2 activity (eCK2)	34.400 ± 1.340	0.040 ± 0.001	860.00
Constitutive intracellular CK2 activity (iCK2)	4.800 ± 0.610	0.290 ± 0.002	16.55
Constitutive secreted CK2 activity (CsCK2)	7.290 ± 0.320	0.060 ± 0.006	121.50
Inducible secreted CK2 activity (IsCK2)	13.960 ± 1.010*	0.070 ± 0.006	199.43

The reactions were performed as described in Section 2. The values represent the mean of 3 independent experiments conducted in triplicate (∗ indicates $P \leq 0.005$ in relation to CsCK2; one-way ANOVA, Tukey's post hoc test).

Table 2: Secreted kinase activity of *Leishmania braziliensis* promastigotes using different substrates.

Substrates	Secreted kinase activity (pmol·Pi mg^{-1}·min^{-1})	Percentage (%)[a]	Percentage (%)[b]
Casein	7.282 ± 0.476	100	—
CK2-specific Peptide	5.790 ± 0.245	79.510	100
CK2-specific peptide + heparin	0.327 ± 0.210*	4.490	5.648

The reactions were performed as described in Section 2. The values are presented as the mean ± SEM of at least 3 independent experiments, which were performed in triplicate (* $P < 0.05$ in relation to CK2-specific peptide; one-way ANOVA, Tukey's post hoc test).

[a]The kinase activity was arbitrarily considered to be 100% when the value was obtained using casein as substrate. Based on the secreted kinase activity using CK2-specific peptides, the percentage was estimated by comparison with that obtained using casein, whereas the activity sensitive to heparin (1 μg/mL) was considered CK2 activity.

[b]The kinase activity was arbitrarily considered to be 100% when the value was obtained using the CK2 peptide as substrate.

2.8. Statistical Analysis. All results are presented as the mean and standard error of the mean (SEM). Normalised data were analysed with one-way analysis of variance (ANOVA), and differences between groups were assessed by Tukey's post hoc test. A P value of <0.05 was considered significant. All the experiments were performed in triplicate in 3 independent experimental sets.

3. Results

Virulent and avirulent parasites exhibited differences in protein kinase activity in the supernatant of promastigotes incubated in a specific buffer (Table 1). The virulent live promastigotes presented high specific activity for CK2 (34.400 ± 1.340 pmol Pi·mg/min) compared with the avirulent strain (0.040 ± 0.001 pmol·Pi mg^{-1}·min^{-1}). The sCK2 activity of the virulent sample was increased (91.5%) compared with the previous incubation of intact cells with extracellular dephosphorylated casein, indicating that the total enzyme levels secreted to the medium could be enhanced by the previous presence of a classical *in vitro* CK2 substrate, such as casein. Thus, from this point, we will make reference to general sCK2 activity, and whenever necessary, the different pools of the enzyme obtained by constitutive secretion will be referred to as CsCK2. The increase in enzyme activity exhibited by substrate induction will be referred to as IsCK2 (Table 1). In the virulent sample, at least 62% of the secreted kinase activities or 53% of ectokinase activity measured in the conditions described here corresponded to CK2 activity. In the avirulent sample, this value was 50% (data not shown). The secreted kinase activity of the virulent sample was arbitrarily considered to be 100% of the nonspecific kinase activity when using dephosphorylated casein as substrate, and the activity sensitive to heparin (1 μg/mL) was considered CK2 activity (Table 2). Based on the secreted kinase activity using the CK2-specific peptide, the actual percentage of specifically CK2 activity was estimated by comparison with that obtained using casein. Thus, under the conditions used in the present study, 79.51% of the secreted kinase activity was attributed specifically to CK2. However, the difference between kinases activities measured using dephosphorylated casein and CK2-specific peptide as a substrate was not significant. The activity measured using the CK2 peptide as a substrate was abolished by heparin (inhibition of 94.35%). The viability of *L. braziliensis* promastigotes did not significantly change during the course of all the experiments presented in this study, as assessed by the Trypan blue dye exclusion method.

The CK2 activities assayed in the virulent strain were at least 121-fold more pronounced than the activities exhibited by the avirulent sample. Dephosphorylated casein stimulated sCK2 in approximately 92% of the virulent parasites and did not affect the avirulent parasites (Table 1).

We tested the ability of traditional CK2 inhibitors, TBB, DRB, and heparin, to interfere with parasite growth [20, 26]. These inhibitors had no effect on the growth of avirulent

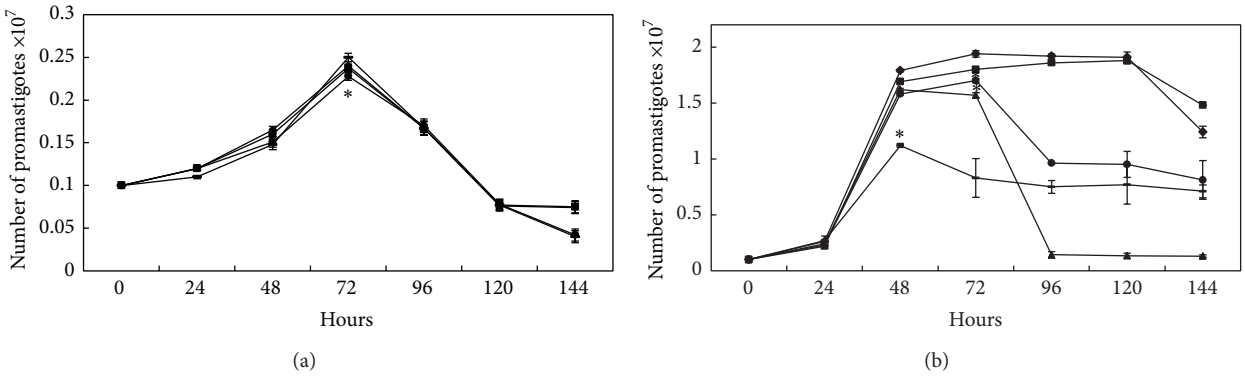

Figure 1: Effect of specific CK2 inhibitors (heparin, TBB, and DRB) on the proliferationof *L. braziliensis* avirulent (a) and virulent (b) samples. The assay was performed as described in Section 2. The values are presented as the mean of 3 independent experiments conducted in triplicate. In (a), (*) heparin and DRB significantly inhibited proliferation ($P \leq 0.05$) after 72 hours. In (b), (*) heparin significantly inhibited proliferation ($P \leq 0.002$) after 48 hours, and TBB and DRB significantly inhibited proliferation ($P \leq 0.01$) after 72 hours (one-way ANOVA and Tukey's post hoc test) (◆ Control; ■ 1 mM Casein; ▲ 1 μM TBB; — 10 μg/mL Heparin; ● 6 μM DRB).

Table 3: Effect of polyamines on the constitutive and inducible secreted CK2 activity of the *Leishmania braziliensis* virulent strain.

Addition	Constitutive secreted CK2		Inducible secreted CK2	
	nmol Pi/mg·min^{-1} ±SE	% kinase activity	nmol Pi/mg·min^{-1} ±SE	% kinase activity
None	7.70 ± 0.63	100.00	13.96 ± 0.64	100.00
Putrescine (500 μM)	14.48 ± 0.52**	188.01	21.50 ± 1.15**	154.01
Spermidine (500 mM)	18.00 ± 0.84**	233.77	25.32 ± 0.24*	181.38
Spermine (500 mM)	26.75 ± 0.72*	347.40	32.76 ± 0.26*	234.67

The reactions were performed as described in Section 2. The values are presented as the mean of 3 independent experiments conducted in triplicate (* indicates $P \leq 0.0002$, ** indicates $P \leq 0.007$, and *** indicates $P \leq 0.05$ compared with control without addition; one-way ANOVA, Tukey's post hoc test).

promastigotes (Figure 1(a)). However, such inhibitors triggered significantly decreased virulent promastigote growth (Figure 1(b)). TBB was the most effective inhibitor and completely abolished parasite growth after 96 h (Figure 1(b)). Heparin induced 37% inhibition of cell growth at an earlier phase (48 h) compared with the other inhibitors (Figure 1).

Spermine, a polyamine that activates CK2, induced the greatest effect on these activities, whereas constitutive secreted CK2 activity exhibited a maximum activation of 247% (Table 3). The smallest effect was caused by putrescine, whereas the greatest stimulation occurred with the IsCK2 (54%; Table 3). Inactivated human serum and fixed macrophages increased secreted CK2 activity by 58% and 87%, respectively (Figure 2). BSA did not interfere with this activity (Figure 2).

The parasite supernatant, which was obtained after incubation with 1 mg/mL dephosphorylated casein for 30 min, was fractionated by gel filtration chromatography. The samples recovered from the HPLC column were assayed for CK2 activity as described in Materials and Methods. Only one fraction (fraction 45) presented kinase activity (Figure 3(a)). This kinase activity (9.790 ± 0.040 pmol·Pi mg^{-1}·min^{-1}) was abolished by TBB and heparin (data not shown). This fraction presented a 50 KDa protein (Figure 3(a), *Inset*, Lane 1), which was recognised by polyclonal antibodies against the human α-CK2 subunit (Figure 3(a), *Inset*, Lane 2).

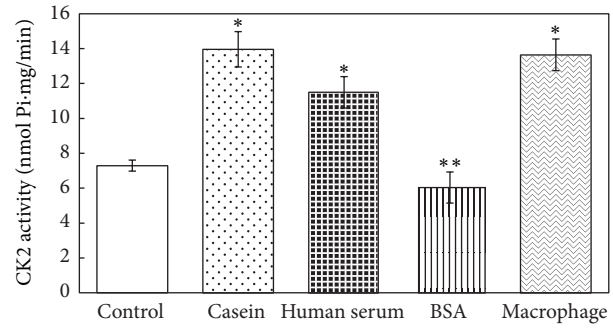

Figure 2: Effect of possible substrates on sCK2 activity in the virulent sample of *L. braziliensis*. CK2 activity was measured as previously described [32], and the conditions of the assay are described in Section 2 (all substrates were used at 1 mg/mL). The values are presented as the mean of 3 independent experiments conducted in triplicate (* indicates $P \leq 0.002$ and ** indicates $P \leq 0.01$; one-way ANOVA and Tukey's post hoc test).

The supernatant obtained from virulent promastigotes incubated in the absence (Figure 3(b), Lanes a, c, e, and f) or presence (Figure 3(b), Lanes b, d, and g) of dephosphorylated casein was phosphorylated *in vitro* and presented a major band with a molecular mass of 55 kDa (Figure 3(b), Line 1). This protein was phosphorylated by the secreted contents of virulent promastigotes (Figure 3(b), Line 2).

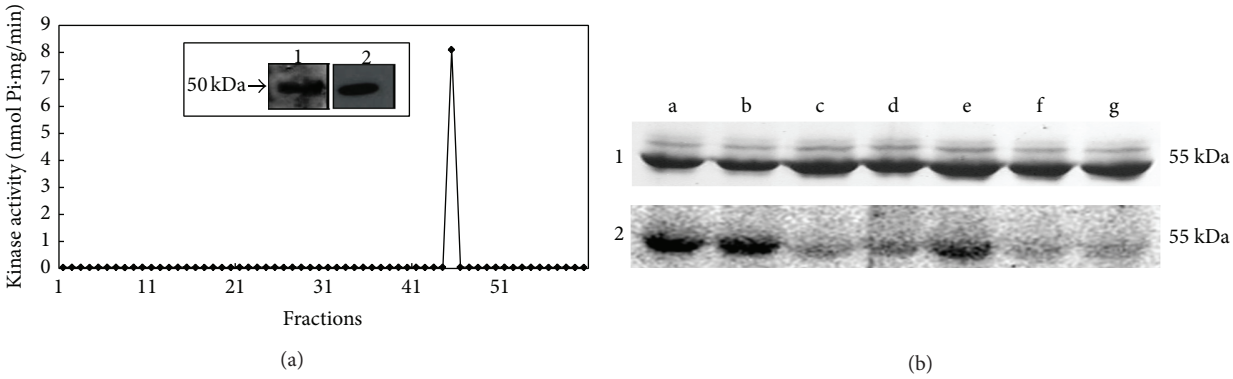

Figure 3: Partial purification of the sCK2 in the virulent sample of *L. braziliensis*. (a) Identification of kinase activity. All of the obtained fractions were assayed for CK2 activity as described in Section 2 [32]. *Inset*. Western blotting of the purified secreted CK2 of the virulent *L. braziliensis* sample. Fraction 45 was submitted to 12% SDS-PAGE (Lane 1), transferred to a PVDF membrane (Lane 2), incubated with an anti-αCK2 antibody, and developed as described in Section 2. (b) Phosphorylation of the supernatant proteins of the virulent *Leishmania braziliensis* sample by the CK2 enzyme secreted by this parasite. After the supernatant was obtained in the absence (control) or presence (experimental) of dephosphorylated casein, the assay was performed as described in Section 2. (1) Proteins stained with Coomassie Brilliant Blue R250. (2) Phosphorylated proteins observed by exposure to a phosphorimager plate (a: control supernatant, b: experimental supernatant, c: control supernatant + TBB, d: experimental supernatant + TBB, e: control supernatant + BIS, f: control supernatant + heparin, and g: experimental supernatant + heparin).

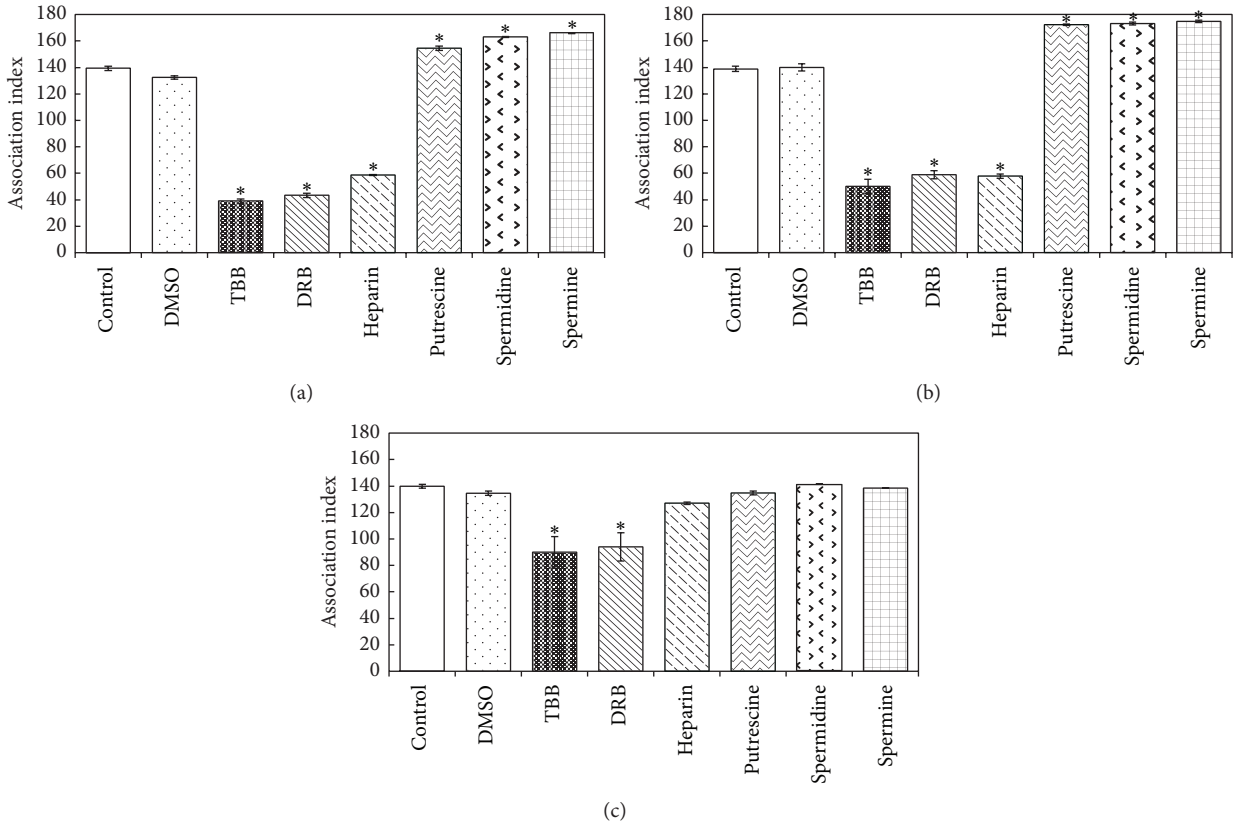

Figure 4: Effect of CK2 inhibitors on virulent *L. braziliensis* sample and macrophages. The assay was performed as described in Section 2. (a) Inhibitors added during the interaction experiment. (b) Promastigotes pretreated with inhibitors. (c) Macrophages pretreated with inhibitors. The values represent the mean of 3 independent experiments conducted in duplicate ($*$ indicates $P \leq 0.0001$ and $**$ indicates $P \leq 0.003$; one-way ANOVA and Tukey's post hoc test).

TBB (Figure 3(b), Line 2, Lanes c and d) and heparin (Figure 3(b), Line 2, Lanes f and g) significantly decreased this phosphorylation. The PKC inhibitor bisindolylmaleimide (Figure 3(b), Line 2, Lane e) did not cause alterations in the phosphorylation profile.

TBB, DRB, and heparin were used as tools to determine the role of CK2 in the host-parasite interaction. Therefore, these inhibitors were added during a cell interaction assay (Figure 4(a)), and either the parasite (Figure 4(b)) or the macrophage (Figure 4(c)) was treated with the inhibitors before the cell interaction assay. All of the inhibitors decreased the association index between virulent promastigotes and macrophages. DMSO, a diluent of drugs, had no effect on this interaction process (Figure 4). The inhibition effect was more pronounced when the inhibitors were added during the interaction experiments. TBB, DRB, and heparin inhibited the interaction process to 72%, 69%, and 58% of control levels, respectively (Figure 4(a)). Similarly, the inhibition was stronger when the parasites were pretreated with these drugs, as the interaction was inhibited to approximately 60% of control levels (Figure 4(b)). In contrast, the pretreatment of macrophages with TBB and DRB caused a weak inhibition (~34%), whereas heparin had no effect (Figure 4(c)), likely due to drug distribution to the intracellular milieu during the previous period of cell incubation with the drugs. Thus, such drugs must still be available in the extracellular environment where sCK2 is secreted. The polyamines putrescine, spermidine, and spermine increased the association index of virulent promastigotes and macrophages by 23% (Figures 4(a) and 4(b)) but had no effect when only the macrophages were pretreated (Figure 4(c)).

The virulent strain showed an association index with host cells approximately 48% higher than that of the avirulent strain (Figures 4 and 5, Table 4). The effect of CK2 modulators in the interaction process between avirulent promastigotes and macrophages was less pronounced (Table 4). Heparin promoted an inhibition of 20% in the pretreated macrophages, 31% when the parasites and macrophages were treated together, and 46% in the pretreated parasites (Table 4). In contrast, TBB promoted an inhibition of only 16% when the parasites and macrophages were treated together and 22% in the pretreated parasites. This inhibitor had no effect when the macrophages were pretreated (Table 4). Spermine promoted a maximum enhancement of only 19% in the pretreated parasites. On the other hand, it caused an inhibition of 17% when the macrophages were pretreated (Table 4).

The supernatant of virulent promastigotes and the purified CK2 fraction (fraction 45) were tested for their ability to promote macrophage invasion. These sources of CK2 caused an enhancement of 18% and 44%, respectively, in the association index between avirulent promastigotes and macrophages. This effect was abolished by TBB (Figure 5). The same effect could be observed on the phagocytosis of latex beads. This enzyme promoted a great enhancement in this process that was totally inhibited by TBB (Figure 5), showing that the effect also occurred in the macrophage.

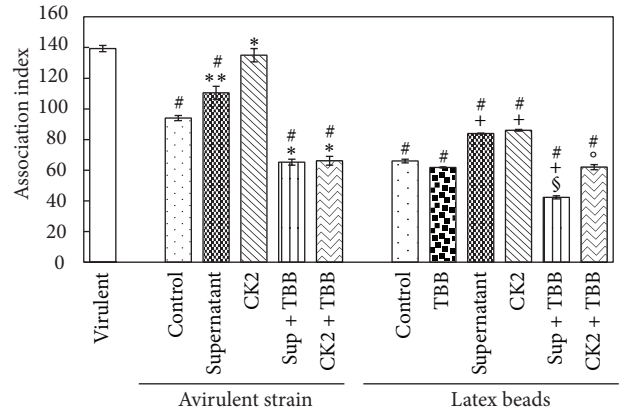

Figure 5: Effect of purified CK2 and the supernatant of the virulent strain on the interaction between the *L. braziliensis* avirulent strain and macrophages and the phagocytosis of latex beads. The assay was performed as described in Section 2. The values are presented as the mean of 3 independent experiments conducted in duplicate (∗ indicates $P \leq 0.001$ and ∗∗ indicates $P \leq 0.03$ in relation to control avirulent strain; # indicates $P \leq 0.003$ in relation to virulent strain; + indicates $P \leq 0.005$ in relation to control latex beads; § indicates $P \leq 0.05$ in relation to supernatant of latex beads; and ◦ indicates $P \leq 0.05$ in relation to CK2 of latex beads; one-way ANOVA and Tukey's post hoc test).

4. Discussion

Posttranslational modifications greatly impact the activity of certain proteins that play key roles in several cellular processes ranging from metabolism to cell growth, proliferation, and differentiation. The reversible phosphorylation of proteins mediated by protein kinases and phosphatases is crucial for the intracellular signal transduction pathways involved in these processes. Several protein kinases [23–30] and phosphatases [34–41] have been identified in trypanosomatids and are likely involved in the regulation of the cell cycle, cell differentiation, and responses to stress during their complex life cycles. These enzymes have frequently been associated with the parasite-host relationship [23–25, 27, 35, 36, 42]. The presence of these enzymes in these parasites was confirmed by sequencing the *T. cruzi, T. brucei,* and *L. major* genomes. These sequencing efforts made the kinome prediction of these parasites possible. Kinome analysis has revealed that the trypanosomatids lack members of the receptor-linked or cytosolic tyrosine kinase families but have an abundance of soluble protein kinases [43]. The presence of CK2 activity has been previously described in trypanosomatids [23–27]. Kinome analysis of *L. major* has revealed the presence of six isoforms of CK1 and two isoforms of CK2 in this species. In addition, this last enzyme was shown to be distributed on the external surface of the parasite (ecto-CK2 activity), in the cytoplasmic content (intracellular CK2 activity), and in the secreted content (secreted CK2 activity) by this parasite. These enzyme activities phosphorylated either casein or phosvitin. However, the amino acid sequences of these enzymes are different from their mammalian homologues [25, 27, 43]. Accordingly, we show

Table 4: Effect of CK2 modulators on the interaction between the *L. braziliensis* avirulent strain and macrophages.

Addition	Addition during interaction course		Avirulent pretreated parasites		Pretreated macrophages	
	Association index ± SE	% interaction	Association index ± SE	% interaction	Association index ± SE	% interaction
None	91.69 ± 2.08	100.00	91.78 ± 2.11	100.00	91.68 ± 1.64	100.00
TBB (1 μM)	76.89 ± 1.27**	83.86	71.83 ± 1.94*	78.26	83.96 ± 1.92	91.58
Heparin (10 μg/mL)	62.99 ± 0.80*	68.70	53.56 ± 0.91*	54.77	73.28 ± 1.34*	79.93
Spermine (500 mM)	97.43 ± 1.11	106.26	109.25 ± 0.72*	119.03	76.35 ± 0.95*	83.28

The reactions were performed as described in Section 2. The values are presented as the mean of 3 independent experiments conducted in triplicate (∗ indicates $P \leq 0.009$ and ∗∗ indicates $P \leq 0.05$ compared with control without addition; one-way ANOVA, Tukey's post hoc test).

here that considering the phosphorylation of casein by the supernatant of live *L. braziliensis* virulent promastigotes, 53% of the ectokinase and 62% of the secreted kinase activities were attributed to CK2. The remaining ectokinases activities (47%) and secreted kinase activities (38%) corresponded to casein kinase activities of unknown identity. These activities could be due to other isoforms of CK1 and CK2 or even other protein kinases. Heparin abolished the phosphorylation of casein and the phosphorylation of a substrate peptide by the CK2 secreted enzyme. Heparin has been used to identify CK2 activity because its IC_{50} for CK1 is 160-fold higher than that for CK2 [44].

L. braziliensis virulent promastigotes exhibited significantly higher activity (at least 121-fold) than the avirulent promastigotes. These protein kinases appear to be important for the growth of virulent parasites because their inhibition resulted in inhibited cell growth. In addition, CK2 inhibitors (heparin, DRB, and TBB) strongly inhibited the growth of the virulent strain but had no effect on the avirulent strain. Similar to heparin, TBB and DRB are specific CK2 inhibitors [8]. The effects of heparin and TBB were more pronounced than DRB, and TBB abolished protozoan growth. Therefore, CK2 activity appears to be extremely important for the survival of the *L. braziliensis* virulent strain, which has also been shown for CK2 activity in *L. chagasi* [45], implying an association between enzyme activity and parasite infectivity. Similarly, this enzyme is important for another parasite, *Plasmodium falciparum*, as gene disruption experiments have shown that CK2 is essential for blood schizogony [46].

This first set of results indicates that these parasites possess a significant array of CK2 enzymes expressed in different cell compartments and that they differ in their ability to respond to extracellular protein targets. In addition, such differences were positively correlated with the infectivity of the parasite and prompted us to investigate the general aspects of CK2 biochemistry in these cells.

Similarly, the induced CK2 secretion of *L. braziliensis* virulent promastigotes was strongly stimulated by incubation with this substrate. In *L. major*, the secreted CK1 activity is constitutive [25], whereas the release of CK2 is only stimulated by the incubation of the parasite with the substrate. *L. major* promastigotes release both constitutive and inducible protein kinases with different activities [25], suggesting that ecto- and secreted protein kinases may play an important role in parasite survival. In *L. donovani*, both enzymes are present constitutively [27]. *L. tropica* secretes CK2 constitutively, whereas the secreted CK2 activity is induced by incubation with dephosphorylated casein [23].

The secretion of CK2 may play a vital role in parasite survival inside the vertebrate host cells, that is, macrophages. Phosphorylation of host serum or host cell proteins may be involved in the regulation of leishmanicidal processes. The components of these substances can be used as substrates by the CK2 activity of the parasite. The presence of inactivated human serum and murine macrophages enhances the secreted CK2 activity of the virulent strain. This stimulation was equivalent to that induced by casein. Foetal calf serum and bovine serum albumin did not influence CK2 activity. The macrophages and human serum may present substrates for secreted CK2 from *L. braziliensis*, whereas exogenous substrates can promote CK2 release [25, 27].

To further investigate the nature of the enzymes responsible for secreted *L. braziliensis* kinase activity, we demonstrated that polyclonal antibodies raised against the mammalian CK2α catalytic subunit recognised a protein secreted by *L. braziliensis* promastigotes. These promastigotes were incubated in the same incubation buffer used for the secreted kinase assays and were partially isolated by HPLC chromatography. Approximately 60 fractions were harvested with this method. Only one fraction (fraction 45) presented kinase activity, which was readily abolished by heparin and TBB, confirming the possibility that this activity was from CK2. This assay showed a single band with a molecular weight (50 kDa) compatible with the subunit of the CK2 enzyme of other trypanosomatids, such as *H. m. muscarum* [26] and *L. tropica* [23].

The secreted CK2 activity of the virulent sample of *L. braziliensis* phosphorylated a protein secreted by this same parasite. This protein weighs approximately 55 kDa, and phosphorylation was strongly inhibited by TBB and heparin, specific CK2 inhibitors, whereas no effect was observed for the PKC inhibitor bisindolylmaleimide. The phosphorylation of macrophage receptors during the *Leishmania* interaction and phagocytosis can act synergistically with other virulence factors, such as lipophosphoglycan (LPG). This major surface glycoconjugate of *Leishmania* is transferred from the parasite to the host macrophage membrane during phagocytosis and seems to promote blockage of macrophage activation, protecting the parasite [47]. The autophosphorylation of some protein kinases, such as PKC, has been observed in mammal

cells [48] and in *T. cruzi* [49]. In addition to autophosphorylation, the phosphorylation of other molecules present on the parasite, such as secreted proteins, can also be part of the complex signalling pathway of this protozoan.

Polyamines such as putrescine, spermidine, and spermine are positively charged molecules and are thus able to increase constitutive and inducible virulent ecto- and secreted CK2 activity. Spermine exhibited the most pronounced effect on constitutive secreted CK2 activity. This effect is likely due to the four positive charges of this molecule [50]. Moreover, its structure is the most compatible with the CK2 structure because the stability of α and β binding is due to four electrostatic interactions [16].

The possible role of ecto- or secreted enzymes in parasite-host interactions has been suggested by several authors [23, 24, 27, 34–36, 42]. The CK2 activity of *L. major* and *L. amazonensis* influences the cell growth, morphology, and infectivity of the promastigotes in murine macrophages *in vitro* and BALB/c mice *in vivo* [24]. Two of the CK2 inhibitors, TBB and DRB, strongly decreased the association index between the *L. braziliensis* virulent sample and murine macrophages when added during the interaction assay, whereas heparin exhibited a minor effect. When these drugs were used to pretreat the parasites or macrophages, some differences were observed. The CK2 inhibitors were more potent in inhibiting the interaction when the parasites were pretreated than when the macrophages were pretreated; however, heparin did not inhibit this process. The polyamines increased the virulent sample index but had no effect when the macrophages were pretreated. These data suggest that the parasite enzymes may play a more important role in this process than the host cell enzymes.

As expected, the association index of the virulent sample was 48% higher than that of the avirulent sample; therefore, the CK2 modulators had a less pronounced effect. Interestingly, the avirulent sample association index increased with the addition of supernatant from the virulent strain or purified CK2. The CK2 isolated from the parasite showed a more potent effect because it increased the association index to the same value as that found in the virulent strain. TBB inhibited the purified CK2 and supernatant effects. The same effect was observed in the association index between *L. tropica* and macrophages with CK2 purified from another source, which enhanced the index by 166%. Heparin, a specific CK2 inhibitor, inhibited the association index by 50% [23]. sCK2 significantly affects the phagocytosis of latex beads by macrophages, indicating that this enzyme interferes with these cells. These results, together with the fact that macrophage extract increased sCK2 activity, suggest that the effect on the macrophage most likely occurs due to phosphorylation of some substrates capable of interfering with the phagocytosis process. These data demonstrate the importance of CK2 activity on the *Leishmania*-host interaction.

The survival of *Leishmania* inside macrophages depends on two inducible enzymes: iNOS and arginase. Both of these enzymes use L-arginine as substrate; iNOS (inducible NO sintase) produces NO (nitric oxide) (the most important leishmanicidal molecule) from L-arginine through two steps, whereas arginase catalyzes the hydrolysis of L-arginine into ornithine, the primary source of polyamines [51]. The *Leishmania* infection can induce host IL-4 production [52], which changes macrophage L-arginine metabolism to polyamine production by arginase activation [51]. These molecules promote parasite growth [51], interfere with signalling pathways, and act as immunosuppressors [53]. Furthermore, these molecules act as activators of leishmanial CK2 activities, promoting parasite-host cell interactions, as observed in the present study (Figure 4).

The maintenance of the virulent status of these parasites and their interaction with the host are essential for their life cycle. These processes are very complex and involve several signal transduction pathways. The study of factors that modulate the *Leishmania*-host interaction and the signalling routes of these processes may be of the great value for controlling the diseases associated with this parasite. The knowledge generated by these studies can be used in the development of novel, potentially less toxic, and more effective drugs than those currently used to treat leishmaniasis. Similarly, this knowledge may ultimately lead to new preventive strategies that target specific parasite molecules involved in the regulation of parasite survival and the infectivity of host cells.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Extraintestinal Helminth Infection Limits Pathology and Proinflammatory Cytokine Expression during DSS-Induced Ulcerative Colitis: A Role for Alternatively Activated Macrophages and Prostaglandins

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Chronic inflammation of the intestinal mucosa is characteristic of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease. Helminth parasites have developed immunomodulatory strategies that may impact the outcome of several inflammatory diseases. Therefore, we investigated whether *Taenia crassiceps* infection is able to decrease the inflammatory effects of dextran sulfate sodium- (DSS-) induced ulcerative colitis in BALB/c and C57BL/6 mice. Preinfection significantly reduced the manifestations of DSS-induced colitis, as weight loss and shortened colon length, and decreased the disease activity index independently of the genetic background of the mice. *Taenia* infection decreased systemic levels of proinflammatory cytokines while increasing levels of IL-4 and IL-10, and the inflammatory infiltrate into the colon was also markedly reduced. RT-PCR assays from colon showed that *T. crassiceps*-infected mice displayed increased expression of Arginase-1 but decreased expression of iNOS compared to DSS-treated uninfected mice. The percentages of T regulatory cells were not increased. The adoptive transfer of alternatively activated macrophages (AAMΦs) from infected mice into mice with DSS-induced colitis reduced the severity of colon inflammation. Administration of indomethacin abrogated the anticolitic effect of *Taenia*. Thus, *T. crassiceps* infection limits the pathology of ulcerative colitis by suppressing inflammatory responses mechanistically associated with AAMΦs and prostaglandins.

1. Introduction

Helminth parasites have developed complex strategies to modulate the immune responses of their hosts through utilizing versatile immunoregulatory mechanisms to avoid immune effector cells and molecules. These parasites bias the immune response toward Th2 and/or a regulatory environment associated with high levels of IL-4, IL-13, IL-9, IL-5, and IL-10; also, infection with helminths compromises immunity to other unrelated infections and may also affect the efficacy of vaccines [1]. Various cell populations are affected by helminth infections, including macrophages, dendritic cells (DCs), T regulatory cells (Treg), mast cells, and neutrophils [2]. Thus, helminths use multiple means to escape or modulate the immune response in their hosts [3]. A growing body of evidence in recent years has shown

that immunomodulatory activities displayed by helminths can impact in different ways the outcomes of several inflammatory diseases, including multiple sclerosis (MS), arthritis, type 1 diabetes (T1D), and inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease [4].

One inflammatory disease of alarming frequency is IBD. Although these diseases were considered rare 50 years ago, today some developed countries report 1 patient for every 250 people [5]. In Europe up to 0.3% of the population suffer from IBD [6]. An association has been suggested between an absence of helminth infection and the increase in cases of IBD [4]. Rodent models of IBD have been used to study the mechanisms underlying the development of these inflammatory diseases [7], and several experimental studies support the idea that IBD (mainly ulcerative colitis) can be regulated by helminth infection [8, 9]. Human trials have demonstrated some efficacy for patients but with undesirable side effects [10–12]. Nevertheless, seven different species of helminths, mainly gastrointestinal ones, have been tested for their role in modulating the development of ulcerative colitis, some with adverse effects [10, 11, 13–15].

Taenia crassiceps (class *Cestoda*) is a helminth parasite that can be found in its adult form in the small intestine of canids and in its larval stage (metacestode) in the muscles and peritoneal and pleural cavities of rodents. An interesting feature of *T. crassiceps* is its ability to reproduce asexually through budding at the larval stage. This characteristic permits the parasite to remain in and colonize its hosts for long periods of time; thus, 6–8 weeks after the intraperitoneal (i.p.) inoculation of 10–20 metacestodes, hosts can harbor hundreds of parasites. In addition, the parasite in its larval stage is innocuous to humans, is macroscopic in size, does not kill the host, and is able to cause chronic infection with a minimum amount of damage in mice. We found an inhibition of proinflammatory responses, induction of Th2-biased immune responses, myeloid-derived suppressor cells, impairment of DC maturation, and lymphocyte proliferative responses, as well as recruitment of alternatively activated macrophages (AAMΦs) during *T. crassiceps* infection, reviewed in [16]. Such immunoregulatory properties of this helminth had important beneficial effects on the development of experimental autoimmune encephalomyelitis (EAE, a murine model for multiple sclerosis) and T1D without side effects on the hosts [17, 18], whereas no effect at all was observed on arthritis [19].

Most of the effects of helminths on colitis have been studied using gastrointestinal infections, and consequently the impact of infection with helminths on organs outside the parasite's location has received much less attention [20]. For this reason and given the anti-inflammatory and immunoregulatory mechanisms of *T. crassiceps* infection, in this work we evaluated the effect of an extraintestinal infection on the development of dextran sulfate sodium- (DSS-) induced colitis.

2. Material and Methods

2.1. Mice. Female BALB/c or C57BL/6 mice 6–8 weeks of age were purchased from Harlan Laboratories (México) for use in some experiments. Mice were maintained in a pathogen-free environment at the FES-Iztacala, UNAM, animal facility according to Faculty Animal Care and Use Committee and government guidelines (official Mexican regulation NOM-062-ZOO-1999), which are in strict accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (USA). Mice were sacrificed using a CO_2 chamber, and all efforts were made to minimize pain. In some experiments C.Cg-*Foxp3*^{tm1Tch}/J reporter mice (Jackson Labs, USA) were used to detect the presence of T regulatory cells.

2.2. Parasites and Infection. Metacestodes of *T. crassiceps* were harvested from the peritoneal cavities of female BALB/c mice after 2–4 months of infection. The cysticerci were washed four times in sterile phosphate-buffered saline (PBS; 0.15 M, pH 7.2). Experimental infection was achieved via i.p. injection of 20 small (approximately 2 mm in diameter) nonbudding cysticerci of *T. crassiceps* suspended in 0.3 mL PBS per mouse (BALB/c). Because C57BL/6 mice are resistant to low doses (10–20 metacestodes) of *T. crassiceps*, we infected all C57BL/6 mice with 40 metacestodes.

2.3. Development and Assessment of DSS-Induced Colitis. DSS (MW: 35,000–50,000; MP Biomedicals, Solon, OH, USA) was administered ad libitum dissolved at 4% in drinking water for 7 to 10 days.

2.4. Assessment of Disease Activity Index (DAI) Score and Weight Loss. To assess the severity of colitis, we monitored DAI scores and weight loss daily. The DAI score was calculated as the sum of the diarrheal score and the bloody stool score as follows: 0 = normal stool and normal-colored stool, 1 = mildly soft stool and brown stool, 2 = very soft stool and reddish stool, and 3 = watery stool and bloody stool.

2.5. Histology. Colon tissue samples were fixed in 10% formalin and embedded in paraffin. Then 5 micrometer-thick tissue sections were prepared and stained with hematoxylin and eosin (HE) to evaluate mucosal damage. The sections were also stained with Alcian blue to evaluate the presence of goblet cells. We calculated the number of Alcian blue-positive goblet cells per five power fields (40x) using an Axio Vert.A1 microscopy (Carl Zeiss, Gottingen, Germany).

2.6. Cytokine Measurement. IL-4, IL-10, IL-17A, IL-17E, and TNF-α levels were quantified in mouse serum at the indicated times. Kits were used according to the manufacturer's instructions (Peprotech México, Mexico City, Mexico, and Biolegend, San Diego, CA, USA, for IL-17E).

2.7. Peritoneal Macrophage Purification, Adoptive Transfer, and In Vitro Suppression. Peritoneal exudate cells were isolated from the peritoneal cavities of *T. crassiceps*-infected mice 8 weeks after infection. Fc receptors were blocked by incubating the cells with mouse serum for 10 min at 4°C. Then cells were labeled with anti-F4/80 APC and anti-mannose receptor (FITC; 0.25 ug/10^6 cells; Biolegend) for 20 min

at 4°C. $F480^+MR^+$ and $F480^+MR^-$ populations were high-speed-sorted using a FACs Aria III flow cytometer. The viability of the cells was 90%, and purity was 95%. One million purified $F4/80^+MR^+$ or $F4/80^+MR^-$ cells were injected intraperitoneally into BALB/c mice 2 days after the start of DSS treatment. In addition, the antiproliferative properties of sorted $F4/80^+MR^+$ or $F4/80^+MR^-$ cells on T cells were tested. Briefly, total CFSE-labeled splenocytes from naïve mice were plated in 96-well plates previously coated with anti-CD3/CD28 (2 μg/mL). After 4 h, $F4/80^+MR^+$ or $F4/80^+MR^-$ cells were added in different ratios. Proliferation was evaluated after 72 h on CD8- or CD4-gated populations by CFSE dilution assay using a FACSCalibur cytometer.

2.8. Flow Cytometry Analysis of Monocytes and T Regulatory Cells. Single-cell suspensions of circulation and lamina propria obtained during the sacrifice were stained with specific antibodies against CD11b (M1/70), Ly6C (HK1.4), Ly6G (1A8; all from Biolegend), and CCR2 (R&D Systems, USA) for 30 min at room temperature. To isolate colonic lamina propria cells, we flushed colons of their luminal content with cold PBS, opened them longitudinally, and cut them into 0.5 cm pieces. Epithelial cells and mucus were removed via 30 min incubation with HBSS containing 2% FBS, 2 mM EDTA, at 37°C and shaking at 50 g. Colon pieces were digested in DMEM containing 2 mg/mL Collagenase VIII (Sigma) and 40 μg/mL DNase I (Invitrogen) for 2 h at 37°C with shaking at 250 rpm. The digested cell suspension was then washed with DMEM with 10% FBS, passed sequentially through 100 and 40 μm cell strainers, and pelleted by centrifugation at 448 g for 10 min. Cells were subsequently separated by centrifugation through Percoll. Finally, cells from the same groups were pooled for analysis. Analyses of cells were performed using the FACSCalibur system and Cell Quest software (Becton Dickinson).

For the analysis of T regulatory cells, single spleen cells and PECs suspensions were obtained from C.Cg-*Foxp3*^{tm1Tch}/J reporter mice and stained with CD4 and CD25 (Biolegend) and gated on the $CD4^+$ cell population. From this population CD25 and FOXP3 expression were analyzed.

2.9. In Vivo Suppression of Prostaglandin E_2. To block the production of prostaglandin E_2 in mice, we daily subjected mice to i.p. injection with indomethacin (3 mg per kg body weight). Controls were injected with DMSO 0.5% in bicarbonate buffer 5% as a vehicle control.

2.10. Statistical Analysis. Data were analyzed either by one-way analysis of variance followed by Tukey's multiple comparisons test or by unpaired two-tailed t-tests with GraphPad Prism 5 (San Diego, CA, USA).

3. Results

3.1. Taenia crassiceps Infection Decreases the Severity of DSS-Induced Colitis Independent of Genetic Background. We found that i.p. injection with the cestode *T. crassiceps* induces immunomodulation in its hosts. To formally assess the possible role of this extraintestinal helminth infection in the modulation of inflammatory responses in the colon, we explored whether the presence of this parasite in the peritoneal cavity of the host would modulate the severity of disease in an experimental model of ulcerative colitis. BALB/c and C57BL/6 mice previously infected (6 weeks) or not with *T. crassiceps* were exposed to DSS 4% or 3%, respectively, in drinking water for 7–9 days. The DAI was assessed daily as an average of loss of body weight and signs of rectal bleeding and diarrhea. Under such experimental conditions *T. crassiceps*-infected mice of both strains did not lose weight, whereas uninfected mice had significant progressive weight loss over time, weighing up to 20% of their initial body weight less at day 7 of exposure to DSS (Figure 1(a) for BALB/c; data not shown for C57BL/6). In line with these observations, DSS-treated *T. crassiceps*-infected mice developed reduced signs of morbidity (DAI) over the course of the disease compared to uninfected mice similarly treated with DSS (Figure 1(b)). Consistent with this, at necropsy, reduced colon shortening was found in *T. crassiceps*-infected mice after exposure to DSS compared to uninfected mice; this observation was similar for both strains of mice (Figures 1(c)-1(d) for BALB/c and Figure 4(a) for C57BL/6).

We next analyzed the architecture of the colonic structure and evaluated the histopathology associated with DSS-induced colitis. *T. crassiceps*-infected mice displayed less inflammatory infiltrate as assessed via histological slides (Figures 2(a)-2(b) for BALB/c and Figure 4(b) for C57BL/6). Helminth infection also inhibited the development of cryptitis and neutrophil accumulation within epithelial crypts and in the intestinal mucosa, as uninfected mice that received DSS showed large numbers of neutrophils and macrophages in the injured mucosa of the colon, which correlates directly with clinical disease activity and epithelial injury in colitis (Figure 2(a) upper panel and Figure 2(b)). Moreover, *T. crassiceps*-infected mice and those exposed to DSS had normal numbers of goblet cells as assessed by Alcian blue staining compared to DSS-treated uninfected mice (Figure 2(a) lower panel and Figure 2(c)). The diameter of the colonic submucosa was also measured as a sign of tissue damage and was significantly smaller for the infected group exposed to DSS than for the DSS-treated group (data not shown).

3.2. Taenia crassiceps Infection Reduces Levels of Systemic Proinflammatory Cytokines during DSS-Induced Colitis. Several proinflammatory cytokines as well as inflammatory cells are associated with the severity of IBD [21]. Here we explored whether *T. crassiceps*-infected mice exposed to DSS may modulate systemically the expected increase in proinflammatory cytokines. As shown in Figure 3 for BALB/c mice, DSS-induced colitis in uninfected mice generated an increase in circulating levels of TNF-α and IL-17E, two inflammatory cytokines associated with different models of colitis [22]; however, *T. crassiceps*-infected mice displayed lower levels of both cytokines (Figures 3(a)-3(b)). In contrast, infected mice exposed to DSS displayed increased levels of IL-10 and IL-4 compared to uninfected and DSS-treated mice (Figures 3(c)-3(d)). A similar effect was observed in the C57BL/6 strain, in which systemic TNF-α levels were downregulated by the

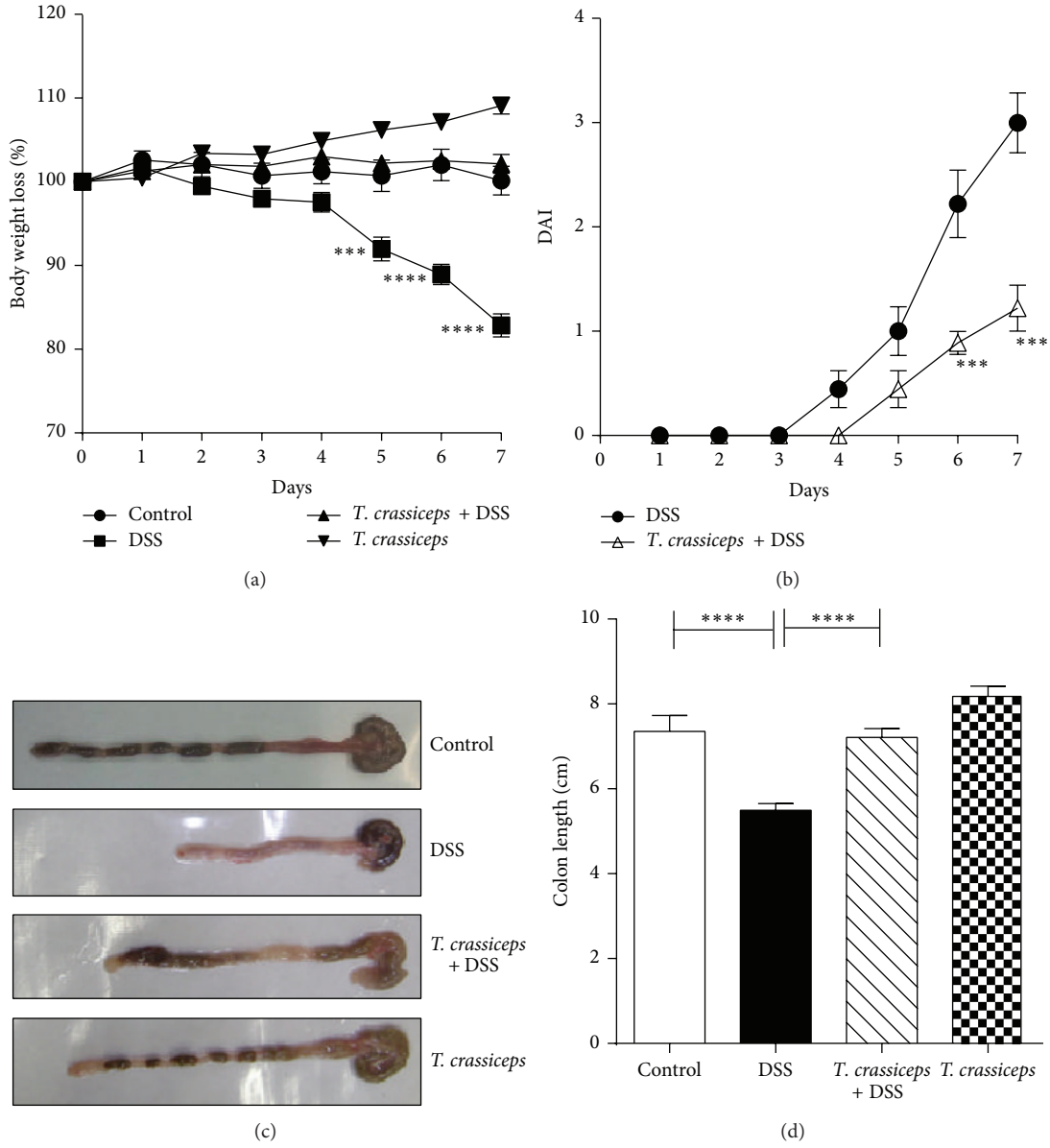


Figure 1: *T. crassiceps*-infected mice efficiently control colitis-associated pathology. Course of ulcerative colitis in *T. crassiceps*-infected and uninfected mice following 7 days of treatment with DSS at 4%. (a) Body weight change. (b) Clinical score. (c) Photograph of gross pathology of colons from different groups of mice. (d) Length of colon of infected and uninfected mice with ulcerative colitis. Bars represent the mean ± SD for six mice per group. $^{*}P < 0.05$, $^{***}P < 0.003$. All data are representative of three independent experiments.

presence of *T. crassiceps* infection (Figure 4(c)) but IL-4 and IL-10 levels were significantly elevated (Figures 4(d)–3(e)). Unexpectedly, *T. crassiceps*-infected mice exposed to DSS displayed significant increased levels of IL-17A compared to uninfected and DSS exposed mice (Figure 3(e)).

3.3. Taenia crassiceps Infection during DSS-Induced Colitis Does Not Modify the Population of T Regulatory Cells. Given reports suggesting that the increases in Treg ($CD4^+CD25^+Foxp3^+$) induced by helminth infections are critically involved in the anticolitic effects of these parasites [23–26], we used a Foxp3 reporter mouse to evaluate the percentages of T regulatory cells in the spleen and peritoneal cavity during *T. crassiceps* infection and DSS-induced colitis. As shown in Figure 5, we did not find significant changes in the percentages of T regulatory cells at either location in infected mice or infected and DSS-treated mice compared to DSS-treated uninfected mice.

3.4. Taenia crassiceps Infection Reduces Levels of Systemic and Colonic Inflammatory Monocytes but Increases AAMΦ Markers in Colonic Tissue during DSS-Induced Colitis. To examine changes in the recruitment of inflammatory monocytes to the colon tissue during DSS-induced colitis and the effect of *T. crassiceps* infection in these populations we examined circulating levels of $CD11b^+Ly6^{hi}CCR2^+$ cells as well as those

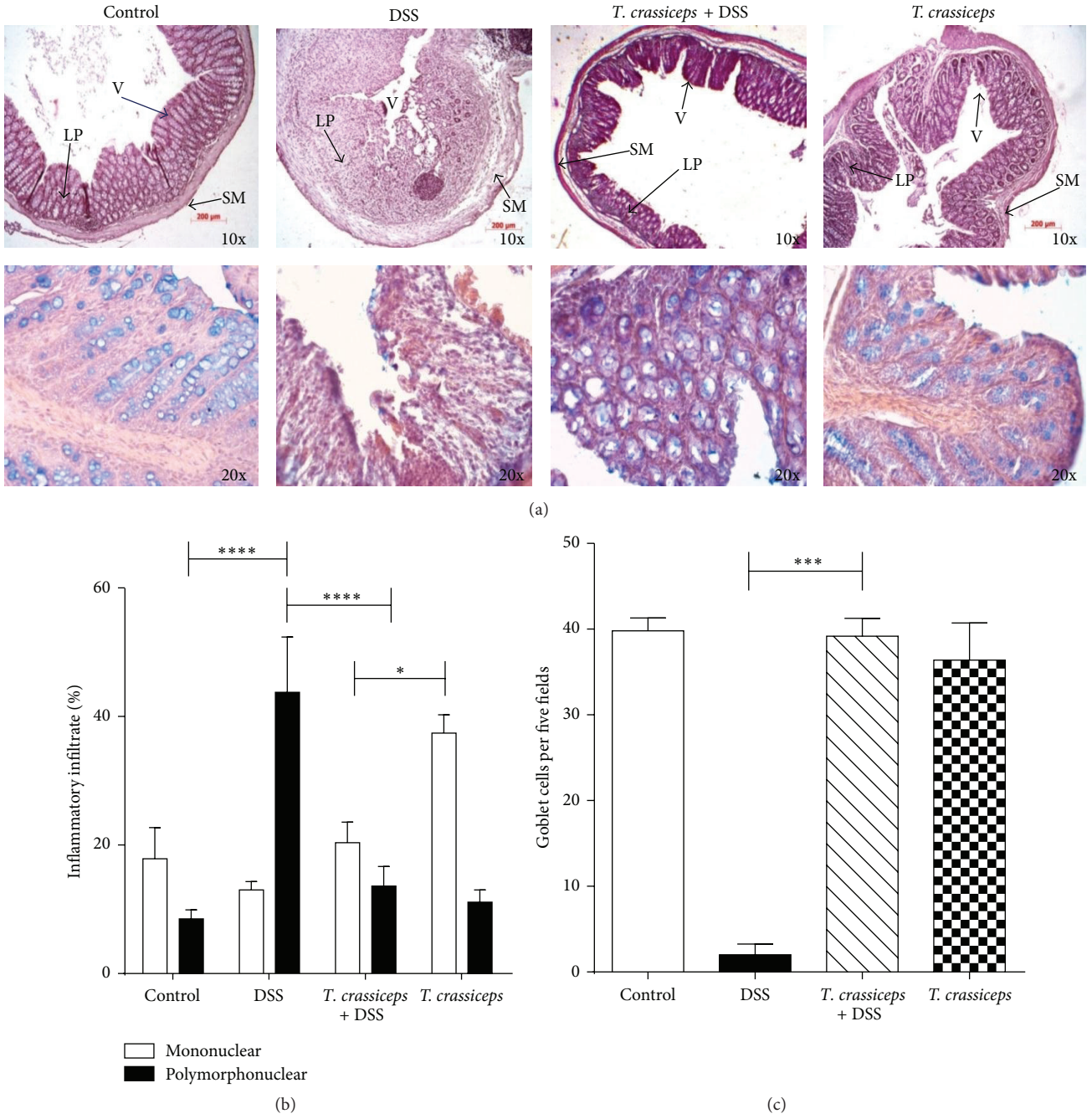


Figure 2: *T. crassiceps*-infected mice do not display severe pathology during ulcerative colitis. (a) Upper panel, colon tissue histology stained with H&E and showing colonic inflammation in different groups: magnification is 10x; bottom panel, Alcian blue-stained goblet cells (blue): magnification is 20x. (b) Percentages of neutrophils and monocytes located in distal colons. (c) Number of goblet cells; these cells were quantified from at least 20 crypts per region in five fields in four different slides per animal. Data are means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

recruited into the colon. As shown in Figure 6(a), by day 8 after DSS treatment we detected an important systemic increase in the inflammatory monocytes CD11b^{+}Ly6^{hi}CCR2^{+} in uninfected mice, whereas mice infected with *T. crassiceps* and exposed to DSS displayed a reduced percentage of these inflammatory monocytes (Figures 6(a)-6(b)). In contrast, CD11b^{+}Ly6^{low}CCR2^{-} cells were increased in these mice (Figure 6(c)).

To further explore the effect of *T. crassiceps* infection on the development of colitis, we looked for markers of AAMΦs locally in the colon tissue. We found that colons from *T. crassiceps*-infected mice that received DSS positively expressed Arginase 1, FIZZ-1, and Ym-1 (Figure 7(a)), all molecules associated with AAMΦs. In contrast, in the same samples inducible nitric oxide synthase (iNOS) was not detected. It is interesting that uninfected mice exposed to

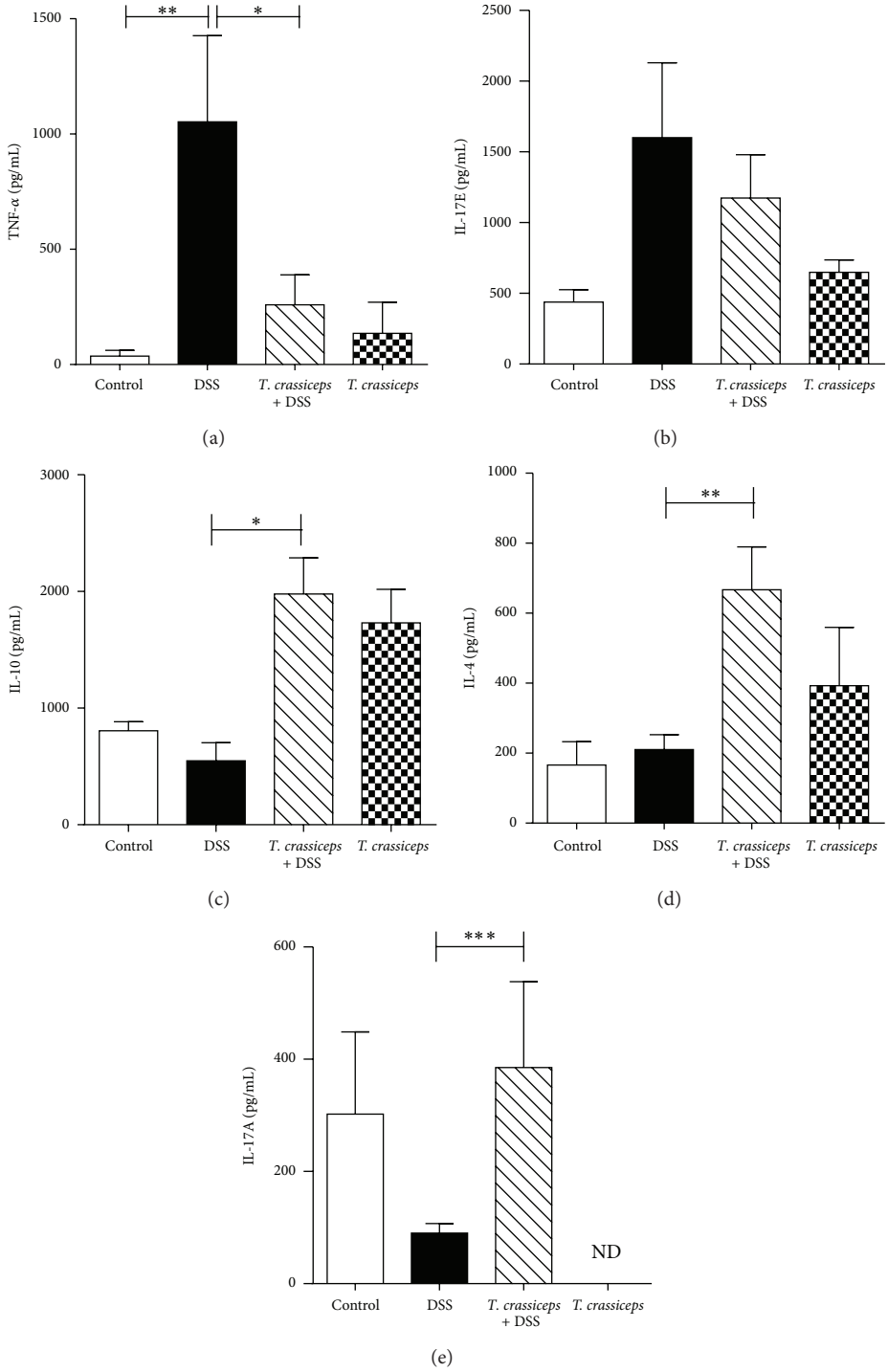


Figure 3: Systemic cytokine profile of *T. crassiceps*-infected and uninfected mice during DSS-induced colitis. (a) Sera TNF-α detection. (b) Sera IL-17E detection. (c) Sera IL-10 detection. (d) Sera IL-14 detection. (e) Sera IL-17A detection. Data are means ± SE and are representative of three independent experiments, $n = 4$ mice per group. $^{*}P < 0.05$ comparing *T. crassiceps*-infected mice and uninfected mice at the end of the experiment.

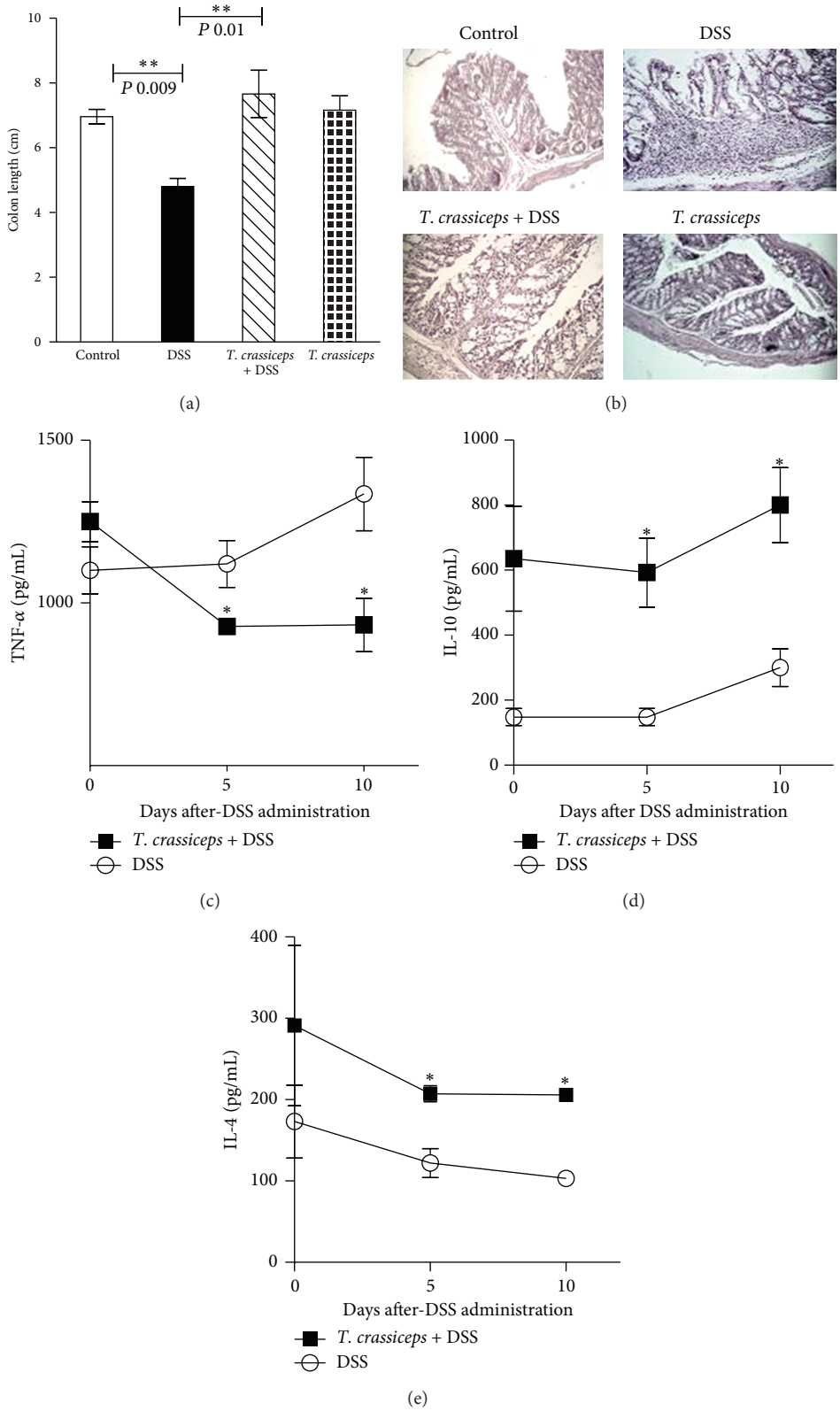


Figure 4: The anticolitic effect of *T. crassiceps* infection is independent of the genetic background of the host. The C57BL/6 mice were infected or not with 40 metacestodes of *T. crassiceps* and ulcerative colitis was induced. (a) Length of the colon for different groups exposed or not exposed to *T. crassiceps* infection. (b) Colon tissue histology stained with H&E and showing colonic inflammation in different groups, magnification = 20x for Control, DSS, and *T. crassiceps* + DSS, 10x for *T. crassiceps*. Serum levels of (c) TNF-α, (d) IL-10, and (e) IL-4 detected by ELISA on different days after exposure to DSS. Data are means ± SE and are representative of two independent experiments, $n = 4$ mice per group. $^{*}P < 0.05$ comparing *T. crassiceps*-infected mice and uninfected mice at the end of the experiment.

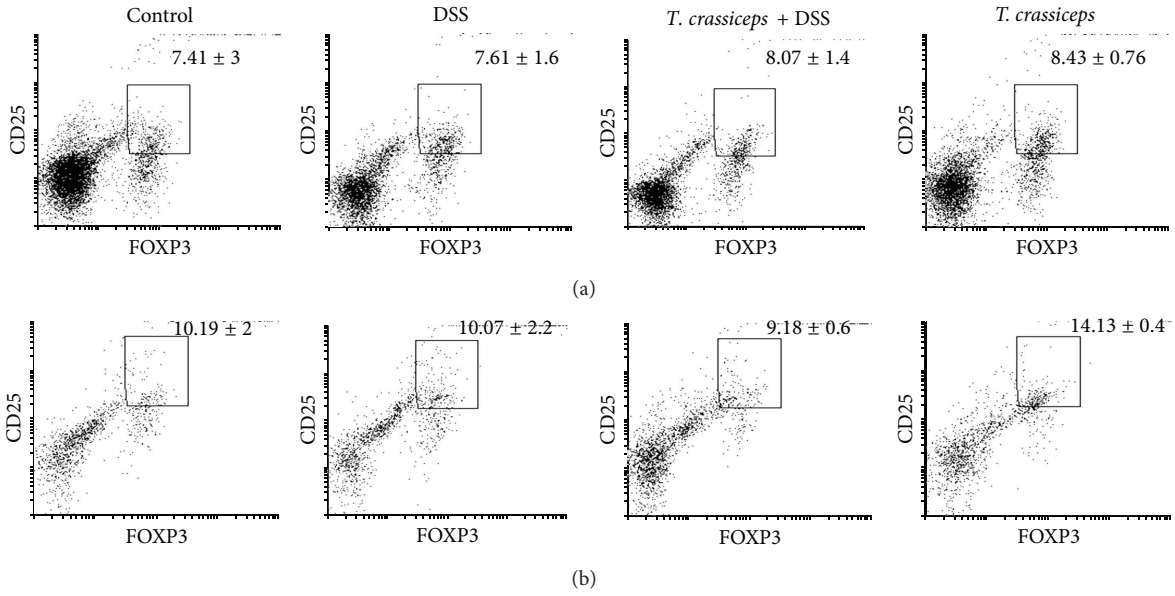


Figure 5: Percentages of T regulatory cells are not altered by *T. crassiceps* infection. Using the Foxp3 reporter mice C.Cg-*Foxp3*^{tm1Tch}/J, we analyzed the expression of CD25 and FOXP3 as indicative of the presence of T regulatory cells in (a) spleen cells and (b) peritoneal exudate cells. No significant differences were found among treatments. $n = 4$ mice per group.

DSS did not express Arginase-1 and Ym-1, but they did express iNOS (Figure 7(a)). Thus, the presence of *T. crassiceps* attenuated the levels of mRNA iNOS, IL-17, and TNF-α in both colon tissue and sera.

A classic side effect of strong Th2-type-biased responses induced by helminths is potential development of fibrosis [9, 27]. Here we found that *T. crassiceps*-infected and DSS-treated mice did not display an excess of collagen in the colon tissue, thus ruling out fibrosis as a side effect of this infection during the modulation of colitis (Figure 7(b).

3.5. Transfer of AAMΦs (F4/80$^+$MR$^+$) with Suppressive Activity from T. crassiceps-Infected Mice Ameliorates DSS-Induced Colitis. Based on our observation that percentages of T regulatory cells were not altered by infection with *T. crassiceps* and because *T. crassiceps* infection recruits AAMΦs into the peritoneal cavity (approximately 35% of peritoneal exudate cells are F4/80$^+$MR$^+$Arg1$^+$) and peritoneal adherent cells suppress T cell proliferation [28], we evaluated whether this population was able to influence the development of DSS-induced colitis. We obtained peritoneal cells from mice previously infected with *T. crassiceps* (6–8 weeks after infection) and sorted them in F4/80$^+$MR$^+$ (AAMΦs) and F4/80$^+$MR$^-$ with purity >90% (see Supplemental Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/563425). Cells (1×10^6 F4/80$^+$MR$^+$ or F4/80$^+$MR$^-$ cells) were adjusted and immediately transferred intraperitoneally to mice previously exposed to DSS. A portion of these purified cells were tested for in vitro suppressive activity on CD4 and CD8 cells from naïve mice. As shown in Figure 8(a), the F4/80$^+$MR$^+$ cells strongly suppressed the proliferation of CD4 cells as well as CD8 cells (Supplemental Figure 2). In contrast, F4/80$^+$MR$^-$ cells were unable to inhibit T cell proliferation in response to anti-CD3/CD28 stimuli. It is important to note that the transfer of F4/80$^+$MR$^+$ cells ameliorated DSS-induced colitis by significantly decreasing bloody diarrhea (Supplemental Figure 3). Moreover, mice that received F4/80$^+$MR$^+$ cells displayed less signs of colitis, such as shortened colon, and tissue architecture was very well conserved in these mice (Figures 8(b)-8(c)) compared to mice that did not receive cells or mice that received F4/80$^+$MR$^-$ cells. The transfer of F4/80$^+$MR$^+$ cells into DSS-treated mice was characterized by much less severe mucosal pathology than in mice that did not receive cells or mice that received F4/80$^+$MR$^-$ cells, as evidenced by marked destruction of the crypt architecture and a greater influx of inflammatory polymorphonuclear cells, which are largely associated with colitis (Figures 8(c)-8(d)); thus mice receiving F4/80$^+$MR$^-$ cells showed even worse pathology, with shorter colons and severe signs of cryptitis and loss of colon tissue architecture.

3.6. In Vivo Indomethacin Treatment Impairs the Anticolitic Effect of T. crassiceps Infection. Previous work from our lab demonstrated that AAMΦs recruited by *T. crassiceps* infection are strong producers of PGE_2 [29]. However, PGE_2 may play a dual role in inflammatory processes, mainly in the gut [30]. Thus, to further elucidate the possible mechanisms involved in the effect of *T. crassiceps* on the amelioration of ulcerative colitis, we injected uninfected and *T. crassiceps*-infected mice with 3 mg/kg indomethacin daily to transiently block PGE_2 production in vivo. Injections began 2 days before the induction of colitis and were maintained throughout the experiment. Loss of body weight was significantly greater in DSS + indomethacin-treated *T. crassiceps*-infected mice than in DSS-treated *T. crassiceps*-infected mice (Figure 9(a)).

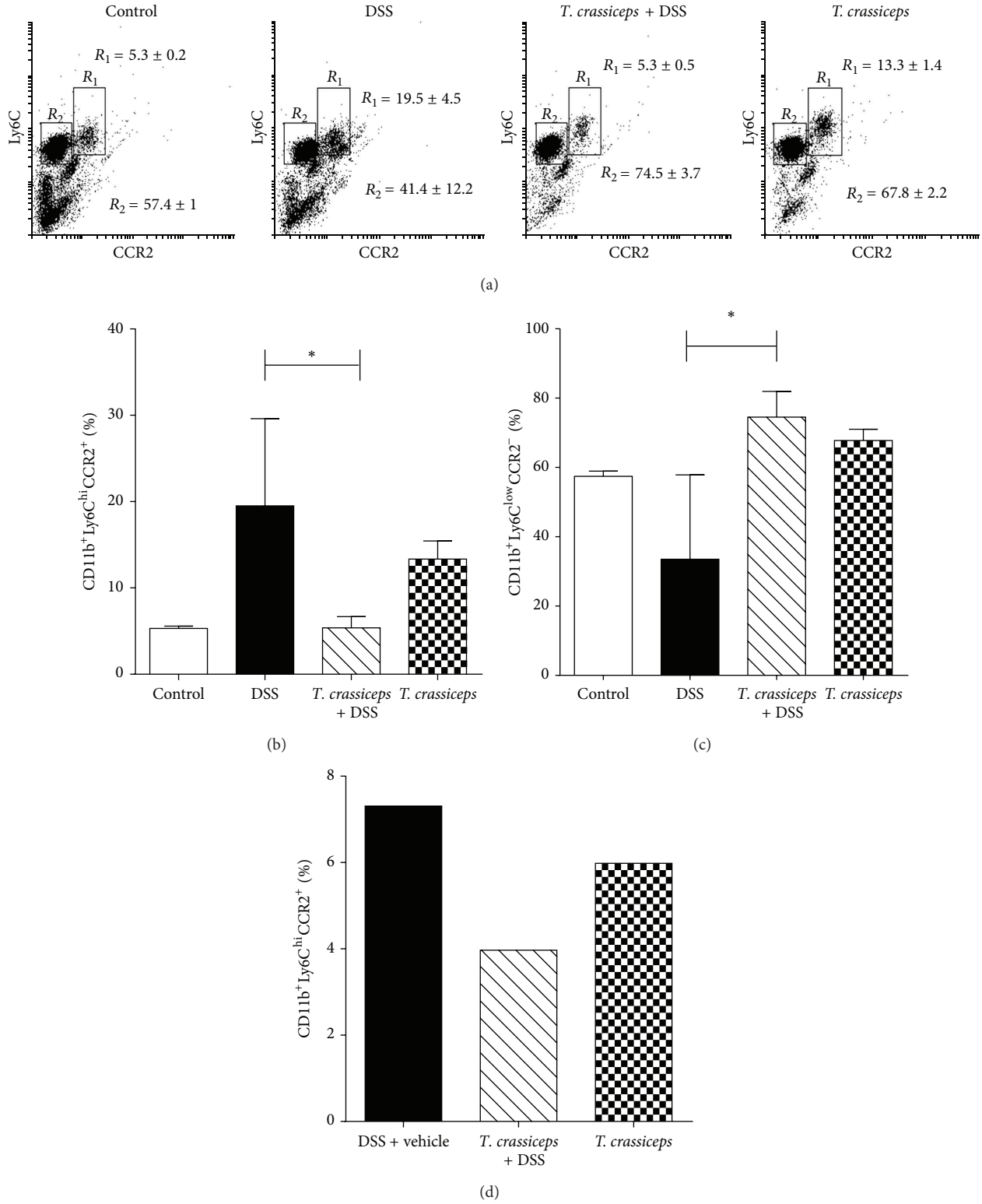


Figure 6: *T. crassiceps* infection reduces the number of inflammatory monocytes during colitis. (a) Representative flow cytometry plots from control mice, DSS-treated mice, and *T. crassiceps* + DSS mice gated on $CD11b^+$ living cells isolated from the circulation. Quantification of circulating (b) $CD11b^+Ly6C^{hi}CCR2^+$ cells and (c) $CD11b^+Ly6C^{lo}CCR2^-$ cells. (d) Percentage of $CD11b^+Ly6C^{hi}CCR2^+$ and $CD11b^+Ly6C^{lo}CCR2^-$ in cells isolated from the colonic lamina propria. Data are representative of two independent experiments. Values are means ± SE ($n = 4$ mice/group). $^*P < 0.05$, pooled cells for (d).

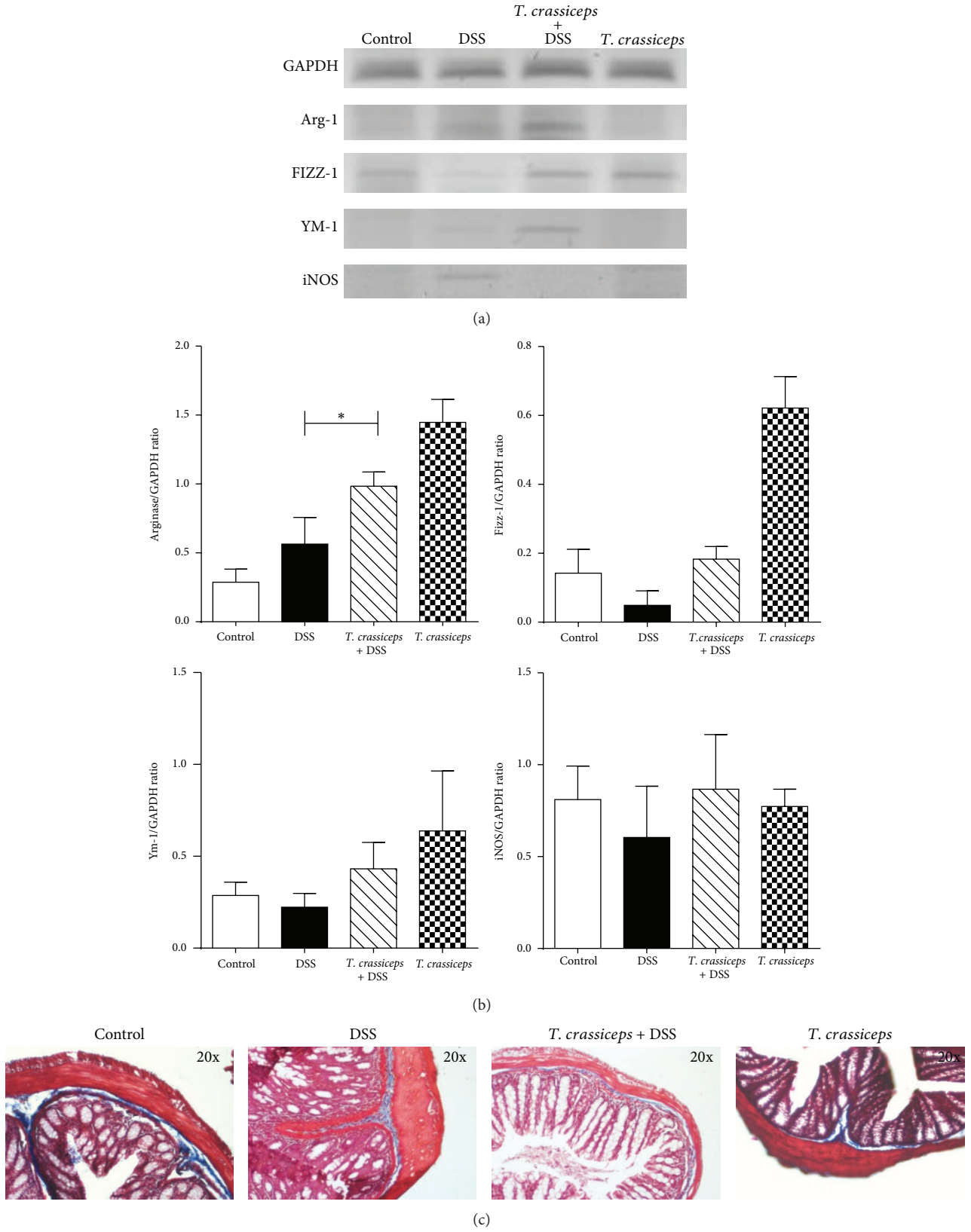
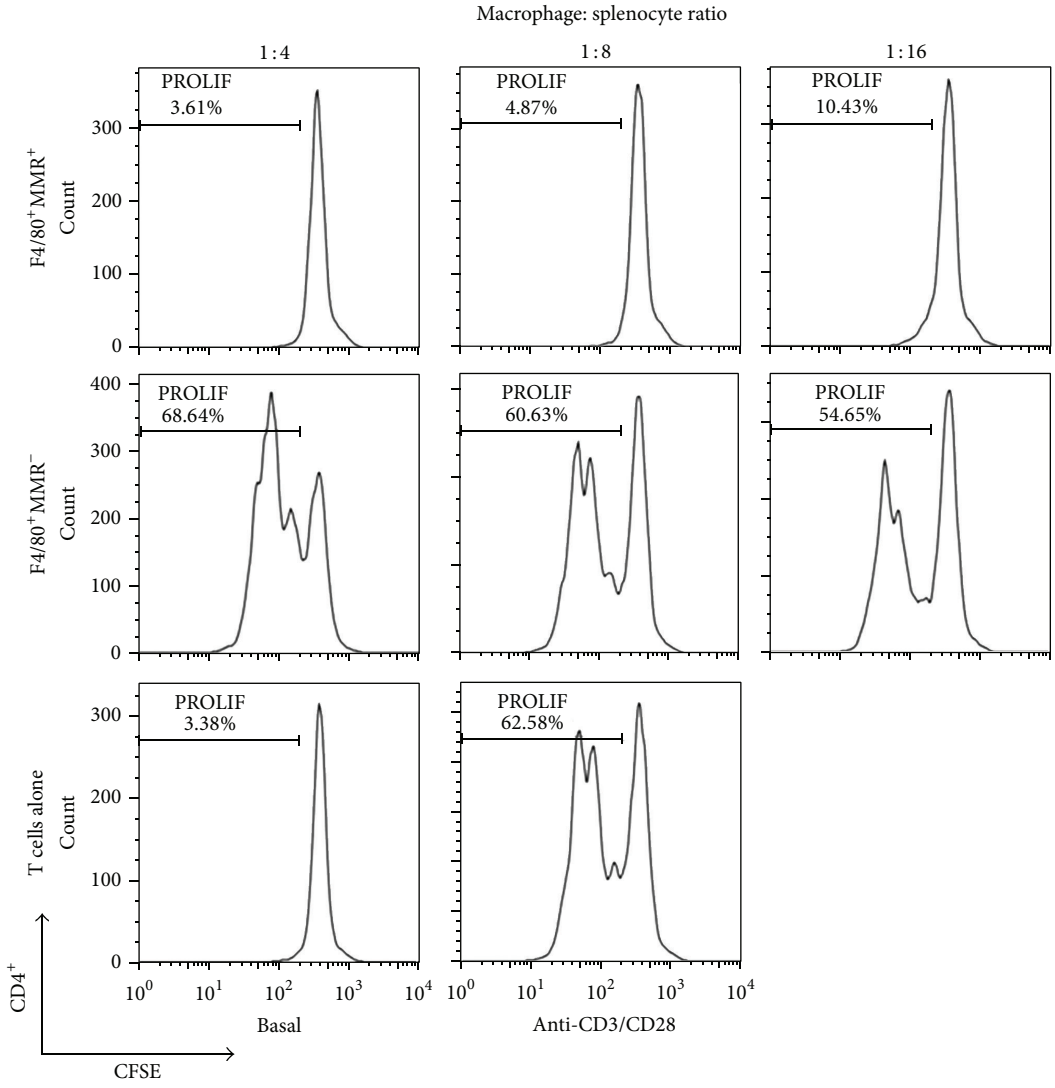
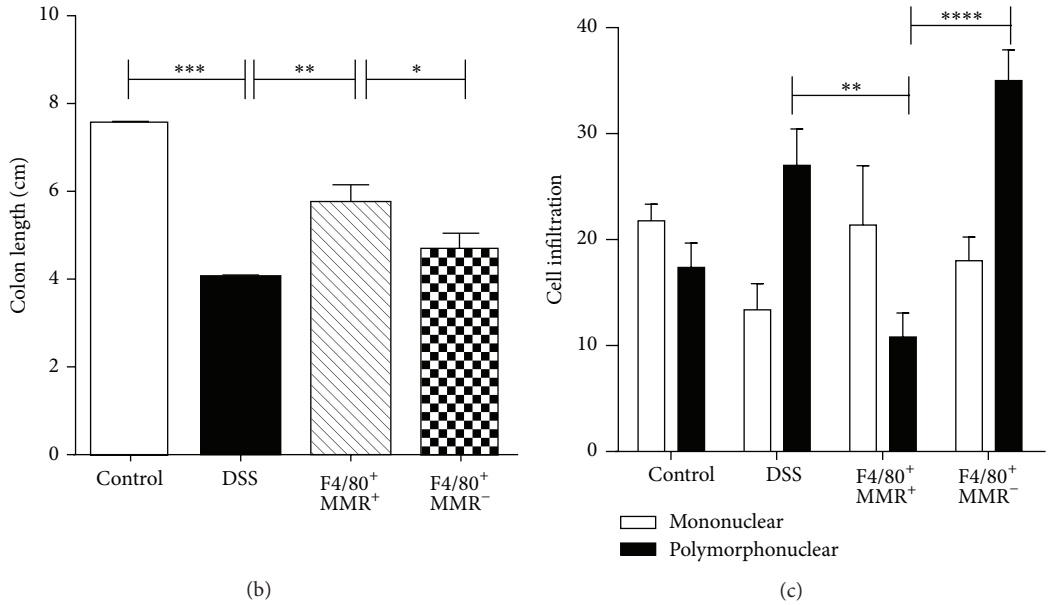


FIGURE 7: Colon tissue from *T. crassiceps*-infected mice and uninfected mice displays different levels of AAMΦ-associated transcripts. (a) Colon tissue was collected at the end of the experiments, and transcript levels of GAPDH, Arginase 1, Fizz1, Ym1, and iNOS were analyzed by RT-PCR. (b) Densitometry of Arg 1, Fizz1, Ym1, and iNOS. (c) Histology with Mason stain for the identification of collagen deposition as a sign of fibrosis. *T. crassiceps* infection does not induce fibrosis during colitis (collagen in dark blue). Magnification = 10x.



(a)



(b) (c)

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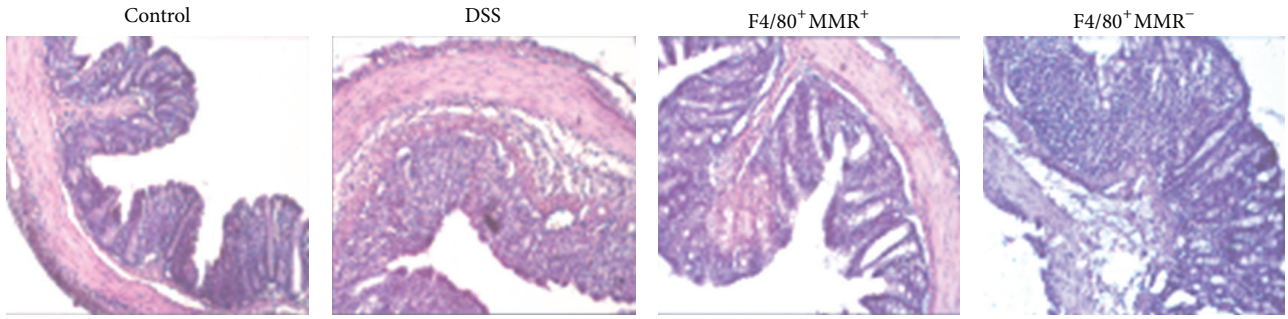


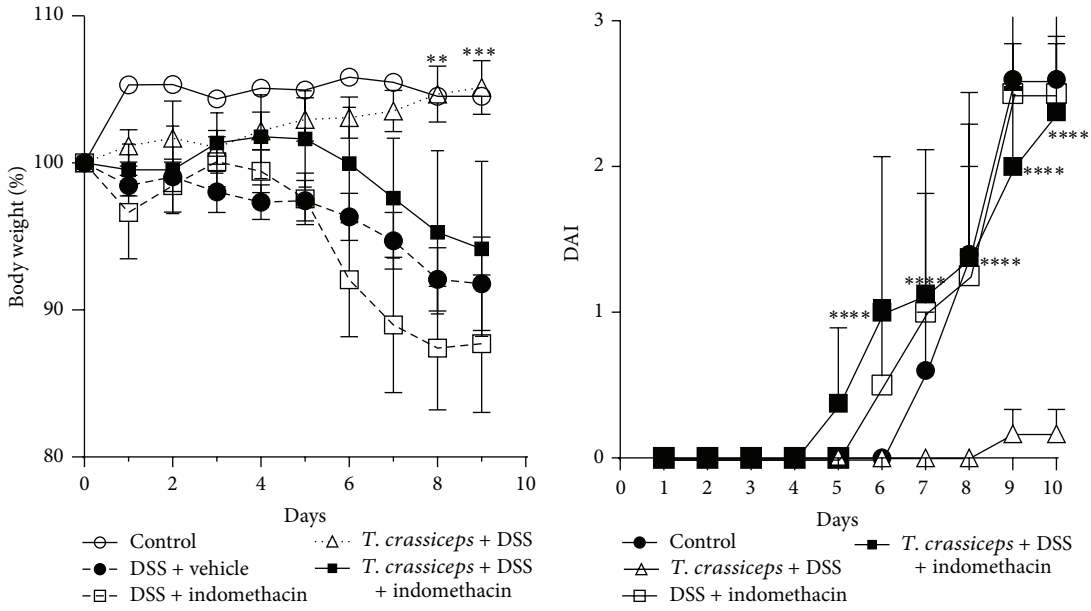
Figure 8: F4/80$^+$MR$^+$-sorted peritoneal macrophages from *T. crassiceps*-infected mice transferred intraperitoneally into naïve mice inhibit the development of colitis. (a) F4/80$^+$MR$^+$ peritoneal macrophages sorted from *T. crassiceps*-infected mice inhibit CD4 and CD8 T cell proliferation (data not shown for CD8). In contrast, sorted F4/80$^+$MR$^-$ macrophages from the same mice do not suppress CD4 cell proliferation. (b) Colon length of mice with ulcerative colitis that received F4/80$^+$MR$^+$ and F4/80$^+$MR$^-$ cells. (c) Infiltration of inflammation. (d) Histology of the effect of F4/80$^+$MR$^+$ adoptive transfer during colitis: magnification is 20x for all the slides shown. Bars represent the mean ± SD from three slides per mouse. $^*P < 0.05$, $^{***}P < 0.003$, $n = 5$ mice per group. All data are representative of two independent experiments.

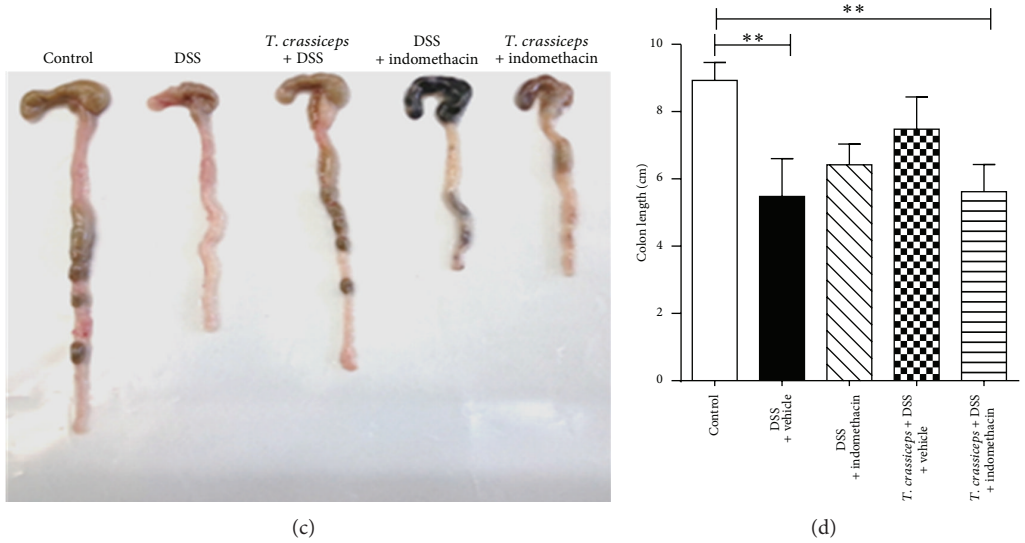
This effect correlated with increased signs of morbidity (DAI) over the course of the disease compared to *T. crassiceps*-infected mice similarly treated with DSS (Figure 9(b)). Moreover, increased colon shortening was found in *T. crassiceps*-infected mice exposed to DSS and indomethacin compared to *T. crassiceps*-infected mice exposed to DSS alone (Figures 9(c)-9(d)). It is interesting that indomethacin treatment eliminated the pathological differences between uninfected and *T. crassiceps*-infected mice associated with the development of colitis, as shown in Figures 9(b)–9(e), as a loss of colon tissue architecture was detected even in the presence of this helminth infection. Moreover, a clear change in inflammatory recruitment was found among the different groups of mice. For example, whereas *T. crassiceps* infection reduced the influx of neutrophils into the lamina propria during colitis, uninfected mice recruited higher numbers of neutrophils. What is interesting is that blockage of PGE_2 by indomethacin treatment significantly increased the influx of neutrophils into the colon of *T. crassiceps*-infected mice (data not shown), and a significant reduction in the number of goblet cells was observed in DSS + indomethacin-treated *T. crassiceps*-infected mice, whereas mice with *T. crassiceps* infection plus colitis retained a higher number of these cells (Figures 9(e)-9(f)). Finally, mice harboring *T. crassiceps* and treated with indomethacin displayed increased production of TNF-α and IL-17 compared to *T. crassiceps*-infected mice (data not shown), whereas IL-10 was not affected either at the systemic level or in colonic extracts (data not shown).

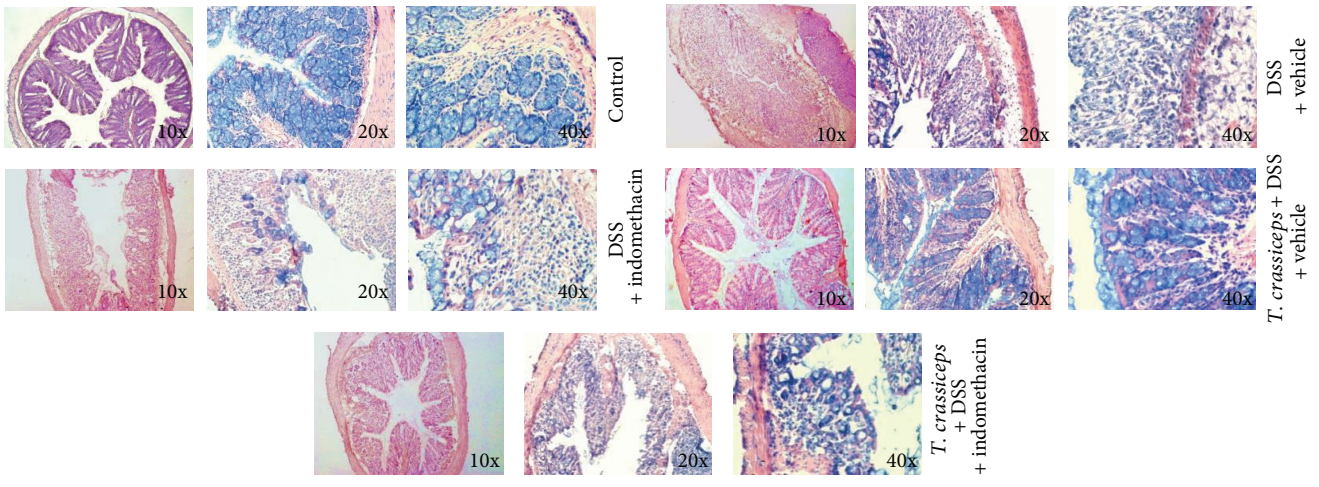
4. Discussion

The frequency of autoimmune and inflammatory diseases such as multiple sclerosis, type 1 diabetes, and IBD has increased enormously in the past few years, a situation attributed to a lack of exposure to pathogens, especially helminths [4]. Among such inflammatory diseases, IBD has increased at an alarming rate in the past decade, accompanied by improved hygiene, sanitation, and medical conditions and, of course, less infectious diseases, including helminths [4, 11]. Symptoms of IBD are the result of complex interactions among genetic and environmental factors and the immune response [31]. Immunomodulatory effects exerted by helminth parasites on their hosts help to prevent or ameliorate such diseases [14]. Although a large body of evidence indicates that regulatory mechanisms triggered by helminth infections may help to modulate colitis, the precise mechanisms involved are not yet very well understood. Mainly gastrointestinal and transient infections of helminths induce higher levels of Th2 cytokines, induction of T regulatory cells, recruitment and expansion of AAMΦs, and reduction of inflammatory cytokines that results in amelioration in different murine models of colitis [14, 24, 32]. All of these observations suggest that distinct helminths may trigger different pathways to modulate this particular inflammatory disease.

Here we demonstrated that extraintestinal infection with the larval stage of *T. crassiceps* can be added to the growing list of helminth infections with the capacity to modulate colitis; this is just the second cestode reported to induce such protection [14]. Besides the high levels of IL-4 expected with this infection we also found elevated IL-10 levels associated with a downregulation of proinflammatory cytokines. However, *T. crassiceps* infection did not induce higher numbers of T regulatory cells, which is in line with a previous finding on the effect of this infection on experimental autoimmune encephalomyelitis [18]. Therefore, we think that T regulatory cells may play a minor role in our system, even though several authors have found that increased levels of T regulatory cells are associated with improvement in colitis during helminth infections [25, 26]. Here, using T regulatory cell reporter mice we did not find increased levels of T regulatory cells during *T. crassiceps* infection and colitis, but we did observe an improvement in colitis when mice were infected. Based on these observations, our data point to a greater role for AAMΦs (as opposed to regulatory T cells) in the anticolitic







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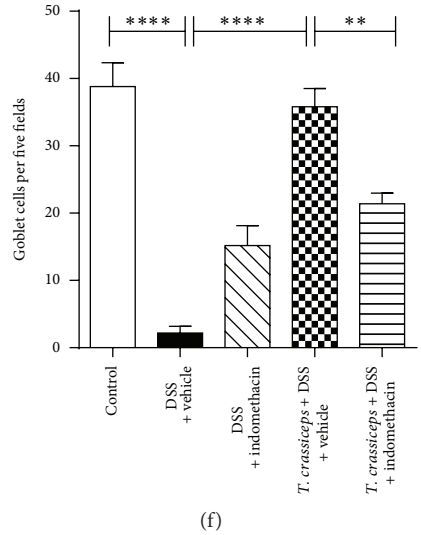

(f)

Figure 9: The anticolitic effect of *T. crassiceps* is abrogated by indomethacin treatment. (a) Percentage of weight loss. (b) Disease index. (c) Photograph of gross pathology of colons from different groups of mice. (d) Length of colon in infected and uninfected mice with ulcerative colitis treated or not with indomethacin (3 mg/kg). (e) Histology of colons, left panel colon tissue microphotography (10x) stained with H&E, all other panels are tissue colon stained with Alcian blue to detect goblet cells (20x and 40x, resp.). (f) Number of goblet cells for all the groups. $^{*}P < 0.05$, $^{***}P < 0.003$, $n = 5$ mice per group. Similar results were observed in two independent experiments.

effect of *T. crassiceps* infection. Furthermore, *T. crassiceps* infection in DSS-treated mice was characterized by much less severe mucosal pathology than that seen in uninfected control mice, as evidenced by marked destruction of the crypt architecture and an increased influx of inflammatory cells. Furthermore, we demonstrated that in vivo transfer of purified AAMΦs ($F4/80^{+}MR^{+}$) recruited for this infection is able to modulate ongoing colitis; thus, it is possible that these cells not only prevent the development of colitis but also may play a curative role. These observations are in line with those recently reported for another cestode, *Hymenolepis diminuta,* which induces AAMΦs associated with the prevention of colitis [9]. However, more studies are needed to confirm whether AAMΦs are the most potent immunoregulatory pathway induced by helminths to reduce colitis.

Neutrophil infiltration is a key event in inflammation of the colon [33, 34]. Here we found that *T. crassiceps* infection during DSS-induced colitis generates a significant change in the populations of cells that infiltrate the colon. We generally observed greater infiltration by monocytes than neutrophils, the latter being the main cell population detected in the absence of this helminth infection. Monocytes can be divided into two subsets: patrolling monocytes that express $CD11b^{+}CD115^{+}CX3CR1^{hi}CCR2^{lo}Ly6C^{lo}$ and inflammatory monocytes that express mainly $CD11b^{+}Ly6C^{hi}CCR2^{+}$ [35]. Inflammatory monocytes accumulate in response to infection or tissue injury, and in most cases they help to clear pathogens [36]. However, in some pathology and especially in IBD, the recruitment of inflammatory monocytes into damaged tissue frequently worsens the inflammation [35]. The recruitment of inflammatory $Ly6C^{hi}$ monocytes into adult mucosa is dependent on CCR2 expression [37]. Here we found that the circulating levels and recruitment of $CD11b^{+}Ly6C^{hi}CCR2^{+}$ inflammatory monocytes into the colon were significantly reduced by the presence of *T. crassiceps* infection; in contrast, $CD11b^{+}Ly6C^{lo}CCR2^{-}$ cells increased during infection. This is the first time that it has been reported that an extraintestinal helminth infection is able to modulate these cell populations. Such modulation may have an impact on the development of colitis and also may favor development into AAMΦs, as demonstrated by the expression of Arg 1, FIZZ 1, and YM1 in colon tissue. Thus, a reduction in the recruitment of inflammatory monocytes together with an increase in AAMΦs in the colon may be a strong anticolitic mechanism triggered by *T. crassiceps* infection.

An interesting finding is that indomethacin treatment to inhibit PGE_2 production in vivo during *T. crassiceps* infection and exposure to DSS completely abrogated the anticolitic effect of this infection. This result suggests that the in vivo capacity of *T. crassiceps* infection to suppress DSS-induced colitis is highly dependent on the ability of the host to produce PGE_2, maybe in response to commensal or parasite-derived stimulation. These findings are in line with those reported by Bao et al. [38], who found that PGE_2 plays a fundamental role in regulating the immune response to colitis as well as modulating Th1/Th2 responses [38]. Specifically, when we blocked in vivo PGE_2 using indomethacin, the anticolitic effect of *T. crassiceps* infection was clearly abrogated, but IL-10 levels still remained elevated, indicating a major role for PGE_2 during *T. crassiceps*-mediated amelioration of colitis. In line with these data, other authors have found that even in the absence of IL-10, colitis can be modulated [39]. As further support of this idea, our group previously reported that macrophages obtained from *T. crassiceps*-infected mice are able to produce significantly elevated levels of PGE_2 in response to

stimulation with LPS [29], a molecule to which epithelial and intestinal macrophages are highly exposed.

Moreover, we found the number and size of goblet cells increased in infected animals with DSS-induced colitis compare with colitic mice, suggesting that *T. crassiceps*, like other helminths, can help in preserving these cells [40, 41]. Goblet cells are involved in regulating both the mucosal barrier and the relative composition of the luminal microbiota by mucin production [42]. The high expression of IL-4 in *T. crassiceps* + DSS mice suggests that this infection may maintain the numbers of goblet cells. The production of mucus by these cells could limit bacterial access to epithelial cells and prevent chronic inflammation [43]. Thus, an increase or recruitment of AAMΦs in the lamina propria seems to be necessary for anti-inflammatory activity. We demonstrated that *T. crassiceps* infection during colitis is able to promote the polarization of AAMΦs, thereby attenuating the expression of inflammatory cytokines, preventing damage to the colon and the development of colitis. The relationship between infiltrating AAMΦs and prognosis in colitis has not been analyzed; therefore, the distribution and function of macrophages in experimental ulcerative colitis need to be evaluated further. Another interesting finding here is a trend to reduce IL-17E by *T. crassiceps* infection in colitis, but surprisingly IL-17A levels were increased in the same group; in line with these data are several findings indicating that the presence of IL-17A has an anticolitic effect, given that IL-17AKO mice became dramatically susceptible to DSS-induced colitis [44] and other researchers reported similar findings in distinct models of colitis, these data are in this moment very difficult to explain, and suggest that Th17 family has complex functions during different inflammatory diseases [45]. The mechanisms regarding the differential IL-17 modulation by *T. crassiceps* infection remain to be elucidated. Finally, the modulation of Th1- and Th17-type cytokines observed here accords with Tao et al. [22], who suggested that inactivation of STAT1 and STAT3 may contribute to resolving different models of colitis. We recently found that infection with *T. crassiceps* or its excreted/secreted products are able to inhibit STAT1 phosphorylation in macrophages and splenocytes in response to IFN-γ [46]. Thus, it appears that multiple mechanisms can be triggered by *T. crassiceps* infection or its products to modulate inflammatory responses.

5. Conclusion

We found that extraintestinal infection with *T. crassiceps* significantly reduced both symptoms and colonic inflammation associated with ulcerative colitis independently of the genetic background. Moreover, AAMΦs and prostaglandins may play a critical role in avoiding colonic inflammation and perhaps inhibiting recruitment of inflammatory monocytes $CD11b^+Ly6C^+CCR2^+$ into the lamina propria of the colon.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Yadira Ledesma-Soto and Blanca E. Callejas contributed equally to this work.

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Review Article

The Dialogue of the Host-Parasite Relationship: *Leishmania* spp. and *Trypanosoma cruzi* Infection

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The intracellular protozoa *Leishmania* spp. and *Trypanosoma cruzi* and the causative agents of Leishmaniasis and Chagas disease, respectively, belong to the Trypanosomatidae family. Together, these two neglected tropical diseases affect approximately 25 million people worldwide. Whether the host can control the infection or develops disease depends on the complex interaction between parasite and host. Parasite surface and secreted molecules are involved in triggering specific signaling pathways essential for parasite entry and intracellular survival. The recognition of the parasite antigens by host immune cells generates a specific immune response. *Leishmania* spp. and *T. cruzi* have a multifaceted repertoire of strategies to evade or subvert the immune system by interfering with a range of signal transduction pathways in host cells, which causes the inhibition of the protective response and contributes to their persistence in the host. The current therapeutic strategies in leishmaniasis and trypanosomiasis are very limited. Efficacy is variable, toxicity is high, and the emergence of resistance is increasingly common. In this review, we discuss the molecular basis of the host-parasite interaction of *Leishmania* and *Trypanosoma cruzi* infection and their mechanisms of subverting the immune response and how this knowledge can be used as a tool for the development of new drugs.

1. Host-Parasite Interaction

Parasitic diseases are some of the greatest public health problems in developing countries. Several of these diseases are neglected, either because of their incidence in countries with little purchasing power or their low visibility. In general, the majority of these countries are located in the tropical zone. The climates of these areas contribute to the development of parasitic infections because humidity and high temperatures provide the necessary conditions for vector and protozoan growth [1].

All mammalian hosts are at risk of infection by viruses, bacteria, fungi, and parasites. The host-parasite relationship is the most important factor in determining whether an infection is successful or is resolved by the host. Several mechanisms are involved in this complex interaction, and

aspects of both the host and the parasite are essential. Some parasites have evolved evasive mechanisms, such as intracellular infection, as in the case of the genus *Leishmania* and *Trypanosoma cruzi*, protozoa parasites belonging to family Trypanosomatid, order Kinetoplastida. These parasites are among the most important agents of neglected tropical diseases [2]. They are heteroxenic and infect two host types: vertebrates and invertebrates [3, 4]. Throughout their life cycle, they progress through several forms, including epimastigotes and metacyclic trypomastigotes, which are found inside the Triatominae vector of *T. cruzi* and procyclic and metacyclic promastigotes, which are found inside the Phlebotominae vector of *Leishmania* genus [3, 4]. Amastigotes are the intracellular form of the both parasites and are found inside the vertebrate host. Additionally, *T. cruzi* presents the blood trypomastigote forms in this host [3, 4].

Leishmania is responsible for a group of cutaneous and visceral infections known as leishmaniasis. These parasitoses are endemic in 98 countries distributed in Latin America, South and Central Asia and sub-Saharan Africa [5], where approximately 350 million people are threatened with contracting this infection. The annual incidence is estimated at 1.6 million, and the prevalence is 12 million [6].

Trypanosoma cruzi causes Chagas disease. An estimated 10 million people are infected by *T. cruzi*, mostly in Latin America, where Chagas disease is endemic, and more than 25 million people are danger of contracting this parasitosis [6].

The first step in the interaction between the host and these intracellular protozoa parasites is the binding of the parasite to the host cell. These protozoa have a variety of surface and secreted molecules used to attach and enter mammalian cells. Several of these molecules are involved in triggering specific signalling pathways essential for parasite entry and intracellular survival. Scientific advances in this area have identified factors critical to parasite virulence and the disease pathogenesis.

2. Molecular Basis of Trypanosomatid-Host Cell Interaction

Metacyclogenesis is an important process for parasite virulence. This mechanism allows trypanosomatids to infect their vertebrate host and thus their host cells [7]. Inside the vector gut, *Leishmania* parasites transform from procyclic promastigotes to metacyclic promastigotes during metacyclogenesis [7], whereas *T. cruzi* transitions from epimastigotes to metacyclic trypomastigotes [8].

For a long time, *Leishmania* spp. was believed to be obligatory intracellular pathogens of macrophages. However, recent studies have shown that these protozoa infect a large range of host cells [9–11]. Various groups have shown that these parasites can infect multiple cell types *in vitro* as well as *in vivo*, from haematopoietic cells that arise from a common myeloid precursor to nonhaematopoietic cells, such as fibroblasts [10].

Early in infection, neutrophils are recruited in response to a bite from the insect vector due to the release of the alarmins (signal for tissue damage), cytokines, and chemokines [10, 12]. These cells can act against the intracellular microorganisms through reactive oxygen species (ROS) [13, 14], neutrophil elastase (NE), and neutrophil extracellular traps (NETs) [15]. Nevertheless, if these mechanisms can be evaded, neutrophils may serve as host for *Leishmania* parasites. They are infected by promastigotes during the first 18 hours. These cells undergo apoptosis, and the apoptotic bodies are phagocytized by macrophages, triggering anti-inflammatory signal pathways. This results in the silent entry of the parasites inside macrophages, which promotes infection success [16]. It is interesting to note that neutrophils readily phagocytized promastigotes, but recognition or uptake of amastigotes has not been detected yet [17].

The initial binding and internalization of the *Leishmania* promastigotes is a classical receptor-mediated endocytic event that involves serum-derived factors as well as parasites and host cell molecules. The major macrophage plasma membrane structures involved in this interaction are (1) receptors for the complement component 3 subunits C3b and C3bi, which bind to CR1 and CR3, respectively; (2) Fc receptors; (3) lectin receptors, which mediate connections with carbohydrate molecules; and (4) the integrin family of molecules that recognize specific amino acid sequences. The major surface molecules of *Leishmania* that may also participate in this interaction include gp63 or promastigote surface protease (PSP), the primary parasite surface protein; lipophosphoglycan (LPG), the main promastigote glycoconjugate; and glycosyl inositol phospholipids (GPIs), which are present in large numbers in both promastigotes and amastigotes [18].

The parasite surface molecules responsible for the independent binding of serum are LPG, gp63, and glyco inositol phospholipids (GIPLs). In *L. major*, LPG is involved in the invasion of both promastigotes and amastigotes, although this molecule is absent in amastigotes of certain parasite species. Proteophosphoglycan (PPG) is particularly important in the invasion of macrophages by a number of *Leishmania* amastigotes [19, 20].

Both LPG and GIPLs are capable of binding to a mannan-binding serum protein (MBP), which is able to activate the complement system in an antibody-dependent manner. This mechanism may be particularly important in the case of amastigotes that have little or no LPG and gp63 on their surface [18]. On the other hand, gp63 and LPG act as acceptor sites for the complement component 3 (C3) and interact with CR3 and p150, 95, members of the CD18 family of integrins [21, 22]. Meantime, some studies demonstrated that internalization of promastigotes of LPG-defective *Leishmania* is higher than of wild-type (WT) promastigotes [23–26]. Thus, it seems unlikely that LPG plays an essential role in promastigote adhesion to macrophages, but it appears that may interfere with the process of phagocytosis. For accommodating the plasma membrane extension that occurs during the phagocytosis of large particles, as the parasites, focalized exocytosis of endomembrane occurs at the phagocytic cup [27–29]. Several intracellular compartments, including endoplasmic reticulum, late endosomes, and recycling endosomes

may contribute to membrane formation of the phagosome through fusion events regulated by soluble N-ethylmaleimide sensitive factor attachment protein receptors (SNAREs), such as VAMP3, VAMP7, and syntaxin 18 [30–35]. The activity of SNARE is regulated by synaptotagmins (Syts), a family of transmembrane proteins that act as sensors of Ca^{2+} [36, 37]. The first Syt protein characterized in phagocytosis is the lysosomal Syt VII, which regulates Ca^{2+}-dependent exocytosis of lysosomes [38] and directs the lysosomal membrane to the phagosome [39]. Another protein was posteriorly identified as Syt V, a recycling endosome associated protein recruited to forming phagosome and controls the phagocytic process [40]. After *Leishmania*-host cell contact, LPG is transferred from the parasite to the macrophage membrane during phagocytosis and seems to promote blockage of macrophage activation, protecting the parasite [41]. This insertion promotes disruption of existing lipid microdomains and alters the formation of these structures after promastigote internalization [42, 43], causing the exclusion of Syt V [44]. Consequently, LPG impairs the recruitment of Syt V to the nascent phagosome, resulting in a reduction in the phagocytic capacity of host macrophages [45]. However, the Syt V exclusion from phagosomes promoted by LPG abrogated the recruitment of the vacuolar ATPase and, consequently, their acidification [44], creating a hospitable intracellular niche for *Leishmania* (Figure 1). Thus, although the entry of parasites into macrophages is reduced, their higher survival is reached due to lack of the phagosome acidification and this may represent a larger gain in overall adaptation of these protozoa.

In addition to vector transmission, infection by *T. cruzi* can also occur through organ transplantation [46], blood transfusion [47], congenital transmission [48], oral transmission [49], or laboratory accidents [50]. The literature has suggested that host cell invasion requires the activation of signal transduction pathways that lead to an increase in cytosolic calcium concentration in both the parasite and the host cell and the recruitment and fusion of host perinuclear lysosomes to the site of invasion [51, 52]. According to Andrews [53], the trigger for host cell calcium production is the recruitment of perinuclear lysosomes to the *T. cruzi* invasion site. At this site, lysosomes are incorporated immediately into the parasitophorous vacuole without polymerized actin accumulation, and invasion is facilitated by disruption of microfilaments. However, the recruitment of lysosomes is not currently believed to be essential in this process but is essential for parasite persistence in the host cell. In professional phagocytes, parasite internalization occurs by conventional phagocytosis. Following the adhesion of the parasite to the host cell membrane, molecular signals are triggered, initiating this process. The invasion efficiency in nonphagocytic cells varies among the different developmental forms, that is, *T. cruzi* strains and phylogenetic lineages. Extracellular amastigotes, for example, are potent inducers of phagocytosis in nonprofessional phagocytes, a process that may facilitate parasite persistence in infected hosts [54].

Trypomastigotes adhere to host cells using surface receptors. Surface glycoproteins such as gp82 and gp35/50, which induce calcium-mediated signaling, are utilized differently among different strains of *T. cruzi*. Isolates that enter the host cell in a gp82-dependent manner (*T. cruzi* II—endemic areas) activate a protein tyrosine kinase and a parasite phospholipase C, which releases Ca^{2+} from inositol-1,4,5-triphosphate (IP3) sensitive reservoirs, possibly the endoplasmic reticulum (Figure 2(a)). However, *T. cruzi* isolates that bind to target cells using gp35/50 (*T. cruzi* I—Amazon region) appear to stimulate adenylate cyclase activity that seems to participate in Ca^{2+} release from acidocalcisomes [55, 56] (Figure 2(b)). Metacyclic trypomastigotes trigger Ca^{2+} release from intracellular stores sensitive to IP3 in the host cell and induce Ca^{2+}-dependent disorganization of actin cytoskeleton. The Ca^{2+} release also mobilizes perinuclear lysosomes to the site of *T. cruzi* invasion. Some studies report that lysosomes that fuse directly to the vacuole are already in the plasma membrane. Thus, Ca^{2+} would act only in the fusion and not in the recruitment in this case. Ca^{2+}-dependent lysosomal exocytosis is regulated by cAMP and is increased by isoproterenol, a β-adrenergic agonist that activates adenylate cyclase. This mechanism appears to be used by the cell to repair cellular membrane (Figure 2) [57]. In the early 2000s, some studies suggested that Syt VII, which is located on the membrane of lysosomes and regulates exocytosis of these organelles, appears to participate in the invasion process of *T. cruzi* [58].

A new lysosome-independent route of host cell invasion has recently been described. In this route, the parasite enters into host cell by creating an invagination in the plasma membrane, which accumulates phosphatidylinositol-3,4,5-triphosphate (PIP_3), the main product activation of phosphatidylinositol-3-kinase class I (PI_3K) (Figure 2) [56]. In a quantitative analysis of the ways in which trypomastigotes of *T. cruzi* penetrate into the host cell, 20 to 25% of trypomastigotes were observed to enter the lysosome exocytosis- and Ca^{++}-dependent pathway, approximately 50% invaded via the PI_3K-dependent pathway and remained in a vacuole formed only by the plasma membrane for an initial period, and approximately 20% entered using the PI_3K-dependent pathway and quickly associated with primary endosomes [56]. However, independently of the entry mechanism, all parasites are found in parasitophorous vacuole-associated lysosomes within 60 minutes because this fusion is essential for *T. cruzi* survival [59]. Unlike in many other intracellular parasites that avoid fusion with host cell lysosomes [60], this process is a prerequisite for the survival of *T. cruzi* [56]. If the parasite does not associate with these organelles, the persistence of parasites in the host cell is seriously compromised, and the entry process is reversed [56]. The exposure of trypomastigotes to this acidic environment is essential for the activity of the porin-like protein TcTOX; this protein is responsible for parasitophorous vacuole lysis and parasite escape into the cytoplasm, which is necessary for the differentiation of trypomastigotes into amastigotes that begins within the low vacuole pH (Figure 3) [55, 61].

Because *T. cruzi* is unable to synthesize sialic acid, the only mechanism for sialylation of their membrane glycoproteins is to transfer the sugar from host cell glycoconjugates through the action of enzymes. This phenomenon is

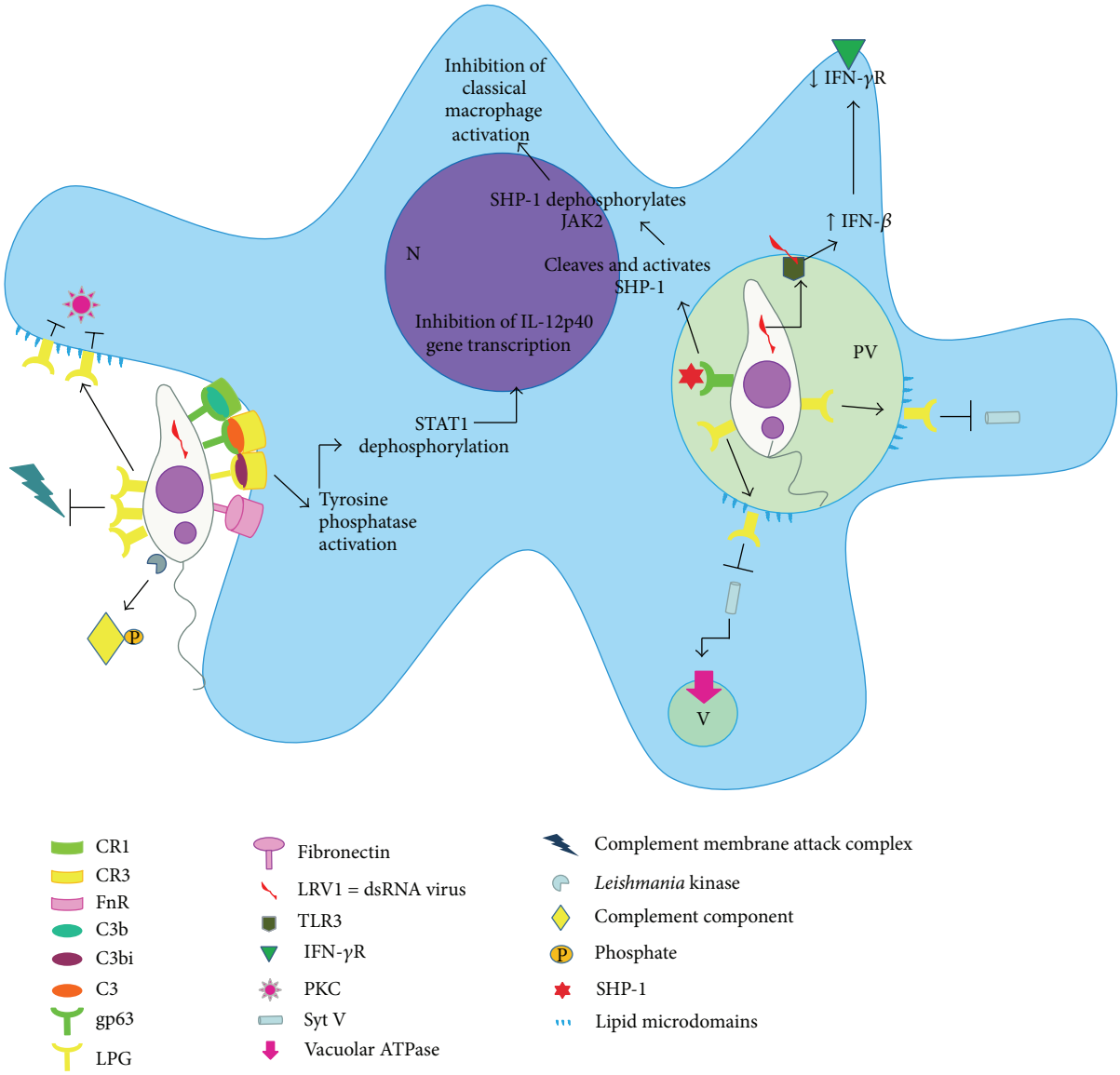

FIGURE 1: *Leishmania* survival and host cell modulation: The LPG coating of the parasites prevents the complement membrane attack complex insertion. In addition, the promastigote kinase phosphorylates the components of the complement, inhibiting its activation. Promastigote opsonized by C3bi interacts with macrophage membrane CR3 activating tyrosine phosphatase that dephosphorylates STAT-1 leading to inhibition of transcription of the IL-12p40 gene. During the process of promastigote internalization LPG is transferred from parasite membrane to host cell membrane promoting lipid microdomain disruption inhibiting PKC activation and ROS generation (burst oxidative). Inside the parasitophorous vacuole (PV) membrane, this disruption causes the exclusion of synaptotagmins V (Syt V), abrogating the recruitment of the vacuolar ATPase and, consequently, PV acidification allowing the survival of promastigotes. The *Leishmania* dsRNA virus (LRV1) binds toll-like receptor 3 (TLR3) triggering strong IFN-β production and downregulation of IFNγ-R. Already gp63 cleaves SHP1 prevents classical macrophage activation by IFN-γ. N—nucleus, PV—parasitophorous vacuole and V—vacuole.

important for host cell interaction and parasite internalization [62, 63]. Trypomastigotes express large amounts of a protein with transsialidase (TcTS) activity on their surface [64]. The transsialidase is bound to the parasite membrane through a glycosylphosphatidylinositol (GPI) anchor and acts to specifically catalyze the transfer of sialic acid from glycoconjugate proteins from the extracellular environment to mucin-associated surface protein (MASP) that cover the surface of the parasite (Tc-mucins) [65], which is important to promote the parasite entry and persistence in the mammalian host cells (Figure 3). These protein domains are rich in threonine residues [66, 67]. These residues can be modified by protein glycosylation, which is an important posttranslational modification for host-parasite interactions. The *O*-glycosylation of *T. cruzi* mucins (Tc-mucins) is initiated by enzymatic addition of α-*O*-N-acetylglucosamine (GlcNAc) to threonine (Thr) by the UDP-GlcNAc: polypeptide α-N-acetylglucosaminyltransferase (pp-α-GlcNAcT) in the Golgi

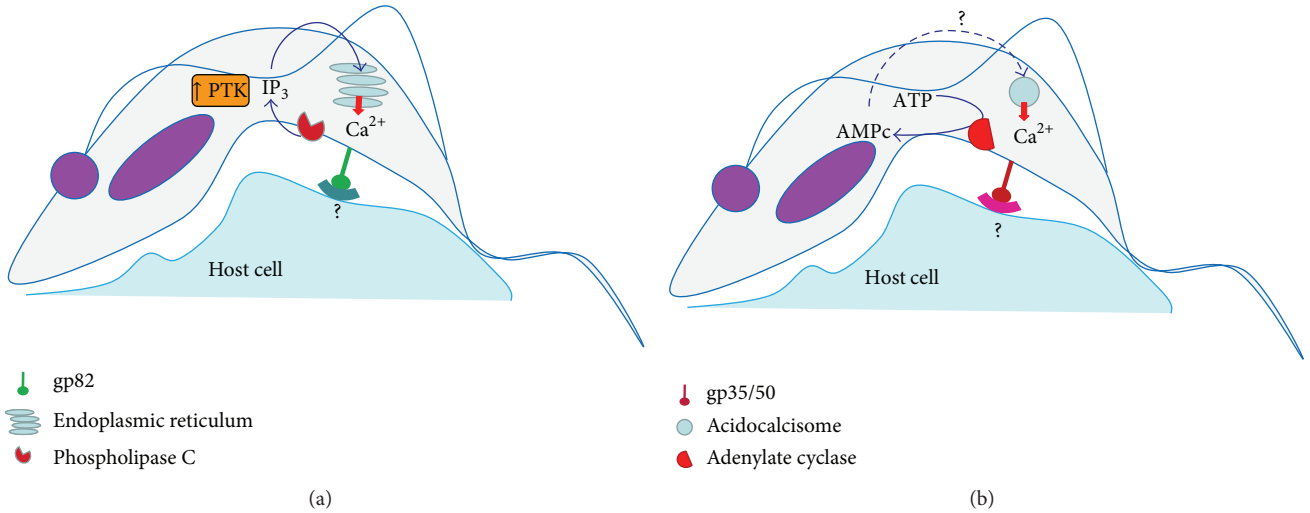

FIGURE 2: Activation of different signaling pathways for host cell invasion by *T. cruzi* II (a) and *T. cruzi* I (b). (a) The ligation gp82-receptor activates a protein tyrosine kinase and a parasite phospholipase C, which releases Ca^{2+} from inositol-1,4,5-triphosphate (IP3) sensitive reservoirs, possibly the endoplasmic reticulum. (b) The gp35/50-receptor binding appears to stimulate adenylate cyclase activity that seems to participate in Ca^{2+} release from acidocalcisomes.

[68]. These *O*-glycans are the acceptors of sialic acid, as already cited by the literature [69, 70]. The different evolutive forms of *T. cruzi* present different molecular masses of Tc-mucins. Epimastigotes and metacyclic trypomastigotes (MT) present Tc-mucins with molecular mass varying from 35 to 50 kDa, while the Tc-mucins from trypomastigotes derived from cell culture (TCT) the variation range is between 60 and 200 kDa [71, 72]. These masses are compatible with glycosylated protein containing sialic acid, which is essential for host cell binding and invasion [72]. These differences seem to contribute for differential susceptibility of MT and TCT to pepsin digestion. The mucin-like molecules that covered the MT are resistant to proteolysis and protect the parasites from lysis in the gastric milieu [73], in the meantime TCT are susceptible to peptic digestion and are mostly lysed (90%) when incubated with pepsin at pH 3.5 for 30 min [74]. In addition Tc-mucins from TCT are capable to induce NO, IL-12 and TNF-α by activated macrophages [75], modulating the immune response during *T. cruzi* infection.

The gene superfamily gp85/trans-sialidase (TS) encodes several glycoproteins that are present on the surface of the parasite and can participate in cell invasion. One of these glycoproteins is called gp83 and plays an important role in the interaction of *T. cruzi* with the host cell interacting with the p74 receptor present on the surface of the host cell and acting as a universal ligand for *T. cruzi* infection of both, phagocytic and non-phagocytic cells [76]. The Tc85 molecules are involved in the adhesion of parasites to the host cell by laminin and other extracellular matrix proteins (ECMP), which can be anchored to the plasma membrane (Figure 3) [77].

A synthetic peptide based on the conserved FLY domain (VTVXNVFLYNR) present in all members of the gp85/TS family promotes dephosphorylation of an intermediate filament protein (cytokeratin 18) that leads to cytoskeletal reorganization facilitating entry of the parasite [78]. This mechanism also promotes activation of the ERK1/2 signaling cascade, resulting in an increase in parasite invasion in epithelial cells [79]. However, an inactive form of TS from TCT that binds sialic acid has been shown to trigger NF-κB activation, the expression of adhesion molecules on endothelial cells and upregulation of parasite entry in an FLY-independent and carbohydrate-dependent manner [80].

The gp90 protein, an N-glycosylated protein [81] with a GPI anchor [82, 83], as well as cruzipain are also involved in host cell invasion of metacyclic trypomastigotes. Secreted cruzipain cleaves host kininogen to liberate bradykinin, and the triggering of the host bradykinin receptor activates host cell PLC, contributing to Ca^{2+} release (Figure 3) [81, 84]. In epimastigotes, cruzipain appears to be linked to degradation processes and localization in the endosomal-lysosomal system [85] but has been described as playing a role in adhesion [86] and cell invasion in trypomastigotes [87].

3. Immune Response against *Leishmania* and *Trypanosoma cruzi* and Their Evasion Mechanisms

The immune system recognizes and responds to a broad spectrum of pathogens, including microorganisms such as viruses, bacteria, fungi, and protozoan parasites, and multicellular parasites, such as helminthes and ectoparasites. Vertebrates possess two types of immunity: innate and adaptive. The innate immune response involves the innate lymphoid cells (ILCs), which are lymphoid cells that do not express rearranged receptors. These cells present essential effector and regulatory functions in innate immunity and tissue remodeling. Two model members of ILC family are natural killer (NK) cells and lymphoid tissue inducer (LTi) cells.

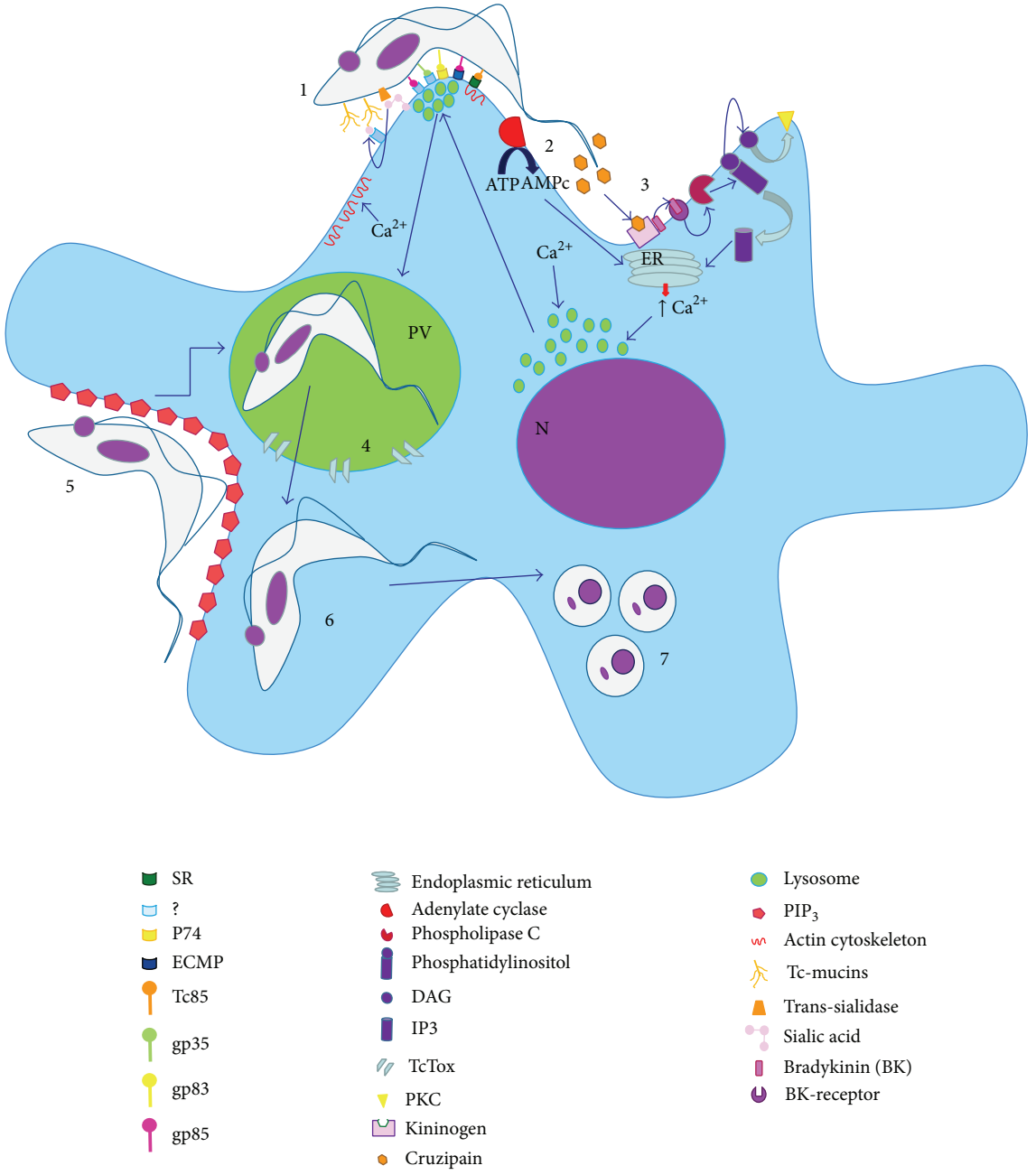

Figure 3: General molecular mechanisms for host cell invasion by *Trypanosoma cruzi*. (1) Several receptor-ligand complexes seem to participate in *T. cruzi* internalization by the host cell. The enzyme transsialidase transfers sialic acid from host cell membrane to Tc-mucins. These molecules can interact with host cell receptor. Some host cell receptors remain unknown. Glycoproteins from the parasite (gp 83 and gp 85, resp.) can bind to host cell receptors, such as P74 or EMCP. Members of Tc85 family can bind to specific receptor (SR) promoting cytoskeletal changes and facilitate parasite invasion. (2) During the process, the host cell adenylate cyclase is activated promoting an enhancement of AMPc that contributes to Ca^{2+} release from endoplasmic reticulum. (3) The cruzipain secreted by the parasite cleaves the host kininogen to liberate bradykinin, and the triggering of the host bradykinin receptor activates host cell PLC, contributing to Ca^{2+} release, via IP_3. (4) Ca^{2+} seems to promote recruitment of perinuclear lysosomes that contributes to formation of the parasitophorous vacuole. In addition, Ca^{2+} promotes disorganization of actin cytoskeleton, and invasion is facilitated by disruption of microfilaments. (5) In another route, the parasite enters into host cell by creating an invagination in the plasma membrane, which accumulates PIP3. (6) After the PV formation, TcTox promotes pores in their membrane and the trypomastigotes escape for cytoplasm. (7) Trypomastigotes transform into amastigotes, multiplying inside the cytoplasm from host cell. N—nucleus, PV—parasitophorous vacuole.

The ILCs are divided into 3 groups. This classification is based on their pattern of cytokines produced and the transcription factors required for their development. Group 1 ILCs (ILC1s) produce interferon γ and depend on Tbet, group 2 ILCs (ILC2s) produce type 2 cytokines like interleukin-5 (IL-5) and IL-13 and require GATA3, and group 3 ILCs (ILC3s) include lymphoid tissue inducer (LTi) cells, produce IL-17 and/or IL-22, and are dependent on RORγt [88]. NK cells were classified into ILC1s group because they produce IFN-γ [89] and recent information about ILCs development in mouse suggests that NK cells can be considered as the innate form of TCD8 (T cytotoxic) cells as well as CD127+ILCs, the innate form of TCD4 (T helper) cells [90].

The innate response is based on the recognition of pathogen-associated molecular pattern molecules (PAMPs), which are present in diverse organisms but are absent in the host and function as an exogenous signal that alerts the host to the presence of pathogens [91]. The major PAMPs include microbial nucleic acid, lipoproteins, surface glycoproteins, and other membrane components. They are recognized by pattern recognition receptors (PPRs), such as toll-like receptors (TLRs), retinoic acid-inducible gene I- (RIG-1-) like receptors (RLRs), AIM2 like receptors (ALRs), and nucleotide-binding oligomerization domain- (NOD-) like receptors (NLRs) [92]. During infection, PAMPs are recognized by PPRs that initiate signaling cascades that lead to the activation of transcriptions factors in innate immune cells. Macrophages, dendritic cells (DCs), mast cells, and neutrophils are important cells involved in the innate immune response. Innate immune effectors mechanisms include phagocytosis, cytokine and chemokine production, and expression of costimulatory molecules on antigen present cells (APCs) and have an influence on T lymphocyte differentiation [93, 94].

The adaptive immune response involves T and B lymphocytes that recognize a large spectrum of antigens using highly specific receptors. The two major populations of T cells, TCD4 (T helper) and TCD8 (T cytotoxic) cells, have T cell receptors (TCR) that recognize antigens bound to the major histocompatibility complex (MHC) on APC (MHC class II) or target cell (MHC class I) surfaces. Antigen presenting cells such as DCs, macrophages, and B cells express MHC class II molecules and costimulatory molecules on their membranes and present antigen to naive TCD4 cells, whereas MHC class I cells can be also expressed by others cell present antigen to TCD8 cells. After an antigen is recognized, T cells proliferate and differentiate into effector T cell subsets. TCD4 cells orchestrate the immune response by the differentiation into a T helper cell population that secretes distinct sets of cytokines, providing help to B lymphocyte and TCD8 cytotoxic cells. Naive TCD4 cells differentiate into at least four T helper (Th) cell subsets: Th1, Th2, Th17, and regulatory T cells (T_{reg}) [95]. DCs play a critical role not only in processing and presenting antigens to naive TCD4 cells but also in secreting cytokines such as IL-12 that induce Th1 effector lymphocyte differentiation. Although DCs are important in the development of Th2 response, other cells such as mast cells, basophils, natural killer cells, and monocytes, secrete cytokines like IL-4 that induce TCD4 differentiation to Th2 [96, 97]. A recent study demonstrates that lung ILC2s enhance effector functions of Th2-type $CD4^+$ T cells when they are cultured together *in vitro*. The interaction between ILC2s and $CD4^+$ T cells appears bidirectional and likely requires both OX40L and IL-4 and perhaps other molecules. These findings suggest that lung ILC2s and $CD4^+$ T cells cooperate to mediate robust Th2-type immune responses in mice [98].

Type 1 responses are characterized by the induction of Th1 cells; these cells secrete cytokines such as interleukin-2 (IL-2) and IFN-γ, which are indispensable for host immunity to intracellular parasites (e.g., *Trypanosoma cruzi*). In contrast, type 2 responses are characterized by Th2 cells that secrete IL-4, IL-5, IL-9, and IL-13 and are induced by and confer immunity to extracellular parasites (e.g., helminths). IFN-γ induces cytotoxic TCD8 cell differentiation and macrophage activation, which stimulates the expression of nitric oxide (NO) synthase enzyme (iNOS or NOS2) and the production of NO, the main microbicidal agent able to destroy intracellular parasites such as *Leishmania*. Th2 cells promote B cell responses and immunoglobulin E (IgE) secretion through IL-4 production. In addition to antibody production, B cells have other important functions, such as presenting antigens to T cells and cytokine production. As with T cells, B cells contain functionally distinct subsets with regulatory functions, such as the production of anti-inflammatory IL-10 [99, 100]. The immune system must adjust the magnitude and duration of response because uncontrolled inflammation may lead to immune-mediated tissue injury. Treg cells are important anti-inflammatory cells that are critically involved in limiting the inflammatory response. The suppression of the immune response by Treg cells includes both cell contact- and factor-dependent mechanisms, such as cytokine production (IL-10, TGF-β, and IL-35) [101, 102].

The balance between effector and regulatory T cell responses influences the balance between infection control and pathogenesis. Comparing responses exhibited by susceptible and resistant experimental models has contributed to an understanding of protective immune responses to *T. cruzi* and *Leishmania* spp.

After transmission by sand flies, *Leishmania* parasites infect neutrophils, macrophages, and DCs in the vertebrate host, and the development of a protective immune response requires the coordinated action of cells of the innate and adaptive immune response. Generally, protective immunity against leishmaniasis is associated with an inflammatory Th1 response, while disease is associated with an anti-inflammatory Th2 response [103].

Six major *Leishmania* species (*L. tropica*, *L. major*, and *L. donovani*, in the Old World and *L. infantum*, *L. braziliensis*, and *L. mexicana*, in the New World) cause the three main forms of the disease in humans, dermal cutaneous leishmaniasis (CL), visceral leishmaniasis (VL), and mucocutaneous leishmaniasis or mucosal leishmaniasis (MCL or ML). The form and severity of the disease depend on the *Leishmania* species causing the infection and the immune status of the host [104]. Some of these species have metastatic characteristics and up to 10% of CL cases progress to MCL forming destructive secondary lesions in the mucosa of nose

and mouth, in South America. This clinical complication is associated with *Leishmania (Viannia)* subgenus, since it is promoted by species inside of this group, predominantly *L. (Viannia) braziliensis* but also *L. (Viannia) guyanensis* and *L. (Viannia) panamensis* [105]. A common characteristic in the cases of metastatic infection of *Leishmania* is the destructive hyper-inflammatory immune response, caused by numerous activated immune cells, promoting swelling and destroying local tissue [106, 107]. Thus, in ML/MCL exacerbated inflammatory immune response induces tissue injury, and patients present higher levels of proinflammatory cytokines, such as IFN-γ, and low levels of anti-inflammatory cytokines, such as IL-10, even after cure compared to the benign cutaneous clinical form of disease [108]. This exacerbated reaction generally is associated to a parasite factor. Although endosymbiont dsRNA virus already have been described on the subgenus *Leishmania (Viannia)* a long time ago [109–111], just recently the presence of this endosymbiont was associated with leishmanial virulence and metastasis [112, 113]. The nucleic acid of *Leishmania* dsRNA virus (LRV1) behaves as a strong innate immunogen, inducing a hyper-inflammatory immune response by pathway of toll-like receptor 3 (TLR3). This pathway induces production of a type 1 IFN response: IFN-β-mediated antiviral response. Type I IFNs promote downregulation of IFNγ-R on the surface of macrophages. Consequently, the macrophages become insensitive to classical activation, which promotes the reactive nitrogen species (NO) production responsible to kill intracellular pathogens (Figure 1) [114]. In this way, this mechanism is proposed as promoter of the exacerbated MCL phenotype, triggering an increase in the disease severity and parasite persistence [115].

The dsRNA virus seems to interfere with NO production for another via: metastatic *L. braziliensis* species induce higher levels of insulin-like growth factor (IGF) which is able to promote upregulation on the arginase activity [116]. However, this kind of report does not have to investigate the possible influence of LRV infection on oxidative resistance [115].

VL exhibits a mixed type 1 and type 2 cytokine profiles. Studies in experimental models show that neither IL-4 nor IL-13 (typically Th2 cytokines) is able to induce disease exacerbation. However, a consensus exists in relation to the suppressive effect of IL-10 on the immune response in VL and its correlation with disease severity, as this cytokine is an important immunosuppressant and inhibitor of macrophage microbicidal activity in both mice and humans with VL [103, 117]. The control of VL is also dependent on the development of type 1 cytokines and effector antileishmanial molecules such as reactive nitrogen and oxygen intermediates for parasite control in the spleen [118].

In the case of Chagas disease, the existence of a large spectrum of clinical manifestations is associated with parasite heterogeneity and the host immune response. The acute phase is followed by the development of effective acquired immunity, leading to the control of parasitaemia and parasitism levels in tissues. Some authors have shown that in experimental models of acute *T. cruzi* infection, the T helper type 1 response (Th1) appears to have a critical role in infection control. This response can occur through toll-like receptor- (TLR-) mediated and TLR-independent cytokine production [119–121]. Early studies indicated that this infection promotes a certain degree of immunosuppression; however, subsequent data using new approaches demonstrate a substantial antiparasite response during acute experimental infection [122]. Peripheral blood mononuclear cells (PBMCs) from children with acute phase infections present mRNA profiles of interferon (IFN)-γ, interleukin (IL)-2, and IL-10, with low levels of IL-4 [123]. Interestingly, children with asymptomatic chronic Chagas disease present upregulated IL-4 mRNA, suggesting that following Th1-mediated parasite clearance, a balance of both Th1 and Th2 immune responses occurs to suppress parasite load and protect against immunopathology [122, 123]. Another study showed that children with acute infection present higher serum levels of tumor necrosis factor (TNF)-α, sIL2R, sCD8, sCD4, and IL-6, but no change in IL-2, IL-12, and IL-8 is observed compared to healthy controls or children with asymptomatic Chagas disease in the chronic phase [124].

The lifelong chronic phase is maintained with low parasitaemia and tissue parasitism. The first source of IFN-γ seems to be natural killer (NK) cells. This cytokine augments IL-12, TNF-α and other cytokines synthesized by classically activated macrophages [125–127]. IFN-γ produced by NK cells and IL-12 produced by macrophages have been suggested to induce the differentiation of T-helper cells to a predominant protective Th1 phenotype [128, 129]. IFN-γ produced by Th1 cells activates effector mechanisms in macrophages. These effector mechanisms destroy both amastigotes and phagocytized trypomastigotes, whereas cytotoxic activity displayed by $CD8^+$ T cells destroys cells containing intracellular amastigotes [130–137]. *T. cruzi* antigen-specific CD8 T cells are frequently present in infected mice and humans [137]. Antibodies produced by B cells lyse the extracellular trypomastigote form, facilitate the phagocytosis of parasites opsonized with IgG [138], and promote the complement-dependent killing of the parasites [139]. During severe cardiomyopathy of the chronic phase of Chagas disease, the occurrence of intense inflammatory response is correlated with the production of type 1 cytokines, such as TNF-α and IFN-γ. In this disease phase, the level of these cytokines is higher than during indeterminate phase of Chagas disease [140]. Indeterminate patients seem to have a more regulated response by Treg cells, limiting tissue damage with the maintenance of improved cardiac function, but apparently the mechanism is not IL-10- or CTLA-4-dependent [141]. These essential responses to Chagas disease have been clearly shown using experimental models or natural human infections in that the absence or the reduction in any of these immune responses (via targeted depletion, immunosuppressive treatments, or infection-induced immunosuppression) can exacerbate parasitaemia [142–144]. In summary, the literature indicates that the persistence of protozoans is related to the delayed kinetics of $CD8^+$ T cell development and the balance between Th1 and Th2 responses. An efficient protective response against *T. cruzi* requires the Th1 response, activation of phagocytes, T-helper cells and cytotoxic T lymphocytes, and lytic antibodies.

The ability of parasitic protozoa to interfere with effector mechanisms of the immune response has been studied over decades. The complex life cycle of *Leishmania* sp. and *T. cruzi* involves the emergence of a number of characteristics that allow its survival in different microenvironments in the insect vector and the vertebrate host. Polyclonal lymphocyte activation is an example of an immune evasion mechanism found in some pathogens. In a mouse model of *T. cruzi* infection, reduced levels of polyclonal lymphocyte responses correlate with infection and control of cardiomyopathy. The enzyme proline racemase was described in *T. cruzi* (TcPRAC) and is expressed either as a cytoplasmic, membrane-associated protein [145] or as a secreted isoform, which can be detected at all stages of the parasite life cycle. The secreted form is shown to be a B cell mitogen, which contributes to parasite evasion of the host immune system and its persistence in the vertebrate host [146].

The vertebrate infective forms have developed several strategies to survive in the hostile host environment. For example, bloodstream *T. cruzi* trypomastigotes express molecules on their surface that are capable of interfering with the activation of the classical and alternative complement pathways [147]. Several membrane glycoprotein-specific trypomastigotes participate efficiently and prevent complement activation on the surface of the parasite. Some of these molecules, such as gp160, gp58/68, and T-DAF, regulate complement by inhibiting the development and/or accelerating the decay of C3 convertase, a central enzyme in the complement cascade [148]. Recent studies [149] provide evidence that metacyclic trypomastigotes induce the formation of vesicles derived from the host cell, which form a complex released from the parasite surface, leading to stabilization and inhibition of C3 convertase resulting in increased survival of the parasite.

Another mechanism for the attachment-independent invasion of trypomastigotes involves the activation of the TGF-β signaling pathway [150]. A protease secreted by the parasite is likely to activate latent TGF-β associated with extracellular matrix (ECM) components, allowing activation of Smad 2/3 pathway through the TGF-β receptors (I and II) present on the surface of host cells. The pivotal role of this pathway in infections of heart tissues and, consequently, in the chagasic cardiomyopathy has previously been described [150].

T. cruzi has a gene, FL-160, encoding the C-terminus of flagellar protein. This protein has a twelve amino acid epitope similar to nervous tissue proteins present in the sciatic nerve plexus and mesenteric SNC. This gene belongs to a family of highly related genes that are encoded in more than 750 copies in the parasite genome; sequential analyses reveal that all copies of this gene have the 12 amino acids that mimic the human sequence, which may be a prevalence and immunosuppression factor in Chagas disease [151].

In the *T. cruzi* experimental murine acute infection model, several changes are observed in lymphoid organs, including the thymus, where intense and severe thymic atrophy due to depletion of $CD4^+8^+$ double-positive cells (DP) thymocytes by apoptosis in the cortical area of the thymus occurs [152]. A recent study shows that *T. cruzi* trans-sialidase (TcTS) induces thymic atrophy that affects the dynamics of intrathymic thymocytes, resulting in an increase in the number of $CD4^+8^+$ DP recent thymic emigrants in the spleen. TcTS is also capable of activating MAPK JNK signaling in thymocytes, modulating their adhesion to thymic epithelial cells and their migration toward the extracellular matrix. These data suggest the possible involvement of this enzyme in abnormal thymocyte trafficking inside the thymus of animals acutely infected by *T. cruzi*, which could influence the escape of immature thymocytes to peripheral blood in Chagas disease. The authors report that the frequency of DP T cells in chronic patients presenting high antibody titles against TcTS with the cardiac form of Chagas disease is increased. Thus, the presence of peripheral activated DP cells with potentially autoreactive TCRs may contribute to the immunopathological events found in this disease [153].

T. cruzi can also interfere with signaling via new members of the B7 family, such as the programmed death ligand 1 (PD-L1). This ligand binds to the programmed death 1 (PD-1) receptor, which is expressed on activated T cells, B cells, and myeloid cells. Their interactions result in downmodulation of the T-cell response [154, 155]. *T. cruzi* infection promotes an increase in expression of PD-1 and its ligands on peritoneal macrophages as well as during *in vitro* infection. Macrophages from mice infected by this protozoan are able to promote suppression of T cell proliferation. This suppression is restored when anti-PD-1 and anti-PD-L1 antibodies are added. Additionally, the blockage of PD-1 and PD-L1 increases iNOS expression and NO production on peritoneal macrophages from *T. cruzi*-infected mice [156].

T. cruzi infection promotes the formation of lipid bodies in macrophages through TLR2 signaling, which is amplified by the uptake of apoptotic cells in a mechanism dependent on integrins and TGF-β synthesis and results in an increased parasite survival and proliferation [157].

For leishmanial infection to be successful, the parasite must resist the hostile environment inside the host and survive to its innate and acquired immune response. Initially these include complement system, followed by phagocytosis, acidification of the phagolysosome, ROS release and, finally, NO production. *Leishmania* promastigotes abundantly express LPG (lipophosphoglycan), which forms a great glycocalyx surround the parasite and interferes with the insertion of membrane attack complex [158]. Promastigotes also present specific kinases able to deactivate the classical and alternative complement pathway by phosphorylating of complement components [159]. In addition gp63 can convert C3b (complement subunit 3), attached to parasite surface, into its inactive form, iC3b, which prevents parasite lysis via the complement system [160]. Furthermore, iC3b can opsonize *Leishmania* and allow entry in the host cell by binding the receptors CR1 and CR3 (complement receptors) (Figure 1) [161, 162].

Inside the neutrophils, the first cell to phagocyte the promastigotes, LPG and an acid phosphatase resistant to tartrate, present on the parasite cell surface, inhibit lysosome fusion and the respiratory burst [163–165]. Promastigotes release a chemotactic substance, lipid *Leishmania* chemotactic factor (LCF) to attract more neutrophils [166].

The LCF can interact with lipoxin A4 receptors (ALX), resulting in the deactivation of oxidative burst of neutrophils [167]. Furthermore, *Leishmania* presents an inhibitor of serine peptidase capable to inhibit the serine peptidase released by neutrophils, the neutrophil elastase. This is crucial for intracellular parasite survival [168].

The Trojan horse theory supports the idea that apoptotic neutrophils infected by *Leishmania*, when taken up by macrophages, allow a silent entrance of the parasite, contributing to the success of infection [169]. Peters et al. [16] showed robust neutrophil infiltration after the bite of the sand fly (vector insect of leishmaniasis) infected with *L. major*. These parasites survive and appear to be better adapted to resist macrophages that were committed during the clearance of apoptotic neutrophils and thus with impaired inflammatory functions when released by neutrophils. Apoptotic *Leishmania* parasites seem to be essential for disease development, because when these cells were depleted from a population of virulent *L. major*, experimental infection was controlled both *in vitro* and *in vivo*. This is due to the increased production of TGF-β induced by apoptotic promastigotes [170].

After neutrophils, *Leishmania* promastigotes can enter into dermal macrophages, which lack the respiratory burst machinery. Thus, promastigotes have opportunity to ameliorate their ability to transform into amastigotes and grow inside the host cell [171]. In the same way, promastigotes and amastigotes can be actively ingested by the skin fibroblasts [172], where they find a safe environment for up to 7 days after infection, since these cells produce low levels of NO even in the presence of interferon-γ, compared to macrophages [172].

Inside the macrophage, LPG can inhibit the oxidative burst initiated by NADPH oxidase at the time of their entry into the host cell. This phenomenon seems to be another strategy used by *Leishmania* to buy time to transform into the resistant amastigote form. LPG can inhibit PKC (protein kinase C), a key enzyme for initiation of the oxidative burst. LPG is known to inhibit the translocation of this enzyme to the cell membrane due to its transference from parasites membrane to the macrophage membrane during phagocytosis and binds to the regulatory domain of PKC, inactivating the production of reactive oxygen species (ROS) (Figure 1) [173–175]. Furthermore, LPG can inhibit the production of IL-12 by macrophages, thus impairing Th1 differentiation, though the mechanism has not been elucidated [175].

The metalloprotease gp63 is also involved in parasite protection within the phagolysosome. This protease activity appears to protect against host proteolytic enzymes [176]. This important protease is also involved in processes of immune evasion. Gp63 cleaves and activates the protein tyrosine phosphatase SHP-1, interfering with the IFN-γ signaling pathway by dephosphorylating Janus kinase 2 (JAK2) (Figure 1) [177]. This JAK2 dephosphorylating negatively interferes with ERK1/2 (extracellular signal-regulated kinase 1/2), MAPK (mitogen-activated protein kinase), nuclear factor-κB (NFκB), IRF-1 (interferon regulatory factor-1), and AP-1 (activator protein 1), inhibiting classical macrophage activation and impairing the production of IL-12, NO, and immunoproteasome formation [178, 179]. NFκB is a key transcription factor that mediates innate and adaptive immunity and is involved in the transcription of adhesion molecules and chemokines that leads to the recruitment and activation of effector cells. GP63 in *Leishmania* promastigotes can cleave the $p65^{RelA}$ subunit of this transcription factor, resulting in the $p35^{RelA}$ fragment that is associated with promotion of certain chemokines (MIP-1β and MIP-2—macrophage inflammatory protein) and favors the recruitment of phagocytic cells but does not induce other macrophage products such as iNOS and IL-12, essential for a protective response [180, 181]. *Leishmania* may also stimulate the degradation of STAT-1 (signal transducer and activator of transcription) in host cells by modulating signaling pathways through receptors CR3 and FcyR, which inhibits iNOS expression and NO production, leading to parasite survival [182, 183].

In summary, *Leishmania* parasites have a very complex repertoire of strategies to escape the immune system by interfering in a range of signal transduction pathways in host cells (mainly macrophages), inhibiting the protective response and continuing the life cycle.

4. Therapeutic Targets

There are large differences between the trypanosomatids and mammalian cells, so different biochemical pathways of parasites from the hosts would be excellent targets for the new drugs design [184]. With the post genomic era, the discovery of new targets can be amplified and supporting the development of drugs more specific for the parasite and less toxic for the host. Among these possible targets include (a) mitochondrial markers; (b) fatty acids, sterols, carbohydrates, and folate biosynthesis [185, 186]; (c) recovery and metabolism of purines, pyrimidines [187], and aminoacids (as proline) [145]; (d) biosynthesis, transport, and metabolism of polyamines [188]; (e) the cell cycle [189, 190]; (f) proteases [191] and (g) proteasomes [192, 193].

Current therapeutic strategies in leishmaniasis and trypanosomiasis are far from satisfactory. The efficacy is variable, toxicity is high, and the emergence of resistance is increasingly common.

The trypanosomiasis treatment dates back over 50 years and is based on nifurtimox and benznidazole, which belong to the class of nitroaromatic compounds. These agents function as pro-drugs and must depend of enzyme-mediated activation inside parasites. The nitroreductases mediate reduction of the nitro-group generating an unstable nitroradical that, in presence of oxygen, generate superoxide. The *T. cruzi* sensibility depends on its capacity detoxification of free radicals as well as associated to downregulation of type I nitroreductases of the parasites [194]. Although benznidazole is considered to be better tolerated than nifurtimox, various adverse effects are attributed to their use, such as neuropathy and agranulocytosis. These drugs are active against blood forms of *T. cruzi* and effective in treating the acute phase of infection; however their efficiency in chronic phase is controversial. Actually, studies are being done to make nitro drugs selectively toxic to the parasite and more effective

in chronic phase [195]. Fexinidazole is a nitroheterocyclic effective oral treatment of acute and chronic experimental *T. cruzi* infection [196]. Recently a study showed the efficacy of the metabolites fexinidazole in a mouse model of acute infection, leading to reduced inflammation in heart tissue associated with the chronic phase of Chagas disease [197].

In general, the first line leishmaniasis treatments are pentavalent antimonial that have been developed over 50 years ago. To exert its antileishmanial activity, the pentavalent antimony needs to be reduced to its trivalent form inside macrophages. The mechanism of action of antimonials is not completely clarified, but is known to involve inhibition of glycolitic pathway, fatty acids and trypanotione reductase [198, 199]. Pentamidine, amphotericin B, miltefosine, and paramomycin are used as second-line drugs [200–203]. The action mechanism of pentamidine is not well characterized, although there is evidence that involves mitochondrial functions interference [204]. The paromomycin mechanism is based on inhibition of protein synthesis of the parasite [203], covalently bound to protein and effects translation and vesicle-mediated trafficking [205]. The miltefosine acts on the cell signal transduction pathway by inhibiting protein kinase B, which makes an important role in the biosynthesis of sterols and phospholipids [206]. Already amphotericin B binds to ergosterol, a major component of the cellular membrane of *Leishmania*, forming transmembrane channels that release monovalent ions (K^+, Na^+, H^+ and Cl^-) leading to cell death [207]. To minimize the adverse events of amphotericin B, various lipid formulations have been introduced leading rapidly concentrated into organs such as liver, spleen and increase the antileishmanial activity with selectivity to macrophage reticular-endothelial system [208]. Sitamaquine (8-aminoquinoline) is oral drug for the treatment of visceral leishmaniasis which has completed Phase II trials in India and Kenya [208, 209]. The molecular targets of sitamaquine are still unknown, however it was shown that upon binding to transiently membrane sterols is found in the cytoplasm and induces changes mitochondrial membrane potential [210].

The sterol biosynthesis is a potential drugs target in trypanosomatids since there are some differences between parasite and host. Parasite is entirely dependent of endogenously sterols for survival and growth and cannot use the supply of the host cholesterol. The major product sterol biosynthesis of trypanosomatids is ergosterol and other 24-methyl sterols and the 14α-demethylase (CYP51) is key of pathway inhibited by azoles. These azoles have antiparasite action same the antifungal because block the biosynthesis accumulating toxic methylated precursors [211, 212]. Besides the main targets are membranes of mitochondria, the protozoan cell body and flagellum, other important changes take place in the organization of the kinetoplast DNA network and on the protozoan cell cycle leading to cell death [213]. Azoles as ketoconazole and fluconazole demonstrate the efficacy to treat some clinical forms of leishmaniasis [214–216], while posaconazole and ravuconazole have been reported on clinical trials (phase I or II) on *T. cruzi* infection [217–219].

The TcPRAC is a promising target for the development of a new therapy against Chagas disease since parasites are no longer viable when PRAC genes are knocked down or more virulent if PRAC genes are over expressed [220]. Two compounds, which are irreversible competitive inhibitors of TcPRAC, were able to inhibit the mammalian host cell infection [221].

Recently, protease inhibitors used in antiretroviral therapy regimen of high efficiency (HAART) to treat HIV-infected patients have been used to treat trypanosomiasis and leishmaniasis. Studies show that the strategy had good results against *T. cruzi* and different species of *Leishmania* genus [222]. It is believed that these inhibitors affect the proteasomes of parasites responsible for the proliferation, differentiation and intracellular survival of microorganisms [223].

Aspartic peptidase inhibitors used in the current chemotherapy against HIV were able to inhibit the aspartic peptidase activity produced by different species of *Leishmania* spp and induced an increase in the level of reactive oxygen species, triggering parasite death pathways such as programed cell death (apoptosis) and uncontrolled autophagy [224, 225].

Finally, the possibility of different therapies combination against trypanosomatids can be result ameliorates to the low efficiency, high toxicity and especially reduce possibility of parasite resistance [226]. Thus emerged over the last decade this strategy has been tested successfully against others parasites included malaria and tuberculosis agents [227]. The multitarget compounds use against trypanosomatids is another way to reduce resistance to treatment, since drugs with a single target are susceptible to high level of resistance, a result of the mutation of the target protein [197, 228, 229]. Besides the combination chemotherapy, another important therapeutic approach is immunotherapy. The immunotherapy includes the use of biological substances or molecules to modulate the immune responses for the purpose of achieving a prophylactic and/or therapeutic success. The immunotherapeutic agents can exert their effect by directly or indirectly augmenting the host natural defenses, restoring the impaired effectors functions or decreasing host excessive response. The combination of immunotherapy with chemotherapeutic drugs (immunochemotherapy) is showing promise in the treatment of visceral and mucosal leishmaniasis [230–233].

5. Concluding Remarks

The complexity of the relationship between intracellular *Leishmania* spp. and *T. cruzi* and their human hosts is a limitation to vaccine and drug design. Heterogeneity within the same parasite species and our limited experimental models make it even more challenging to understand this complex association. However, knowledge is accumulating regarding the molecular machinery of these parasites and the host immune response can be used to change our paradigms and develop new strategies for the treatment and control of these diseases. Treatments that target different points in parasite metabolism are an important strategy to improve efficacy and prevent resistance. In this sense, a combination of drugs for treatment should be encouraged. The use of

immunomodulators may also be relevant to restore homeostasis of the host and attenuate tissue damage contributing to the therapeutic success.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Silvia Amaral Gonçalves Da Silva and Patrícia Maria Lourenço Dutra share equal contribution in authorship.

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Corrigendum

Corrigendum to "Somatostatin Negatively Regulates Parasite Burden and Granulomatous Responses in Cysticercosis"

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In the paper titled "Somatostatin Negatively Regulates Parasite Burden and Granulomatous Responses in Cysticercosis" there was an error in the Discussion and Bibliography section.

The changes made to the references section are as follows. Reference 26 has been added: H. Wu, X. Chen, Y. Deng, X. Huang, and Z. Zhang, "Effect of somatostatin on modulation of IL-10 and TGF-beta 1 during acute pancreatitis," *Journal of Sichuan University (Medical Science Edition)*, vol. 34, pp. 315-316, 373, 2003.

Therefore, references from 26 to 31 have been shifted to references 27 to 32: A. Hernanz, E. Tato, M. De la Fuente, E. de Miguel, and F. Arnalich, "Differential effects of gastrin-releasing peptide, neuropeptide Y, somatostatin and vasoactive intestinal peptide on interleukin-1 beta, interleukin-6 and tumor necrosis factor-alpha production by whole blood cells from healthy young and old subjects," *Journal of Neuroimmunology*, vol. 71, pp. 25–30, 1996; J. Komorowski and H. Stepien, "Somatostatin (SRIF) stimulates the release of interleukin-6 (IL-6) from human peripheral blood monocytes (PBM) *in vitro*," *Neuropeptides*, vol. 29, pp. 77–81, 1995; D. Paran, D. Kidron, and A. Mayo et al., "Somatostatin analogue treatment attenuates histological findings of inflammation and increases mRNA expression of interleukin-1 beta in the articular tissues of rats with ongoing adjuvant-induced arthritis," *Rheumatology International*, vol. 25, pp. 350–356, 2005; A. Lang, E. Sakhnini, H. H. Fidder, Y. Maor, S. Bar-Meir, and Y. Chowers, "Somatostatin inhibits pro-inflammatory cytokine secretion from rat hepatic stellate cells," *Liver International*, vol. 25, pp. 808–816, 2005; B. L. Spangelo, S. Horrell, A. L. Goodwin, S. Shroff, and W. D. Jarvis, "Somatostatin and gamma-aminobutyric acid inhibit interleukin-1 beta-stimulated release of interleukin-6 from rat C6 glioma cells," *Neuroimmunomodulation*, vol. 11, pp. 332–340, 2004; Y. Chowers, L. Cahalon, and M. Lahav et al., "Somatostatin through its specific receptor inhibits spontaneous and TNF-alpha- and bacteria-induced IL-8 and IL-1 beta secretion from intestinal epithelial cells," *The Journal of Immunology*, vol. 165, pp. 2955–2961, 2000.

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M. Rodriguez-Sosa, R. Saavedra, E. P. Tenorio, L. E. Rosas, A. R. Satoskar, and L. I. Terrazas, "A STAT4-dependent Th1 response is required for resistance to the helminth parasite *Taenia crassiceps*," *Infection and Immunity*, vol. 72, pp. 4552–4560, 2004. This should be changed in the last 3 paragraphs of the Discussion section as follows.

Somatostatin is known to be an inhibitory neuropeptide [3, 10, 11, 26]. The inhibitory role of somatostatin is depicted in studies wherein it has been shown to downmodulate production of cytokines such as IFN-γ and IL-10. For example, studies using human peripheral blood mononuclear cells demonstrated that somatostatin inhibited the secretion of IFN-γ [11]. Similarly, other studies have shown that somatostatin decreased the production of IFN-γ by splenocytes and T lymphocytes isolated from murine schistosome granulomas *in vivo* and *in vitro* [3]. Furthermore, validation of the inhibitory role of somatostatin is seen in studies wherein somatostatin has been shown to depress the increase of IL-10 that is noted in response to acute pancreatitis [26] or systemic lupus erythematous [10]. Therefore our studies demonstrating more IFN-γ and IL-10 in the absence of somatostatin coincide with the above studies, wherein somatostatin has been shown to have an inhibitory role in IFN-γ and IL-10 production.

The finding of reduced IL-1β in the absence of somatostatin is not completely unexpected. Earlier studies examining the ability of somatostatin to modulate production of IL-1β, along with TNF-α and/or IL-6, are conflicting. Some studies showed that somatostatin stimulates the production of IL-1β, TNF-α, and/or IL-6 by human blood cells [27, 28], as well as the expression of IL-1β in articular tissues of rats with ongoing adjuvant-induced arthritis [29]. However, other studies showed that somatostatin decreased secretion of one or more of these cytokines [7, 9, 29–32]. Thus our results demonstrating lesser IL-1β levels in the absence of somatostatin coincide with the first set of studies, wherein somatostatin has been shown to have a stimulatory role in IL-1β production.

There are various studies performed using knockout mice by Dr. Terrazas' group [33–36]. The protective role of Th1 responses has been evident in these knockout studies. For example, *T. crassiceps*-infected STAT6$^{-/-}$ mice have been shown to mount a strong Th1 response in the absence of Th2 development and to control the infection [35]. Similarly, other studies wherein knockout mice with decreased Th1 responses such as IL-12 p35$^{-/-}$ mice have been shown to demonstrate increased susceptibility to the larval stage of *T. crassiceps* [34].

The above studies using various knockout animals and other studies wherein IFN-γ and anti-IFN-γ antibody administration, respectively, led to lower and higher parasite levels [21] indicated that a Th1-type response is essential for resistance against experimental cysticercosis, which coincides with our observation that Th1 cytokine IFN-γ plays an important role in limiting infection in the somatostatin knockout mice.

In contrast to Th1 cytokines, Th2 cytokines are implicated with susceptibility with this parasitic infection. For example, studies done by Dr. Terrazas' group have shown that IL-10 increases parasite load [21]. Similarly, studies using toll-like receptor 2 knockout mice demonstrated a reduction in the production of proinflammatory cytokines, resulting in a Th2 bias and significantly impaired resistance to *T. crassiceps* infection [33]. Furthermore, another study showed that STAT4$^{-/-}$ mice that mount a strong Th2 response were highly susceptible to infection and displayed large parasite loads [36]. However our studies showing lower parasite levels in the somatostatin knockout mice that have higher IL-10 levels implicate that IL-10 does not have a role to play in the parasite levels in our somatostatin knockout mice or additionally and/or alternatively it may implicate that other cytokines such as IFN-γ and IL-1β may be the predominant players in inducing parasite reduction and may far outweigh the parasite-stimulating effects of IL-10.

Our findings have potential implications for treatment and prevention of the detrimental effects of granulomatous inflammation induced as a result of antihelminth treatment in patients with neurocysticercosis and viable cysts. Current options for management of patients with viable cysts include antihelminth treatment along with corticosteroid administration aiming at reducing granulomatous inflammation. However, corticosteroids can have severe side effects and cannot be used in patients with concurrent latent tuberculosis, strongyloidiasis, and optical cysticercosis. Our finding that somatostatin downmodulates granuloma growth suggests the possibility of using somatostatin analogues, instead of corticosteroids, to downmodulate granulomatous inflammation in the brain of neurocysticercosis patients.

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Research Article

Morphological Findings in Trophozoites during Amoebic Abscess Development in Misoprostol-Treated BALB/c Mice

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During amoebic liver abscess (ALA) formation in susceptible animals, immune response is regulated by prostaglandin E_2 (PGE_2) dependent mechanisms. The aim of this study was to analyze the effect of misoprostol (MPL), a PGE_1 analogue, on ALA formation in BALB/c mice. Male mice from BALB/c strain were intrahepatically infected with 7.5×10^5 trophozoites of *E. histolytica* strain HM1:IMSS and treated with 10^{-4} M of MPL daily until sacrifice at 2, 4, and 7 days postinfection (p.i.). ALA formation was evaluated at 2, 4, and 7 days postinfection; trophozoite morphology was analyzed using immunohistochemistry and image analysis. Results showed an increase in frequency of ALA formation in infected and MPL-treated mice only at 2 days p.i. ($P = 0.03$). A significant diminution in the size of trophozoites was detected in abscesses from mice independently of MPL treatment (from 5.8 ± 1.1 μm at 2 days p.i. to 2.7 ± 1.9 μm at 7 days p.i.) compared with trophozoites dimensions observed in susceptible hamsters (9.6 ± 2.7 μm) ($P < 0.01$). These results suggest that MPL treatment may modify the adequate control of inflammatory process to allow the persistence of trophozoites in the liver; however, natural resistance mechanisms cannot be discarded.

1. Introduction

Amoebic liver abscess (ALA), produced by *Entamoeba histolytica* infection, is a common complication of invasive intestinal amoebiasis. Hamsters and gerbils are susceptible to hepatic amoebiasis [1, 2] while mice are resistant to infection either intracecally or intrahepatically [3]. Although basis for mice resistance is not totally known, several studies in susceptible animals have demonstrated the importance of macrophage function in host resistance during ALA formation [4]. Prostaglandin E_2 (PGE_2) is an important mediator of inflammation that can modulate T helper (TH) cells towards a preferential TH2 subgroup [5] and in this way regulate cooperator and effector macrophage functions.

The increase in PGE_2 plasmatic levels and induction of the type 2 cyclooxygenase enzyme (COX-2) in infected liver, as well as the expression of COX-2 mRNA in macrophages and neutrophils, was reported by our group using the hamster, a susceptible experimental model [6, 7]. In these studies we found that the inhibition of PGE_2 biosynthesis by indomethacin treatment had a beneficial effect limiting the inflammatory process [6]. Additionally, studies done in SCID mice in which a human intestinal xenograft was implanted have provided evidence that the infection with *E. histolytica* promotes an intense acute inflammatory reaction, accomplished by neutrophil infiltration with an increase in PGE_2-COX-2 enzyme dependent levels [8]. These results suggest that PGE_2 could be a host-dependent factor involved

in susceptibility or resistance. Immunosuppressive therapy with PGE_2 is limited by poor oral bioavailability and short half-life. However, several studies using the PGE_1 analogue, misoprostol (MPL), have demonstrated that MPL shares, with PGE_2 and PGE_1, the ability to inhibit mitogenic activity of IL-1, tumor necrosis factor (TNF) (α and β), IFN-γ, IL-12, and IL-18 production [9, 10]. Moreover, MPL has been used to restore immune responsiveness and the expression of surface class II antigen and IL-2 receptors in lymphocytes [11, 12]. In this study we hypothesized that exogenous prostaglandin analogue (MPL) could induce, in a resistant model such as BALB/c mice, an increase in susceptibility. Results reported here demonstrate that MPL can modulate the inflammatory process to allow the persistence of trophozoites in the liver. Additionally, we report significant changes in trophozoites' morphology, which could be related with the mice natural resistance to *E. histolytica* infection.

2. Materials and Methods

2.1. Chemicals. Adult bovine serum was obtained from Equitech-Bio, (Ingram, TX, USA). Ethanol was obtained from Baker (Edo. de México, México); sodium pentobarbital (Anestesal®) was obtained from Pfizer S.A de C.V. (Toluca, Edo. De México, México). Gelfoam was obtained from Upjohn (Kalamazoo, MI, USA); suture 3/0 Curex was obtained from International Pharmaceutics (México); formalin and hematoxylin and eosin (H&E) were obtained from Merck (Darmstadt, Germany). For immunohistochemistry Histostain-plus kit (Zymed Lab. Inc., San Francisco, CA, USA). Misoprostol (MPL) was a gift from the G.D. Searle, Co. (México).

2.2. Entamoeba histolytica Cultures. E. histolytica strain HM1:IMSS was kindly provided by Dr. V. Tsutsumi (Center for Research and Advanced Studies, National Polytechnical Institute, México City, México) and passed three times through hamster liver to preserve virulence. The strain was maintained in our laboratory by subculturing twice a week in axenic TYI-S33 medium [13]. Cultures for inoculation were grown in 15 mL screw-capped tubes. Log-phase cultures were chilled on ice for 5 min. Trophozoites were pelleted by centrifugation at 500 ×g for 5 min in a Sorvall RT6000B refrigerated centrifuge, counted on a hemocytometer, and resuspended in the same medium to yield a cell density of 7.5×10^6 cell/mL and 1.5×10^7 cell/mL for inoculation in hamsters and mice, respectively. Sterile screw cap vials containing amoebas were kept on ice, pending inoculation.

2.3. Amebic Liver Abscess Development. Male inbred hamsters (*Mesocricetus auratus*) weighing approximately 100 g were used as controls for amoeba virulence, liver damage, and trophozoite morphology. BALB/c mice weighing approximately 30 g were divided in four groups: infected or not and with or without MPL treatment. Hamsters and mice were infected intrahepatically with 7.5×10^5 trophozoites of *E. histolytica* in mid-log phase as described previously [6]. Briefly, animals were anaesthetized with sodium pentobarbital diluted 1 : 10 in phosphate buffer saline pH 7.4 and applied i.p. (1.5 mL/100 g of body weight). After a longitudinal midline incision, approximately 1.5 cm in length, the liver was exposed and the inoculum (0.1 mL for hamsters and 0.05 mL for mice) was slowly injected using a tuberculin syringe equipped with 29G × 13 mm needle. Gelfoam was used to prevent hemorrhages and was removed before suturing, using 3–0 normal suture. Aseptic precautions were observed throughout infection procedure and during postinfection time. At 2, 4, and 7 days postinfection (p.i.) animals were anaesthetized and killed by exsanguination, and livers and abscesses were dissected and weighted to determine the percentage of damage as the ratio between abscess and liver weight before abscess removal. Liver and abscesses were fixed with 3.8% phosphate-buffered formalin, and paraffin embedded. Paraffin sections were stained with hematoxylin and eosin (H&E) to confirm amoebic invasion.

2.4. Treatment. Treatment was used only in mice groups; for this, 2 mg of MPL was dissolved in 1 mL of ethanol and further diluted with sterile water to obtain a final concentration of 1×10^{-4} M of MPL. From this solution, 0.1 mL (128 μg/100 g of body weight) was applied 1 h previous to infection in treated groups and daily until sacrifice. This dose was considered by other studies as immunosuppressive [9, 12]. A treated and noninfected group was included to discard morphologic features due to MPL effects in hepatic tissue. Consumption water for MPL-treated animals, infected or not, was added with 0.25 μg/mL of MPL, to avoid variation in MPL plasmatic levels.

2.5. Immunohistochemical Analysis. Paraffin liver sections (6 μm thick) were obtained from each group and processed for immunohistochemical (IHC) analysis to detect trophozoites. IHC was performed as described previously [7]. Briefly, paraffin sections (4 μm thick) were deparaffinized with xylene, rehydrated in a graded series of ethanol, and equilibrated in phosphate-buffered saline bath for 5 min. Endogenous peroxidase quenching treatment was done by incubating samples for 10 min at room temperature in absolute methanol containing 1% hydrogen peroxide. Blocking step was performed following instructions of Histostain-plus kit. The primary antibody, a polyclonal rabbit anti-amoeba antiserum (1 : 500 dilution), was detected using the secondary affinity-purified biotinylated goat anti-rabbit IgG antibody, avidin-peroxidase, and freshly prepared diaminobenzidine substrate. All these reagents were used as provided by the manufacturer. Nonrelated or preimmune sera were used as negative controls.

2.6. Image Analysis and Trophozoites Measurements. Trophozoite measurements were done using a BX41 Olympus microscope equipped with a Pixera-CCD camera and analyzed with the IMAGE Pro Plus 4.1 software (Media Cibernetics, Silver Spring, Maryland, USA). The size of trophozoites present in ten microphotographs taken from damaged areas of each liver (40x) was measured as major (MD) and minor diameter (md) using the IMAGE software measure tool, previously calibrated. Microscope calibration was done using a stage micrometer (1 mm/100 parts; Euromex Microscopes Holland

Table 1: Percentage of damage obtained in *E. histolytica* infected animals treated or not with MPL.

Specie	Treatment	Days p.i.	Weight (g)		Damage (%)
			Liver	Abscess	
Hamster	—	2	5.8 ± 1.3*	0.6 ± 0.1	10 ± 2
	—	4	6.5 ± 1.5*	1.3 ± 0.8	18 ± 7
	—	7	11.5 ± 4*	4.1 ± 0.4	35 ± 2
Mice	—	2	1.9 ± 0.4	0.227 ± 0.09	11 ± 5
	MPL	2	2.4 ± 0.4	0.215 ± 0.01	12 ± 3
	—	4	2.38 ± 0.9	0.236 ± 0.09	10 ± 2
	MPL	4	2.3 ± 0.4	0.273 ± 0.10	12 ± 2
	—	7	2.3 ± 0.4	0.191 ± 0.10	8 ± 0.1
	MPL	7	2.3 ± 0.2	0.192 ± 0.04	8 ± 0.1

* $P < 0.01$ versus liver weight from noninfected hamsters.

BV, Arnhem, Holland) with a value of 5.240 pixels/μm. All determinations were made the same day to diminish calibration or lighting errors.

2.7. Statistical Analysis. Liver damage data were analyzed as quantitative variables using an ANOVA test; differences between groups were considered significant when $P \leq 0.05$ by Wilcoxon analysis. Trophozoites' measurements data are presented as means ± standard deviation. The differences in trophozoites' dimension between groups and among species were analyzed by Student's t-test and considered significant when $P < 0.05$. Comparisons between experimental and control groups were performed using an analysis of variance (ANOVA), where appropriate. Data analyses were carried out with the STATA 9.0 program for Windows (Stata Statistical Software, Release 9.0., Stata Corporation, College Station, Texas, USA).

3. Results

Intrahepatic inoculation of *E. histolytica* trophozoites in hamsters induced ALA formation in 100% of the animals, which corroborated the virulence of the amoeba strain. A significant increase in liver weight was observed in infected hamsters in comparison to liver weight of noninfected hamsters; however, in the case of infected BALB/c mice groups, liver weight did not suffer significant changes neither in MPL-treated animals nor in untreated mice (Table 1). Liver damage in hamsters increased significantly until it reached 35% at 7 days p.i, while damage observed in BALB/c mice was less than 15% (Table 1) at different times p.i., and no significant difference was detected among infected mice due to MPL treatment. Although ALA formation was more frequently observed in MPL-treated and infected mice in comparison to untreated and infected mice, ALA development was significantly different only at two days p.i. ($P < 0.05$) compared with untreated and infected mice (Table 2).

Abscesses in liver from infected hamsters had macroscopic characteristics similar to those described previously [1]. Microscopically, the hepatic parenchyma showed multiple foci of acute inflammatory reaction, enlarged granulomas, and intense necrosis areas. Cellular infiltrate was composed by polymorphonuclear leukocytes and eosinophils and histiocytes were present as an inner layer. Livers from infected hamsters at 2, 4, and 7 days p.i. showed the presence of numerous trophozoites mainly at the periphery of necrosis which was corroborated by IHC (Figure 1).

Table 2: Frequencies of ALA presentation in *E. histolytica* infected BALB/c mice treated or not with MPL.

Group	Presence of ALA		
	2 days % (ratio)	4 days % (ratio)	7 days % (ratio)
Infected	36 (9/25)	50 (13/26)	61 (8/13)
Infected + MPL	76.9 (10/13)*	65 (13/23)§	60 (6/10)#

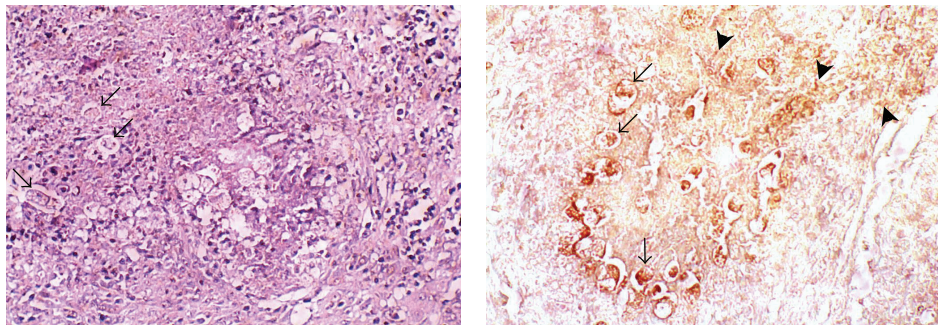
* $P = 0.03$.
§ $P = 0.37$.
# $P = 0.63$.

Livers from infected mice treated or not with MPL had normal surface and color; abscesses were grossly observed and frequently showed a grey-white coloration. To confirm ALA formation in mice, microscopic examination was performed to demonstrate foci of inflammatory infiltrate, trophozoites, and/or necrosis.

Histological examination of livers from infected mice showed small foci of inflammatory infiltrate in some samples, mainly at 2 and 4 days p.i. (Figures 3(a) and 3(b), resp.); trophozoites were detected, although smaller than normal and with a circular morphology (Figure 2(a)).

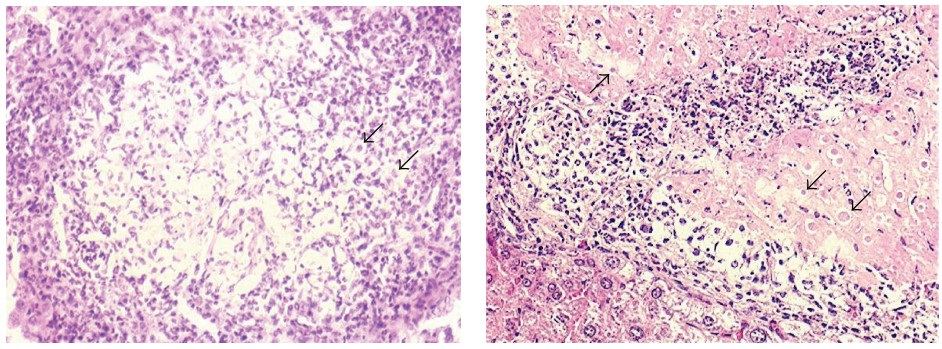
Microscopic examination of lesions in infected and MPL-treated mice showed small necrosis areas accompanied by chronic granulomatous inflammation foci (Figures 3(c) and 3(d)). Cellular exudates included polymorphonuclear leukocytes and lymphocytes; multinucleated giant cells were observed in one preparation. Abundant trophozoites, with different morphology, were found at 2 days p.i. (Figure 2(b)), decreasing at 4 and 7 days p.i.

Moreover, in infected mice, trophozoites showed an apparent decrease in size in relation to that observed in trophozoites present in hamster. To analyze this, trophozoites were detected by IHC and a morphometrical analysis was performed to compare them with those trophozoites present in hamster liver lesions.



(a) (b)

Figure 1: Microphotography of histological changes observed in liver from hamsters infected with *E. histolytica* at four days postinfection. (a) Numerous amoebic trophozoites (arrows) present in liver tissue. H&E stain; original magnification 10x. (b) Immune detection of trophozoites showing a strong peroxidase reaction (arrows); multiple signals of amoebic proteins were observed in areas adjacent to trophozoites (arrow heads). Original magnification 10x.



(a) (b)

Figure 2: Effect of MPL treatment on ALA formation in BALB/c mice at two days postinfection. (a) Histological changes in mice infected without MPL treatment. Original magnification 10x. (b) Histological changes in infected mice and MPL-treated mice. Original magnification 10x. Arrows indicate the trophozoites in the hepatic tissue. H&E stain.

Trophozoites detected in liver from infected hamsters looked elongated with pseudopodia or rounded, and in most of the analyzed samples, peroxidase stain was strong and clearly defined (Figure 4(a)). In contrast, in nontreated infected mice, trophozoites were scarce and, at all times of postinfection tested, looked smaller and with a circular morphology (Figure 4(b)), while in MPL-treated and infected mice, standing trophozoites showed a diminution in size regardless of postinfection time (Figures 4(c) and 4(d)).

Results of morphometrical analysis of trophozoites found in the different experimental groups are described in Table 3. Trophozoites present in ALA from hamster, at different time p.i., did not have significant changes in major diameter (MD) or minor diameter (md). At two days postinfection a significant difference in the size was detected in trophozoites from mice with ALA compared with those detected in hamsters (Table 2, $P < 0.01$). However, no differences were observed in the size of trophozoites from infected mice treated or not with MPL (Table 2). Similar results were obtained at four and seven days p.i.; a significant diminution in the size of the trophozoites was observed in liver from mice, independently of the treatment, compared to trophozoites size from hamster.

Additionally, the size of the trophozoites present in liver from infected mouse treated or not with MPL showed a significant diminution in MD as well as in md ($P < 0.01$; Table 2) at 4 and 7 days p.i. in relation to the size observed in trophozoites found in samples from 2 days p.i. Trophozoites present in the livers from infected hamsters did not have significant differences in their diameters among postinfection times ($P = 0.13$ for MD and $P = 0.32$ for md).

4. Discussion

The mechanisms for murine host resistance to *E. histolytica* infection to develop hepatic abscesses are unknown. Some evidences with rabbit anti-mouse thymocyte globulin pretreated mice infected intracecally and intrahepatically suggest that cell mediated immunity plays a crucial protective role in this parasite infection [14]. However, studies realized by Stern et al. [4] demonstrated that resistance of nu/nu mice to infection only could be abolished with previous silica treatments, suggesting that macrophages provide the critical host defense in response to *E. histolytica*.

Table 3: Morphometric analysis of trophozoites detected in liver abscesses from experimental groups.

Days p.i.	BALB/c mice				Hamster	
	Infected		Infected + MPL		Infected	
	MD	md	MD	md	MD	md
2	$5.8 \pm 1.1^{*}$	4.9 ± 1.4	$5.8 \pm 1.4^{*}$	4.8 ± 1.3	9.6 ± 2.7	7.3 ± 2.1
4	$4.1 \pm 0.9^{*\S£}$	3.4 ± 0.8	$3.0 \pm 0.6^{\S£}$	2.6 ± 0.60	8.9 ± 1.8	6.9 ± 0.62
7	$2.7 \pm 1.9^{*\S£}$	2.5 ± 0.5	$2.1 \pm 0.3^{\S£}$	1.9 ± 0.28	8.8 ± 1.0	6.3 ± 1.26

MD: major diameter; md: minor diameter; MPL: misoprostol (10^{-4} M).
$^{*}P < 0.01$ difference among species.
$^{\S}P < 0.01$ difference between treatments.
$^{£}P < 0.01$ difference between p.i. times.

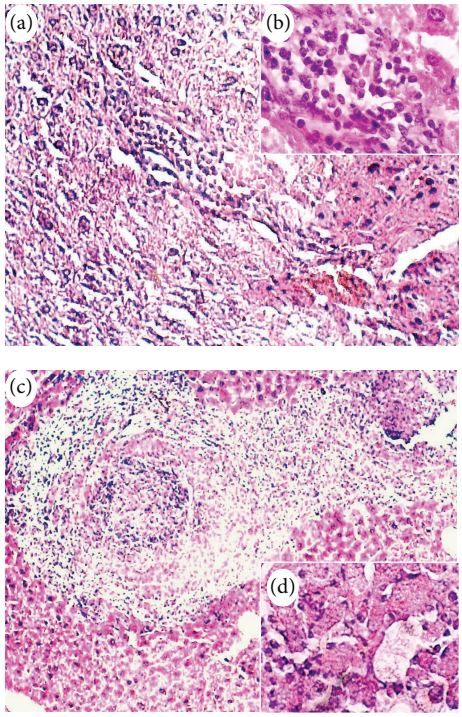


Figure 3: Effect of MPL treatment in ALA formation in BALB/c mice at four days postinfection. Histological changes in mice infected without MPL treatment. (a) Small foci of inflammatory infiltrate in the liver. Original magnification 10x. (b) Cellular infiltrate composed mainly by polymorphonuclear leukocytes. Original magnification 40x. Histological changes in infected mice and MPL-treated mice. (c) Granuloma with a necrotic center, inflammatory infiltrate, and trophozoites in the periphery. Original magnification 10x. (d) Cellular infiltrate near to trophozoites. Original magnification 40x. H&E stain.

In our studies, despite the fact that damage percentage in MPL-treated mice was <15%, we observed that immune manipulation with MPL was able to induce an inflammatory process and hepatic features similar to those produced in susceptible animals. The mechanism by which MPL could participate in ALA development is unclear, but since MPL shares many effects on the immune system with PGE_1, the mechanism could be explained as cytokine induced immunomodulation of macrophage function [9].

PGE_2 can modulate immune responses preferentially towards TH2-cell subpopulations which inhibit macrophage activation by inhibition of IFN-γ production [5]. Previous work in hamsters has suggested that PGE_2 plays an important role in immunomodulation of host's response in infection with this parasite, since *E. histolytica* infection in hamster increases plasmatic and local levels of PGE_2 [6].

MPL shares with PGs the capability to regulate inflammatory cytokines and macrophage functions and may be an example of a natural feedback mechanism for controlling inflammation, which is consistent with the mechanism proposed by Bonta and Parnham [15]. Administration of MPL could be supplying the natural production of PGs in mice and modifying the adequate response against the amoeba.

In addition, the fact that MPL-treated mice did not develop bigger abscesses could be explained by the number of trophozoites used to inoculate mice, which was under the number usually used in resistant models. However, the participation of additional natural resistance mechanisms cannot be discarded.

Regarding morphological alterations in trophozoites, although diverse studies exist in which ALA development in susceptible and/or resistant models has been used, to date a study that describes the morphological changes that occur in trophozoites during the invasion has not been reported. Chavez-Munguia et al. described the ultrastructure of the trophozoites recovered from hamster ALA, using electronic microscopy [16]. They found a great diversity of sizes among recuperated trophozoites, which varies between 10 and 60 μm. In our study, the MD of the trophozoites detected in hamsters had an average of 9.6 ± 2.7 μm; nevertheless, we did not detect trophozoites with bigger dimensions in livers. Our observations suggest that trophozoites could modify their morphology as a consequence of microenvironment changes and tissue aggression by cells and/or components of the innate immune response of the host; differences between this study and that from Chavez-Munguia et al. could be due to the fact that, before their analysis, recovered trophozoites were transferred to axenic culture, which could support their growth and development. Moreover, data about trophozoites size in mice models are rather scarce. Cieslak et al., who obtained successful hepatic infection in SCID mice, reported

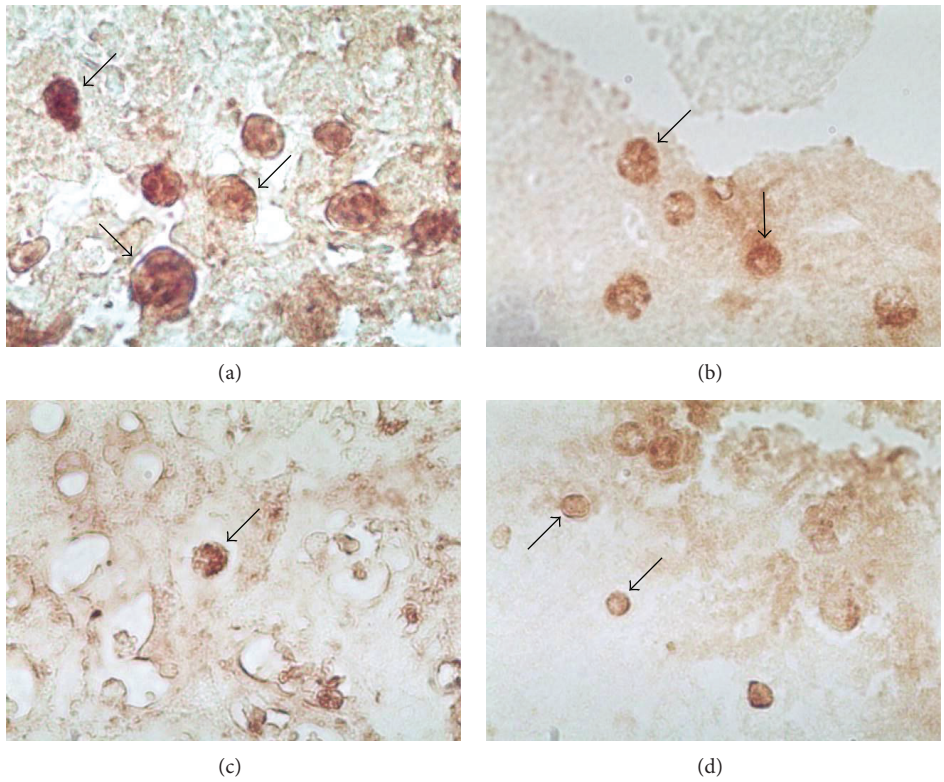


Figure 4: Morphological differences detected in trophozoites present in liver sections from hamster and BALB/c mice treated or not with MPL. Trophozoites were localized by IHC using a rabbit polyclonal antiserum against *E. histolytica*. (a) Trophozoites (arrows) present in liver tissue from infected hamster at four days p.i. Original magnification 40x. (b) Trophozoites present in liver from mice infected without MPL treatment at four days p.i. Original magnification 40x. (c) and (d) Trophozoites detected in liver from infected mice and MPL-treated mice at two and four days p.i., respectively. Original magnification 40x.

trophozoites with a similar morphology as we described in this paper; however, authors did not discuss it [17]. Notwithstanding in our study, since a significant difference was not detected in the size of trophozoites present in mice treated with MPL in comparison to infected and untreated mice, this discarded a direct effect of MPL on parasites.

Concerning amoebic infection in BALB/c mice, although several studies have reported that, in resistant models, such as mice, trophozoites disappeared by 48 h p.i., we were able to detect trophozoites at longer times p.i., probably due to the use of IHC methodology; however, trophozoites were more abundant at 2 days p.i. decreasing by 4 and 7 days p.i., which agree with previously reported results [18, 19].

Finally, our results on infected and MPL-treated mice are consistent with Tsutsumi's discussion related to the proposal that the inflammatory process contributes to ALA formation.

Additional research could be focused to understand the role of an adequate control of the inflammatory process in the natural resistance to *E. histolytica* infection in BALB/c mice and its participation in regulation of the immune response.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Dendritic Cell Profile Induced by *Schistosoma mansoni* Antigen in Cutaneous Leishmaniasis Patients

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The inflammatory response in cutaneous leishmaniasis (CL), although responsible for controlling the infection, is associated with the pathogenesis of disease. Conversely, the immune response induced by *S. mansoni* antigens is able to prevent immune-mediated diseases. The aim of this study was to evaluate the potential of the *S. mansoni* Sm29 antigen to change the profile of monocyte-derived dendritic cells (MoDCs) from subjects with cutaneous leishmaniasis (CL) *in vitro*. Monocytes derived from the peripheral blood mononuclear cells of twelve patients were cultured with GM-CSF and IL-4 for differentiation into dendritic cells and then stimulated with soluble *Leishmania* antigen (SLA) in the presence or absence of Sm29 antigen. The expression of surface molecules associated with maturation and activation (HLA-DR, CD40, CD83, CD80, and CD86), inflammation (IL-12, TNF), and downregulation (IL-10, IL-10R) was evaluated using flow cytometry. We observed that the frequencies of HLA-DR, CD83, CD80, and CD86 as well as of IL-10 and IL-10R on MoDCs were higher in cultures stimulated with Sm29, compared to the unstimulated cell cultures. Our results indicate that the Sm29 antigen is able to activate regulatory MoDCs in patients with cutaneous leishmaniasis. It might be useful to control the inflammatory process associated with this disease.

1. Introduction

Leishmaniasis is endemic in 88 countries with approximately 12 million people infected and 350 million at risk worldwide, with an incidence of 1.5 million cases per year [1, 2]. A variety of disease manifestations are associated with *Leishmania* spp. infection, primarily determined by the infecting species. Cutaneous leishmaniasis (CL) is the most common clinical manifestation of tegumentary leishmaniasis, characterized by one to several skin lesions in exposed areas, with small number of parasites [3, 4]. The immune response is characterized by a Th1-inflammatory profile with macrophage activation and parasite killing. During the innate immune response monocyte-derived dendritic cells (MoDCs) which interact with the pathogen may differentiate at the inflammation site and act as local tissue resident APCs or as a source of inflammatory cytokines [5, 6]. The initial events determine the cytokine environment and consequent adaptive immune response to certain pathogens, such as *Leishmania*. For instance, this parasite leads to an exacerbated Th1-inflammatory immune response associated with tissue injury in LC. On the other hand, the immune response induced by the parasite *Schistosoma mansoni* is able to downregulate the inflammatory response in immune-mediated diseases [7–11]. Studies have shown that chronic helminths infections, especially *Schistosoma mansoni*, possess the ability to

modulate the inflammatory response associated with both Th1 [7, 9] and Th2 [8, 11–13] immune-mediated diseases. These findings have provided the rationale for the use of recombinant *S. mansoni* proteins in *in vitro* studies with cells from patients with leishmaniasis in an attempt to modulate the inflammatory response associated with pathogenesis. Previous studies performed by our group have shown that the addition of the *S. mansoni* antigens Sm29, PIII, and TSP-2 in cultured PBMC from cutaneous leishmaniasis patients stimulated with soluble *Leishmania* antigen (SLA) caused a reduction in the levels of IFN-γ and TNF in a significant number of individuals, with an increase in the levels of IL-10 [9]. In other studies from our group [7, 9, 11] Sm29 has been the better inducer of IL-10 among the *S. mansoni* antigens tested, a cytokine with the property to prevent inflammatory process associated with immune-mediated diseases. The aim of this study was to evaluate the potential of the *S. mansoni* antigen Sm29 to induce a regulatory profile in monocyte-derived dendritic cells (MoDCs) from individuals with CL in an attempt to prevent or minimize the inflammatory response associated with the disease.

2. Material and Methods

2.1. Study Design. We included twelve patients with CL who reside in an endemic area, named "Corte de Pedra," located in the southeast region of the state of Bahia, Brazil. Six of them were male and six female with a mean age of 33 ± 6 years. The diagnostic criteria comprised a clinical presentation characteristic of CL, parasite isolation or positive delayed-type hypersensitivity (DTH) in response to *Leishmania* soluble antigen (SLA), and histological features of CL.

The Ethics Committee of the University of the State of Bahia (UNEB) approved the present study (License Number 0603110287514). Three stool samples from each individual were examined using the Hoffman sedimentation method to exclude individuals infected with *S. mansoni*.

2.2. Antigen Stimulation. The *Schistosoma mansoni* tegument antigen Sm29 used in this study was provided by Dr. Sergio C. Oliveira from the Institute of Biological Science, Department of Biochemistry and Immunology, UFMG, Brazil. The recombinant proteins were cloned in *E. coli* and were tested for the presence of lipopolysaccharide (LPS) using a commercially available LAL Chromogenic Kit (CAMBREX). The level was below the detection limit (data not shown). The SLA was prepared from a *L. braziliensis* strain as previously described [14].

2.3. In Vitro Generation of Monocyte Dendritic Cells (MoDCs). MoDCs were obtained from PBMCs from cutaneous leishmaniasis patients as reported in previous studies [15]. Briefly, PBMCs were obtained from the Ficoll-Hypaque gradient method and cultured in 6-well plates with complete medium RPMI 1640 medium containing 10% inactivated fetal bovine serum, 100 mg/mL gentamicin, 2 mM L-glutamine, and 30 mM HEPES (Gibco-BRL Life Technologies, Gaithersburg, MD) at a concentration of 5×10^6 cells per well. They were incubated for 2 hours with 5% CO_2 at 37°C to allow for monocyte adherence to the plate. After this period, the supernatant was removed, and cells were washed three times with PBS to remove nonadhered cells. The adhered monocytes were harvested with a solution containing PBS1x, EDTA (10 nM), and glucose (3 nm) and then adjusted to 3×10^5 cells/mL and cultured with complete medium in the presence of IL-4 (800 IU/mL) and GM-CSF (50 ng/mL) for 6 days with the replacement of medium and cytokines at day 3 to allow for differentiation into dendritic cells. Subsequently, 3×10^5 MoDCs were cultured with Sm29 (10 μg/mL) and SLA (5 μg/mL) for 20 hours. At this point, the MoDCs were assessed by flow cytometry. Next, we determined the percentage of $CD11c^+$ cells by flow cytometry, and for all experiments, the frequency of these cells was ≥80%.

2.4. Flow Cytometry. For flow cytometry, MoDCs were harvested and stained with fluorochrome-conjugated antibodies for surface and intracellular markers. DCs treated with different antigens or controls (without stimulation) were collected by centrifugation at 1100 rpm for 10 min and resuspended in RPMI 1640 containing 10% fetal bovine serum (FBS, heat inactivated) (GIBCO, INVITROGEN). Cells were stained with fluorescently conjugated mouse anti-human monoclonal antibodies against CD11c-APC (clone 3.9), CD1a-FITC (clone HI149), IL-10Rα-PE (polyclonal), CD40-PerCP-e Fluor 710 (clone 5C3), CD80-PerCP-e Fluor 710 (clone 2D10.4), CD86-PE (clone IT2.2), CD83-PE-Cy7 (clone HB15e), and HLA-DR-PerCP-Cy5.5 (clone LN3) (all from eBioscience, California) and then analyzed for 100000 events per sample using a flow cytometer (FACSCanto, Becton Dickinson). Limits for the quadrant markers were set based on negative populations and controls isotype (data not shown).

Intracellular staining was performed with PE-labeled monoclonal antibody against human IL-10 (clone JES3-19F1, BD Pharmingen), IL-12 (clone C8.6, eBioscience), and TNF (clone Mab11, eBioscience) in saponin buffer (PBS, supplemented with 0.5% BSA and 0.5% saponin). During the last 4 hours of culture, Brefeldin A (10 μg/mL; Sigma, St. Louis, MO) was added to the cultures. Afterwards, the cells were washed in PBS and fixed in 2% formaldehyde for 20 minutes at room temperature.

The frequency of positive cells was analyzed using the program FlowJo (Tree Star, USA) in two regions. The monocyte-derived dendritic cell region was defined by non-specific fluorescence with forward scatter (FSC) and side scatter (SSC) as parameters of cell size and granularity, respectively. The cells were gated based on their granularity and expression of CD11c (Figure 1).

2.5. Statistical Analysis. Data were analyzed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). The differences among MoDCs stimulated with SLA in the presence or absence of Sm29 antigen were assessed using Friedman exact test. The frequencies of positive adherents cells were expressed as percentages and mean fluorescence intensity

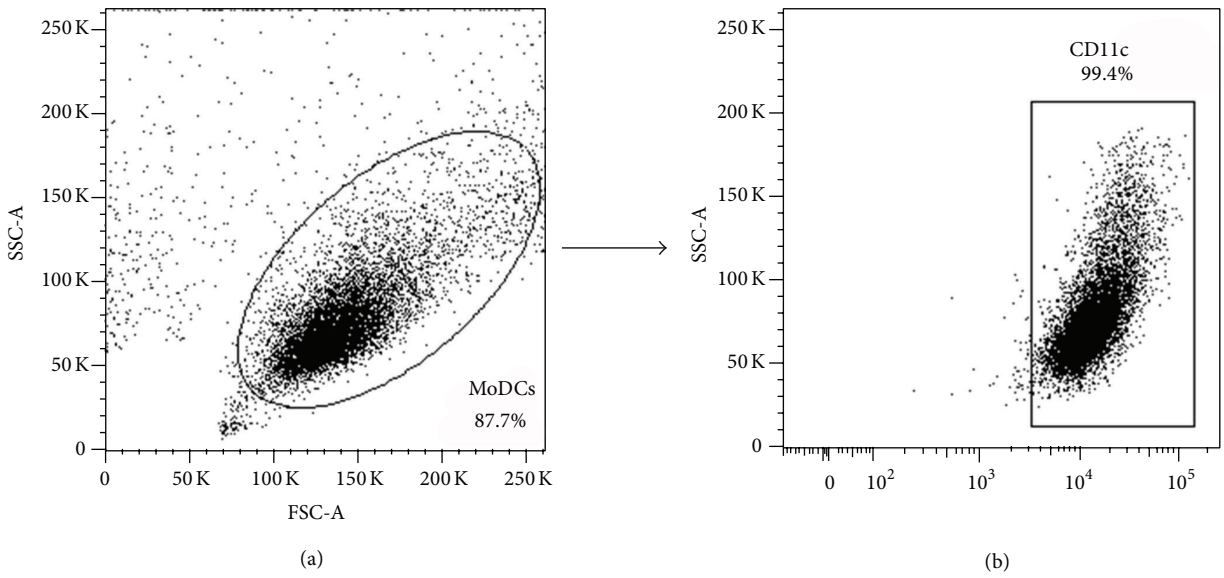

Figure 1: Gate strategy for the identification of monocyte-derived dendritic cells (MoDCs) (a). Frequency of cells expressing CD11c (b). Representative graph of one experiment.

(MFI), respectively. Statistical significance was established at the 95% confidence interval.

3. Results

3.1. Frequency and Maturation Status of Monocyte-Derived Dendritic Cells (MoDCs) Stimulated with the S. mansoni Antigen Sm29. The frequency and maturation status of MoDCs stimulated with SLA in the presence or absence of Sm29 were evaluated *in vitro* by the expressions of CD1a and CD83 molecules on $CD11c^+$ cells (Figure 2). The addition of Sm29 to SLA stimulated cultures did not alter the frequency of the CD1a molecule on MoDCs (Figure 2(a)). The frequency of $CD11c^+CD1a^+$ cells was similar among cultures stimulated with SLA (98% (93–99%)), SLA + Sm29 (98% (90–99%)), and Sm29 alone (97% (92–99%)). Regarding the maturation status evaluated through the expression of CD83 molecule, it was observed that the addition of Sm29 to the cultures of MoDCs leads to an increase in the frequency of CD83, being 27.5% (16–68%) in SLA + Sm29 cultures and 42 (16–75%) in cultures with Sm29 alone compared to cultures without stimulation (19% (11–39%), $P < 0.005$, Figure 2(b)).

3.2. Activation Status of MoDCs after Addition of Antigen Sm29. The mean fluorescence intensity (MFI) of activation marker HLA-DR on MoDCs after *in vitro* addition of Sm29 in cultures stimulated with SLA was also evaluated (Figure 3(a)). The addition of Sm29 antigen in cultures stimulated with SLA leads to an increase in the expression of HLA-DR by MoDCs (325 (83–1452) MFI) when compared to the unstimulated cultures (185 (62–927) MFI, $P < 0.05$; Figure 3(a)). The expression of costimulatory molecules CD80, CD86 was also affected by the presence of antigens (Figures 3(b) and 3(c)). The addition of Sm29 to the cultures stimulated with SLA led to an increase in the frequency of cells expressing CD80 (13.5% (3–39%)) compared to unstimulated cultures (3.9% (2–12%), $P < 0.0001$) or those stimulated with SLA alone (6.3% (2.4–17%), $P < 0.05$; Figure 3(b)). Regarding the frequency of MoDCs expressing CD86, the addition of Sm29 to the cultures stimulated with SLA showed an increase in the frequency of these cells (95.5% (81–99.5%)) compared to unstimulated cultures (80.5% (40–95%), $P < 0.005$; Figure 3(c)). The frequency of MoDCs expressing CD86 was also higher in cultures stimulated with SLA (93.5% (50–99%)) compared to unstimulated cultures (Figure 3(c)). The frequency of MoDCs expressing CD40 was similar among the groups, being 25.25% (11.10–72.90%) in cultures without stimulation, 31.35% (11.5–63.5%) for cultures stimulated with Sm29, 36.55% (12.2–65.3%) for cultures stimulated with SLA, and 56% (13–88%) in cultures stimulated in the presence of SLA plus Sm29 (Figure 3(d)).

In cultures stimulated with LPS there was an increase in the frequency of cells expressing CD40 (60.1% (45.3–71.8%)), compared to cultures without stimulus (25.25% (11.10–72.90%), $P < 0.05$) or stimulated with SLA (36.55% (12.2–65.3%), $P < 0.05$). The mean fluorescence intensity HLA-DR was higher in cultures with LPS (352 (116–2097) MFI) compared to the unstimulated cultures (82 (55–97) MFI, $P < 0.05$), data not shown.

3.3. Inflammatory and Regulatory Status of MoDCs Induced by Sm29. The expression of inflammatory cytokines IL-12 and TNF by MoDCs from patients with cutaneous leishmaniasis induced by the presence of Sm29 is shown in Figures 4(a) and 4(b). The frequency of MoDCs expressing IL-12 did not differ among all stimulated cultures, being 1.4% (0.4–3.05%) in cultures without stimulation, 1.45% (0.3–3.3%) in cultures stimulated with SLA alone, 1.6% (0.5–3.0%) in cultures stimulated with SLA + Sm29, or 1.75% (0.5–4.3%) in cultures stimulated with Sm29 (Figure 4(a)). The

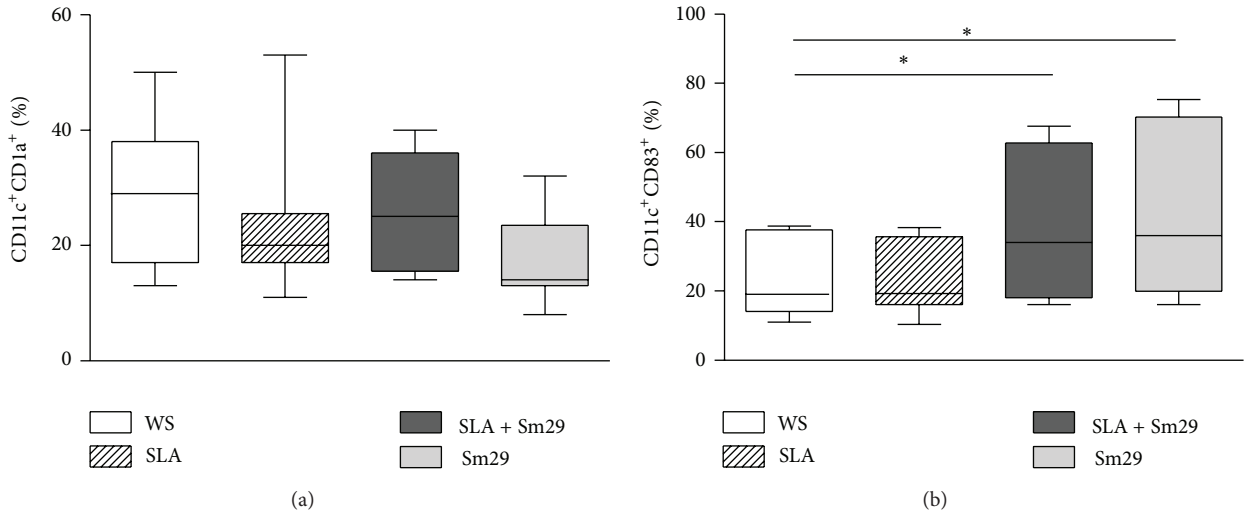

Figure 2: Maturation status of MoDCs from CL patients stimulated with Sm29 antigen. Frequency of MoDCs expressing CD1a (a) and CD83 (b) from individuals with CL ($n = 12$). WS = without stimulation, SLA = soluble *Leishmania* antigen, and Sm29 = *S. mansoni* Sm29 antigen. The results were expressed as median, min-max values, and percentiles. $^{*}P < 0.05$, Friedman test.

frequency of the MoDCs expressing TNF was also similar among all groups, being 1.45% (0.5–2.7%), 1.75% (0.6–3.1%), 1.40% (0.3–2.8%), 1.5% (0.7–3.1%), or 0.8% (0.4–2.7%) in the cultures without stimulation or stimulated with SLA, SLA + Sm29, or Sm29 alone, respectively (Figure 4(b)).

It has been demonstrated that the balance between inflammatory and regulatory networks is important to control the parasites and suppress the clinical manifestation of disease as observed in subclinical forms [16]. We then assessed the ability of Sm29 antigen to induce a regulatory profile by the MoDCs from patients with CL. The frequencies of MoDCs expressing the regulatory cytokine IL-10 in cultures stimulated with SLA + Sm29 (2.3% (0.8 to 4.1%)) or with Sm29 alone (1.8% (from 0.5 to 3.8%)) were higher when compared to the unstimulated cultures 1.3% (0.3–2.3%, $P < 0.05$; Figures 5(a) and 5(b)). Regarding the expression of IL-10 receptor (IL-10R) by MoDCs, we observed that in cultures stimulated with SLA + Sm29 there was a higher frequency of cells expressing this molecule (4.3% (3.3–9.8%)) compared to unstimulated cultures (1.4% (0.2–3.7%), $P < 0.005$) and cultures stimulated with SLA alone (2.5% (1.2–3.8%)) and with Sm29 alone (2.7% (2.2–4.8%); Figure 5(c)).

In cultures stimulated with LPS there was a reduction in the frequency of IL-10 (0.5% (0.3–1.2%), $P < 0.005$) and IL-10R (1.3% (1.0–1.8%), $P < 0.05$) when compared to cultures stimulated with SLA + Sm29 ($P < 0.05$). There was no significant difference in the frequency of MoDCs stimulated with LPS regarding the expression of IL-12 or TNF compared to unstimulated cells (data not shown).

4. Discussion

Cutaneous and mucosal leishmaniasis diseases result from the exacerbation of the Th1-inflammatory immune response. Additionally, the Th1 response with the production of IFN-γ and TNF represents the most important mechanism of *Leishmania* elimination by the activation of macrophages. However, once exacerbated, this response is associated with tissue damage, resulting in cutaneous and mucosal leishmaniasis [17]. The early events in *Leishmania* sp. infection involve dendritic cells and cytokine production, determining the host response and the course of the infection [18]. Thus, the cellular environment associated with the proinflammatory and anti-inflammatory balance is important to control parasite growth and prevent damage to the host [19–22]. This is observed in subclinical forms of *Leishmania* infection in endemic areas [16, 23].

There are evidences in the literature that infection with *Schistosoma* sp. or its products protects against the development of Th1 and Th2 mediated diseases as reviewed by Khan and Fallon (2013) and Elliott et al. (2007) [24, 25]. We have studied the relationship between inflammatory diseases and schistosomiasis in an attempt to characterize the ability of *S. mansoni* antigens to modulate the inflammatory process associated with immune-based diseases, such as asthma [11, 13], HTLV-1 [7], and leishmaniasis [9, 10]. We are currently interested in characteristics of parasite antigens with modulatory properties, able to downmodulate the inflammatory process in CL patients. The mechanisms underlying the regulatory property of *S. mansoni* antigens may include induction of cells and regulatory molecules, such as $CD4^{+}CD25^{+}$ T cells, CTLA-4, and IL-10 molecules [9, 11, 13, 26–29].

In a study performed by our group, Bafica and colleagues (2011) showed that the addition of Sm29 antigen to the cultures of PBMC from CL patients resulted in an increase of IL-10 production in a significant number of patients, coincidental with a reduction in the production of the inflammatory cytokines TNF and IFN-γ. Since in leishmaniasis dendritic cells seem to be important to orchestrate the initial immune

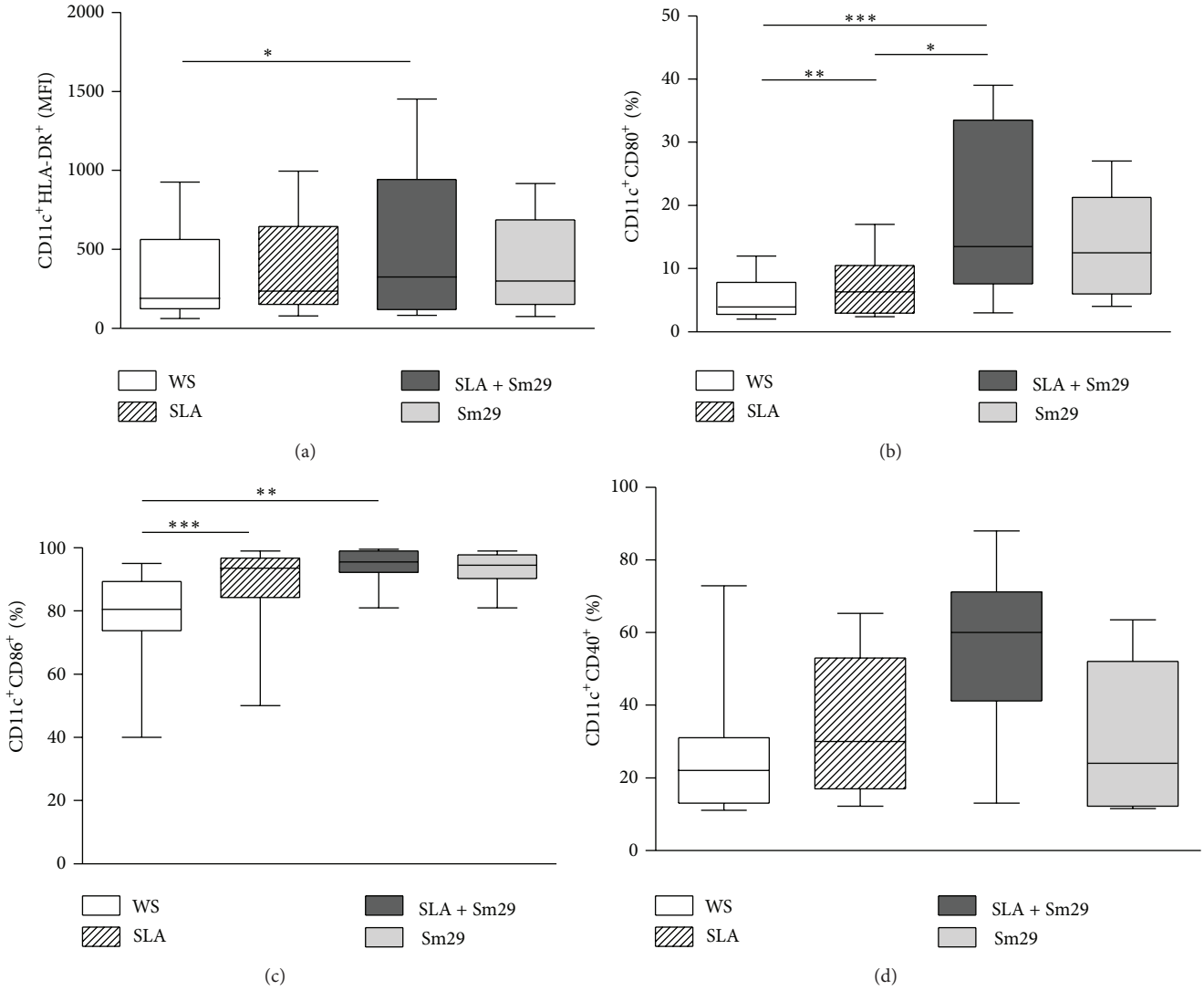

Figure 3: Activation status of MoDCs. Mean florescence intensity of the expression of HLADR (a) and frequency of MoDCs expressing CD80 (b), CD86 (c), and CD40 (d) in cell cultures from patients with cutaneous leishmaniasis ($n = 12$). WS = without stimulation. SLA = soluble *Leishmania* antigen. Sm29 = *S. mansoni* antigen Sm29. The results are expressed as median, min-max values, and percentiles. $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$, Friedman test.

response, the use of an *S. mansoni* antigen able to modulate the inflammatory response of these cells is an important strategy to control the exacerbated response observed in patients with cutaneous leishmaniasis.

The addition of Sm29 to the cultures did not alter the frequency of MoDCs expressing $CD1a^{+}$. It is in agreement with a study performed by Donovan et al. (2007) that showed that infection by *L. major* or *L. donovani* was capable of inhibiting the expression of $CD1a^{+}$ in dendritic cells, decreasing their ability to recognize pathogens and thus respond to their stimuli [30]. Moreover, human monocytes in the presence of *L. amazonensis* showed a decreased expression of CD1a *in vitro* leading to an incomplete differentiation into dendritic cells [30].

Studies have shown that infection by helminths or the use of parasite antigens in *in vitro* studies results in low rate of dendritic cell differentiation, low CD1a expression, and impaired maturation status due to decreased expression of CD83 in MoDCs, both in individuals infected with helminths and in healthy controls [31, 32].

When we assessed the influence of Sm29 antigen on the expression of CD83 on MoDCs, we observed an increase in the frequency of this marker in cells in the presence of this antigen. Different pathogens can influence the maturation and activation status of dendritic cells and affect the outcome of infection [33, 34]. Terrazas et al. (2010) showed that the addition of an antigen of *T. crassiceps* (TCEs) to the cultures stimulated with LPS led to an increase in the expression of CD83 by MoDCs. However, the presence of TCEs alone did not alter the maturation status of these cells. Favali et al. (2007) showed that infecting MoDCs with *L. amazonensis* or stimulation of these cells with SLA did not alter the

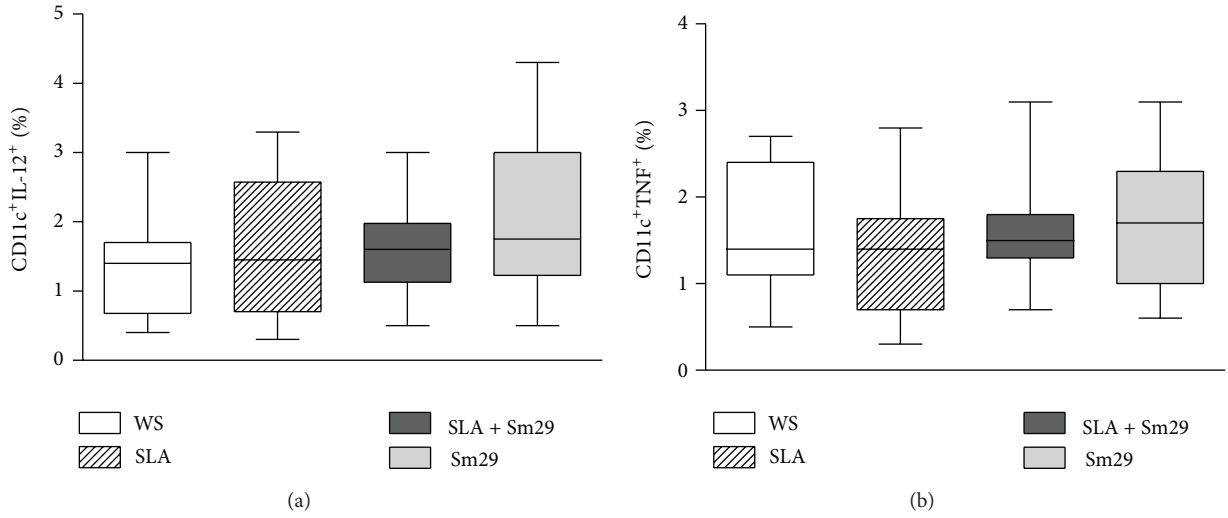

Figure 4: Inflammatory cytokines expressed by MoDCs from patients with cutaneous leishmaniasis ($n = 12$). Frequency of MoDCs expressing IL-12 (a) and TNF (b). WS = without stimulation. SLA = soluble *Leishmania* antigen. Results were expressed as median (min-max values and percentiles).

maturation status of DCs [35]. The maturation process is essential to make DCs capable of presenting antigens to T cells, as well as increasing their ability to produce cytokines. Studies have shown that the exposure of DCs to parasite antigens, including those derived from helminths, results in a limited maturation of these cells [36, 37].

In studies with experimental models, an increase was demonstrated in the frequency of activation markers (HLADR, CD80, and CD86) on dendritic cells infected with *Leishmania* sp. [38, 39]. However, Carvalho et al. (2008) showed that bone marrow-derived DCs from uninfected mice (bystander) present in the environment of DCs infected with *L. braziliensis* showed a higher activation status compared to those cells infected with *L. braziliensis*. It suggested that bystander dendritic cells are better responders to the parasite antigens than the infected ones [39]. Moreover, it has been demonstrated that SLA stimulation of MoDCs of healthy subjects infected with *L. amazonensis* does not alter the expressions of HLA-DR, CD80, and CD86 when compared to uninfected or unstimulated DCs [35].

In this study the frequency of MoDCs expressing CD40 did not alter in the presence of Sm29. A study conducted by Dowling et al. (2011) demonstrated that stimulation of DCs with the antigen ABF from *A. lumbricoides* also did not affect the frequency of the CD40 expression on these cells [40].

It is well known that the cytokines IL-12 and TNF have an important role in mounting the Th1-inflammatory response and control *Leishmania* infection. On the other hand, it is also known that an increased inflammatory response with high production of these cytokines is associated with the development of clinical manifestations of CL [17].

Since it has been shown that there is an impairment of IL-10 production by cells of CL patients [17] and that *S. mansoni* antigens induce the production of this cytokine [8, 9], we decided to evaluate the expression of IL-10 and its receptor on DCs stimulated with Sm29 antigen. We observed that the Sm29 antigen led to an increase in the frequency of MoDCs expressing IL-10 in patients with CL. It has been shown that IL-10 inhibits the differentiation of dendritic cells and suppresses the production of inflammatory chemokines and cytokines [41, 42]. A balance in the immune response, where activated macrophages continue to kill *Leishmania*, without harm to the host is desirable. In this context, DCs stimulated with Sm29 antigen could theoretically provide the necessary regulation to control the inflammatory process.

In this study, we showed that MoDCs are an important source of IL-10 in cutaneous leishmaniasis. Previous studies have documented that the macrophages and regulatory T cells ($CD4^+$ $CD25^+$ $Foxp3^+$) are the main source of IL-10 in the lesions and in supernatants of PBMC from patients with CL [43–45]. IL-10 may act in the control of cell-mediated lesion development in leishmaniasis [46, 47]. In mucosal leishmaniasis (ML) there is a lack of IL-10 response, in part explained by the downregulation of IL-10 receptor [48].

It has been demonstrated that PD-L1 and PD-L2 molecules induce $CD4^+CD25^+$ regulatory cells [49, 50]. PD-L1 and PD-L2 are able to control DC activation during antigen presentation through a variety of mechanisms [51, 52]. A study published by Wang et al. [53] showed that the upregulation of PD-L1 molecule was independent of IL-10 production, suggesting that there are other mechanisms of immune response to pathogens. In leishmaniasis, it has been demonstrated that PD-L1 and PD-L2 have distinct roles in regulating immunity to infection and that they are associated with the outcomes of infection. PD-L1 was associated with resistance and PD-L2 with susceptibility to mice infection with *L. mexicana* [54]. Studies about PD-L1 and PD-L2 are

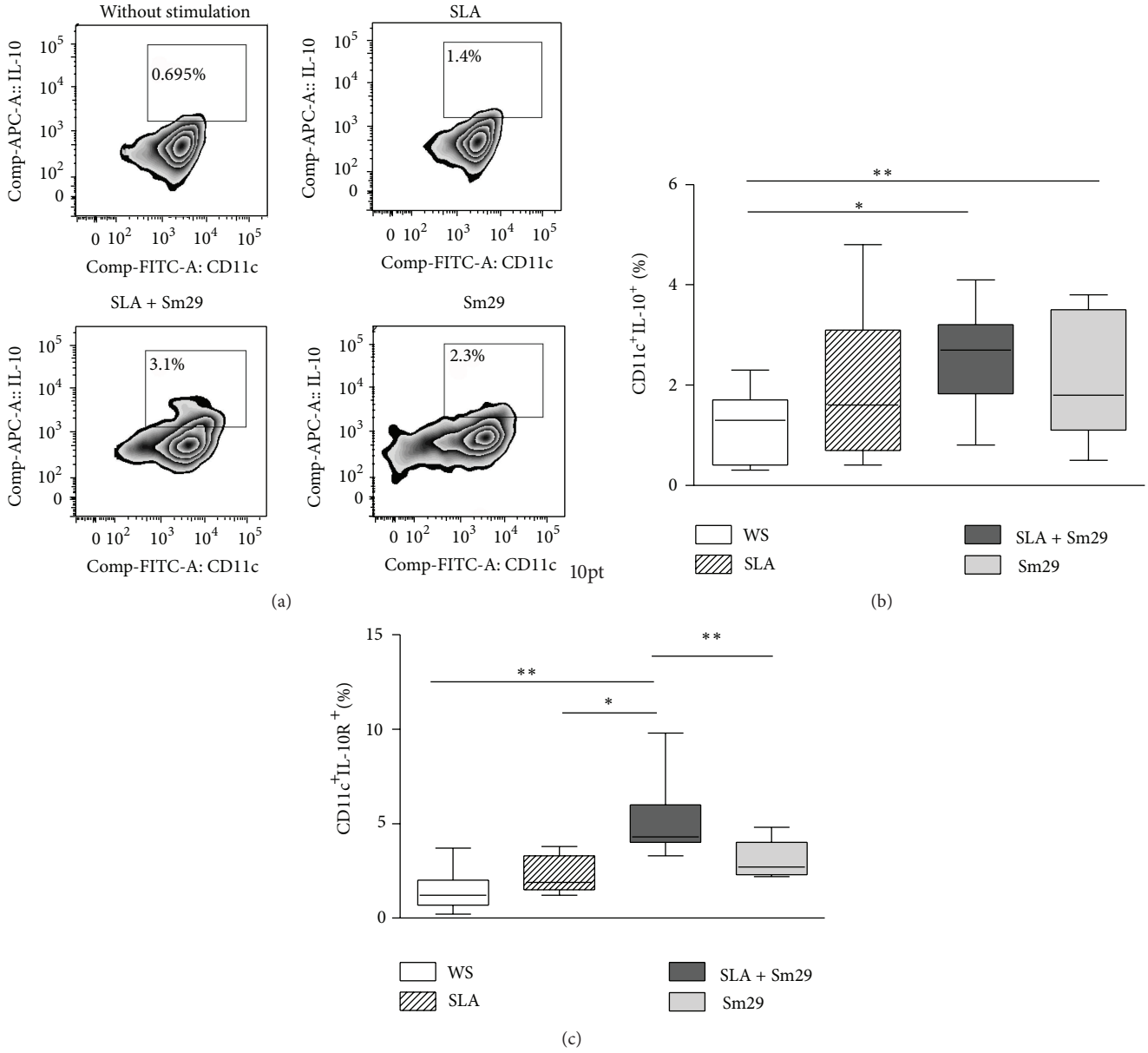

Figure 5: Regulatory markers induced by Sm29 antigen on MoDCs. Frequency of MoDCs expressing IL-10 ((a)-(b)) and IL-10R (c) in individuals with cutaneous leishmaniasis ($n = 12$). WS = without stimulation. SLA = soluble *Leishmania* antigen. Results were expressed as median (min-max values and percentiles). $^{*}P < 0.05$ and $^{**}P < 0.005$, Friedman test. (A) Representative plot of one experiment.

still controversial and have been performed basically using murine models.

The regulatory role of DCs in the overall immune response against parasitic worms is still unclear. It is known that worms promote local immunosuppression in the host, allowing the parasite to achieve long-term survival, which is usually associated with chronic infections [55]. Li and colleagues (2011) described a subset of DC that occurs naturally with regulatory activity in a murine model of *Heligmosomoides polygyrus* infection. These protective regulatory DCs promoted *in vitro* differentiation of Treg cells [56]. However, more studies are needed to understand the mechanisms that lead DCs to present regulatory functions.

Regarding the frequency of cells expressing the IL-10 receptor (IL-10R), in this study we showed a higher expression of this molecule in cultures stimulated with Sm29, even in the presence of SLA. Faria and colleagues (2005) demonstrated that the impaired expression of IL-10R in lesions from patients with ML was associated with the exacerbated immune response observed in this clinical form of disease.

Other studies have associated a decrease in the expression of IL-10 receptor with parasite persistence and with an increase in the healing time of lesion [57–59].

5. Conclusion

Our study indicates that the *S. mansoni* antigen Sm29 has the potential to induce a desired regulatory response in CL patients. Recombinant Sm29 induced higher frequency of IL-10 and IL-10R on MoDCs compared to the unstimulated cell cultures. This molecule has a great potential to be used as a therapeutic agent to modulate inflammatory diseases. Our results may contribute to the development of new strategies for the treatment of diseases that are caused by excessive or inappropriate activation of the immune response, such as leishmaniasis.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Helminth Parasites Alter Protection against *Plasmodium* Infection

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More than one-third of the world's population is infected with one or more helminthic parasites. Helminth infections are prevalent throughout tropical and subtropical regions where malaria pathogens are transmitted. Malaria is the most widespread and deadliest parasitic disease. The severity of the disease is strongly related to parasite density and the host's immune responses. Furthermore, coinfections between both parasites occur frequently. However, little is known regarding how concomitant infection with helminths and *Plasmodium* affects the host's immune response. Helminthic infections are frequently massive, chronic, and strong inductors of a Th2-type response. This implies that infection by such parasites could alter the host's susceptibility to subsequent infections by *Plasmodium*. There are a number of reports on the interactions between helminths and *Plasmodium*; in some, the burden of *Plasmodium* parasites increased, but others reported a reduction in the parasite. This review focuses on explaining many of these discrepancies regarding helminth-*Plasmodium* coinfections in terms of the effects that helminths have on the immune system. In particular, it focuses on helminth-induced immunosuppression and the effects of cytokines controlling polarization toward the Th1 or Th2 arms of the immune response.

1. Introduction

Currently, it is estimated that approximately one-third of the almost three billion people who live on less than two US dollars per day are infected with one or more helminths [1]. Human infections with these organisms remain prevalent in countries where the malaria parasite is also endemic [2]. Consequently, coinfections with both parasites occur frequently [3, 4]. These interactions could have potential fitness implications for both the host (morbidity and/or mortality) and the parasite (transmission). Several studies have shown that the ability of a parasite to successfully establish an infection will depend on the initial immune response of the exposed host [5, 6]. When entering the host, a parasite will experience an "immune environment" potentially determined by both previous and current infections [7–9]. It is widely recognized that, in the presence of Th2 effector response, Th1 response is suppressed and vice versa [10]. Thus, Th2-type response evoked in response to helminth infection would in theory have the ability to suppress proinflammatory Th1 response that generates immunopathology in *Plasmodium* infection.

Despite the fact that helminth parasites cause widespread, persistent human infection that results in a Th2 immune response, the influence of helminths on the duration of episodes of malaria in humans is not clear. The questions of how the coexistence of helminths and *Plasmodium* parasites within the same host might influence the immunological responses to each species and whether interactions affect resistance, susceptibility, and the clinical outcome of malaria has yet to be answered.

In this review, we attempt to answer these questions and particularly address whether the preexistence of a Th2/T regulatory response induced by helminths could affect the immune response against *Plasmodium*.

Before analyzing the influence of helminths infection on malaria, we must first briefly outline the immune response to *Plasmodium* infection and later outline the immune response to helminths parasites, as this is important to subsequent analyses of how malaria can be modified by the helminths.

2. *Plasmodium*

Malaria is caused by protozoan parasites belonging to the genus *Plasmodium*; it is transmitted by female *Anopheles* mosquitoes. *Plasmodium* is still one of the most successful pathogens in the world and is a major cause of morbidity and mortality in tropical countries. Five species of *Plasmodium* (i.e., *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, and *P. knowlesi*) are responsible for all human infections [11, 12].

Plasmodium parasites have a complicated, multistage life-cycle involving an *Anopheline* mosquito vector and a vertebrate host. The parasite develops in two stages in its human host: in the liver (the exoerythrocytic stage) and in the blood (the intraerythrocytic stage). The most characteristic features of malaria in humans are a fever that occurs every 48 to 72 h depending on the species of *Plasmodium*, chills, headache, and gastrointestinal symptoms. In a naive, untreated individual, these can rapidly escalate into cerebral malaria (CM), anemia, severe organ failure, and death [13].

2.1. Immune Response during Plasmodium Infection. The immune response to *Plasmodium* is poorly understood; it depends on the parasite species and the specific stage within the host [12]. In addition, it is dichotomized into the preerythrocytic response, which is directed against the sporozoite and liver-stage parasites, and the blood stage response, which is directed against merozoites and intraerythrocytic parasites.

Although animal models do not fully replicate human malaria, they are invaluable tools for elucidating immune processes that can cause pathology and death [14]. Several mouse strains have been used to study the immune response to different combinations of *Plasmodium* species, such as *P. berghei* [15–20], *P. yoelii* [15, 21–24], *P. chabaudi* [25–30], and *P. vinckei* [31] (Table 1). These malarial models suggest that the efficiency of parasite control requires both a humoral and a cellular immune response, most likely in cooperation, although the importance of each is not entirely clear. For example, immunity to the sporozoite depends on antibodies to surface proteins, such as CSP-2 [32, 33] and liver-stage antigen (LSA-1) [34]; these antigens induce the production of antibodies that neutralize or block the invasion of hepatocytes [35]. Once sporozoites have entered the hepatocyte, the parasite clearance in mice requires $CD8^+$ T cells [36], natural killer cells (NK), and NKT and $\gamma\delta$ T cells that produce IFN-γ to eliminate infected hepatocytes [35]. When the parasite invades red blood cells (RBC), it dramatically alters the physiological and biochemical processes of its host cell. Parasite-infected RBCs (pRBC) express parasite-encoded molecules on their surface that affects the RBCs' mobility and trafficking within the body. The parasite biomass increases very rapidly and activates innate immune mechanisms, including NK cells and $\gamma\delta$ T cells [13].

NK cells play an important role in restricting parasite replication. The absence of NK cells is associated with low IFN-γ serum levels and increased parasitemia in mice infected with *P. chabaudi* [37]. Likewise, the absence of IFN-γ reduces the ability of mice to control and eliminate parasites, eventually resulting in the death of the animals [38, 39]. Interestingly, macrophages (Mφ), but not IFN-γ, play a major role in the control of early peaks in lethal infections with *P. yoelii* [40]. In addition, IFN-γ produced by $CD4^+$ T cells plays a pivotal role in protective immunity against non-lethal strains of *Plasmodium* [41, 42]. In contrast, the infection with *P. berghei* ANKA induces high levels of IFN-γ and TNF-α which are associated with cerebral malaria [43]. However, the peak of parasitemia in athymic mice tends to be similar to the peak in WT mice. These results suggest that extrathymic T cells are the major lymphocyte subset associated with protection against malaria [44].

In a resistant strain of mice, the presence of the parasite induces the production of proinflammatory cytokines, such as IL-1β, IL-6, TNF-α, and IFN-γ. Furthermore, IL-12 is also necessary for elimination of *P. chabaudi* AS [45], *P. berghei* XAT [20], and *P. yoelii* XNL [46].

Besides, the inflammatory cytokine MIF (macrophage migration inhibitory factor) induces pathogenesis and susceptibility on BALB/c mice infected with *P. chabaudi*, high serum levels of MIF correlated with severity of disease [47]. In addition, infection of MIF knockout mice with *P. chabaudi* increases survival [48].

$CD4^+$ T cells, together with B cells, are crucial to develop efficient protection in murine experimental models [49, 50]. Whereas IFN-γ, produced by TCD4+, activates Mφ-mediated responses [51], the antibodies produced by B cells inhibit invasion of RBCs by the parasites [52], opsonize parasitized RBCs, block pRBC adhesion to the vascular endothelium, and neutralize parasite toxins [35]. In addition, mice rendered B cell deficient by treatment with anti-μ antibodies or B cell knockout mice (μMT) are unable to clear the erythrocytic infection of *P. chabaudi* [50, 53, 54]. Specifically, the early acute infection is controlled to some extent, giving rise to chronic relapsing parasitemia that cannot be cleared. Finally, parasitemia can be reduced by adoptive transfer of B cells [50].

Antibodies also induce pathology due to parasite antigens that are freed and adhere to healthy erythrocytes; this generates anemia or autoimmune reactions that cause damage to the kidneys and other tissues [55–58]. For example, pathogenesis of malaria nephropathy is linked to subendothelial deposits of immune complexes containing IgG and IgM [59, 60]. The antibodies involved in the elimination of the parasite mainly belong to cytophilic subclasses (IgG1 and IgG3) [50, 61]. In addition, high levels of immunoglobulin E (IgE) correlate with protection against severe malaria [62–64].

Interestingly, after the peak of parasitemia, cellular immune responses should switch from Th1- to Th2-type response in *P. chabaudi* infected mice [65], because the malaria pathogenesis is caused by inappropriate or excessive inflammatory responses to eliminate the parasite [43, 66].

Table 1: Mouse models of malaria infection. ECM: experimental cerebral malaria, PvAS: *P. vinckei* petteri arteether sensitive, PvAR: *P. vinckei* arteether resistant, *Py*: *P. yoelii*, and KO: knockout.

Species	Subspecies: clone	Mouse strain and anemia	Mouse strain and CM	Useful in research	Ref.
P. berghei	*P. berghei ANKA*	C57BL/6: lethal CD-1: lethal C57BL/6J: non-lethal BALB/c: lethal	C57BL/6: susceptible CBA: susceptible BALB/c: resistant	Used as a model of ECM; there is genetic variation in the development of ECM between inbred strains	[15–17]
	P. berghei K173	C57BL/6: lethal		Used to study pathogenesis; differs in some aspects of pathogenesis, indicating the influence of parasite genetic variation	[18]
	P. berghei NK65	C57BL/6: lethal		Is a murine noncerebral malaria strain; induces a progressive increase in parasitemia, intense hepatic inflammation, and death	[19]
	P. berghei XAT	Spontaneously cleared in immune competent mice		Irradiation-induced attenuated variant from lethal strain *Pb* NK65; comparison of immune responses induced by these lethal and attenuated parasites lead us to elucidate the mechanisms of protective immunity and pathogenesis	[20]
P. yoelii	*P. yoelii 17 NXL*	BALB/c: non-lethal	Most strains resistant	Used to study immune mechanisms and pathogenesis; *Py*; line A1 is a mild line which is restricted to reticulocytes	[15]
	P. yoelii 17XL	BALB/c: lethal C57BL/6: lethal	Most strains susceptible	Used to identify vaccine-induced immune response	[21, 22]
	P. yoelii YM	CBA: lethal		*Py*-YM is virulent infection which multiplies in both immature and mature erythrocytes	[23]
	P. yoelii YA	CBA: non-lethal		YM parasites are responsible for normocyte invasion, increased virulence compared to mild line *Py* YA parasites; lines YM and A/C differed additionally in enzyme and drug-sensitivity markers	[24]
P. chabaudi	*P. chabaudi AS*	A/J: lethal C57BL/6: non-lethal BALB/c: non-lethal	C57BL/6 IL-10KO: susceptible	Used to study immune mechanisms and immunoregulation by cytokines, to identify susceptibility loci, and to study the immune basis of pathology	[25–28]
	P. chabaudi AJ	BALB/c: non-lethal		Used to study experimental vaccines and immunological processes that control hyperparasitaemia	[25, 27]
	P. chabaudi adami DS	C3H: lethal C57BL/6: non-lethal		Is fast-growing and high pathogenicity, induces more anemia, weight loss, and is less infective to mosquitoes than DK strain	[29, 30]
	P. chabaudi adami DK	BALB/c: non-lethal C3H: non-lethal		Is slower growing and less pathogenic and more selective in its invasion of subset of RBCs than DK	[29, 30]
P. vinckei	*P. vinckei vinckei*	BALB/c: lethal AKR: lethal		Used to study pathogenesis and for chemotherapy studies; it causes aggressive, overwhelming hyperparasitaemia	[31]
	P. vinckei petteri	AKR: lethal (PvAS) AKR: non-lethal (PvAR)		Used for drug screening and immunological studies	[31]

Interestingly, *Plasmodium* can modulate the response of antigen presenting cells, such as Mφ and dendritic cells (DC), which leads to suppression of the immune response [67]. In the infection with *P. yoelii* YM, the DC function is affected by the presence of TNF-α [68]. Wykes et al. suggested that damage to the activity of DCs is due to a virulence factor that is present in certain parasite strains because, when DCs were transferred from mice infected with a "nonlethal" strain to mice infected with a "lethal" parasite strain, the mice were protected [46].

The regulatory T cells are extremely important to control the inflammatory process in malaria, the number of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Treg) increases in mice infected with *P. yoelii* [69] or *P. berghei* [70]. In addition, mice infected with the lethal *P. yoelii* XL17 show higher levels of IL-10 and TGF-β compared to mice infected with the nonlethal strain *P. yoelii* XNL, at early time points during infection [71]. Furthermore, the suppression of T cells induces lethality in mice infected with *P. yoelii*, while neutralization of TGF-β and IL-10 decreases parasitemia and prolongs the survival of infected mice [71, 72]. Accordingly, Couper et al. reported that the main sources of IL-10 in lethal infection with *P. yoelii* are Treg cells [73]. Finally, the ablation of Treg cells from *P. yoelii*-infected DEREG-BALB/c mice significantly increases T cell activation and decreases parasitemia [74]. In addition, in mice infected with nonlethal strains of *P. yoelii*, the presence of cytokines such as IL-10 and TGF-β during the chronic phase of infection was detected [71]. Thus, these data together suggest that the outcome of malaria infection could be determined by the balance of proinflammatory and regulatory immune responses, which could inhibit pathology (Figure 1).

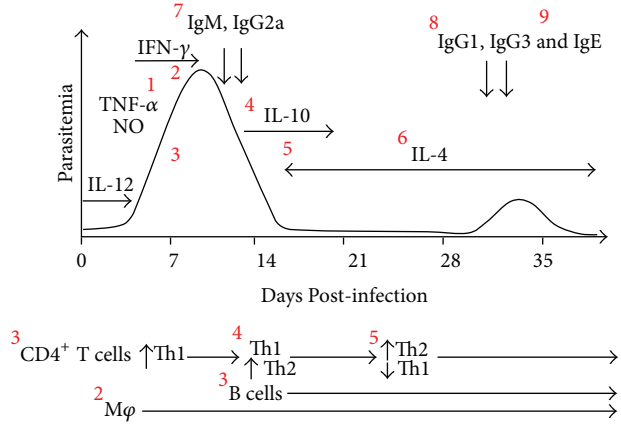

Figure 1: Representation of the course of *Plasmodium chabaudi* infection. Early infection with the erythrocytic stage is characterized by the production of proinflammatory cytokines, such as IL-12 and TNF-α, and a pronounced IFN-γ response. In addition, NO produced by Mφ helped control parasitemia (1). IFN-γ activates Mφ-mediated responses, in particular phagocytosis and elimination of pRBC (2). $CD4^+$ T cells, together with B cells, are crucial for developing efficient protection (3). Th1 production is downregulated later by an increased Th2-type immune response following primary infection (4). In a later stage of infection, after the peak parasitemia has been reached, CD4 T cells switch from a Th1 to a Th2 cytokine profile (5). This switch helps B cells produce antibodies (6). The antibodies inhibit the invasion of RBCs by the parasites, opsonize parasitized RBCs, or block pRBC adhesion to the vascular endothelium (7, 8). The slow late switch from noncytophilic (IgM and IgG2a) (7) to cytophilic subclasses (i.e., IgG1 and IgG3) (8) is involved in parasite elimination (9). However, IgE correlates with protection against severe malaria. Figure modified from Langhorne et al. 2004 [75] and Stevenson and Urban 2006 [67].

3. Helminths

Helminths are multicellular worms, some of which have adapted successfully to a parasitic lifestyle. They can be classified into three taxonomic groups: cestodes (e.g., *Taenia solium*), nematodes (e.g., *Ascaris lumbricoides*), and trematodes (e.g., *Schistosoma mansoni*). Helminths vary in their biology in terms of size, lifecycle, and the diseases they cause. However, despite this complexity, helminths usually cause asymptomatic and chronic infections [76]. Helminths are among the most widespread infectious agents in human populations, especially in developing countries; they affect more than a third of the world's population, and more than 20 species infect humans (Table 2) [1, 77–83].

3.1. Immune Response during Helminth Infections. Infection of mammals by helminth parasites typically results in a conserved series of immune events that are orchestrated and dominated by T helper cell type (Th2) events, characterized by the activation of eosinophils, basophils, and mast cells; high levels of immunoglobulin E (IgE); and the proliferation of T cells that secrete IL-4, IL-5, IL-9, and IL-13 [84, 85]. Despite this response, helminths are able to modulate and suppress the host immune response to promote their own survival and their persistence in the host for a long time, resulting in chronic infection [76, 86, 87]. These mechanisms include the ability to induce regulatory responses via regulatory T cells (Treg) which express molecules that inhibit the immune response, such as glucocorticoid-induced TNF-R-related protein (GITR) and the receptor cytotoxic T lymphocyte antigen 4 (CTLA-4) [88–91]. Treg cells also secrete suppressive cytokines, such as IL-10 and TGF-β [92]. On the other hand, B regulatory cells (Breg) also contribute to immune modulation and can release IL-10 and restrict proinflammatory responses [93]. Helminths also induce the differentiation of anti-inflammatory Mφ, called alternatively activated Mφ (AAMφ) [94, 95], as well as regulatory dendritic cells (DCreg), which are characterized by the expression of the regulatory cytokines IL-10 and TGF-β [96, 97] (Figure 2).

This anti-inflammatory or regulatory response could be potentially detrimental to the host if it interferes with the development of protection against other infections that require an inflammatory response, such as *Leishmania major* [9, 98] or *Trypanosoma cruzi* [8].

The hyporesponsive immune response induced during chronic helminth infection affects not only the response to helminth antigens but also to other antigens. Several studies have examined the effect of infections on the immune response to other unrelated antigens. In particular, it has been

Table 2: Prevalence of common helminths in the world. These are estimates of the number of people with active infections. The number of people potentially exposed or with subclinical helminthic infections is much higher.

	Helminth	Estimated number of infected people	Ref.
Nematodes	*Ascaris lumbricoides*	1450 billion	[77]
	Trichuris trichiura	1050 million	[77]
	Ancylostoma duodenale	740 million	[78]
	Trichinella spiralis	600 million	
	Necator americanus	576 million	[1]
	Brugia malayi	157 million	[1]
	Wuchereria bancroftiand Brugia malayi	120 million	[79]
	Strongyloides stercoralis	100 million	[80]
	Onchocerca volvulus	37 million	[81]
	Loa loa	13 million	[1]
Trematodes	*Schistosoma spp.*	207 million	[82]
	Fasciola hepatica	17 million	
Cestodes	*Taenia spp.*	0.4 million	[81]
	Hymenolepis nana	75 million	
	Echinococcus spp.	2–3.6 million	[83]

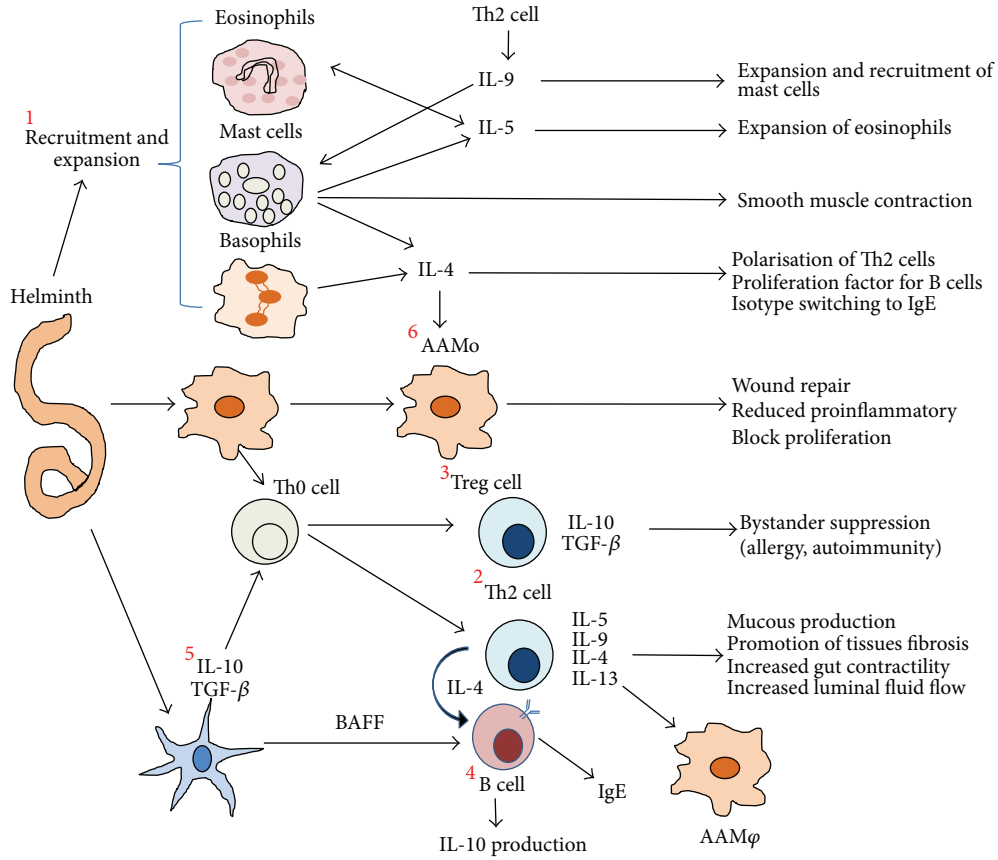

Figure 2: Helminth infections are strong inducers of a Th2-type immune response. These infections are characterized by the expansion and activation of eosinophils, basophils, and mast cells (1). Their upregulation due to high levels of immunoglobulin E (IgE) and the proliferation of T cells that secrete IL-4, IL-5, IL-9, and IL-13 are part of the host immune response against the parasite (2). However, helminth infections tend to be long-lived and largely asymptomatic because helminth infections are sustained through a parasite-induced immunomodulatory network, in particular through activation of regulatory T cells (3) and systemically elevated levels of IL-10 produced by B regulatory cells (4). They are additionally affected by the expression of the regulatory cytokines IL-10 and TGF-β, produced by regulatory dendritic cells (5) and alternatively activated Mφ (AAMφ) (6).

shown that the response to vaccines can be modified by the presence of concomitant helminth infection. For example, chronic *Onchocerca* infection [99], *Lymphatic filariasis* [42], or *Schistosoma* [100] reduces the effectiveness of the tetanus vaccine. Likewise, chronic *Onchocerca* infection affects *Bacillus Calmette-Guérin* and *Rubella* vaccinations [101]. Similarly, *Ascaris lumbricoides* reduces the response to the oral cholera vaccine, which can be restored by albendazole treatment [102]. However, helminthic infections are beneficial in the control of excessive inflammatory reactions, such as Crohn's disease [103] and ulcerative colitis [104], as well as in allergic diseases [105–107] and autoimmune diseases, such as encephalomyelitis [108, 109] and arthritis [110].

Despite the widespread acceptance that helminthic infections influence each other directly or indirectly, little attention has been paid to helminth-*Plasmodium* coinfections. One reason is that the interactions involved are complex and difficult to understand. Here, we will try to discuss several reports about helminth-malaria coinfections to clarify the consequences of this interaction.

4. Human *Plasmodium*-Helminth Coinfection

Plasmodium spp. infect between 349 and 552 million people and kill over one million each year; approximately 40% of the world's population is at risk of being infected [2, 111]. Importantly, people living in malaria-endemic regions are exposed to other pathogens, especially those associated with poverty, such as helminths.

Several studies have been carried out to explore the influence of helminths on *Plasmodium* infection in humans (Table 3). However, the evidences described in these researches are controversial. While some studies have reported that helminth infection favors protection because reduces the *Plasmodium* parasite density [112], promotes protection against clinical malaria [113, 114], reduces anemia [113, 115, 116], cerebral malaria [117] and renal failure [118] (Table 3(a)). Other studies showed no influence of helminths on the curse of *Plasmodium* infection [119–121] (Table 3(b)). In contrast, others showed an increased susceptibility to *Plasmodium* infection [114, 122], increased risk of complications [123–125], anemia [125, 126], hepatosplenomegaly [127, 128], and increased *Plasmodium* parasite load [129, 130] (Table 3(c)).

Although a Th2 phenotype is a conserved response to helminth infection in human and mice, the nature of the host immune response varies considerably between species of helminths; in some cases Th1 immune response predominates, depending on both the time of infection and the helminth development stage [131, 132]. The time that Th1 immune response is sustained until it polarizes toward Th2, could vary between species [133–135]. Thus, the controversial results related to helminth-*Plasmodium* coinfection in humans could be explained because many studies did not consider critical features of the helminth parasite biology. For example, the biological niche or parasite stage within the host. Neither the previous time of infection with the helminth nor the nutrition state and age of the host were taken into account.

Because all of these variables were not considered in existing studies in humans and in order to establish a possible consensus, we review in detail the murine *Plasmodium-helminth* coinfections, which in theory, controlled variables more rigorously.

5. Experimental Models of Coinfection

Although helminth infections in mice are a questionable model for chronic helminth infections in humans, the fact is that many intraintestinal helminths can reach large biomass which can change the cytokine environment and therefore the possible mechanisms of response. By establishing chronic infections and inducing strong Th2-type responses, helminths could have a potentially significant influence on the nature of the immune response in infected individuals and hence modify their susceptibility to subsequent infections with other important pathogens, at least those that require a Th1-type or mixed Th1-/Th2-type immune response, such as *Plasmodium* sp.

5.1. Schistosoma-Plasmodium Coinfection. According to the theory that Th2-type response evoked in response to helminth infection would have the ability to suppress proinflammatory Th1 response that generates immunopathology in *Plasmodium*-infected individuals, there are some reports of experimental models of coinfection with *Plasmodium berghei* ANKA (*Pb*) after *Schistosoma mansoni* (*Sm*) infection in ICR mice or with *Schistosoma japonicum*- (*Sj*-) *Pb* in C57BL/6 mice 7 or 8 weeks after helminthic infection, respectively; both coinfections showed a delay in death of mice [136, 137]. Interestingly, there was a reduction in the brain pathology associated with high levels of the anti-inflammatory cytokines IL-5, IL-10, and IL-13 [136–138].

In contrast, similar coinfection with *Pb* 7 or 8 weeks after *Sm* infection showed an increase in mortality and parasitemia in Swiss albino and C57BL/6 mice [138, 139]. Moreover, the coinfections in Swiss albino mice reduced the effectiveness of antimalarial treatments and delayed elimination of the parasite [139] (Table 4(a)). In these reports neither evidence of immune response nor pathology data were shown. Thus, we speculate that increase parasite load was probably due to the presence of helminth than inhibited Th1-type immune response which was able to contain the replication of *Plasmodium*, and the increased mortality was due to parasite load rather than a pathological Th1-state dependent. In line with this hypothesis, coinfection with *Plasmodium chabaudi* (*Pc*) at 8 weeks after *Sm* infection in C57BL/6 mice allowed high *Pc* replication. This increase was associated with low levels of the proinflammatory TNF-α [140].

It is known that the immune response against *Schistosoma* shifts from an early helminth-protective Th1-type immune response to a late helminth-permissive Th2-type response during the course of infection [134]. Thus, the moment when the second infection is acquired (2, 4 and 6 weeks post-helminth infection) would be critical for disease outcome and pathology [141]. These findings could be supported by the fact that chronically *Sm*-infected BALB/c mice coinfected (6 weeks) with the nonlethal strain *Plasmodium yoelii* NXL

Table 3: Human studies of coinfection. ARF: acute renal failure, MSM: moderately severe malaria, *S*: *Schistosoma, A*: *Ascaris*, and *T*: *Trichuris*.

(a)

Study area	Age of group	Sample (size)	Study design	Helminth type	Outcome for malaria diseases in coinfection	Ref.
Senegal (Niakhar)	Children	178	Over a 2-year followup period	*S. haematobium*	Children with a light *S. haematobium* infection presented lower *P. falciparum* parasite densities than children not infected by *S. haematobium*	[112]
Mali (Tieneguebougou and Bougoudiana)	Children and young adults	62	Followed prospectively through a malaria transmission season	*Wuchereria bancrofti Mansonella perstans*	Pre-existent filarial infection attenuates immune responses associated with severe malaria and protects against anemia, but has little effect on susceptibility to or severity of acute malaria infection	[113]
Southern Ethiopia	1 to 82 years Mean 18.6 years	1,065 febrile patients	Cross-sectional	*A. lumbricoides T.trichiura, S. mansoni,* and hookworm	The chance of developing non-severe malaria were 2.6–3.3 times higher in individuals infected with helminth, compared to intestinal helminth-free individuals The odds ratio for being infected with non-severe *P. falciparum* increased with the number of intestinal helminth species	[114]
South-central Côte d'Ivoire	Infants (6–23 months), children (6–8 year), and young women (15–25 years)	732 subjects	Cross-sectional survey	Soil-transmitted helminth	Coinfected children had lower odds of anemia and iron deficiency. Interaction between *P. falciparum* and light-intensity hookworm infections vary with age.	[115]
Brasil (Careiro)	School children 5 to 14 years	236	Cohort and cross-sectional	*A. lumbricoides* hookworm and *T. trichiura*	Helminthes protect against hemoglobin decrease during an acute malarial attack by *Plasmodium.*	[116]
Thailand (Bangkok)	Mean 24 years (range 15–62)	537 files	*Retrospective* case-control	*A. lumbricoides*	Percentage protection for mild controls against cerebral malaria ranged from 40% for *Ascaris* (present/absent) to 70% for *Ascaris* medium infection. For intermediate controls protection against cerebral malaria was 75% for *Ascaris* (present/absent).	[117]
Thailand (Bangkok)	19–37 years 22 patients with malaria-associated ARF and 157 patients with MSM	179	*Retrospective* case-control	*A. lumbricoides, T. trichiura,* hookworm, and *Strongyloides stercoralis*	Helminths were associated with protection from renal failure Helminth-infected controls were less likely to have jaundice or to have peripheral mature schizonts than controls without helminths	[118]

(b)

Study area	Age of group	Sample (size)	Study design	Helminth type	Outcome for malaria diseases in coinfection	Ref.
Kenya (Kingwede)	8 years and older	561	cross-sectional	*S. haematobium*	Children had 9.3 times the odds of coinfection compared to adults	[119]
Nigeria (Osun)	preschool children (6–59 months)	690	Double-blind and randomized	*A. lumbricoides*	There was no significant difference in the severity of anaemia.	[120]

(b) Continued.

Study area	Age of group	Sample (size)	Study design	Helminth type	Outcome for malaria diseases in coinfection	Ref.
Kabale, Uganda	All ages (856)	856	Retrospective; 18 months	*A. lumbricoides, T. trichiura,* and hookworm	Non evidence for an association and risk of malaria	[121]

(c)

Study area	Age of group	Sample (size)	Study design	Helminth type	Outcome for malaria diseases in coinfection	Ref.
Senegal (Niakhar and Bambey)	Children, mean 6.6 years	105	Prospective case-control	*A. lumbricoides*	Prevalence of *A. lumbricoides* infection was higher in cases of severe malaria	[123]
Northern Senegal	Children aged 6–15 years	512	Cohort	*S. mansoni*	The incidence rate of malaria attacks was higher among *S. mansoni*-infected individuals carrying the highest worm loads. In contrast, the rate of malaria attacks were lower in medium grade *S. mansoni* infections	[124]
Ghana (Kumasi)	Women (15–48 years) mean 26.8 years	746	Cross-sectional	*A. lumbricoides, T. trichiura, S. stercoralis,* and *E. vermicularis*	Coinfection resulted in increased risks of anemia, low birth weight, and small for gestational age infants	[125]
Ethiopia (Alaba Kulito)	Children <5 years, children 5–14 years, and adults ≥15 years	1802 acute febrile patients	case-control	Hookworm, *A. lumbricoides*, and *T. trichiura*	Coinfection is associated with higher anaemia prevalence and low weight status than single infection with *Plasmodium* in children	[126]
Kenya (Makueni)	Primary school children 4–17 years	(221 and 228)	Cross-sectional	*S. mansoni*	Hepatosplenomegaly due to proinflammatory mechanism exacerbated by schistosomiasis	[127]
Kenya (Mangalete)	Children 4–17 years	79	Cross-sectional	*S. mansoni*	Hepatosplenomegaly is associated with low regulatory and Th2 response to *Schistosome* antigens	[128]
Zimbabwe (Burma Valley)	Children 6–17 years	605	12-month followup of a cohort of children	Schistosome	Increased prevalence of malaria parasites and had higher sexual stage malaria parasite in children coinfected with schistosomiasis	[129]
Cameroon (Bolifamba)	9 months to 14 years	425 children		*A. lumbricoides*, *T. trichiura*, and hookworm	Coinfections in which heavy helminth loads showed high *P. falciparum* parasite loads compared with coinfections involving light helminth burden	[130]

Table 4: Helminthic infection drives immune response to challenge with *Plasmodium*. *The time after helminth infection when the *Plasmodium* challenge was performed. ND: nondetermined, wks: weeks, KO: knockout, ECM: experimental cerebral malaria.

(a)

Background mouse	*Plasmodium* strain	Helminth type	Coinfection time*	Malaria disease outcome	Ref.
ICR HSD	*P. berghei* ANKA	*S. mansoni*	7 wks	Low rates of ECM (30%), delay in death associated with high levels of IL-4, IL-10	[136]
C57BL/6	*P. berghei* ANKA	*S. japonicum*	8 wks	Increased survival rate and reduction of the brain pathology. Th2 response induced by worm plays an important role in protecting against ECM	[137]
C57BL/6	*P. berghei* ANKA	*S. mansoni*	8-9 wks	Increased parasitemia, mortality, weight loss, and hypothermia; decreased pathology in the brain associated with high levels of IL-5, IL-13 and low serum IFN-γ	[138]
Swiss albino	*P. berghei* ANKA	*S. mansoni*	7 wks	Increased parasitemia and mortality Delayed reduction/elimination of the parasite followed by administration of antimalarial treatment	[139]
C57BL/6	*P. chabaudi*	*S. mansoni*	8 wks	Increased parasitemia associated with a deficiency in the production of TNF-α	[140]
BALB/c	*P. yoelii* NXL (non-lethal)	*S. mansoni*	2, 4, and 6 wks	Increased parasitemia and death at 6 wks of coinfection. Hepatosplenomegaly was more marked in coinfected mice compared to either disease separately	[141]
A/J	*P. chabaudi*	*S. mansoni*	8 wks	Mice escape death and showed high production of IFN-γ	[142]

(b)

Background mouse	*Plasmodium* strain	Helminth type	Coinfection time*	Malaria disease outcome	Ref.
C57BL/6	*P. chabaudi*	*H. polygyrus*	2, 3, or 5 wks	Increased parasitemia and mortality associated with low levels of IFN-γ and high levels of TGF-β, IL-10	[143]
C57BL/6	*P. chabaudi AS*	*H. polygyrus*	2 wks	Increased parasitemia; however, it ameliorates severe hypothermia and hypoglycaemia; besides this, it induced earlier reticulocytosis than *Pc*-infected WT mice	[144]
C57BL/6 IFN$^-$/_ IL-23$^-$/_	*P. chabaudi AS*	*H. polygyrus*	At the same time	Increased mortality and severe liver disease, associated with increased IFN-γ, IL-17, and IL-22 in the liver. The coinfected IFN$^-$/_ and IL-23$^-$/_ mice survive	[145]
C57BL/6 BALB/c	*P. chabaudi AS*	*H. polygyrus*	2 wks with AgPc + adjuvant	Suppresses the protective efficacy of the malaria vaccine. Deworming treatment before antimalarial immunization restored the protective immunity to malaria challenge	[146]
C57BL/6	*P. yoelii* 17 XNL	*H. polygyrus*	2 wks	Increased pathology due to reduced response against *Py* (low levels of IFN-γ) in the spleen cells, as a result of higher activation of Treg	[147]
BALB/c	*P. yoelii* 17 NXL	*H. polygyrus*	3 wks	Reduction of pathology, low levels of IFN-γ, and high levels of IL-4 induced by helminthes	[148]
C57BL/6	*P. berghei* ANKA	*H. polygyrus*	2 wks	*Hp* infection did not alter ECM development, despite accelerated *P. berghei* growth *in vivo*	[149]

(b) Continued.

Background mouse	*Plasmodium* strain	Helminth type	Coinfection time*	Malaria disease outcome	Ref.
C57BL/6 BALB/c	*P. berghei* ANKA	*H. polygyrus*	2 wks	No differences	[150]

(c)

Background mouse	*Plasmodium* strain	Helminth type	Coinfection time*	Malaria disease outcome	Ref.
BALB/c	*P. yoelii* 17 NXL	*E. caproni*	3 wks	*Ec* showed counterregulatory antiparasite cytokine responses to non-lethal strain *Py*NXL (less IFN-γ and high IL-4 levels induced by *Ec*)	[148]
BALB/c	*P. yoelii* 17 NXL	*E. caproni*	5 wks	Increased mortality and pathology; the pathology was reversible through clearance of *Ec* by praziquantel treatment	[151]
BALB/c	*P. yoelii* 17XL	*E. caproni*	5 wks	*Ec* does not alter the course of *Py*17XL infection	[151]

(d)

Background mouse	*Plasmodium* strain	Helminth type	Coinfection time*	Malaria disease outcome	Ref.
C57BL/6	*P. yoelii* 17NXL	*Strongyloides ratti*	1 wk	Did not altered cytokine response	[152]
BALB/c	*P. berghei* ANKA	*Strongyloides ratti*	1 wk	The coinfection did not change the efficacy of vaccination against *Pb*	[153]

(e)

Background mouse	*Plasmodium* strain	Helminth type	Coinfection time*	Malaria disease outcome	Ref.
BALB/c	*P. chabaudi*	*Nippostrongylus brasiliensis*	Same day	Reduction of anemia and parasitemia. Th2 response was inhibited by *Plasmodium*	[154]
C57BL/6	*P. berghei*	*Nippostrongylus brasiliensis*	3 wks	Delayed peak parasitemia, increased survival	[155]

(f)

Background mouse	*Plasmodium* strain	Helminth type	Coinfection time*	Malaria disease outcome	Ref.
BALB/c	*P. chabaudi*	*L. sigmodontis*	8 wks	Increased severity of the anemia and weight loss associated with increased IFN-γ	[156]
C57BL/6 IL-10KO	*P. berghei* (ANKA)	*L. sigmodontis*	8 wks	Reduction of ECM associated with increased IL-10 IL-10KO mice coinfected with *Pb-Ls* die of ECM	[157]
BALB/c	*P. berghei* ANKA	*L. sigmodontis*	2 wks	Reduced protection against *P. berghei* challenge infection for low frequencies of CSP-specific CD8 T cells, CSP-specific IFN-γ and TNF-α production	[153]

(g)

Background mouse	*Plasmodium* strain	Helminth type	Coinfection time*	Malaria disease outcome	Ref.
CBA	*P. berghei* (ANKA)	*Brugia pahangi* irradiated attenuated	1 wk	Increased survival and protected them against the ECM development; increase synthesis of IFN-γ, IL-4, IL-5, and IgE	[158]

(h)

Background mouse	*Plasmodium* strain	Helminth type	Coinfection time*	Malaria disease outcome	Ref.
C57BL/6	*P. berghei*	*Trichinella spiralis*	1–4 wks	Partially subdued parasitaemia and prolonged survival	[159]

(*Py*NXL) showed high mortality. In contrast, no mortality was observed in acutely (2 or 4 weeks) coinfected mice, although they developed high parasitemia and hepatomegaly was higher in coinfected mice compared with mice infected with each parasite separately [141] (Table 3(a)). Therefore, the time of previous infection may influence the response against *Plasmodium*.

Together, these reports suggested that the Th2 response, induced by *Schistosoma*, plays an important role in protecting against immunopathology in cerebral malaria. However, the presence of *Schistosoma* does not appear to modify the virulence of *Pb* and, consequently, it does not alter the lethality of *Plasmodium* infection.

Finally, one report scape to the theory that Th2-type immune response evoked by the helminth infection would possess the ability to suppress the proinflammatory Th1-type response in its host. *Sm*-infected A/J mice coinfected at 8 weeks with *Pc* were protected by the presence of concomitant *Sm* infection. The mice escaped death due to malaria; this effect was accompanied by enhanced levels of IFN-γ [142] (Table 4(a)).

5.2. Heligmosomoides polygyrus-Plasmodium Coinfection. Several studies used mice of the same genetic background. Additionally, equivalent helminth and *Plasmodium* strains have been used to explain whether previous helminthic infection plays an important role in the immune response against *Plasmodium*. Su et al. showed that C57BL/6 mice previously infected with *Heligmosomoides polygyrus* (*Hp*) and challenged with *Pc* either 3 or 5 weeks after helminthic infections developed high *Pc*-parasitemia and mortality, which was associated with low levels of IFN-γ and high levels of TGF-β and IL-10 [143]. However, *Hp-Pc* coinfection at 2 weeks resulted in less severe pathology (i.e., less hypothermia and hypoglycemia) and induced earlier reticulocytosis compared with mice infected only with *Pc* [144] (Table 4(b)).

Helmby in 2009 showed that mice developed high mortality in the *Hp-Pc* model when the two infections were introduced simultaneously. The mortality was due to severe liver pathology associated with increased IFN-γ, IL-17, and IL-22. Interestingly, when using an IFN-γ and IL-23 knockout strain, the mice survived the coinfection [145]. Thus, simultaneous *Hp-Pc* coinfection increased mortality, which may be a consequence of a synergistic effect that increased the inflammatory response (Table 4(b)).

In fact, in the first case, in which *Hp-Pc* coinfection was performed at 3 or 5 weeks after the initial helminthic infection, the high mortality observed may have been due to the anti-inflammatory response generated by the previous helminthic infection, which inhibited the inflammatory response necessary for control of the *Plasmodium* infection. However, when the coinfection was performed at the same time, mice developed a stronger inflammatory response, which generated greater pathology and mortality. This susceptibility is supported by the observation that chronic helminthic infection suppresses effective vaccine-induced protection against *Plasmodium*. However, when mice were administered with antihelminthic *Hp* treatment before malaria vaccination, the protective immunity against *Pc* was restored [146]. Therefore, the timing of the infection with *Hp* plays an important role in the type of immune response that is generated within the host, and it determines the susceptibility following challenge with *Plasmodium*.

The genetic background of mice infected with helminths has a crucial role in the outcome of the immune response to *Plasmodium*. For example, coinfection with the nonlethal *Py*NXL strain at 2 weeks after *Hp* infection in C57BL/6 mice resulted in exacerbated pathology and poor survival of mice. This susceptibility was associated with a reduced response against *Py*NXL (i.e., low levels of IFN-γ) in the spleen cells. As a consequence, it increased the activation of Treg cells [147]. However, the same coinfection at 3 weeks in BALB/c mice decreased the pathology associated with low levels of IFN-γ and increased levels of IL-4, but not IL-10 [148]. Therefore, the genetic background of mice infected with the helminth determines the outcome of *Py*NXL infection (Table 4(b)).

What happens when a lethal strain of *Plasmodium* was used in coinfection with *Hp*? The *Pb* ANKA infection in C57BL/6 mice induced typical symptoms of ECM [160, 161]. Coinfection with *Hp-Pb* ANKA 2 weeks after initial helminthic infection did not modify the development of ECM despite accelerated *Pb* growth *in vivo* [149]. Likewise, other results from the same model of coinfection in BALB/c and C57BL/6 mice showed no differences in parasitemia, anemia, or body weight in relation to mice infected only with *Plasmodium* [150]. Therefore, *Hp* infection does not affect the outcome of *Pb* ANKA (Table 4(b)).

5.3. Echinostoma caproni-Plasmodium Coinfection. Studies in BALB/c mice infected for 3 weeks with *E. caproni* (*Ec*) and then coinfected with the nonlethal strain *Py*NXL showed that exacerbation of *Plasmodium*-induced pathology was associated with a deficit in IFN-γ production [148]. Similarly, when *Ec*-infected mice were coinfected at 5 weeks, increased mortality was observed. The exacerbated pathology was reversible through the clearance of *Ec* worms via praziquantel treatment [151]. However, coinfection at 5 weeks with the lethal *Py*XL strain did not alter the course of infection; all mice infected with *Py*XL (i.e., alone, in combination with *E. caproni*, or praziquantel treated) died on day 10 after infection [151] (Table 4(c)). Therefore, *Ec* infection does not affect the outcome of lethal *Py*XL, but *Ec* infection affects the protective response against a nonlethal *Plasmodium* strain.

5.4. Strongyloides ratti-Plasmodium Coinfection. Murine *Strongyloides ratti* (*Sr*) infection is a transient helminthic infection that is resolved spontaneously within 3-4 weeks. This infection induces a strong Th2-type immune response at day 6 after infection [135]. When BALB/c mice were coinfected with the nonlethal strain *Py*NXL at day 6 after *Sr* infection, *Sr* induced a slightly enhanced peak of *Plasmodium* parasitemia and loss of body weight. In contrast, in C57BL/6 mice coinfected at day 6, parasitemia level and body weight were not altered. Interestingly, the Th2-type immune response induced by *Sr* was significantly reduced upon *Py*NXL coinfection [152]. In addition, *Py*NXL clearance was not affected by previous infection with *Sr* in either C57BL/6 or BALB/c mice. Moreover, infection with *Sr* in BALB/c

mice did not change the efficacy of vaccination against *Pb* ANKA [153]. Therefore, infection with *Sr* does not affect the protective response against *Plasmodium*, although it generates small changes in parasitemia levels; which is not decisive for the outcome of *Plasmodium* infection (Table 4(d)).

5.5. Nippostrongylus brasiliensis-Plasmodium Coinfection. BALB/c mice infected with *Nippostrongylus brasiliensis* (*Nb*) exhibit a strong Th2-type immune response [162]. Even so, when BALB/c mice were coinfected with *Nb* and *Pc* simultaneously, the Th2 response against *Nb* was impaired by *Plasmodium*. Interestingly, the *Nb-Pc* coinfection had a beneficial effect; it slightly ameliorated the severity of malarial anemia (SMA) and decreased parasitemia levels [154]. Similarly, C57BL/6 mice infected for 3 weeks with *Nb* and then coinfected with *Pb* showed a delayed peak parasitemia and an increased survival time [155]. Thus, the presence of concomitant *Nb* infection plays an important role in inhibiting pathology associated with a challenge with *Pc* or *Pb* (Table 4(e)).

5.6. Coinfection with Other Helminths. Experimental models of coinfection with *Litomosoides sigmodontis* (*Ls*) 8 weeks and *Pc* infection in BALB/c mice showed increased SMA and weight loss associated with increased levels of IFN-γ [156]. In contrast, coinfection with *Ls* 8 weeks and *Pb* infection in C57BL/6 mice showed significantly reduced ECM rates associated with increased levels of IL-10. This protection was inhibited in IL-10 KO mice [157]. High levels of IL-10 were important in reducing pathology but also interfered with the protective response to *Plasmodium* in the liver. In particular, chronic infection with *Ls* interfered with the protective efficacy of a vaccine against sporozoite *Pb* in the liver [153]. Therefore, infection with *Ls* exacerbates the pathology of a *Pc* infection. In contrast, infection with *Ls* inhibits pathology in *Pb* infection due to an anti-inflammatory cytokine response (Table 4(f)).

In addition, CBA/J mice infected with *Brugia pahangi* (*Bp*) for 1 week and then coinfected with *Pb* displayed a low mortality rate, and mice were protected against the development of ECM. This protection was associated with increased serum IgE levels and Th2 cytokine production [158] (Table 4(g)). Similarly, infection with *Trichinella spiralis* (*Ts*) for 1 or 4 weeks in C57BL/6 mice greatly enhanced their resistance against the fatal coinfection with *Pb* [159]. Therefore, these observations suggest that the Th2-type immune response reduces brain pathology and increases survival in *Bp*- or *Ts-Pb* coinfection, perhaps due to the anti-inflammatory environment generated by the previous helminth infection (Table 4(h)).

The studies described above lead to different conclusions, while some of them suggest that prior infection with helminths induces resistance to *Plasmodium* [136, 137, 142, 148, 154, 155, 157–159], other studies do not show effects [149–153] and finally some others demonstrated an increased susceptibility to *Plasmodium* infection [138–141, 143–148, 151, 153, 156]. These contrasting results may partially be explained because this interaction is affected by the timing between the hosts' exposure to the helminth and *Plasmodium*. In addition, the strain of each parasite is also important, coinfection with nonlethal *Plasmodium* strains in the early stages of a helminthic infection delayed the onset of parasitemia due to early, specific high production of IFN-γ, but this response increased pathology. In contrast, a significant increase in susceptibility to nonlethal *Plasmodium* was observed when mice were coinfected with *Plasmodium* in the late stages of helminthic infection, when the Th2-type immune response is predominant.

Coinfection with lethal strains of *Plasmodium* in the late stages of a helminthic infection inhibits severe pathology and increases the survival of mice due to a decrease inflammatory response (mainly IFN-γ and TNF-α). In addition, the presence of a late anti-inflammatory Th2-type immune response induced by helminthic infection extended the survival of mice susceptible to *Plasmodium* infection; this may be due to a reduced pathological Th1-type immune response or may be due to induction of protective mix of Th1 and Th2 immune response. Recruitment and activation of Mφ are essential for the clearance of malaria infections, but these have also been associated with adverse clinical outcomes [163]. Specifically, immunopathology of severe malaria is often originated from an excessive inflammatory Th1-type immune response. The expansion of Treg cells and the alternative activation of Mφ by helminth infections may modulate the excessive inflammatory response to *Plasmodium*. Therefore, the chronic helminth infections inhibited pathology and increased survival in the challenge with lethal strains of *Plasmodium*.

6. Conclusions

The findings in this review demonstrate that the immune environment generated by a previous helminthic infection influences the response against *Plasmodium*. A helminth that persists in its host is able to significantly modify the host's susceptibility to or protection from *Plasmodium*. These modifications are dependent on the genetic background of mice, the type of helminth, and the time-course of the initial helminthic infection, which is crucial to the resulting immune response to *Plasmodium*.

The impact of helminth-*Plasmodium* coinfection on acute helminthic infection increased or synergized the Th1-type immune response. This might be successful in inducing a response that inhibits *Plasmodium* replication, but it increases the pathology and mortality in the host. Alternatively, chronically helminth-infected mice showed a shift toward Th2-type immune responses. This could render the host more susceptible to *Plasmodium* infection and favor their replication; however, this response protected the host from severe malaria (Figure 3). Overall, these results suggest that malarial immunity is influenced by helminth infections. Therefore, the study and manipulation of antimalarial immunity seems difficult in the absence of any information concerning the effects of helminths on this response.

7. Perspectives

The helminth-*Plasmodium* interaction may have undesirable implications for global public health; for example, malaria

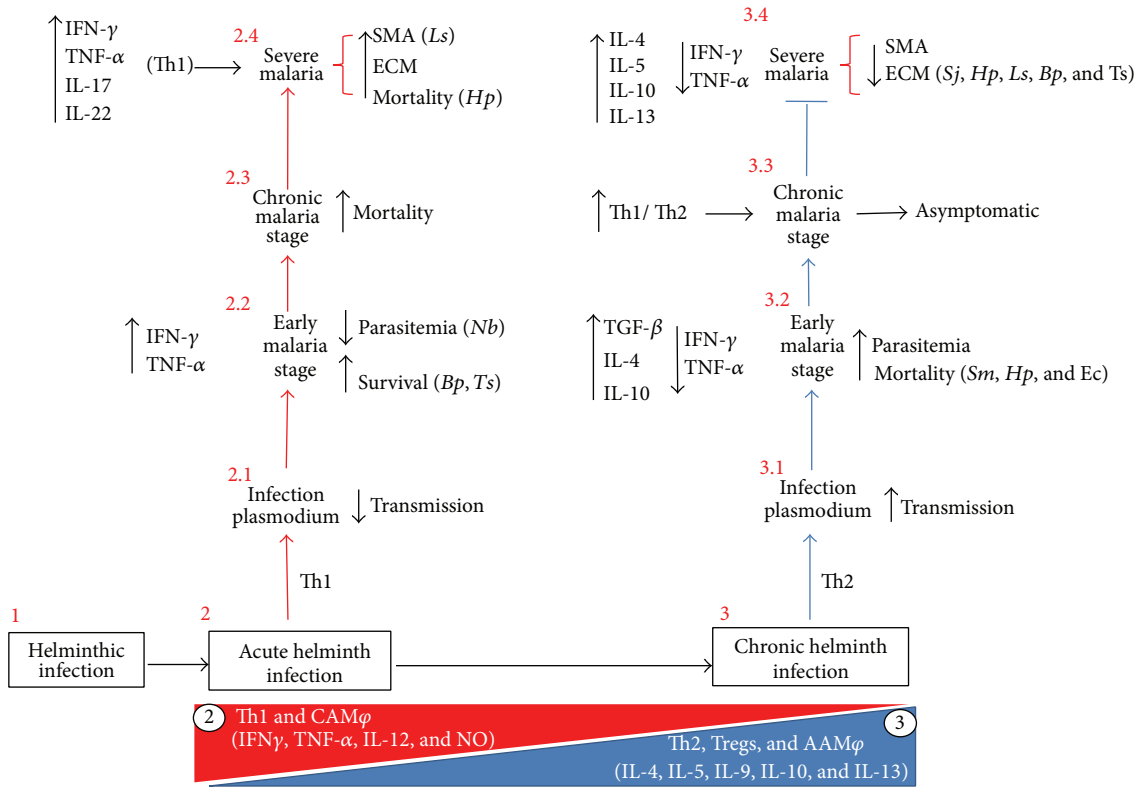

Figure 3: Concomitant helminth infection modified the immune response and susceptibility to *Plasmodium* infection. Helminth parasites have developed complicated strategies to infect and successfully colonize their host. (1) In an acute helminth infection, an initial Th1-like immune response (i.e., IFN-γ, IL-12, and classical activation macrophage (CAMφ)) is associated with low parasite growth. (2) However, as the parasite colonizes the host, the immune response rapidly shifts toward a Th2-dominant response (IL-4, IL-5, IL-10, IL-13, and AAMφ) in parallel with increased helminth parasitemia. (3) This "immune environment" determined by helminth infection modifies the immune response and the susceptibility to *Plasmodium*. That is, acutely helminth-infected mice exhibited (2) decreased transmission of *Plasmodium* (2.1), decreased parasitemia and increased survival (2.2) due to high levels of IFN-γ and TNF-α in the early stage. However, this immune response increased mortality during the chronic stage of malaria (2.3) and increased severe pathology, such as ECM and severe malaria anemia (SMA) (2.4). In contrast, chronically helminth-infected mice (3) increased the transmission of *Plasmodium* (3.1), parasitemia and mortality (3.2) due to high levels of IL-4, IL-10, and TGF-β and low levels of IFN-γ and TNF-α. However, during the course of the coinfection, the Th1 response against *Plasmodium* was increased. In fact, a mixed Th1/Th2 response during the chronic stage induced low levels of parasitemia and was asymptomatic (3.3). Interestingly, chronic helminth infections inhibited severe pathologies caused by *Plasmodium*, such as ECM and SMA (3.4), and increased the survival due to a decreased inflammatory response. Abbreviations: *Schistosoma mansoni* (*Sm*), *Heligmosomoides polygyrus* (*Hp*), *Echinostoma caproni* (*Ec*), *Strongyloides ratti* (*Sr*), *Nippostrongylus brasiliensis* (*Nb*), *Litomosoides sigmodontis* (*Ls*), *Brugia pahangi* (*Bp*), and *Trichinella spiralis* (*Ts*).

vaccines trials do not consider the immune response to helminths, and this could result in decreased performance or cause adverse effects. Thus, a better understanding of helminth-induced regulation in the antimalarial response is indispensable for the rational development of effective antimalarial vaccines and novel therapies to alleviate or prevent the symptoms of severe malaria. The risk that entire populations may have an increased susceptibility to *Plasmodium* should invite study regarding the possible epidemiological relevance of helminth infections and the impact of controlling them on malaria incidence. The presence of helminth infections could represent a much more important challenge for public health than previously recognized. Therefore, we would emphasize that it is extremely important to carry out experiments in animal models that use more rigorous criteria to define exhaustively all the ramifications of immune regulation and potential side effects of helminth infection in the context of malaria. These results would allow extrapolate the observation in human populations presenting malaria.

Conflict of Interests

The authors have no financial or other conflicts to declare.

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Research Article

Protective Effect of a Prime-Boost Strategy with the Ts87 Vaccine against *Trichinella spiralis* Infection in Mice

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Trichinellosis is a widespread zoonosis primarily caused by *Trichinella spiralis*. Mucosal immunity is crucial for preventing *Trichinella spiralis* infection. In our previous study, a DNA vaccine with the *Trichinella* antigen Ts87 delivered by an attenuated *Salmonella typhimurium* elicited partial protection against *Trichinella spiralis* infection in mice. In the current study, to elicit a more robust immune response and develop a potent vaccination strategy against trichinellosis, a heterologous prime-boost vaccination regimen for Ts87 was used in mice and the protective efficacy was evaluated compared to the homologous DNA prime-boost or protein prime-boost immunization alone. The results revealed that the DNA-prime/protein-boost vaccination with Ts87 induced higher levels of both humoral and cellular immune responses. The challenge results showed that mice with the DNA-prime/protein-boost vaccination displayed higher muscle larval reduction than those immunized with DNA prime-boost or protein prime-boost. The results demonstrated that mice vaccinated with Ts87 in a DNA-prime/protein-boost strategy effectively elicited a local IgA response and mixed Th1/Th2 immune response that might be responsible for improved protection against *Trichinella spiralis* infection.

1. Introduction

Trichinellosis is a major food-borne zoonosis and human infection has been reported in 55 countries around the world [1]. Human trichinellosis is characterized by high fever, facial edema, and myositis, which may be serious, particularly in elderly patients [2]. The nematode *Trichinella spiralis* is the most common cause of human trichinellosis [3]. Outbreaks of trichinellosis have been regularly reported during the past two centuries and this parasitic disease is emerging or reemerging in some areas of the world [4–6]. Trichinellosis is not only a public health hazard but also an economic problem for livestock production and food safety [7]. Consequently, there is an urgent need for vaccines to control the infection.

The occurrence of trichinellosis in humans is strictly related to cultural food practices, including the consumption of raw or undercooked meat containing encapsulated *Trichinella* parasite larvae [7]. The infective muscle larvae are released from the muscle tissue in the stomach and migrate to the small intestine where the larvae develop into adult worms. The adult females produce newborn larvae, which penetrate the intestine and migrate to muscle tissue where they form cysts. Therefore, the intestinal mucosa is likely to be the first barrier in protecting the host against *Trichinella* infection. In our previous studies, an immunodominant antigen, Ts87, was cloned from *T. spiralis* [8], and vaccination with the recombinant Ts87 protein (rTs87) produced partial protection in immunized mice [9, 10]. To induce an IgA response in the intestinal mucosa, the Ts87 DNA was transformed into attenuated *S. typhimurium*. Mice vaccinated orally with the attenuated *Salmonella*-delivered Ts87 DNA vaccine exhibited a strong local IgA response and partial protection against *T. spiralis* infection [11].

Although the mucosal immunity induced by the attenuated *Salmonella*-delivered DNA vaccine produced partial protection against *Trichinella* infection, the systemic immune response to the Ts87 DNA vaccination was not high enough and the elicited protection was limited [11]. An effective

Table 1: Immunization regimen.

Group (prime_boost)	Prime	1st boost	2nd boost
DNA + P	SL7207/pVAX1-Ts87 (orally)	rTs87 (intramuscularly)	rTs87 (intramuscularly)
DNA + DNA	SL7207/pVAX1-Ts87 (orally)	SL7207/pVAX1-Ts87 (orally)	SL7207/pVAX1-Ts87 (orally)
P + P	rTs87 (intramuscularly)	rTs87 (intramuscularly)	rTs87 (intramuscularly)
PBS	PBS	PBS	PBS

vaccine usually requires a more optimal immunization regimen in the form of a prime-boost. A heterologous prime-boost regimen can be more immunogenic than a homologous prime-boost regimen [12]. In recent years, many promising results and significant protection have been reported for viral, bacterial, and parasitic infections using the heterologous prime-boost regimen [13–15]. In this study, to elicit a more robust immune response, including local mucosal IgA production and a more potent vaccination strategy against Trichinellosis, a heterologous prime-boost vaccination regimen with Ts87 DNA and rTs87 was used and the protective immunity induced by this regimen was evaluated.

2. Materials and Methods

2.1. Parasites. *T. spiralis* (ISS 533) parasites were originally isolated from a swine source in the Heilongjiang province of China and maintained by serial passage in female ICR mice. Each mouse was orally infected with 400 *T. spiralis* larvae. The muscle larvae (ML) were recovered from infected mice using a modified pepsin-hydrochloric acid digestion method as described by Gamble et al. [16, 17].

2.2. Mice/Ethics Statement. Female, 6-7 week-old BALB/c mice were purchased from the Laboratory Animal Services Center of Capital Medical University (Beijing, China). All experimental procedures were reviewed and approved by the Capital Medical University Animal Care and Use Committee and were consistent with the NIH Guidelines for the Care and Use of Laboratory Animals.

2.3. Ts87 DNA Vaccine. DNA encoding the full-length Ts87 was cloned into the eukaryotic expression vector pVAX1, and the recombinant pVAX1-Ts87 plasmid DNA was transformed into an attenuated *S. typhimurium* SL7207 strain as a DNA vaccine (SL7207/pVAX1-Ts87) as described previously [11].

2.4. Recombinant Ts87 Protein (rTs87). The rTs87 was expressed in *E. coli* BL21 (DE3) with a His-tag at the C-terminus and was purified using Ni-affinity chromatography (Novagen, USA) as described previously [11].

2.5. Immunization Regimens. In this study, BALB/c mice were vaccinated with either rTs87 or Ts87 DNA transformed attenuated *S. typhimurium* with different prime-boost strategies. For the DNA prime-protein boost regimen, a group of 12 mice were immunized orally with 1×10^8 cells of SL7207/pVAX1-Ts87 as described previously [11] and then boosted twice at 2-week intervals with 100 μg rTs87 emulsified with the water-in-oil adjuvant ISA 50 V2 (SEPPIC, France) intramuscularly [18]. All prime-boost regimens are described in Table 1. Two weeks after the last boost, six mice from each group were sacrificed. The serum, intestinal lavage fluid, spleen, and mesenteric lymph nodes (MLNs) were collected to evaluate the humoral and cellular immune responses. Mice immunized three times with PBS were used as a blank control.

2.6. Antibody Responses. The levels of antigen-specific total IgG and subtype IgG1 and IgG2a antibodies in the sera of the immunized mice were determined using a modified indirect enzyme-linked immunosorbent assay (ELISA) as described previously [19]. Briefly, 96-well microtiter plates (Costar) were coated with rTs87 (10 μg/mL) and blocked with 5% fetal bovine serum (FBS) in PBS. For total IgG detection, the plates were incubated with sera at different dilution and then incubated with HRP-conjugated goat anti-mouse IgG. For the isotype-specific ELISA, after incubation with the mouse sera samples (1 : 200 dilution), the plates were incubated with goat anti-mouse IgG1 or IgG2a (BD Pharmingen, USA). Then, HRP-conjugated rabbit anti-goat IgG antibodies (BD Biosciences, USA) were added. The ELISA plates were developed with o-phenylenediamine dihydrochloride substrate (OPD, Sigma, USA) and read at 492 nm.

2.7. Measurement of Total IgA in Intestinal Washes. The intestinal lavage fluid was prepared as described previously [11]. Briefly, for each sacrificed mouse, 10 cm of the small intestine beginning at the gastroduodenal junction was cut, and the interior of the small intestine was flushed twice with a total of 2 mL of cold PBS. After centrifugation at 800 ×g for 10 min, the supernatants were harvested and stored at −80°C until use. The total intestinal IgA was assessed with a sandwich-type ELISA by trapping the intestinal mucosal IgA as described previously [20].

2.8. T Cell Proliferation. A T cell proliferation assay was performed using the CellTiter 96 Aq_{ueous} One Solution Cell Proliferation Assay (Promega, USA). Briefly, 5×10^5 splenocytes in 100 μL of RPMI-1640 were *in vitro* stimulated with 100 μL of rTs87 (10 μg/mL) for 72 h. Then, 40 μL of the CellTiter 96 AQ_{ueous} One Solution Reagent was added to each well and incubated for 1–4 hours at 37°C. The stimulation index (SI) was calculated as the ratio of the mean absorbance of the stimulated/unstimulated wells.

2.9. Cytokine Assays. An enzyme-linked immunospot assay (ELISPOT) was used to detect IFN-γ, IL-4, IL-6, and IL-10

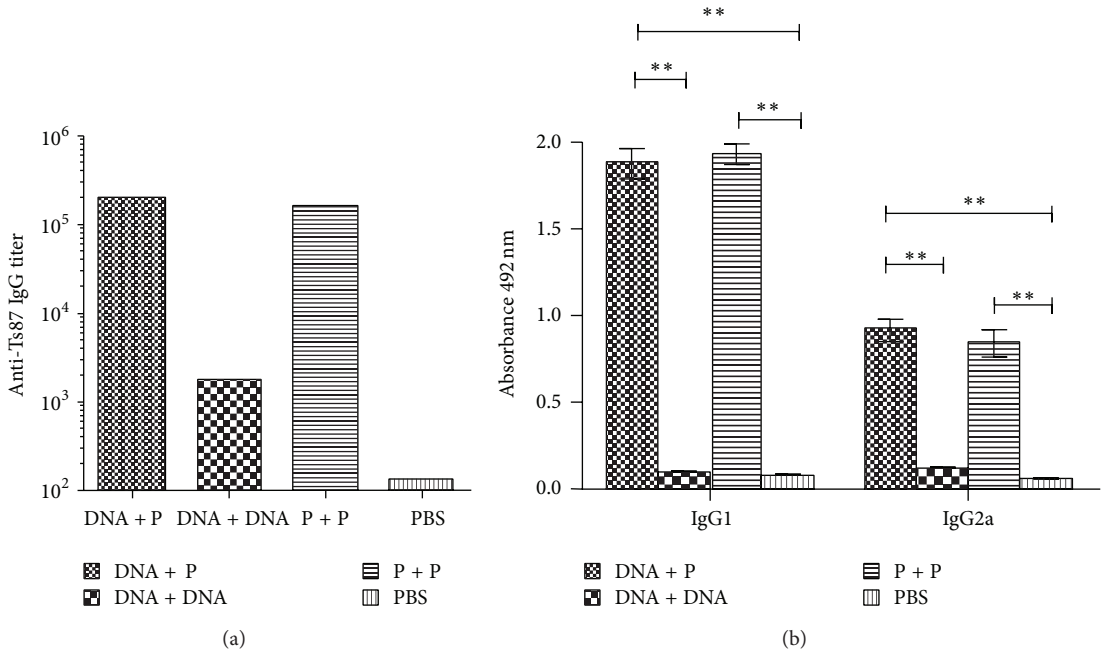

Figure 1: rTs87-specific total IgG and subtype IgG1 and IgG2a levels detected in the sera of immunized mice. The mouse sera were collected two weeks after the 3rd immunization and were measured by ELISA. The rTs87-specific total IgG is shown as the geometric mean titers within the group (Figure 1(a)). $^{**}P < 0.01$. The subtype IgG1 and IgG2a levels are shown as the mean absorbance values ± SE.

secreted by the lymphocytes isolated from the spleen and MLNs of immunized mice according to the manufacturer's instructions (BD Biosciences, USA) [21]. Briefly, the mice were sacrificed two weeks after the last boost and the lymphocytes from the spleen and the MLNs were aseptically isolated. The wells of MultiScreen-IP Filter Plates for ELISPOT (Millipore, USA) were coated with the capture antibody (anti-mouse IFN-γ, IL-4, IL-6, and IL-10; BD Biosciences, USA) at a 1 : 200 dilution in PBS and incubated overnight at 4°C. The plates were washed once with RPMI 1640 medium (Gibco, USA) with 10% FBS and blocked with the same medium for 2 h at room temperature. A total of 1×10^6 lymphocytes for IL-4, IL-6, and IL-10 or 5×10^5 lymphocytes for IFN-γ were added to each well. The rTs87 was added to the well at a final concentration of 10 μg/mL and stimulated for 48 h. Concanavalin A (ConA, Sigma, USA; 5 μg/mL) was used as a nonspecific positive control. The detection antibody (biotinylated anti-IFN-γ, IL-4, IL-6, and IL-10 antibody; BD Biosciences Pharmingen, USA) was added at 1 : 200 in 100 μL of dilution buffer (PBS containing 10% FBS) and incubation was continued for 2 h. After incubation with 100 μL of streptavidin-HRP for 1 h (BD Biosciences, USA), the plates were developed with 100 μL of a 3-amino-9-ethylcarbazole substrate solution (20 μL of an AEC chromogen for each 1 mL of substrate, BD ELISPOT AEC substrate set; BD Biosciences, USA) for 1–5 min. The spots corresponding to the number of IFN-γ, IL-4, IL-6, and IL-10-secreting cells were counted automatically with a CTL ELISPOT reader and analyzed using the ImmunoSpot image analyzer software v4.0.

2.10. Evaluation of Larval Burden. Two weeks after the final boost, the remaining 6 mice from each group were challenged with 400 *T. spiralis* muscle larvae. Six weeks after the challenge, the mice were sacrificed. The larvae in the muscle from each mouse were collected and counted as described previously [17]. Reductions in the larval burden were calculated as follows: worm burden reduction rate (%) = (1 − mean number of larvae per gram muscle in vaccinated mice/mean number of larvae per gram muscle in control mice) × 100%.

2.11. Statistical Analysis. All of the data were evaluated by one-way ANOVA using the SPSS 17.0 software. The data are expressed as the means ± standard error (SE). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Serological Immune Response. Mice immunized orally with Ts87 DNA-attenuated *S. typhimurium* (DNA) and then boosted intramuscularly twice with rTs87 (P) produced much higher levels of total IgG, IgG1, and IgG2a compared to the group boosted with Ts87 DNA alone ($P < 0.01$, Figure 1). The group immunized with rTs87 and boosted with the same protein twice also produced high levels of total IgG, IgG1, and IgG2a. The production of IgG1 and IgG2a indicates a mixed Th1(IgG2a) or Th2-like (IgG1) responses, with Th2 predominant.

3.2. Mucosal IgA Response. The total intestinal mucosa IgA was measured by sandwich ELISA. The secretory IgA level was significantly increased in the mucosa of mice immunized orally with the Ts87 DNA vaccine, either boosted with the same DNA (DNA + DNA) or with the recombinant protein

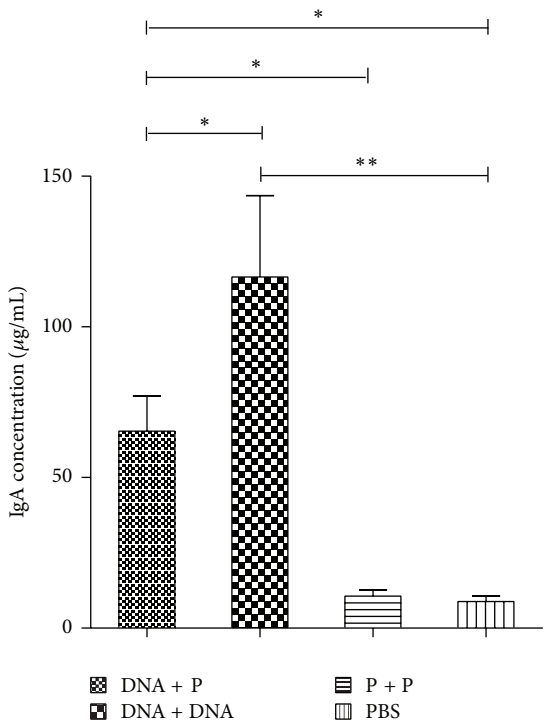

Figure 2: The total secretory IgA in the intestinal washes of vaccinated mice measured by sandwich ELISA. The IgA level was increased in the mice immunized with the DNA-prime/protein-boost (DNA + P) compared to those immunized with rTs87 (P + P) or PBS ($P < 0.05$). The IgA level was significantly higher in mice with the DNA prime-boost (DNA + DNA) compared with the DNA-prime/protein-boost (DNA + P) ($P < 0.05$). $^{**}P < 0.01$. $^{*}P < 0.05$. The results are presented as the mean ± SE for 6 mice per group.

(DNA + P) compared to the group with the protein prime-boost (P + P). However, the highest level of secretory IgA was observed in the DNA immunized group boosted with the same DNA carried with *Salmonella* bacteria compared to the protein boosted group ($P < 0.05$) (Figure 2). There was no significant secretion of mucosal IgA in protein immunized group compared to the PBS control group.

3.3. Cytokine Profiles. The ELISPOT assay was used to detect the cytokines IFN-γ, IL-4, IL-6, and IL-10 secreted by the lymphocytes isolated from the spleen and MLNs two weeks after the 3rd immunization. The IFN-γ and IL-6 levels were significantly increased in the lymphocytes isolated from both the spleen and MLNs of mice immunized with the Ts87 DNA-prime/protein-boost compared to homologous prime-boost immunization regimens (Figures 3(a), 3(c), 4(a), and 4(c)). The IL-4 level was significantly increased in the groups immunized with the DNA-prime/protein-boost or the protein prime-boost in splenocytes compared to the groups immunized with the DNA prime-boost or PBS control (Figure 3(b)). However, the secretion of IL-4 was hardly tested in the MLN cells (less than five spots, Figure 4(b)). Although a higher level of IL-10 was consistently observed in the group immunized with the rTs87 protein prime-boost compared to the other immunization groups, this change was not statistically significant (Figures 3(d) and 4(d)). No spots were detected in the unstimulated lymphocytes, whereas the positive spots were all high without exception in the ConA stimulated control groups (up to $400/5 \times 10^5$ cells, data not shown).

3.4. Proliferative Responses of T Cells. The rTs87-stimulated T cell proliferation of the splenocytes isolated from the DNA-prime/protein-boost mice was significantly higher than the other three groups ($P < 0.01$), indicating that heterologous immunization with a DNA vaccine-prime and recombinant protein-boost greatly enhanced the antigen-specific T cell proliferative response against rTs87 (Figure 5).

3.5. Protective Immunity. In comparison to the PBS control group, mice immunized with DNA prime-protein boost, DNA prime-boost, and protein prime-boost experienced 46.1%, 36.2%, and 24.6% reduction in muscle larval burden, respectively (Figure 6). There is a significant difference between the heterologous prime-boost vaccination regimen and the homologous DNA prime-boost ($P < 0.05$) or between the heterologous prime-boost vaccination regimen and homologous protein prime-boost immunization ($P < 0.01$). These results indicate that the DNAprime/ protein-boost vaccination induced significantly better protective immunity than the homologous DNA prime-boost or protein prime-boost regimens against T. spiralis infection in BALB/c mice.

4. Discussion

DNA vaccination becomes more attractive because of its ability to induce a broad range of immune responses and long-lasting immunity. However, DNA vaccines remain poorly immunogenic compared to protein vaccines [22]. An effective vaccine usually requires more than one immunization in the form of a prime-boost. Traditionally, the same vaccines are administered multiple times as homologous boosts. New findings suggest that the prime-boost can be performed with different types of vaccines containing the same antigens. This type of heterologous prime-boost can be more immunogenic than the homologous prime-boost and may elicit unique immune responses allowing for improved immunogenicity and/or protection against viral, bacterial, and parasitic infections [21, 23–25].

The DNA plus protein vaccination strategy utilizes the benefits of DNA and protein vaccines to effectively induce both cell-mediated immunity and antibody responses against invading organisms [26]. Human studies have also shown superior immune responses during mixed modality prime-boost [27, 28]. The objective of the present study was to explore the protective efficacy and characteristics of the immune response elicited by a DNA prime followed by a protein boost compared to a homologous DNA or protein immunization alone. The kinetics of the mucosal and systemic antibody secretion, patterns of antibody subtype production, cytokine production by the spleen, MLN lymphocyte, and

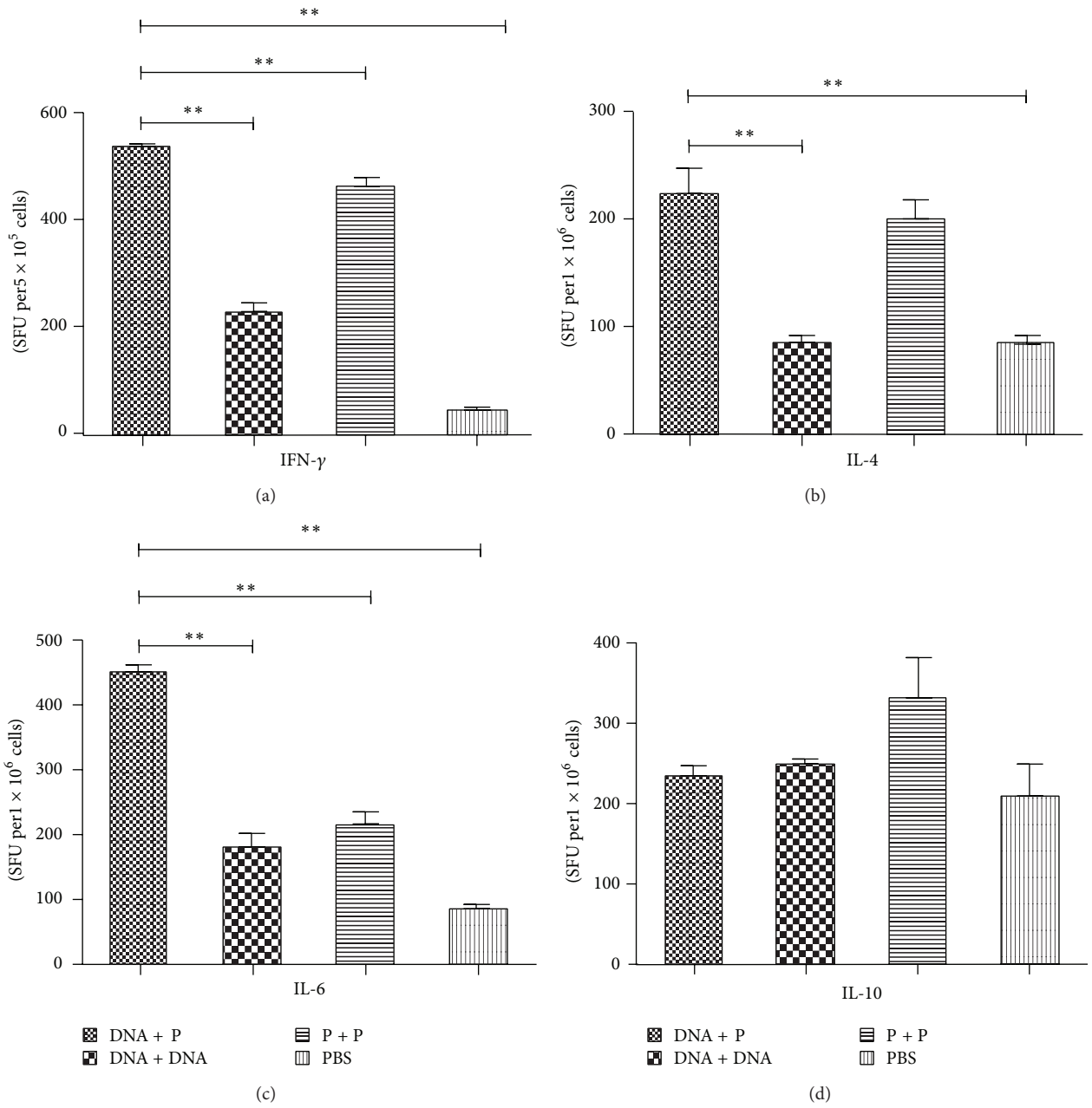

Figure 3: IFN-γ, IL-4, IL-6, and IL-10 secreted by splenocytes isolated from mice immunized with Ts87 in different prime-boost regimens were detected by ELISPOT. $^{**}P < 0.01$. The results are presented as the mean ± SE for 6 mice per group.

protective effect of this DNA-prime/protein-boost regimen against *T. spiralis* infection were evaluated in mice in this study.

Mucosal immune responses act as the first barrier of defense against *T. spiralis*. The mucosal IgA response, when adequately induced, can impede the establishment of infective *Trichinella* parasites in the mouse intestine [29]. Intranasal immunization with a 30-mer peptide of a 43 kDa *Trichinella* antigen induced protective immunity against *T. spiralis* infection accompanied by the secretion of mucosal IgA [30]. Intraperitoneal injection of an IgA monoclonal antibody against the *Trichinella* parasite also protected mice from infection with infective larva [29]. The DNA vaccine delivered by attenuated *S. typhimurium* produced long-lasting mucosal IgA and systemic immune responses and provides an efficient vaccination platform, particularly for intestinal infections in which local immunity is essential for protection [31–33]. In our previous study, oral vaccination with Ts87 DNA delivered by *S. typhimurium* induced significant intestinal IgA secretion and considerable protective immunity against the challenge of *T. spiralis* infective larva [11]. Compared to the homologous DNA prime-boost vaccination, the heterologous Ts87 DNA-prime and protein-boost regimen examined in this

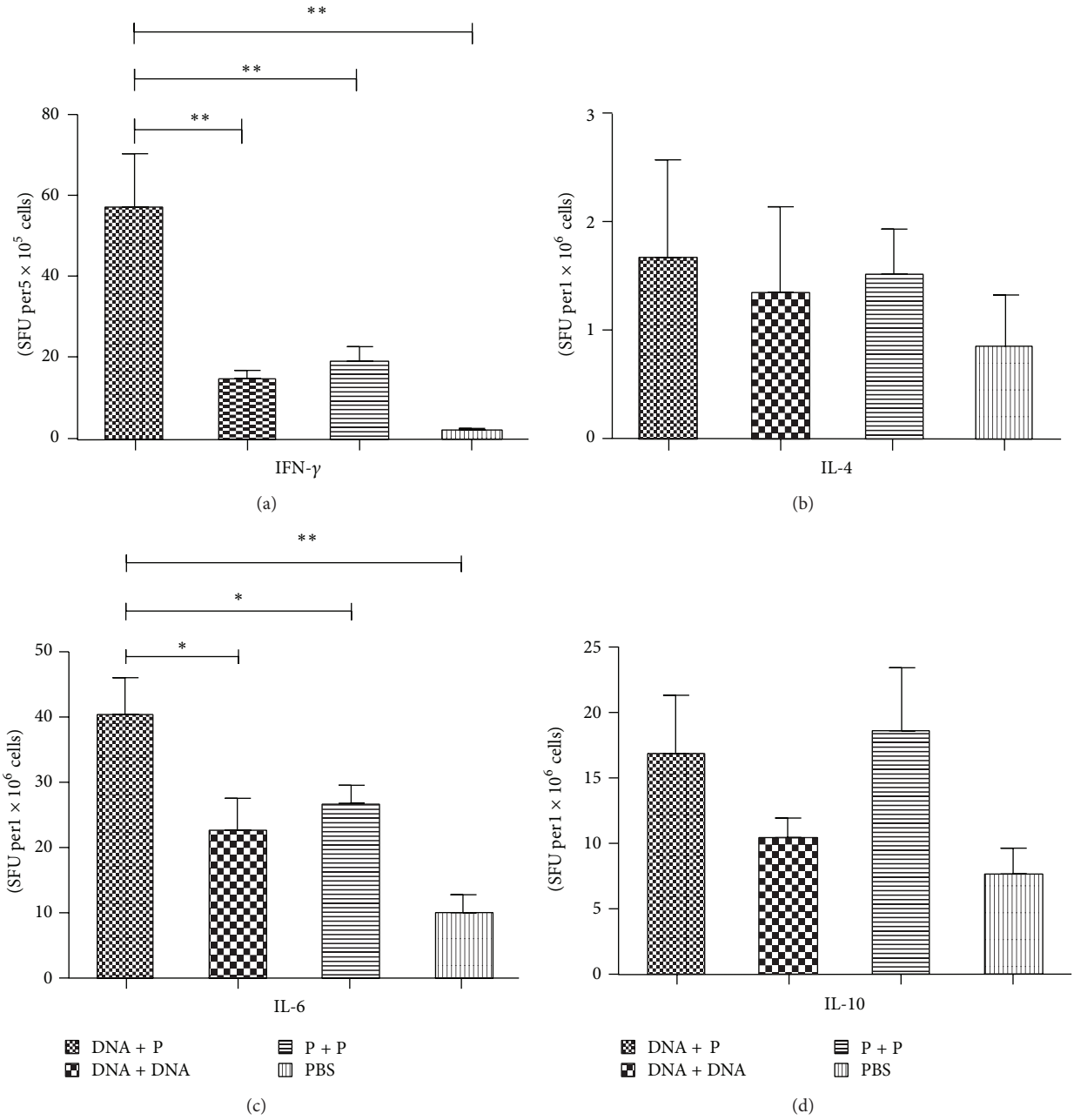

Figure 4: IFN-γ, IL-4, IL-6, and IL-10 secreted by lymphocytes of MLNs isolated from mice immunized with Ts87 in different prime-boost regimens were detected by ELISPOT. The IL-4 levels in the MLN cells of all four groups were low (less than five spots, Figure 4(b)). $^{**}P < 0.01$. $^{*}P < 0.05$. The results are presented as the mean ± SE for 6 mice per group.

study produced significant high level of systemic antibody responses, including increased total IgG and subtypes IgG1 and IgG2a and significantly greater protection against *T. spiralis* larval challenge compared to homologous DNA or protein prime-boost regimens. The greater protection in the mice immunized with the Ts87 DNA-prime and protein-boost regimen (46.1%) compared to the mice immunized with the homologous DNA prime-boost (36.2%) is also associated with more robust cellular responses demonstrated by greater lymphocyte proliferation upon specific antigen stimulation and the higher level of INF-γ secreted by both splenocytes and MLNs. It has been demonstrated that the combined Th1 and Th2 immune responses are important for immunity against *T. spiralis* infection [19, 34, 35], even though it is believed that the Th2 response is essential for protective immunity to gastrointestinal (GI) helminth infections [36]. In this study, mice immunized with the DNA-prime/protein-boost produced not only a stronger Th2-associated immune response (IgG1 antibody, secretory mucosal IgA, IL-4, and IL-6), but also a Th1-like response evidenced by high titers of

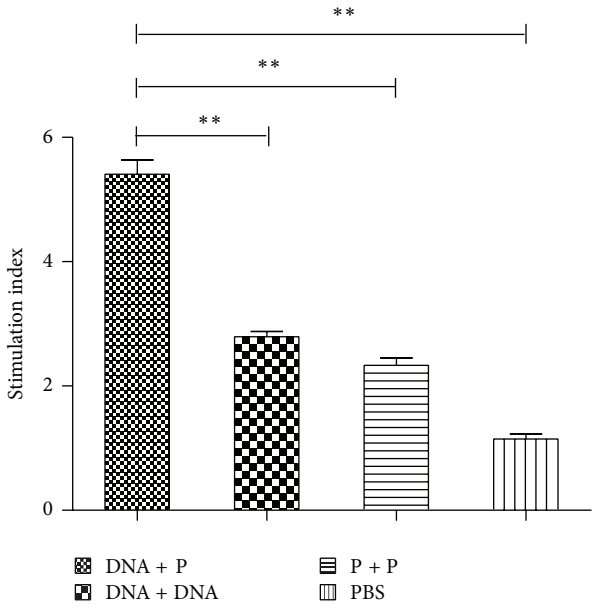

Figure 5: Proliferative responses of splenocytes upon stimulation of rTs87 *in vitro*. The rTs87-stimulated T cell proliferation of splenocytes isolated from the DNA-prime/protein-boost (DNA + P) mice was significantly higher than that in other homologous prime-boost groups. $^{**}P < 0.01$. The results are presented as the mean ± SE for 6 mice per group.

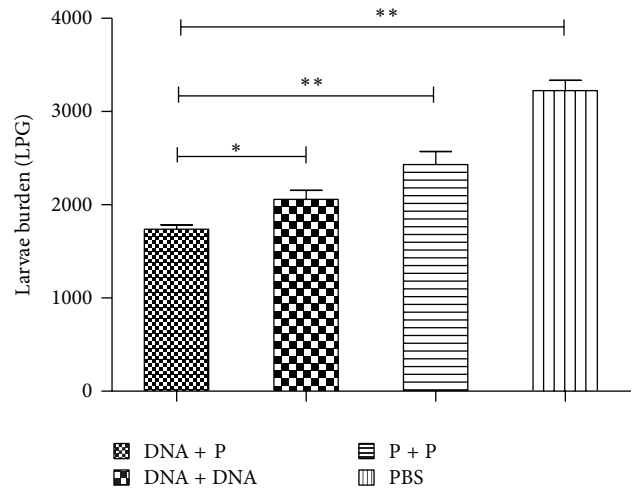

Figure 6: Protection elicited by immunization with different vaccination regimens. The larvae per gram muscle (LPG) were counted in the muscles of mice 6 weeks after a challenge with 400 *T. spiralis* larvae. The mice immunized with the DNA-prime/protein-boost (DNA + P) displayed a 46.1% reduction in the muscle larval burden compared to the groups immunized with the homologous DNA prime-boost (36.2%, $P < 0.05$) and homologous protein prime-boost (24.6%, $P < 0.01$). $^{**}P < 0.01$. $^{*}P < 0.05$. The results are presented as the mean ± SE for 6 mice per group.

IgG2a antibody and IFN-γ. The results indicate that this heterologous immunization regimen of a DNA-prime/protein-boost with a Ts87 vaccine produced a mixed Th1 and Th2 immune response that may contribute to greater protection than homologous DNA or protein prime-boost alone.

High levels of IgA secretion in the mucosal tissue were also observed in the mice vaccinated with the oral Ts87 DNA-prime and intramuscular protein-boost, although the IgA level was not as high as those orally vaccinated three times with Ts87 DNA. IL-4, IL-6, and IL-10 are associated with murine IgA responses [37]. IL-6 has been identified to be the most effective terminal differentiation factor for IgA-committed B cells to become IgA-producing cells in both human and mouse systems [38]. In this study, we also observed high levels of IL-6 secreted by splenocytes and MLNs from mice vaccinated with the Ts87 DNA-prime/protein-boost than other groups with homologous prime-boost vaccination regimens. Significantly higher levels of IL-4 were secreted by the splenocytes in the mice vaccinated with the DNA-prime/protein-boost and protein prime-boost than those vaccinated with the DNA prime-boost or PBS control. Although it is believed that IL-10 plays an essential role in IgA B-cell differentiation in humans [39], in the present study, there was no significant difference in the level of IL-10 secreted by lymphocytes from the mice vaccinated with the DNA-prime/protein-boost regimen and the other immunization regimens. High levels of IL-6 correlated with the elevated intestinal mucosal IgA level upon DNA-prime/protein-boost immunization, indicating that IL-6 may contribute more to the intestinal mucosa IgA response.

In conclusion, the objective of this study was to improve the efficacy of the Ts87 vaccine using a heterologous prime-boost vaccination strategy. The results revealed that the DNA-prime/protein-boost vaccination regimen for Ts87 induced both humoral and cellular immune responses against *T. spiralis* infection, which was associated with high levels of mucosal secreted IgA, serological IgG (total IgG, IgG1, and IgG2a), and lymphocyte secreted IFN-γ, IL-4, and IL-6. Challenge experiments further demonstrated that the DNA-prime/protein-boost vaccination with Ts87 produced significantly greater muscle larval reduction than the traditional homologous prime-boost vaccination. Therefore, the Ts87 vaccine using DNA-prime/protein-boost vaccination produced more effective vaccine efficacy against trichinellosis. Additional studies are needed including optimizing the inoculation dosage, route, immunization sequence, and timing of delivery for this prime-boost vaccination strategy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Diethylstilbestrol Exposure in Neonatal Mice Induces Changes in the Adulthood in the Immune Response to *Taenia crassiceps* without Modifications of Parasite Loads

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Industrial growth has increased the exposition to endocrine disruptor compounds (EDC's), which are exogenous agents with agonist or antagonist action of endogenous steroid hormones that may affect the course of parasite infections. We wanted to determine if the exposure to diethylstilbestrol (DES), an estrogen agonist, to both male and female mice affected the immune response and their susceptibility to *T. crassiceps* cysticercosis. In all infected groups, females showed higher parasite loads than males, and neonatal DES administration did not modify this pattern. In the spleen, noninfected mice showed sex-related differences in the percentage of the CD8+ subpopulation, but DES decreased the percentage of CD3+, CD19+, and CD8+ subpopulations in infected mice. In the mesenteric lymphatic node (MNL), DES showed a dimorphic effect in the percentage of CD19+ cells. Regarding estrogen receptor alpha (ER-α) expression, DES treatment induced a reduction in the expression of this receptor in both noninfected female and male mice in the spleen, which was decreased only in males in CD3+ and CD8+ lymphocytes in MNL cell subpopulations. Our study is the first one to demonstrate that DES neonatal treatment in male and female mice affects the immune cell percentage, without effect on the susceptibility to *T. crassiceps* cysticercosis.

1. Introduction

Endocrine disruptor compounds (EDC's) are exogenous agents that interfere with the synthesis, secretion, transport, binding, action, or elimination of natural hormones in the body with agonist or antagonist action of endogenous hormones. EDCs are from natural sources such as xenoestrogens or have a chemical origin such as diethylstilbestrol (DES), Bisphenol A (BPA), TCDD, and DTT among others [1]. In particular, DES was administered to millions of pregnant women to prevent miscarriages caused by progesterone deficiency between 1940 and 1971 [2]. Studies on neonatal treatment with DES in animal models have reported negative effects on the normal morphology and physiology of the reproductive tract [3, 4]. Several studies have also demonstrated that DES exposure during the fetal and prenatal stages induces tumor formation on estrogen-sensitive tissue in several mice and hamsters models. In adult mice, DES administration also induces cancer in mammary gland, cervix, and uterus. It can also increase the incidence of leukemia and lymphoid tissue tumors [2, 5].

The effect of EDC's on the immune cell function has been barely studied. In humans, prenatal exposure to some EDC's such as DES increased lymphocyte proliferation in response to some chemical mitogens such as Concanavalin A or phytohemagglutinin [5]. DES administration at gestational eighteen day in mice also reduces thymocyte number without changes in thymocyte subpopulations [6].

In experimental murine cysticercosis caused by *Taenia crassiceps* [7, 8], females of all strains of mice studied sustain larger intensities of infection than males [9]. 17β-estradiol (E_2) promotes cysticercus growth by interfering with the thymus dependent cellular immune mechanisms [10]. In addition, gonadectomy alters the resistance pattern of males and produces similar intensities of infection in both sexes [11]. When gonadectomized males receive hormonal replacement with testosterone (T_4), parasite loads are reduced, while E_2 replacement in female mice increased parasite loads [10].

The immune cells mature during fetal development and are exposed to their first external antigens during the neonatal stage. Thus, exposure to EDCs at this early stage of development may affect effectors of the immune response. We have previously demonstrated that neonatal exposure (postnatal day 4) to E_2 increases the resistance to *T. crassiceps* infection in both male and female mice during adulthood. This resistance was accompanied by an increase in the expression of IL-4 and IFN-γ in the serum of experimentally infected neonatally estrogenized animals [12].

At present, however, it is not known whether the administration of DES during the critical period of sexual differentiation of the brain affects the activity of the immune system.

Experimental murine *T. crassiceps* cysticercosis has contributed to revealing the complexities of the interactive network that regulates infection, which is formed by the immune and neuroendocrine systems of the host and the parasite [13]. Briefly, remarkable sex-associated susceptibility to *T. crassiceps* cysticercosis occurs in mice, with females developing larger parasite loads than males during early infection [14]. After 4 weeks of infection, the parasite loads in males slowly increase and within a few months they approximate the parasite loads found in females [15]. Concomitantly, a feminization process occurs in chronically infected male mice, in which serum E_2 levels progressively increase until they almost reach those observed in females. After 6 weeks of infection, T_4 levels drop to 10–15% of normal male levels [15–17] and infected male mice progressively lose their normal aggressive mating behavior [16, 18]. High E_2 levels correlate with an increase in *c-fos* transcription factor and mRNA expression in different areas of the brain at different times of infection [13].

Because sex hormones play a fundamental role in the development of the *T. crassiceps* infection, we hypothesized that a change in the hormonal microenvironment of the host induced by a neonatal injection of DES could determine the proportion of immune cell subpopulations and its estrogen receptors expression during adulthood. This should result in a change in susceptibility to infection in mice, through the modification of immunity of the host towards the parasite. Thus, the aim of this study was to investigate changes in the proportion of immune cells in spleen, mesenteric nodes, and expression of estrogen receptors after a single neonatal dose of DES during *T. crassiceps* infection and to correlate these data with the parasite burden in male and female Balb/c mice. Our findings support the key role of neonatal exposure to DES in controlling immune system function, but not susceptibility to infection in mice.

2. Materials and Methods

2.1. Ethics Statement. The Animal Care and Use Committee at the Instituto de Investigaciones Biomédicas evaluated animal care and experimentation practices according to the official Mexican regulations (NOM-062-ZOO-1999) in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) of the USA. The Ethics Committee of the Instituto de Investigaciones Biomédicas approved this protocol (Permission Number 2009–13).

2.2. Animals. Male and female Balb/cAnN (H2-d) inbred mice obtained from Harlan (Mexico City) were used in all experiments. Animals were housed in the animal care facilities at Instituto de Investigaciones Biomédicas, (UNAM), under controlled conditions of temperature and 12 h dark-light cycles with lights on between 0700 and 1900.

2.3. Neonatal Injection of DES. One single dose of DES was subcutaneously injected (5 μg per mouse) into 4-day-old mice of both sexes. The dose and time of infection were based on previous report by our group [12]. Vehicle—mineral oil—was similarly administered to another group of mice. Intact age matched mice were kept as untreated controls.

2.4. Experimental Infections. At 6 weeks of age, ten non-budding *T. crassiceps* larvae of the fast-growing ORF strain [19] (approximately 2 mm in diameter) were suspended in 0.3 mL sterile phosphate-buffered saline (PBS: 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.2) and intraperitoneally injected into each male and female mouse using a 0.25 gauge needle. Noninfected mice of each sex were used as age-matched controls. Mice were rapidly euthanized by sevoflurane inhalation (Abbott, Mexico) at 8 weeks of infection. Peritoneal cysticerci were collected and counted after rinsing the peritoneal cavity with PBS. Spleen and mesenteric lymphatic nodes were collected immediately after rinsing, to use in flow cytometry assays.

2.5. Flow Cytometry. Briefly, splenocytes from BALB/c mice were purified and stained with the following antibodies: anti-mCD3-FITC, mCD3-biotin, mCD4-APC-Cy7, mCD8-PECy5, and mCD19-PE (from Biolegend). Streptavidin-APC was used as a secondary reagent for CD3-biotin. Cells were fixed with 500 μL of Fixation buffer (4% Paraformaldehyde, Ix PBS, pH. 7.4) and then permeabilized with 200 μL of Perm Buffer (0.2% Saponin, 4% FBS, 1 mM NaN_2). After washing, cells were incubated with Fc blocking reagent (CD16/CD32-FcgammaIII/II Receptor) and incubated with purified anti-ER-α (estrogen receptor alpha) (Santa Cruz Biotech) in Perm Buffer. Primary antibodies were detected with Alexa488- or Alexa647-coupled secondary antibodies (Biolegend) incubated in Perm Buffer. Cells were finally washed with FACS buffer (2% FBS, 0.02% NaN_2, Ix PBS, pH. 7.4) and stored until analyzed. Samples were analyzed by flow cytometry using a FACSAria (BD Biosciences) and data were analyzed with the FlowJo software. Relative ER

expression was calculated as follows: media fluorescence intensity (MFI) MFI = MFI from ER-stained samples/MFI from the secondary antibody-stained sample from the same tissue and mouse. Mean values ± SEM are shown.

2.6. Experimental Design and Statistical Analysis. The experimental design was a four factorial experiment. Independent variables were (1) neonatal DES injection (two levels: yes or no); (2) tissue under study (two levels: spleen, mesenteric lymph nodes); (3) infection (two levels: yes, no); (4) sex (two levels: male or female). The dependent variables were percentage of immune subpopulations, ER expression on each subpopulation, and the number of parasites. Statistical analysis of 2-way ANOVA and Bonferroni's test were performed with the software GraphPad Prism (version 5.0 b for MacOSX, GraphPad Software, San Diego California USA, (http://www.graphpad.com/)).

3. Results

3.1. Female Puberty Onset and Cyclicity. Vaginal opening was not statistically significant, in neonatally DES treated than in vehicle-treated female mice (29.3 ± 0.3 and 30.3 ± 0.4 days, resp.). Immediately after vaginal opening, neonatally DES treated females showed longer estrous cycles particularly arrested at estrous. After 8 weeks of infection, females did not show estrous cycle regardless of neonatal treatment, while noninfected, age-matched, neonatally DES females continued to exhibit longer cycles compared to vehicle-treated mice (not shown).

3.2. Parasite Loads. As others and we have previously reported, we confirm and extend the gender related differences in parasite loads (sexual dimorphism) among male and female mice. In all infected groups, females showed higher parasite loads than males. We did not find differences, either in males or females, produced by neonatal DES administration (Figure 1).

3.3. Spleen Cell Subpopulations. In order to study the effects of DES on lymphocyte subpopulations in the spleen, we decided to determine the proportion of each cell type present in this organ and to analyze if their pattern was sexually dimorphic and if it was affected by DES treatment or infection. As shown in Figure 2, noninfected mice showed no differences in the percentage of any of the analyzed subpopulations when comparing between sexes. Regarding DES treatment, it decreased the percentage of CD3+, CD19+, and CD8+ subpopulations in infected mice when comparing to their noninfected counterparts (Figures 2(a), 2(b), and 2(d)). It is interesting to note that DES effect was more pronounced in the CD19+ cell subpopulation and it was higher in females than in males (when comparing control mice versus DES-treated mice) (Figure 2(b)).

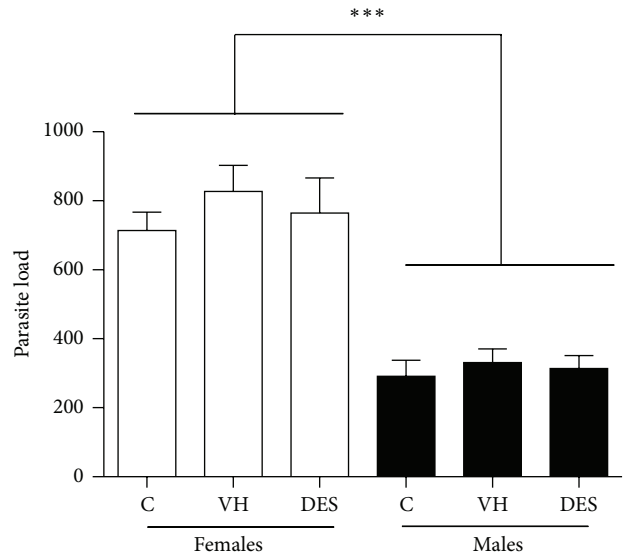

Figure 1: Effect of neonatal administration of diethylstilbestrol (DES) on parasite load. Female and male 4-day old Balb/c mice were administered with a single dose of DES or vehicle (VH) and infected with 10 cysticerci of *Taenia crassiceps* at 6 weeks of age. Data show the number of parasites recovered from the peritoneum at 8 weeks after infection. All groups of animals show the typical sexual dimorphism of this infection. Each bar represents the media ± SEM of parasite loads in 10 infected animals. $^{***}P < 0.001$.

3.4. Mesenteric Lymphatic Node Cell Subpopulations. In order to characterize the subpopulations of lymphocytes present in the mesenteric lymphatic nodes, we decided to determine the percent of each lymphocyte type present in this immune compartment and to analyze if their pattern was dimorphic, affected by DES treatment or the infection. We found no differences in the percentage of the subpopulations in females and males, either in control or infected mice, except for the CD3+ subpopulation in females, where infection induced a significant decrease in this subpopulation (Figure 3(a)). DES treatment showed a dimorphic effect in the CD19+ cells. It induced an increase in infected females while it decreases this subpopulation in infected male mice. However, this difference was not significant. Our FACS analysis showed that infection did not affect the pattern of different subpopulations (Figure 3).

3.5. Expression of Estrogen Receptor Alpha by FACS in Each Spleen Cell Subpopulation. We observed that mice spleen expresses ER-α. We found no differences in the expression of this receptor in any of the analyzed subpopulations in noninfected mice. When the expression of this receptor was measured in DES treated animals, we found that DES induces a reduction in the expression of this receptor in CD3+ and CD19+ cells of both noninfected female and male mice. This reduction is maintained in infected mice when analyzing the CD19+ subpopulation, but in this case, we found no significant differences (Figure 4(b)). We found no differences induced by DES treatment in CD4+ or CD8+ subpopulations

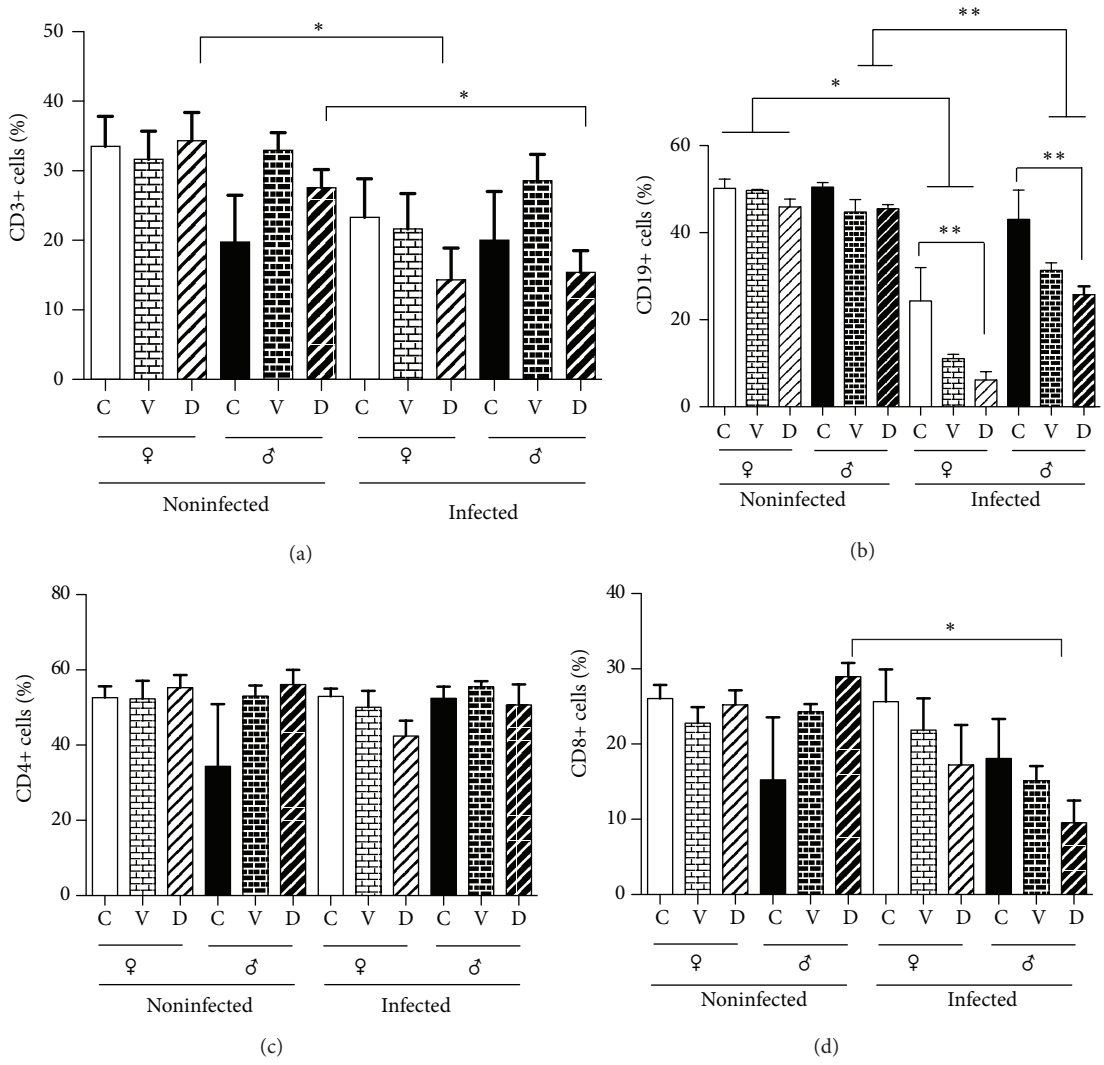

Figure 2: Percentage of lymphocyte populations in spleen of intact (C), vehicle (V), and DES-treated (D) mice, detected by flow cytometry. T lymphocytes (CD3+, CD4+, and CD8+) and B lymphocytes (CD19) were detected in spleen. Each bar represents the media ± SD of parasite loads in 10 infected animals. *Post hoc* individual contrasts of group means by Bonferroni's Exact Test used the sum of residual and three-factor interaction variance to test for significant differences. $^{***}P < 0.001$; $^{**}P < 0.01$.

neither because of the sex nor the infection (Figures 4(c) and 4(d)).

3.6. Expression of Estrogen Receptor Alpha by FACS in Mesenteric Lymphatic Node Cell Subpopulations. Lymphocytes of mesenteric lymphatic node cell subpopulations also express ER-α. We also observed that this receptor is dimorphically expressed, since males show a higher expression than females in noninfected mice and in the T cell subpopulations analyzed. This effect was not observed in the B cell subpopulation. When the expression of these receptors was measured in DES treated animals, we found decreased expression of ER just in males in the total lymphocyte subpopulation (CD3+) and cytotoxic lymphocytes (CD8+). However, these differences did not reach significance. Infection did not affect the expression of the ER-α protein in any sex or DES treated animals (Figure 5).

4. Discussion

In this study, DES administration to neonatal male and female mice was found to produce few changes in immune cell percentage levels, as well as estrogen receptor expression on different immune compartments without affecting the sex-associated susceptibility to *Taenia crassiceps* infection when mice reached the adult phase. Although many reports on neonatal administration of compounds with estrogenic activity have described negative long-term effects on reproductive function [20–24], the impact of neonatal DES treatment on adult mouse susceptibility to helminth parasites had not been studied so far. Since E_2 is an endogenous steroid produced by the ovaries, it is important to study the effects that exposure to analogue compounds, such as DES, on immune functions have. Along the same line, pollutants with estrogenic activity that are released into the environment as a consequence of manufacturing processes could interfere with the normal

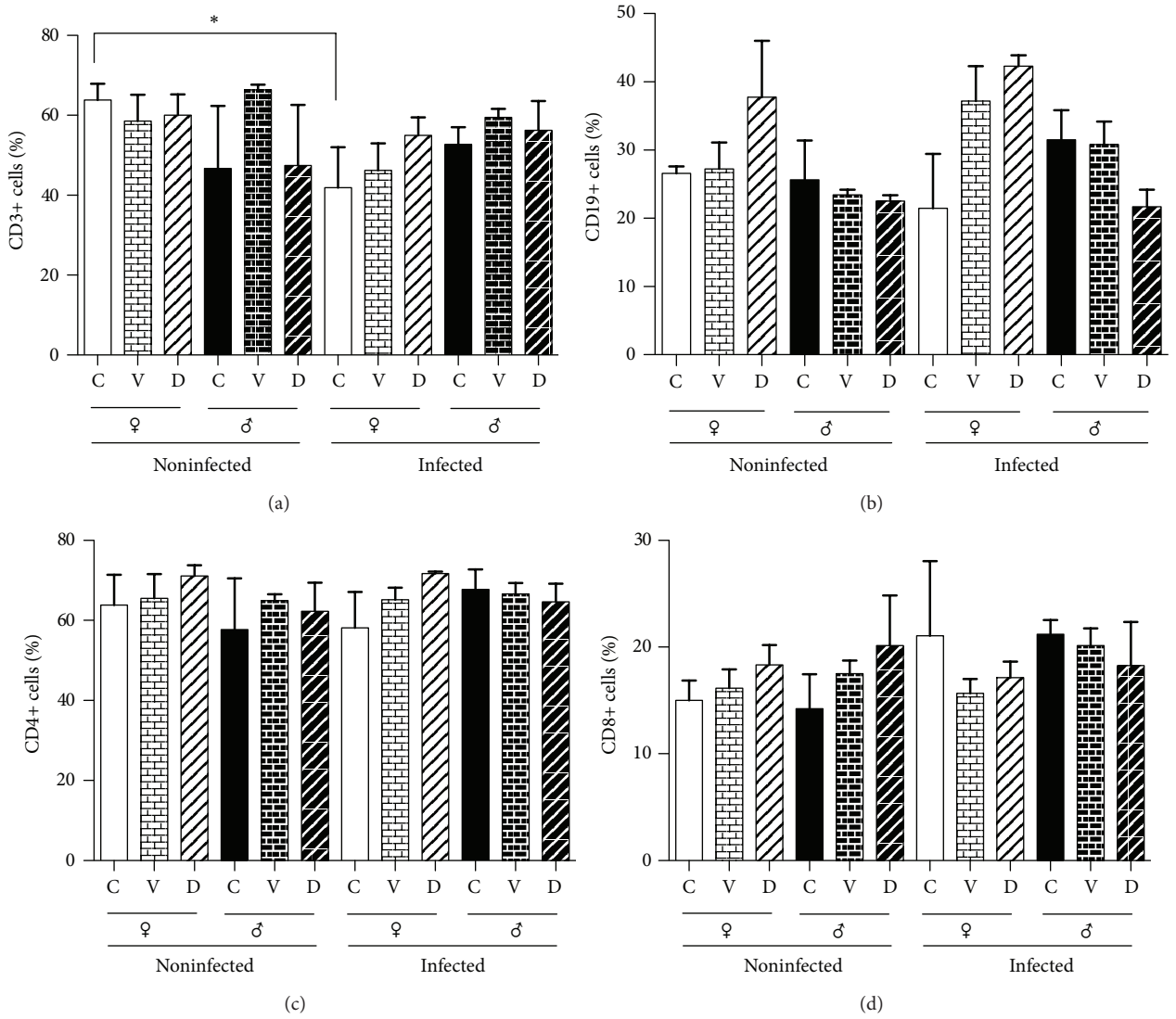

Figure 3: Percentage of immune cell populations in mesenteric lymphatic nodes of intact, vehicle, and DES treated mice, detected by flow cytometry. T lymphocytes (CD3+, CD4+, and CD8+) and B lymphocytes (CD19) were detected in mesenteric lymphatic nodes. Each bar represents the media ± SEM of parasite loads in 10 infected animals. *Post hoc* individual contrasts of group means by Bonferroni's Exact Test used the sum of residual and three-factor interaction variance to test for significant differences. $^{*}P < 0.05$.

development of regular immune functions during adult life. The developmental windows during which they can cause harmful effects are critical. We have previously found that a single-dose of E_2 permanently modified immune functions and immune response to *T. crassiceps*. Exogenous steroid administration showed to upregulate the immune system, specifically the cellular immune response, by increasing IL-4 and IFN-γ serum protein levels in a sexually dimorphic manner as a response to neonatal E_2 treatment [12]. In the present study, we confirm and extend the notion that neonatally administered EDCs affect the immune function.

Some studies suggest that E_2 potentiates the production of cytokines Th1 (IFN-γ) and Th2 (IL-10). High E_2 concentrations were found to stimulate IL-10 secretion by T cell clones, while low concentrations stimulate IFN-γ secretion [24, 25]. In women with a regular menstrual cycle, the immune response tends towards a Th2-type response, which is reflected as an increment in IL-4 production [26]. This suggests that the increased E_2 concentrations during the luteal phase play a role in the deviation of the immune response towards a Th2-type response. This steroid hormone pattern of action was also observed in the lymphocyte proliferation experiments reported by de León Nava et al., 2009 [25]. Lymph node cells were obtained from mice of the two sexes then cultured, activated with anti-CD3 and anti-CD-28, and treated with E_2, P_4, and T_4. E_2 inhibited lymphocyte proliferation. Female cells proliferated more than male cells; however, paradoxically, precisely the steroids associated to female physiology were those to inhibit lymphocyte proliferation. In the specific case of DES, administration into adult rats increases susceptibility to *T. spiralis* infection, when administrated for 5 days (total dosage: 40 mg/kg) before

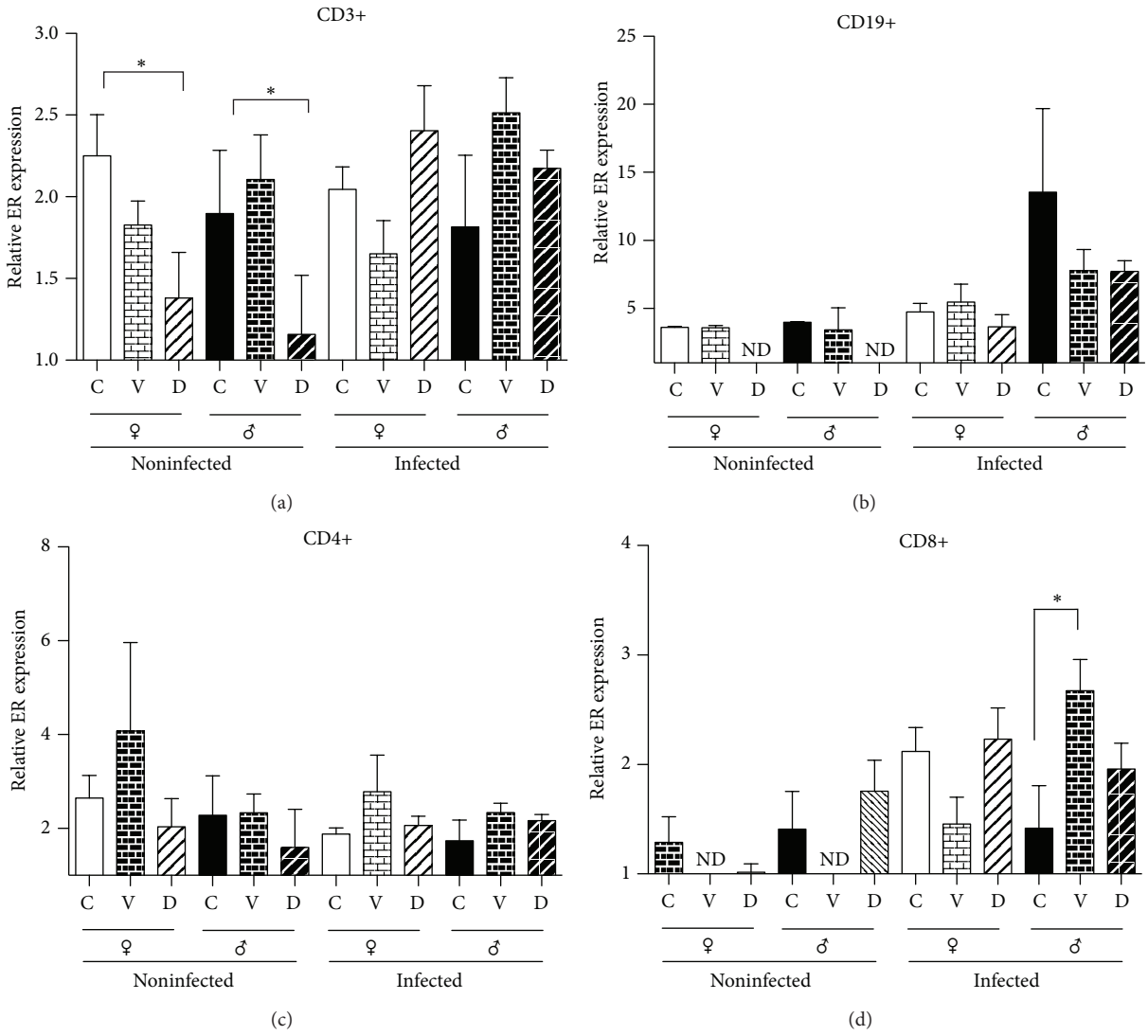

Figure 4: Effects of infection and neonatal DES administration on estrogen receptor α content in splenocytes of mice of both sexes, control, and infected with cysticerci of *Taenia crassiceps* detected by flow cytometry. Estrogen receptor alpha was detected in T lymphocytes (CD3+, CD4+, and CD8+) and B lymphocytes (CD19+). Data are presented as the mean ± SEM of 2 different experiments, $n = 10$ each.

larvae administration [27]. A number of reports on immune and neuroendocrine system interactions indicate that hormones are capable of affecting immune functions [28]. The importance of the interaction between the immune and endocrine systems becomes evident in circumstances such as pregnancy, autoimmune diseases, and some infectious diseases and, as presented in this report, the exposure to endocrine disruptor. In all cases, available evidence underscores the importance of sex steroids as immunoregulators.

The possible mechanisms of action of steroids on immune system cells include, as in any classic endocrine tissue, the genomic and nongenomic pathways. According to the genomic action theory, steroids bind to specific receptors present in the cytoplasm and function as transcription factors. Besides their genomic action, steroids can also act by rapid nongenomic pathways, and the transmission of these effects occurs by specific membrane receptors. Thus, the nongenomic effects on cell function implicate the conventional cascades of second messengers [29]. Although these mechanisms of action have been described in endocrine system organs, evidence has accumulated that they can also operate in the immune system.

Considering the effects that steroids exert on the diverse components of the immune system and that no previous reports are available on the different receptors present in a peripheral organ such as the spleen, we herein aimed to detect the expression of these receptors in the spleen and lymphatic node lymphocyte subpopulations of control, vehicle, DES-treated, control, or infected of both sexes. Ample distribution of ER-α has been found in spleen [30]; however, published reports on the presence of estrogen receptors in spleen contrast with the few publications on estrogen receptors lymphoid mesenteric nodes tissue. In the present work we found the expression of ER-α in the spleen and lymphoid

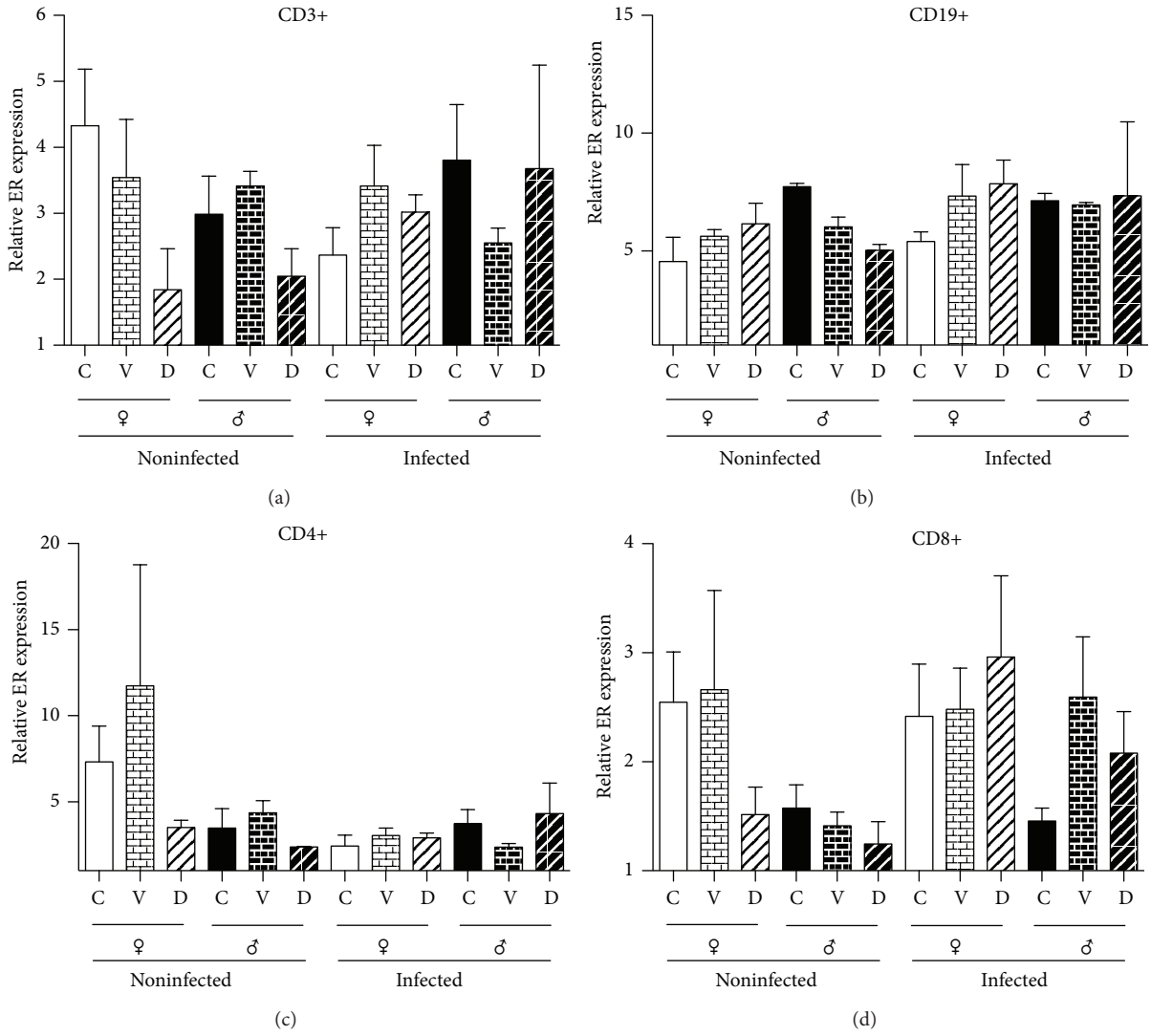

Figure 5: Effects of infection and neonatal DES administration on estrogen receptor α content in mesenteric nodes of mice, control, and infected with cysticerci of *Taenia crassiceps* detected by flow cytometry. Estrogen receptor alpha was detected in T lymphocytes (CD3+, CD4+, and CD8+) and B lymphocytes (CD19+). Data are presented as the mean ± SEM of 2 different experiments, $n = 10$ each. *Post hoc* individual contrasts of group means by Bonferroni's Exact Test used the sum of residual and three-factor interaction variance to test for significant differences.

mesenteric nodes of mice of the two sexes neonatally treated with DES and infected in the adulthood. DES treatment, in turn, had significant sex-associated effect on the expression of this receptor. Although the expression level in spleen is much lower than in endocrine tissue, the presence of this ligand-dependent transcription factor is relevant in a secondary lymphatic organ, since it draws attention to the fact that sex steroids may act not only during the maturation and development of immune cells (in thymus) but also during the effector mechanisms of these cells.

Because the molecular mechanisms by which DES affect the immune system function could be due to their interaction with a specific nuclear receptor, we studied ER-α expression in spleen cells and mesenteric nodes cells of all treatments.

Considering the effects that DES and sex steroids exert on the diverse components of the immune system and that no previous reports are available on the differential expression of this receptor in immune cells in a peripheral organ such as the spleen or mesenteric nodes, as well as its regulation by DES or *T. crassiceps* infection, we herein aimed to detect the expression of this receptor in the spleen and mesenteric nodes of control, vehicle, DES-treated in noninfected and infected mice of both sexes. Ample distribution of ER-α has been found in thymus, bone marrow, and spleen [31–33]; however, published reports on the presence of ER-α in splenic lymphoid tissue contrast with the few publications on its presence on mesenteric lymphatic nodes. In the present work we found the expression of ER-α in the spleen and mesenteric

lymphatic nodes of mice of the two sexes. Although the expression level in spleen and mesenteric lymphatic nodes is much lower than in endocrine tissue, the presence of this ligand-dependent transcription factor is relevant in both immune organs, since it draws attention to the fact that DES may act not only during the maturation and development of immune cells but also during the effector mechanisms of these cells. The question about the population of immune cells that expresses receptors and their regulation was also addressed by flow cytometry analysis.

The possible mechanisms of action of DES on immune system cells, being an agonist or antagonist of estrogens (sex steroids), may include, as in any classic endocrine tissue, the genomic and nongenomic pathways. According to the genomic action theory, steroids or molecules similar to steroids, such as DES, bind to specific receptors present in the cytoplasm and function as transcription factors. Besides their genomic action, steroids and agonists or antagonists, such as DES, can also act by rapid nongenomic pathways, and the transmission of these effects occurs by specific membrane receptors. Thus, the nongenomic effects on cell function implicate the conventional cascades of second messengers [32]. Although these mechanisms of action have been described in endocrine system organs, evidence has been accumulated that they can also operate in the immune system. According to the work by Benten et al. [34], the effects of T4 on T cells are mediated not only by the intracellular androgen receptor, but also by a membrane androgen receptor on the cell surface. Thus, DES and steroid hormones may act through intracellular and membrane receptors of immune system cells by the nongenomic pathway, whenever the regulation of an immune response against a particular pathogen requires their immediate action and by the genomic pathway, when the response needs to be delayed. Speculation ensues on the possibility that the nongenomic pathway predominantly regulates the innate immune response, while the genomic pathway does the same with the adaptive immune response.

In addition to the question of which cell population responds to DES, it would also be interesting to determine the age at which the immune system acquires its dimorphic character [25, 35].

The evidence presented above illustrates the importance of immunoendocrine interactions in an immunocompetent host during *Taenia crassiceps* infection. Interventions aimed at the hormonal network appear as a possible new therapeutic approach to control several immune confrontations, such as parasitic infections.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Gonadal Steroids Negatively Modulate Oxidative Stress in CBA/Ca Female Mice Infected with *P. berghei* ANKA

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We decreased the level of gonadal steroids in female and male mice by gonadectomy. We infected these mice with *P. berghei* ANKA and observed the subsequent impact on the oxidative stress response. Intact females developed lower levels of parasitaemia and lost weight faster than intact males. Gonadectomised female mice displayed increased levels of parasitaemia, increased body mass, and increased anaemia compared with their male counterparts. In addition, gonadectomised females exhibited lower specific catalase, superoxide dismutase, and glutathione peroxidase activities in their blood and spleen tissues compared with gonadectomised males. To further study the oxidative stress response in *P. berghei* ANKA-infected gonadectomised mice, nitric oxide levels were assessed in the blood and spleen, and MDA levels were assessed in the spleen. Intact, sham-operated, and gonadectomised female mice exhibited higher levels of nitric oxide in the blood and spleen compared with male mice. MDA levels were higher in all of the female groups. Finally, gonadectomy significantly increased the oxidative stress levels in females but not in males. These data suggest that differential oxidative stress is influenced by oestrogens that may contribute to sexual dimorphism in malaria.

1. Introduction

Plasmodium species cause more than 200 million cases of malaria each year, with more than 1 million deaths [1]. The mechanisms that are involved in immunity to malaria are extremely complex, with host and parasite factors influencing infection outcomes [2, 3]. Although the prevalence of *P. falciparum* infection does not differ between the sexes, parasite density is 2-fold higher in postpubescent (aged 8–46 years) boys than in girls, suggesting that circulating sex steroids may influence outcomes. Furthermore, sex differences in response to malaria infection have been reported among both adults and children [4–6]. In general, there is evidence that sex-associated hormones can modulate immunity and consequently influence the outcomes of parasitic infections. Males are generally more susceptible to infectious diseases compared with females [7–9]. Under normal circumstances, the levels of sex-associated hormones do not only differ between males and females but also vary according to age and pregnancy progress [10], and the severity of malaria infection is affected by these factors [7]. Specifically, testosterone and 17β-oestradiol are critically involved in the control of sexual dimorphism, affecting the immune response [11]. Typically, oestrogens depress T cell-dependent immune functions and aggravate B cell-dependent diseases, while androgens suppress both T and B cell immune responses. Furthermore, glucocorticoid stress responses, including immune challenges, are enhanced by oestrogens and strongly inhibited by androgens [12, 13].

The elimination of the *Plasmodium* parasite is associated with the increased synthesis of free radicals, which is induced by the immune response or by antimalarial drugs [14, 15]. Testosterone has been shown to increase the production of nitric oxide (NO) in macrophages [16] and has, therefore, been suggested to be a contributing factor to the sexual dimorphism of the immune response [12, 17].

Reactive oxygen species (ROS) are initiators of tissue damage and can upregulate enzyme activity [18]. Antioxidant defences work synergistically to maintain a redox balance. The detoxification of the superoxide anion and hydrogen peroxide, which are catalysed by intracellular superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) enzyme activities, represents a major line of defence [19]. Oestrogens have been shown to upregulate *in vitro* antioxidant activities for membrane phospholipid peroxidation [20]. In malaria, redox status alterations contribute to disease manifestation, including sequestration, cerebral pathology, anaemia, respiratory distress, and placental malaria. In addition, the host immune response to malaria involves phagocytosis and the production of NO and oxygen radicals which also contribute to the pathology of the disease [21]. However, it is not yet clear whether sex steroids modulate the antioxidant enzyme system in malaria-infected individuals.

Gonadectomy is a widely used strategy to analyse the importance of sex steroids in influencing sex dimorphism [11]. In this work, we decreased the levels of gonadal steroids using gonadectomy to study their roles in oxidative stress mechanisms in CBA/Ca mice that were infected with *P. berghei* ANKA.

2. Materials and Methods

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

Plasmodium berghei ANKA parasites were also kindly donated by Dr. William Jarra and were cryopreserved under liquid nitrogen. The parasites were thawed and immediately injected in one mouse; five days later, parasitised blood was obtained to infect CBA/Ca mice. All of the infected animals received an intravenous (i.v.) inoculation of 1×10^3 *P. berghei* ANKA-infected erythrocytes.

2.2. Ovariectomy. One-month-old female mice were anaesthetised with ketamine (80 mg/kg [body mass])-xylazine (8 mg/kg [body mass]) (Phoenix Pharmaceutical Inc., St. Joseph, Missouri, US), and incisions were made in the lower abdomens. The ovaries were removed, and the abdomen was sutured. Sham-operated mice underwent an identical procedure without the removal of the ovaries. The mice were given 4 weeks to recover from the surgery and then infected with 1×10^3 erythrocytes that were parasitised with *P. berghei* ANKA. The mice were sacrificed 9 days following infection, and the lack of ovaries was confirmed by visual inspection.

2.3. Orchiectomy. CBA/Ca male mice were castrated at 3 to 4 weeks of age. The mice were anaesthetised and the testes were pulled out through scrotal incisions. The ductuli efferentes were transected by electrocauterisation, and the testes and epididymis were removed. Sham-operated mice underwent an identical procedure without the removal of the testes.

Mice were allocated into three different groups: (1) intact mice, (2) mice that were bilaterally gonadectomised (Gx), and (3) mice that underwent surgery without gonadectomy (sham-operated). Gx and sham-operated animals were given 4 weeks to recover from surgery prior to parasite infection. The mice were sacrificed by cervical dislocation at 9 days after infection (p.i.), and blood and spleen samples were obtained to assess the antioxidant enzyme activities of SOD, GPx, and catalase as well as to assess NO and malondialdehyde (MDA) levels.

2.4. Parasitaemia. Three days after infection, thin blood smears were prepared daily, fixed with methanol, and stained with a 1 : 10 dilution of Giemsa stain (Sigma, St. Louis, MO, US) in water. Enumeration of the parasitaemia load was performed under a 100x oil immersion lens using a Zeiss Standard 20 microscope (Cark Zeiss LTD, Welwyn Garden City, UK). Parasitaemia levels of 0.5% and above were determined by counting the number of parasitised erythrocytes that were present in a total of 200 red blood cells. Lower levels of parasitaemia were assessed by counting the number of parasitised erythrocytes that were present in 50 fields. The course of infection in each group is shown as the geometric mean of the percentage of parasitaemia.

2.5. Haemoglobin Concentration (Hb). Hb was measured by diluting 2 μL of blood in 498 μL of Drabkin solution (1 g $NaHCO_3$, 0.1 g K_2CO_3, 0.05 g KCN, and 0.2 g $K_3Fe(CN)_6$ in 1 litre of distilled H_2O). The amount of cyanomethaemoglobin formed was detected at 540 nm using a spectrophotometer and converted to mg/mL using a standard curve of rat Hb (Sigma). Measurements were taken on day 0 of infection and then daily (at the same time of day) from day 3 to day 9 after infection.

2.6. Specific Activity of Superoxide Dismutase (SOD). SOD activity was evaluated using the RANSOD kit (Randox Laboratories, Antrim, UK). This kit uses xanthine and xanthine oxidase to generate superoxide radicals, which then react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride to form a red formazan dye. SOD activity was measured by the degree of inhibition of the reaction. On day 9 after infection, the mice were sacrificed, and 125 μL of blood was collected in a heparinised tube. The samples were washed four times with 1 mL of 9.9% NaCl solution and then centrifuged. The pellet of erythrocytes was then brought up to 0.5 mL with cold redistilled water, mixed, and left to stand at 4°C for 15 min. The lysates were diluted 25-fold with 0.01 mmol/L phosphate buffer pH 7.0,

and 12.5 μL of the solution was reacted with the substrates and xanthine oxidase to measure SOD activity levels. All of the steps were performed according to the manufacturer's instructions (Randox Laboratories, Ltd., Crumlin, UK). The reaction kinetics was measured at 505 nm. The inhibition of the amount of chromogen that is produced is proportional to the SOD activity level in the sample. A 50% inhibition is defined as one unit of SOD, and the specific activity is represented as units per mg of protein.

2.7. Specific Activity of Glutathione Peroxidase (GPx). To analyse the GPx activity, we used a method based on the oxidation of glutathione (GSH) by cumene hydroperoxide, catalysed by GPx, in the presence of glutathione reductase and NADPH. Oxidised glutathione (GSSG) is immediately converted to its reduced form with the concomitant oxidation of NADPH to $NADP^+$. Two microliters of heparinised mouse whole blood was diluted with 498 μL of Drabkin reagent to quantify the haemoglobin level, and 50 μL of blood was treated according to the manufacturer's instructions (Randox Laboratories). The GPx activity levels were calculated using the decrease in absorbance at 340 nm using an UV spectrophotometer.

2.8. Specific Activity of Catalase. Catalase activity in the erythrocytes was assessed according to a previously described method [22]. Briefly, peripheral heparinised mouse blood was centrifuged, and the pellet was combined with 4 parts of distilled water. The lysate was diluted 1 : 500 in phosphate buffer (50 mM, pH 7.4), and 1 μL of the resulting solution was mixed with 500 μL of 30 mM H_2O_2. The optical density of the reaction was immediately recorded at 240 nm (A1) and again 60 seconds later (A2) using an UV spectrophotometer. A difference in absorbance/min at 240 nm was indicative of the presence of catalase activity. The results are shown as nmol of H_2O_2consumed per min/mg protein (haemoglobin).

2.9. Nitric Oxide Quantification. Nitrate concentrations were evaluated according to the Griess method, as described previously [23]. Fifteen microliters of serum or homogenised spleen tissue from each mouse was incubated for 3 hours at room temperature with 5 μL of nitrate reductase (5 U/mL; Boehringer Mannheim, Laval, Quebec, Canada) and 15 μL of NADPH (1.25 mg/mL; Boehringer). Following incubation, 100 μL of Griess reagent (1% sulphanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride, and 1% orthophosphoric acid; Sigma Chemical Co. St. Louis, MO, USA) and 100 μL of trichloroacetic acid (10% aqueous solution) were added, and this mixture was incubated for 10 min at room temperature. Subsequently, protein precipitates were removed by centrifugation at 14,000 rpm for 5 min. Next, 100 μL of each supernatant was transferred to a 96-well flat-bottom plate. Concentrations were evaluated in an ELISA reader (Stat Fax Plate Translator, USA, to 540 nm) using a standard curve with sodium nitrate (Sigma), which was diluted in similarly prepared pooled sera from uninfected control CBA/Ca mice.

2.10. Malondialdehyde (MDA) Analysis for Lipid Peroxidation. MDA levels were measured as previously described [14]. Briefly, homogenised spleen or blood samples equivalent to 1 mg of protein or standard (1,1,3,3-tetramethoxypropane (Sigma)) were combined with 100 μL of orthophosphoric acid (0.2 M, Sigma), 125 μL of BHT (2 mmol/L; Sigma), and 12.5 μL of TBA (Fluka Chem, Buchs, Switzerland; 0.11 M in 0.1 mol/L NaOH (Sigma)). Both samples and standard were placed in a water bath, heated for 45 min at 90°C, ice-cooled to stop the reaction, and then extracted once with 250 μL of n-butanol (Sigma). The butanolic phase was separated by centrifugation at 1,500 g for 3 min and the absorbance at 535 nm was measured in a spectrophotometer. The concentration of MDA was calculated using a calibration curve.

2.11. Statistical Analysis. Significant differences between the groups for activities of superoxide dismutase, glutathione peroxidase, and catalase were determined using a one-way analysis of variance. The results are presented as the means ± standard deviations. Statistically significant differences were considered when $P < 0.05$ using the Stat Graphics program for Windows, release 4.

3. Results

3.1. Gonadectomy Modifies Parasitaemia in CBA/Ca Mice Infected with P. berghei ANKA. To determine whether gonadal steroids influence differences in parasitaemia between female and male mice, groups of intact, sham-operated, and gonadectomised (Gx) male and female CBA/Ca mice were infected with *P. berghei* ANKA. Parasitaemia was monitored daily by Giemsa-stained blood smears. Intact female mice developed higher levels of parasitaemia than male mice between days 5 and 7 after infection. However, after day 7, the opposite effect was observed; male mice developed significantly higher levels of parasitaemia than female mice (Figure 1(a)). Gonadectomy significantly increased parasitaemia in female mice compared with sham-operated female mice. In contrast, gonadectomy in male mice only increased parasitaemia levels on day 9 after infection (Figure 1(b)).

3.2. Gonadectomy Influences Body Weight and Anaemia in CBA/Ca Mice Infected with P. berghei ANKA. Body mass and red blood cell turnover are affected by sex steroids [24–26]. In addition, cachexia is an important feature of malaria pathology [27]. Therefore, we evaluated whether the weight loss and anaemic differences that are typically observed between the sexes could be affected by reduction in the levels of gonadal hormones in *P. berghei* ANKA-infected mice. To this end, intact, sham-operated, and Gx female and male mice were infected and then weighed daily. The weight of the mice on the day of infection (day 0) was considered to be 100%, and weight loss or gain was expressed as a percent value compared with day 0. The intact male mice weighed significantly more compared with the intact female mice at the same age (8 weeks). The intact female group lost 4% of their body weight

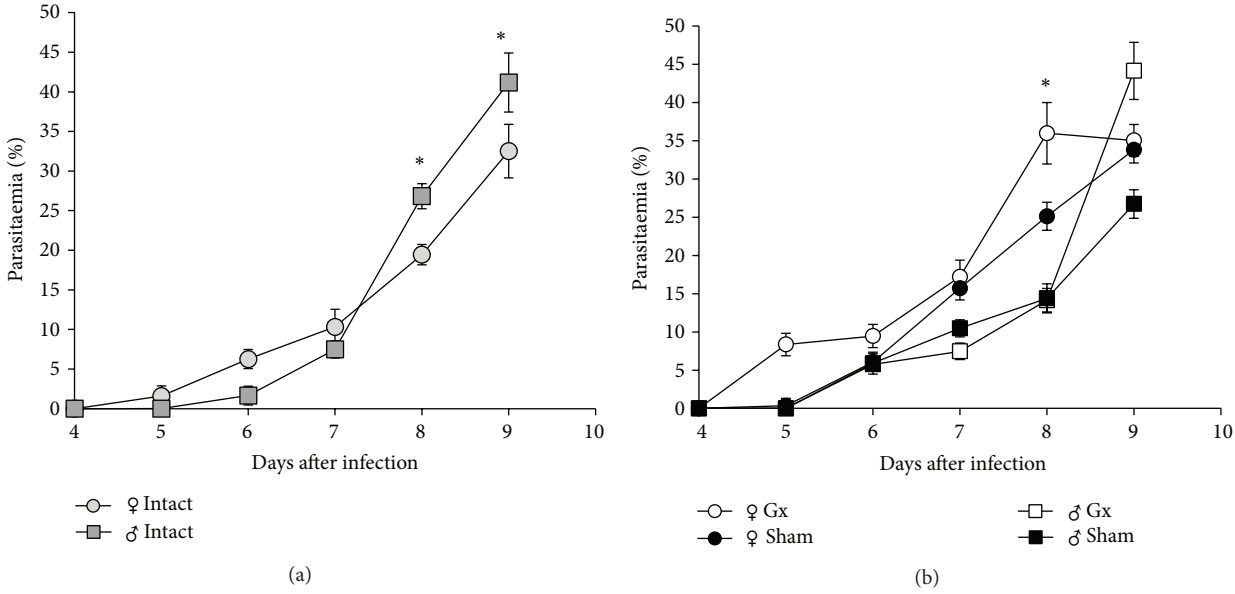

Figure 1: Effect of gonadectomy on parasitaemia levels in CBA/Ca mice infected with *P. berghei* ANKA. Gonadectomised, sham-operated, and intact female and male mice were infected with *P. berghei* ANKA. Parasitaemia levels were measured using Giemsa-stained blood films. Values are presented as the geometric mean ± SD ($n = 10$). Data are representative of two independent experiments. *indicates significant differences ($P < 0.05$).

between days 0 and 2 after infection and then started to increase in weight until day 6, reaching 100%; however, after day 6, the mice displayed a continuous loss of body weight (Figure 2(a)). Interestingly, the sham-operated female group exhibited significantly higher weights than sham-operated male mice for the duration of the entire experiment. On days 8 and 9 after infection Gx female mice significantly decreased body weight compared with the sham-operated female group, corroborating the influence of gonadal hormones on the regulation of corporal mass. In contrast, Gx male mice showed no change in body weight compared with the sham-operated male group. Interestingly, sham-operated female mice had significantly increased body weights compared with both the infected intact and infected sham-operated female groups. These findings suggest that sexual hormones in females are associated with weight increase, while in males sex hormones do not modify body weight in mice infected with *P. berghei* ANKA (Figure 2(a)).

To address whether the anaemia that develops in mice infected with *P. berghei* ANKA differs between the sexes and whether it is influenced by the presence or absence of gonadal steroids, groups of intact, sham-operated, and Gx female and male mice were infected with *P. berghei* ANKA. The peripheral blood concentrations of haemoglobin (Hb) in the mice were quantified daily. Parasitic infection decreased the levels of Hb in all groups. Both intact female and male mice exhibited lower Hb concentrations on days 8 and 9 after infection, and no significant differences were detected between the groups. In general, gonadectomy inhibited the decrease of Hb in the infected mice, but the Hb levels were significantly higher in Gx male mice compared with Gx female mice (Figure 2(b)).

3.3. Gonadectomy Decreases the Specific Activities of Catalase, SOD, and GPx in Both Female and Male Mice. The malaria parasite is highly susceptible to oxidative stress [21]. The mechanisms of the host defence system include phagocytosis and the production of NO and oxygen radicals, which modify the oxidative stress levels during infection. Certain enzymes work as free radical scavengers, which can circumvent the oxidative stress that malaria parasite induces in the host. These enzymes include catalase, SOD, and GPx, which are considered to be some of the most important host protective enzymes [28]. Therefore, to determine whether gonadal steroids could differentially affect oxidative stress levels caused by the parasite, the specific activities of catalase, SOD, and GPx were measured in the blood and spleen of intact, sham-operated, and Gx male and female CBA/Ca mice that were infected with *P. berghei* ANKA.

Intact female mice exhibited higher specific activities of catalase than intact male mice; however, this difference was only significant in the spleen (Figure 3(b)). Gonadectomy decreased the specific activity of catalase in both the blood and the spleen of female mice compared with female sham-operated mice. Gonadectomy in male mice did not significantly modify catalase activity in the blood (Figure 3(a)). In contrast, catalase activity was significantly decreased in the spleen of Gx male compared with sham-operated male mice (Figure 3(b)).

Intact female mice had higher SOD activities than male mice in both the blood and the spleen tissues. These activities were reduced in the blood and spleen of Gx female mice relative to sham-operated female mice. In contrast, no significant alterations in specific activity were detected in Gx male mice

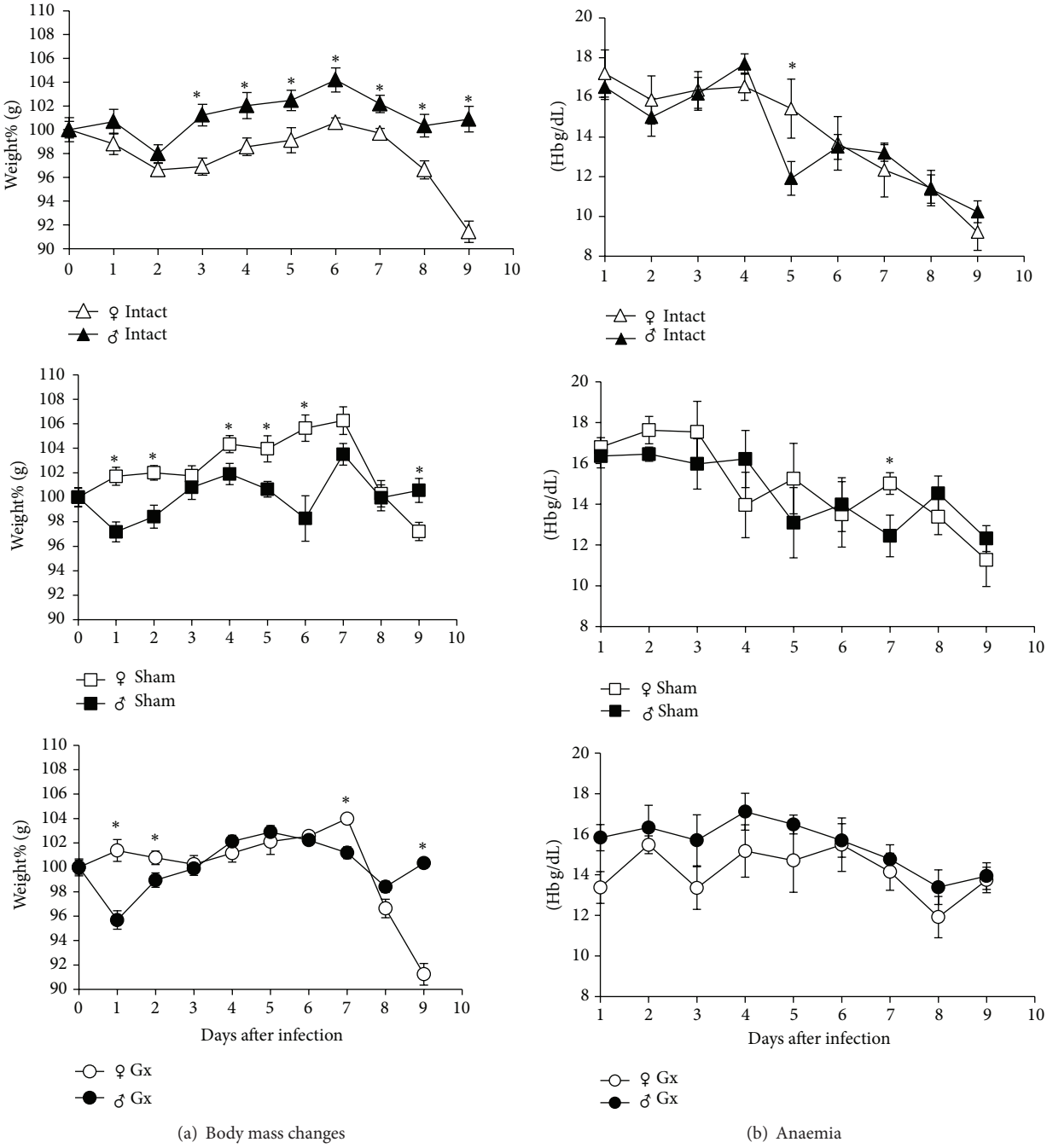

Figure 2: Effect of gonadectomy on body weight and anaemia in CBA/Ca mice infected with *P. berghei* ANKA. (a) Groups of gonadectomised, sham-operated, and intact female and male mice were infected with *P. berghei* ANKA; the weight on the day of infection (day 0) was considered to be 100%. Data represent the averages (means ± SD). * indicates significant differences ($P < 0.05$). (b) Haemoglobin levels were measured by the Drabkin method in the same groups of mice. Values are presented as the means ± SD ($n = 10$); * indicates significant differences ($P < 0.05$). Data are representative of two independent experiments.

in the blood or spleen compared with sham-operated male mice (Figures 3(c) and 3(d)).

Intact female mice displayed slightly higher GPx activities than intact male mice. Gonadectomy reduced these activities in female mice but did not modify them in the blood of male mice compared with the corresponding sham-operated group (Figure 3(e)). Gonadectomy significantly reduced the specific activity of GPx in the spleen of female mice but showed no effect in the spleen of male mice (Figure 3(f)).

3.4. Gonadectomy Increases the Levels of NO in Female Mice. NO in the presence of oxygen is oxidised to different biologically active nitrogen oxides known as reactive nitrogen

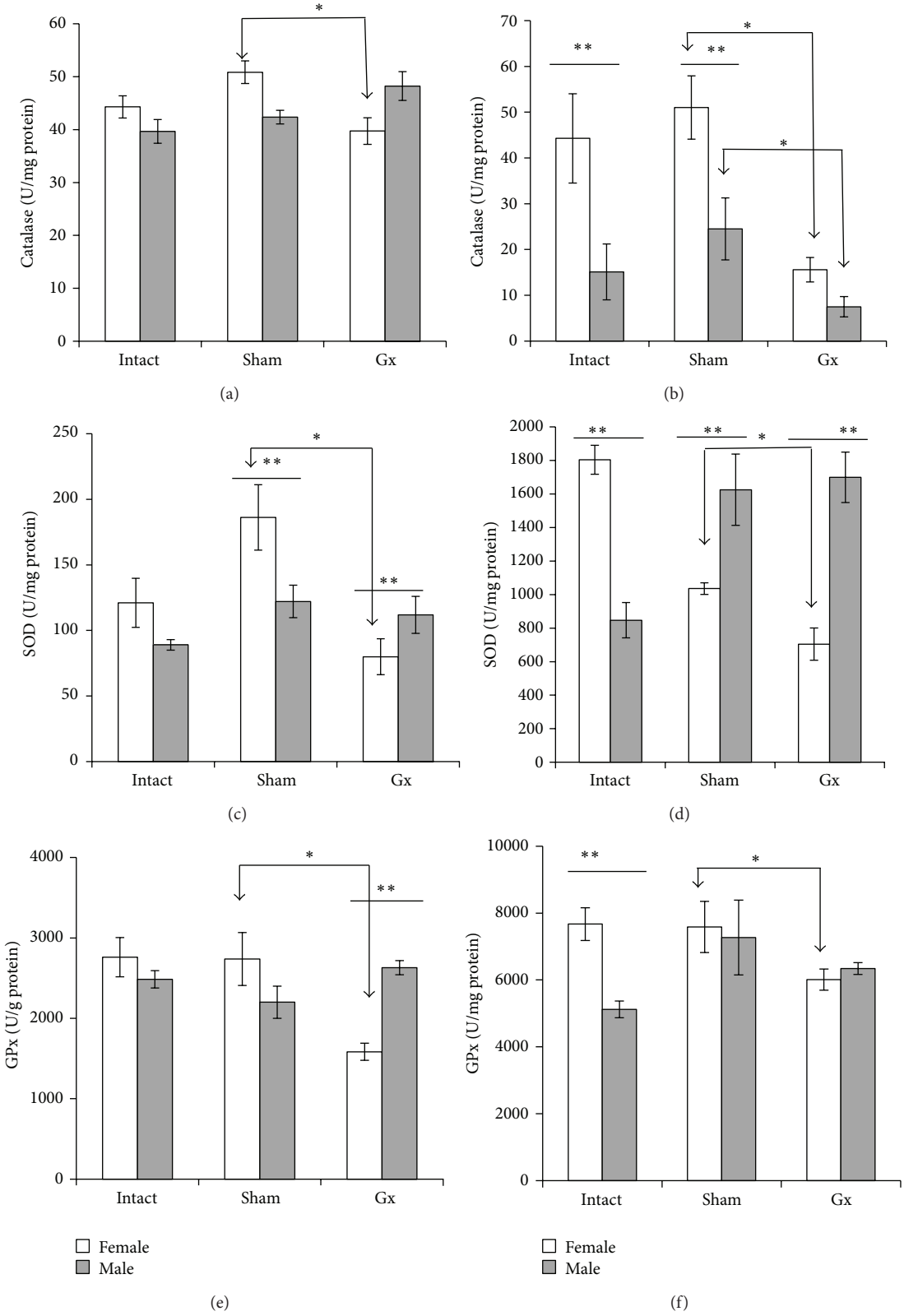

Figure 3: Gonadectomy decreases the specific activity of SOD, GPx, and catalase in female mice infected with *P. berghei* ANKA. Gonadectomised, sham-operated, and intact female and male mice were infected with *P. berghei* ANKA, and on day 9 after infection, the mice were sacrificed, and blood (a) and spleen (b) samples were obtained to quantify the specific activities of catalase, SOD, and GPx. Data represent the average (means ± SD). ($n = 10$), * indicates significant differences ($P < 0.05$). ** indicates significant differences between female and male mice. Data are representative of two independent experiments.

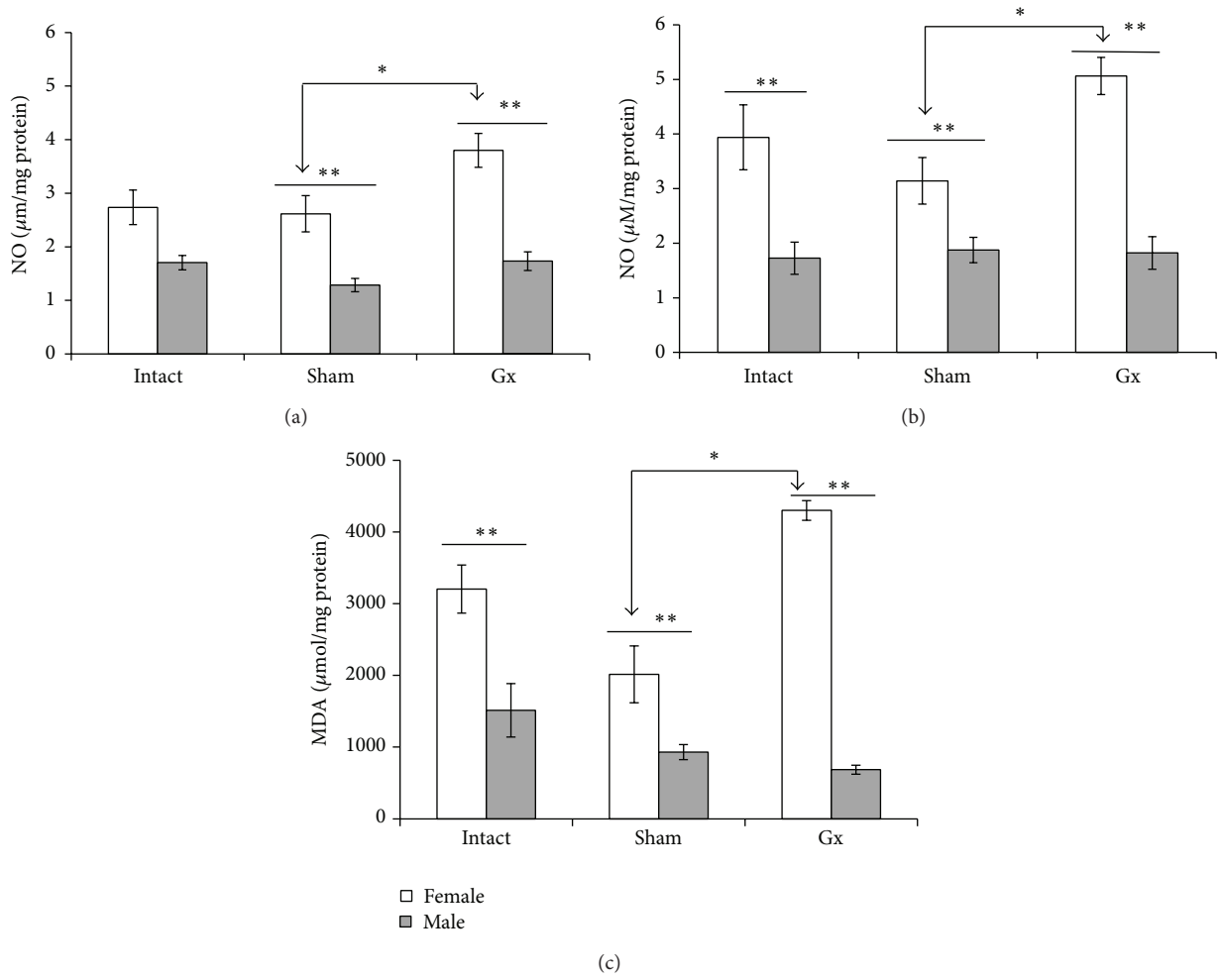

Figure 4: Effect of gonadectomy on nitric oxide and MDA in *P. berghei* ANKA-infected mice. Gonadectomised, sham-operated, and intact female and male mice were infected with *P. berghei* ANKA, and on day 9 after infection, mice were sacrificed, and blood (a) and spleen samples (b and c) were used to quantify the levels of NO and spleen was used to measure the concentration of MDA. Data represent the average (means ± SD). ($n = 10$), *indicates significant differences ($P < 0.05$). **indicates significant differences between female and male mice.

intermediates (RNIs). NO is a potent immune-modulator that has been implicated in both immune response against the malaria parasite [15] and malaria pathology [29]. Therefore, we analysed whether gonadal steroids could differentially affect the NO levels between sexes in CBA/Ca mice infected with *Plasmodium*. To this end, intact, Gx, and sham-operated female and male mice were infected with *P. berghei* ANKA, and on day 9 after infection, NO levels were measured in both serum and spleen tissue cells using the Griess reaction. All of the groups of female mice showed significantly higher levels of NO compared with the respective male groups. Gonadectomy significantly increased NO levels in the serum and spleen of female mice compared with sham-operated female group. Gonadectomy in male mice had no effect on NO levels in the blood or spleen (Figures 4(a) and 4(b)).

3.5. MDA Levels. MDA levels were measured as a proxy for the extent of lipid peroxidation in the spleen, the organ in which the malaria parasite is ultimately eliminated. MDA concentrations were significantly higher in intact female mice compared with intact male mice. Gonadectomy in female mice significantly increased the MDA levels in the spleen compared with the sham-operated female group. In contrast, gonadectomy in male mice decreased the MDA levels; however, this difference was not statistically significant compared with the sham-operated male group (Figure 4(c)).

4. Discussion

The results of the present study show for the first time that gonadal steroids modulate oxidative stress in *P. berghei* ANKA-infected mice. Gx female mice that were infected with the parasite developed significantly higher oxidative stress levels compared with their male counterparts.

The hormonal effects that occur as a result of gender differences do not only affect growth but also exhibit effects

on host immunity. After *Plasmodium* infection, the immune response increases phagocytosis and the production of oxygen radicals and NO, which play key roles in host defence mechanisms against malaria [21] because *Plasmodium* parasites are highly susceptible to oxidative stress [30]. However, the relationship between the redox status of malarial parasites and that of their host is complex, involving both the antioxidant and host defence systems. In fact, the generation of oxidative stress is an important chemotherapeutic strategy against the malaria parasite [14, 31, 32]. However, redox status alterations also contribute to disease manifestations, including sequestration, anaemia, and cerebral pathology [33]. Nevertheless, the relationship between the immune system's oxidative stress response to malarial parasites and gonadal hormones that are involved in sexual dimorphism is complex and has not been elucidated in the parasitised host.

The present study shows that the infection of CBA/Ca mice with *P. berghei* ANKA induced higher parasitaemia levels in intact males compared with intact female mice. However, both anaemia and parasitaemia were exacerbated in Gx female compared with Gx male mice. These findings suggest an involvement of gonadal steroids in the control of parasite proliferation and erythropoiesis in female CBA/Ca mice. In general, anaemia is characterised by the reduction in Hb levels in relation to age, gender, and physiological status. However, the aetiology of anaemia in malaria-infected individuals includes the lysis of infected and uninfected red blood cells [34], dyserythropoiesis, and bone marrow suppression [35, 36]. The increased anaemia that was observed in infected Gx female mice could be potentially explained by their increased NO levels because it has been shown that NO inhibits haem synthesis and iron uptake via the transferrin receptor pathway [37]. In addition, the cytokines TNF-α and IFN-γ induce the suppression of human haematopoiesis, which is mediated by NO [38]. Finally, anaemia could be also increased by the lysis of parasitised erythrocytes.

Sex hormones affect oxidative stress levels. For example, changes in the physiological levels of oestradiol and testosterone alter the production of O_2^- and H_2O_2 in rat macrophages [39]. In addition, the levels of 8-oxo-7,9-dihydro-2′-deoxyguanosine and other modified bases are higher in males than in females [40]. In the present study, the effect of gonadal hormones on oxidative stress in *P. berghei* ANKA-infected mice was supported, particularly in the Gx female mice group, by the significantly decreased catalase, GPx, and SOD specific activities in both blood and spleen of infected female mice compared with sham-operated female or Gx male mice. These findings could be explained by the fact that oestradiol increases the antioxidant activities of these enzymes [41].

The increased NO concentrations that were detected in the blood and spleen of the infected Gx female group correlated with the enhanced oxidative stress that was observed in these mice, particularly when compared with the sham-operated female or Gx male groups. The increased production of NO in *P. berghei* ANKA-infected Gx female mice may be explained by the fact that oestradiol inhibits nitrite release by macrophages [39]. However, even the intact group of infected female mice developed higher levels of NO compared with their intact male counterparts. This increase in NO levels in infected females is consistent with previous reports of female resistance to infection in experimentally induced malaria [42] and also supports the sexual dimorphism that has been described in malaria models [43]. However, our findings with regard to NO in the Gx female mice do not correlate with previous studies [44], and we believe that this divergence may be explained by variations in the numbers of parasitised erythrocytes in the inocula and in differences in the time points at which the NO levels were measured because we have found that NO peak levels only last for two days during infection [15].

In this work, increased lipid peroxidation levels as indicated by MDA concentration were observed in *P. berghei* ANKA-infected Gx female mice compared with both sham-operated female and Gx male groups. The decreased antioxidant enzymatic activity and increased levels of MDA could be explained by the fact that oestradiol, due to its hydroxyphenolic structure, may donate hydrogen atoms to lipid peroxyradicals to terminate chain reactions and act as an antioxidant and free radical scavenger [45]. In addition, oestradiol may upregulate the expression of the antioxidant enzymes catalase, SOD, and GPx [46].

Finally, testosterone suppresses immunity against *Plasmodium chabaudi* in C57BL/10 mice [47–49]. Consistently, testosterone administration induces a lethal outcome of otherwise self-healing blood-stage infections in female mice [50]. Supporting this finding, Gx male mice have been shown to be more resistant to *Plasmodium* infection [49]. These findings are consistent with the results of our previous study in which the administration of testosterone decreased the oxidative stress in female mice infected with *P. berghei* ANKA. This suppression could be partially explained by the fact that testosterone decreases the number of blood leukocytes [51]. The sexual dimorphism in malaria is complex, with oxidative stress being modulated by the direct antioxidant action of oestradiol or via regulation of gene expression. These processes can only partially explain the observed phenomena. Additional biological functions of gonadal hormones also play important roles, including the testosterone-mediated induction of immunosuppression.

5. Conclusions

Gender differences may significantly affect immunological responses. In this work, we presented evidence that *P. berghei* ANKA-induced oxidative stress is higher in female CBA/Ca compared with male mice and that gonadal steroids negatively modulate oxidative stress levels, mainly in female mice. These findings may partially explain the gender differences that are observed in terms of susceptibility to malaria and should be taken into account when considering treatment options or designing future effective vaccines against *Plasmodium*.

Conflict of Interests

The authors have declared that no conflict of interests exists.

Acknowledgments

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Research Article

High Molecular Weight Proteins of *Trypanosoma cruzi* Reduce Cross-Reaction with *Leishmania* spp. in Serological Diagnosis Tests

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Chagas disease is caused by the parasite *Trypanosoma cruzi*. Because of its distribution throughout Latin America, sometimes it can overlap with other parasitic diseases, such as leishmaniasis, caused by *Leishmania* spp. This might represent a problem when performing serological diagnosis, because both parasites share antigens, resulting in cross-reactions. In the present work we evaluated Mexican sera samples: 83.8% of chagasic patients recognized at least one antigen of high molecular weight (>95 kDa) when evaluated by Western blot. Proteins of 130 kDa and 160 kDa are predominantly being recognized by asymptomatic chagasic patients. When the proteins were extracted using Triton X-100 detergent, a larger number of specific *T. cruzi* proteins were obtained. This protein fraction can be used to increase specificity to 100% in Western blot assays without losing sensitivity of the test. High molecular weight proteins of *T. cruzi* include glycoproteins with a great amount of αMan (α-mannose), αGlc (α-glucose), GlcNAc (N-acetylglucosamine), and αGal (α-galactose) content and these structures play an essential role in antigens recognition by antibodies present in patients' sera.

1. Introduction

Trypanosoma cruzi is the causative agent of Chagas disease, endemic to many countries of Latin America and affecting millions of people. The parasite enters the body through broken skin and mucous membranes and causes acute but often mild symptoms. After a few months the chronic stage develops; however it can persist unnoticed for many years before causing abnormal heart rhythm, heart failure, digestive problems, and sudden cardiac death [1]. In this stage the detection of the parasite in peripheral blood is complicated, since *T. cruzi* is able to infect almost every cell of the host. Many serological tests are used for diagnosis, which detect specific antibodies against antigens of *T. cruzi*. In most of these techniques total extract of epimastigotes (insect stage of the parasite) is used as antigen with high levels of sensitivity, due to their easy culture which provides a large number of parasites, not like trypomastigotes' production which can be expensive and inefficient, increasing the cost of the diagnosis tests. But the use of epimastigotes protein extracts might cross-react with other infections, such as leishmaniasis caused by another trypanosomatid: *Leishmania* spp., so purified or recombinant antigens of different stages of *T. cruzi* have been tested [2] and still there is controversy about which antigen is the most efficient. In Mexico there are few reports about the geographic zones where Chagas disease and leishmaniasis may converge, but there have been some attempts to improve specificity of serological tests [3, 4].

The surface of this protozoan parasite is covered with a high density coat of glycoproteins, which contribute to

Table 1: Mexican chagasic patients' sera information.

Sample number	Symptomatic	Western blot (protein bands)	ELISA test (OD/cut-off point)
1	symptomatic	3	1.45
2	US	>10	4.00
3	symptomatic	9	3.65
4	symptomatic	5	3.41
5	symptomatic	10	9.12
6	US	1	1.71
7	US	5	1.00

US: Unknown Status.

both parasite protection and establishment of a persistent infection. Glycosylinositolphospholipids (GIPLs) and mucins represent the most abundant glycoconjugates in *T. cruzi* surface. Mucins are glycoproteins that bear a dense array of *O*-linked oligosaccharides which makes them well-suited for protection. During its life cycle, *T. cruzi* undergoes biochemical and morphological changes, including variation in the surface mucins and so in their biological activity. Other important glycoproteins present in *T. cruzi* surface are the trans-sialidases (TS), molecules able to transfer sialic acid residues from host glycoconjugates to parasite mucins [5]. *Leishmania* spp. surface is covered with glycans as well, in order to survive the hostile environments to which it is exposed during its life cycle, being the most abundant the lipophosphoglycans in the promastigote stage [6]. Because of the antigenic similarities between both parasites, the aim of the present work was to find a protein fraction of a Mexican strain of *T. cruzi* that might reduce cross-reaction observed in serological diagnostic tests, without losing their sensitivity for routine diagnosis.

2. Materials and Methods

2.1. Parasite Culture and Antigen Preparation. Epimastigotes forms of *T. cruzi* (TBAR/MX/0000/Querétaro strain) were grown in liver infusion tryptose medium (LIT) supplemented with 10% of fetal bovine serum previously inactivated at 56°C for 30 min and 25 μg/mL of hemine. Cultures were harvested at the log phase of growth [3].

Promastigotes of *Leishmania mexicana* were cultured in 199 medium supplemented with 10% of fetal bovine serum previously inactivated as described before, 1 M Hepes, 0.25% hemine, 50% triethanolamine, and 200 mM L-glutamine. Cultures were also harvested at the log phase of growth.

For the protein extracts preparation, parasites were collected by centrifugation at 2,000 g for 15 min at 4°C and divided into four fractions for different extraction methods. The parasites were washed and centrifuged twice in phosphate buffered saline (PBS) pH 7.2.

For proteins extraction by sonication, the pellet obtained after the last centrifugation cycle described before, was suspended in 5 mL of 10 mM Tris-HCl, pH 8.2 per gram of humid parasites with protease inhibitors (12 mM EDTA, 1 mM PMSF, 0.1 mM leupeptin, and 0.001 mM pepstatin). The parasites were sonicated three times for 1 min each. The mixture was centrifuged at 10,000 g for 30 min at 4°C. The supernatant was recovered and the protein concentration was determined using DC Protein Assay Kit (Bio-Rad Laboratories). The extract was stored at −20°C until use.

For Triton X-100 extraction, the pellet was suspended (2×10^6 parasites/buffer μL) in 1% Triton X-100 solution with protease inhibitors for 30 min at 4°C. The mixture was centrifuged at 10,000 g for 10 min at 4°C for supernatant recovering and the protein concentration was determined using DC Protein Assay Kit (Bio-Rad Laboratories). The extract was stored at −20°C until use.

For NP40 extraction, the pellet was suspended in 5 mL of 1% NP40 solution per gram of humid parasites with protease inhibitors. A vortex was used to shake the suspension for 15 seconds. The mixture was centrifuged at 10,000 g for 30 min at 4°C. The supernatant was then recovered and the protein concentration was determined using DC Protein Assay Kit (Bio-Rad Laboratories). The extract also was stored at −20°C until use.

For urea-thiourea extraction, the pellet was suspended in 3 mL of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, and 120 μM Tris) per gram of humid parasites with protease inhibitors. A vortex was used to shake the suspension for 3 min and then it was kept in ice for 10 min. The mixture was centrifuged at 10,000 g for 15 min and the supernatant was recovered. The protein concentration was determined using 2-D Quant kit. The extract was stored at −20°C until use.

2.2. Sera. Sera used in this study come from 212 sera from Mexican infected patients who turned up from the *Centro Médico Nacional "La Raza"* of the *Instituto Mexicano del Seguro Social* with a positive result for Chagas disease by a commercially available ELISA test (Chagatest) for a confirmatory diagnosis to the *Laboratorio de estudios sobre Tripanosomiasis* at the *Instituto de Investigaciones Biomédicas*, *Universidad Nacional Autónoma de México* (UNAM). All of the blood donors were informed of the aim of the study and accepted to participate in it, signing an agreement. All sera were evaluated by ELISA and Western blot using sonicated total extract of epimastigotes of *T. cruzi*, according to the protocol described by Sánchez et al. [3]. Information of clinical features of patients used for experiments corresponding to Figures 1, 4, and 5 is shown in Table 1. A pool of well characterized positive patients' sera from Chagatest (Wiener lab; http://www.wiener-lab.com.ar/wiener/catalogo/archivos/6376_chagatest_elisa_recombinante_v3_0_en.pdf) was used as a positive control. As negative

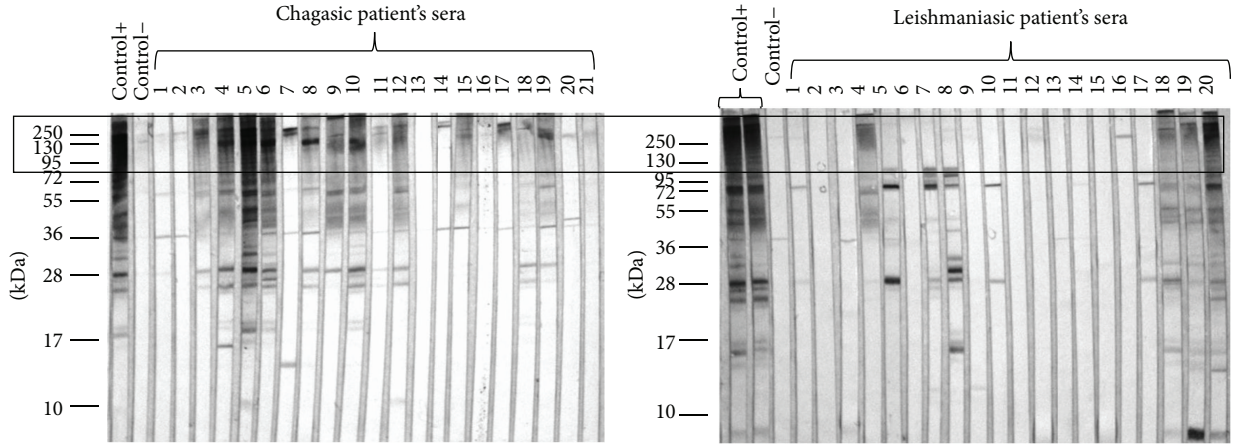

Figure 1: High molecular weight (>95 kDa) antigens predominantly recognized by chagasic patients antibodies. SDS-PAGE was performed in 12% polyacrilamide gel and then transferred into a nitrocellulose membrane for Western blot analysis of chagasic and leishmaniasic patients' sera (dilution 1 : 500) with sonicated total extract of epimastigotes of *T. cruzi* and peroxide-conjugated anti-human IgG (1 : 10000). Predominant recognition of antigens with molecular weights >95 kDa by chagasic patients' antibodies and less recognition by leishmaniasic patients' antibodies of the same antigens is highlighted (black box).

controls, sera of some laboratory members were used. Also a total of 27 sera samples of confirmed leishmaniasic patients were used.

2.3. Electrophoresis and Western Blot. Proteins of the four different *T. cruzi* extracts were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie blue G250 or Silver staining. When required, Western blot assays were carried out by transferring the proteins in the polyacrylamide gel into nitrocellulose membranes. 12% acrylamide concentration was used when proteins of 10 kDa–250 kDa were visualized; 6% acrylamide concentration was used when proteins of high molecular weight (>95 kDa) were visualized. The membrane was cut in strips and left overnight in PBS containing 10% skimmed milk at 4°C with constant shaking. Individually, each strip was incubated with the serum to be evaluated, diluted 1 : 500 or 1 : 750 in PBS containing 10% skimmed milk for 2 h at room temperature. Each strip was washed three times with 0.1% Tween 20 in PBS solution and then incubated with peroxidase-conjugated anti-human IgG diluted 1 : 10000 at room temperature for 2 h. Three more washes were carried out and then the reaction was developed, adding 0.5 mg/mL of 3,3-diaminobenzidine in PBS and 0.02% H_2O_2. Distilled water was used in order to stop the reaction. Positive and negative control sera were included in each assay.

2.4. Lectin Blotting. After electrotransferring proteins into the nitrocellulose membranes, the strips were left for 2 h with 0.1% Tween 20 in PBS solution at room temperature and constant shaking. Each strip was then incubated with 5 μg/mL of biotinilated lectins: *Concavalin A* (Con A), *Triticum vulgaris* (WGA), *Arachis hypogaea* (PNA), *Psophocarpus tetragonolobus* (PT), *Artocarpus integrifolia* (Jacalin), and *Maackia amurensis* (MAA) in 0.1% Tween 20 in PBS solution overnight at 4°C. Three washes were carried out as described in the Western blot method. Afterwards, each strip was incubated with peroxidase-conjugated streptavidin diluted 1 : 2000 in 0.1% Tween 20 in PBS for 2 h at room temperature. Three more washes were carried out and the reaction was developed as described before.

2.5. Enzymatic Deglycosylation. In order to remove all glycans of glycoconjugates present in the different protein extracts of *T. cruzi,* the Enzymatic Protein Deglycosylation kit (Sigma) was used. Briefly, 100 μg of proteins was diluted in 30 μL of miliQ water. 10 μL of 5x reaction buffer was added, as well as 2.5 μL of denaturation solution, then the mixure was gently mixed. Extracts were heated at 100°C for 5 min and cooled to room temperature, after which 2.5 μL of the Triton X-100 solution was added. In order to remove sialic acid residues and *O*-glycans, 1 μL of $\alpha(2 \rightarrow 3,6,8,9)$-neuraminidase and 1 μL of *O*-glycosidase were added. To remove galactose and N-acetylglucosamine residues, 1 μL of both $\beta(1 \rightarrow 4)$-galactosidase and β-N-acetylglucosaminidase was added. Each extract was incubated for 3 h at 37°C and then analyzed by electrophoresis or electrotransferred into a nitrocellulose membrane for Western blot assays.

2.6. Statistical Analysis. Student's *t*-test was carried out using SigmaStat 3.5 and GraphPad Prism 5 software. Significant levels were found at $P < 0.001$; $P < 0.05$.

3. Results

3.1. High Molecular Weight Antigens Are Recognized by Antibodies of Chagas Disease Patients. Sera samples were evaluated by ELISA and Western blot using as an antigen a sonicated total extract of *T. cruzi* epimastigotes to confirm and characterize them as positive to the infection. Only those samples with a positive result in both tests were considered for this study. In the Western blot evaluation,

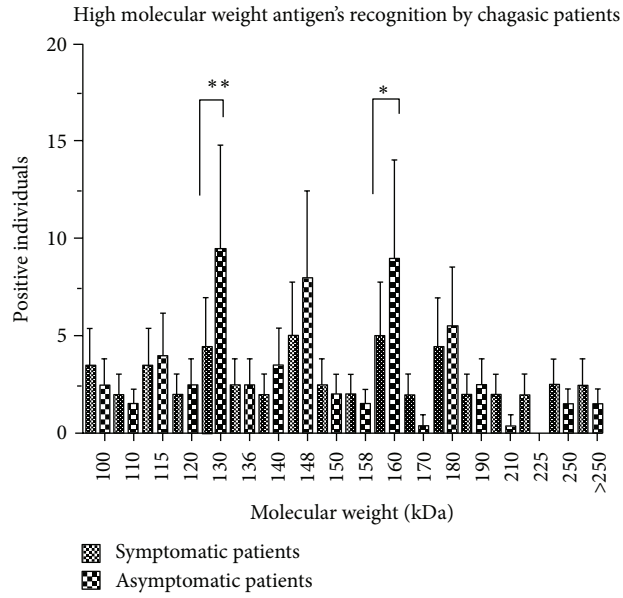

Figure 2: High molecular weight *T. cruzi* antigens' recognition by chagasic symptomatic and asymptomatic patients. Western blot analysis was performed on 173 chagasic patients' sera samples (1 : 500) with total sonicated extract of epimastigotes of *T. cruzi* and peroxide-conjugated anti-human IgG (1 : 10000). Molecular weight of each antigen recognized by symptomatic and asymptomatic patients was determined by comparison of molecular weight markers (Fermentas). Results represent media ± standard deviation. $^{**}P < 0.001$; $^{*}P < 0.05$.

it was found that a major recognition of high molecular weight proteins by the antibodies is present in sera of chagasic patients: from 173 double positive sera samples evaluated, 83.8% (145) recognized at least one protein of high molecular weight (>95 kDa). Recognition in Western blot assays of these proteins by antibodies present in sera of leishmaniasic patients is considerably lower (Figure 1).

Nevertheless, a heterogeneous recognition of high molecular weight proteins (>95 kDa) by antibodies of chagasic patients was observed. We found 18 different proteins recognized. The recognition of antigens by patients with a heart disease diagnosis and those who are asymptomatic was compared (Figure 2). There is a significant difference observed in the 130 kDa and 160 kDa antigens, which are being recognized preferentially by antibodies of asymptomatic patients. The 225 kDa antigen was recognized only by antibodies of 3 symptomatic patients.

3.2. High Molecular Weight Proteins Extracted by Different Methods. High molecular weight proteins are recognized by most of the infected patients. But when the epimastigote sonicated total extract is separated by SDS-PAGE and stained with Coomassie Blue G250, few proteins with molecular weight >95 kDa are visualized (Figure 3(a)). Consequently, proteins were extracted using two detergents (Triton X-100, NP40) and two chaotropic agents (urea-thiourea) in order to try to obtain higher amounts of these proteins. Proteins were separated by SDS-PAGE in a 6% acrylamide concentration in order to improve high molecular weight proteins resolution (Figure 3(b)). Few differences were found between them. With Coomassie blue staining we observed a 190 kDa and a 156 kDa protein present only in the sonicated extract, as well as in the Triton X-100 extract. The Silver staining revealed a 224 kDa and a 215 kDa protein present in the sonicated, Triton X-100, and NP40 extracts but were absent in the urea-thiourea extract.

Then, 7 sera of chagasic patients and 7 sera of leishmaniasic patients were tested using total extract as well as each high molecular weight fraction. Proteins from each extract were separated using a 12% polyacrilamide gel in order to use the total extract and they were also separated in 6% polyacrilamide gels when it was required to maintain only high molecular weight proteins by SDS-PAGE. Then they were transferred into nitrocellulose membranes for Western blot assays. When using sonicated total extract, there were 5 out of 7 samples of leishmaniasic patients that cross-reacted (Figure 4(a)). Thus, we increased the sample dilution from 1 : 500 to 1 : 750 and used each high molecular weight fraction obtained by the different methods. Even though the number of recognized antigens was lower, all cases antibodies of chagasic patients recognized at least one antigen of high molecular weight in each fraction, maintaining the sensibility of the test. However, using these fractions the unspecific antigen recognition observed by the antibodies of leishmaniasic patients was reduced. High molecular weight proteins from Triton X-100 extraction display 100% sensibility and 100% specificity, since none of the leishmaniasic samples antibodies recognized any of the antigens of >95 kDa (Figure 4(b)).

3.3. Glycoconjugates Present in High Molecular Weight Proteins of T. cruzi and Leishmania. To determine the presence of glycoconjugates in the high molecular weight fractions of the different extracts, Lectin blot assays were developed. Molecular weights of those carbohydrates recognized by lectins that correspond to molecular weights of antigens recognized by antibodies of chagasic patients are shown in Table 2.

High molecular weight proteins of sonicated extract show an abundant glycan content; Con A and Jacalin bound several glycoproteins with αMan and αGal, respectively. High molecular weight fraction of Triton X-100 and NP40 extraction turned out to be αMan rich as well. Few glycoproteins are extracted when using urea-thiourea buffer, since Con A and Jacalin bound only two carbohydrates each.

The glycan content in high molecular weight proteins of promastigotes of *Leishmania mexicana* sonicated extract was also determined. This protein fraction, analyzed by Lectin blot, showed a fewer number of glycoproteins. Con A lectin bound to αMan in the 190, 130, and 112 kDa molecular weights. PT lectin bound to carbohydrates in the 210, 200, and 112 kDa molecular weights.

3.4. Deglycosylation of Glycoproteins in the Extracts Diminishes the Antigen-Antibody Reaction in Western Blot. It was important to determine whether the carbohydrates of *T. cruzi* glycoproteins play an important role in antigenicity of the high molecular weight protein fractions. Deglycosylation was

Table 2: Glycoproteins present in high molecular weight extracts fraction.

Extraction agent	Lectin	Recognized carbohydrate	Glycoprotein M.W. (kDa) recognized also by chagasic patients' antibodies
Sonication	Con A	αMan > αGlc > GlcNAc	>250, 190, 160, 130, 120, 110, and 100
	WGA	GlcNAc, NeuNAc	>250, 190
	Jacalin	αGal → Ome	>250, 190, 130, 120, 110, and 100
	PT	GalNAc, βGal	—
	PNA	βGal (1 → 3) galNAc	—
Triton X-100	Con A	αMan > αGlc > GlcNAc	>250, 170, 160, 140, 120, and 110
	WGA	GlcNAc, NeuNAc	>250
	Jacalin	αGal → Ome	—
	PT	GalNAc, βGal	—
	PNA	βGal (1 → 3) galNAc	—
NP40	Con A	αMan > αGlc > GlcNAc	>250, 170, 150, 120, 115, and 100
	WGA	GlcNAc, NeuNAc	—
	Jacalin	αGal → Ome	—
	PT	GalNAc, βGal	—
	PNA	βGal (1 → 3) galNAc	—
Urea-thiourea	Con A	αMan > αGlc > GlcNAc	>250, 100
	WGA	GlcNAc, NeuNAc	—
	Jacalin	αGal → Ome	130, 100
	PT	GalNAc, βGal	—
	PNA	βGal (1 → 3) galNAc	—

Lectin blot assays were performed to determine carbohydrate content in each fraction with a 5 μg/mL concentration of lectins Con A, WGA, Jacalin, PT, PNA, and MAA and the peroxidase-conjugated streptavidin (dilution 1 : 2000).

developed using an Enzymatic Protein Deglycosylation kit (Sigma) from the high molecular weight fraction of Triton X-100 extract, which previously showed to increase specificity when used as antigen in Western blot, and the fraction of the sonicated extract, which is commonly used as antigen in serological tests for diagnosis of Chagas disease.

Sialic acid and *O*-glycans were removed from glycoproteins by adding $\alpha(2 \rightarrow 3,6,8,9)$-neuraminidase and *O*-glycosidase. Galactose (gal) and N-acetylglucosamine (GlcNAc) residues were removed from the glycoproteins by adding $\beta(1 \rightarrow 4)$-galactosidase and β-N-acetylglucosaminidase of both the Triton X-100 and the sonicated extract. Later, extracts were separated by SDS-PAGE in a 6% polyacrylamide gel. The deglycosylated proteins were transferred to a nitrocellulose membrane and incubated with chagasic and leishmaniasic sera samples. PT lectin was used as a positive control. Regardless of the treatment received, the antigens' recognition by the specific antibodies was almost completely lost in both cases (Figure 5).

4. Discussion

Trypanosomatids are the etiological agents of different infections and some of them share antigens recognized by antibodies present in patients' sera. Total extracts of epimastigotes are commonly used as antigens, since it is easier, less expensive, and more efficient to culture this parasite stage than trypomastigotes *in vitro*. Furthermore, since Chagas disease is considered to be a health problem mainly in rural locations of Latin American countries, where sometimes it is hard to count with the suitable equipment for diagnosis, efforts for developing simple, economic, and available serological tests must not be abandoned. It is well established that antibodies in patients sera are able to recognize *T. cruzi* antigens present in epimastigote stage as well [3].

Since high molecular weight proteins seem to be minor components in *T. cruzi* as observed by electrophoresis in the polyacrylamide gel, their purification might not be an easy task. For that reason, it should be considered to produce higher amounts of the antigens recognized by antibodies of Chagasic patients in a heterologous system.

In spite of the efforts for developing a sensible and specific test, high levels of false positive results are still obtained in some of serological diagnosis techniques [7–9]. Thus, it is important to keep looking for an antigen or group of antigens specific of *T. cruzi* that can improve specificity of the diagnostic tests.

When chagasic and leishmaniasic patients' sera were evaluated by Western blot, a predominant recognition of high molecular weight antigens by chagasic patients was found. About 83.8% of the patients recognized at least one antigen of molecular weight >95 kDa. Since each strain has specific characteristics and each patient develops antibodies against different antigens, a great heterogeneity was found when the molecular weight of the antigens recognized in this fraction was determined. Eighteen different proteins were

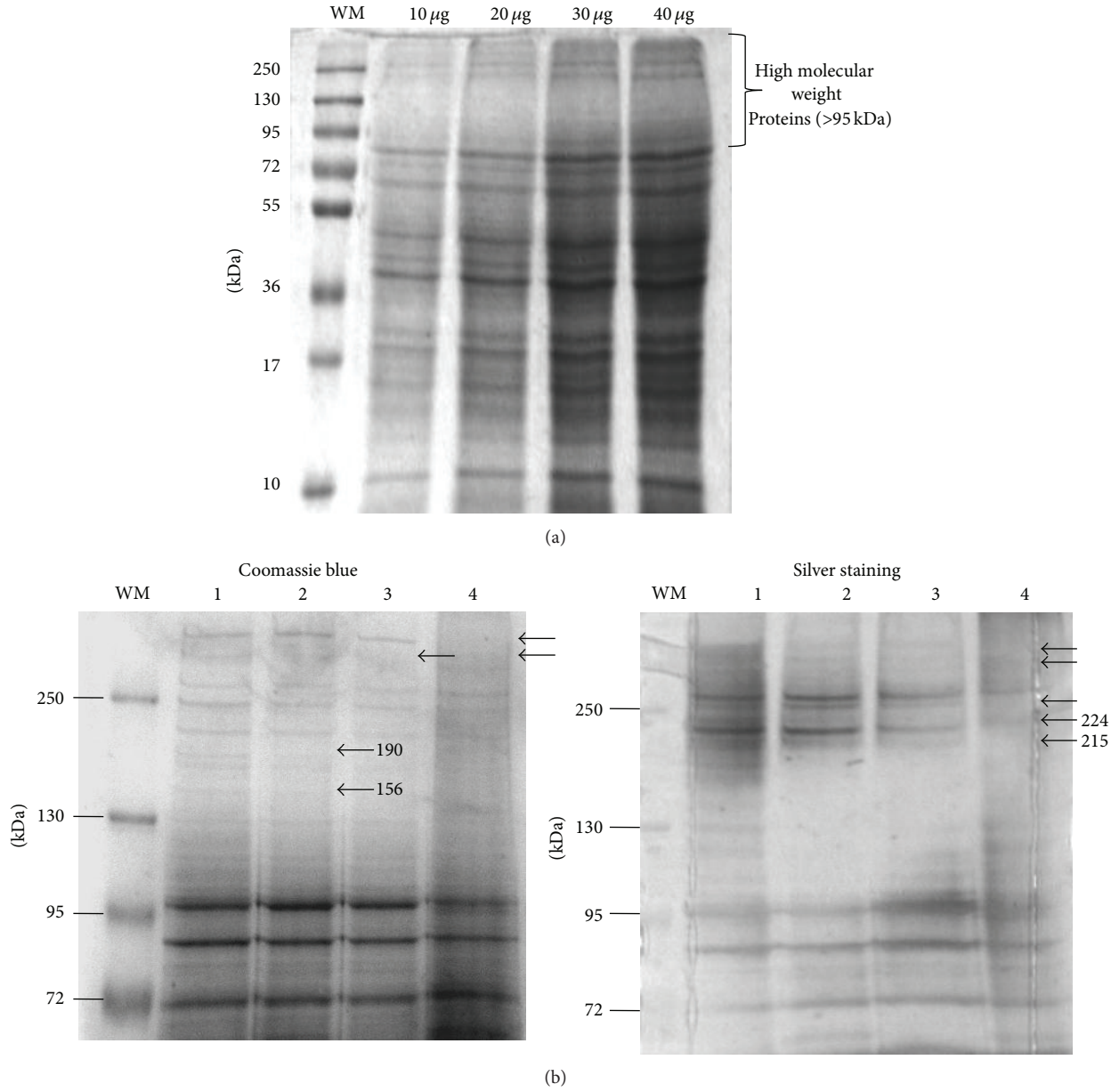

Figure 3: Electrophoresis of *T. cruzi* epimastigotes extracts. (a) SDS-PAGE of sonicated extract was performed in 12% polyacrylamide gel; each concentration has been pointed out. In all cases high molecular weight proteins (M.W. > 95 kDa) were found to be scarce. Gel was stained with Coomassie blue G250. (b) Electrophoresis of high molecular weight protein fractions of *T. cruzi* epimastigotes extracts. (1) Sonication. (2) Triton X-100 extraction. (3) NP40 extraction. (4) Urea-thiourea extraction. Concentration in each case was 15 μg. SDS-PAGE was performed in 6% polyacrylamide gels. Differences between extracts and their molecular weight are highlighted (black arrows). Molecular weight markers (Fermentas) were used (WM), and gel was stained with Coomassie blue G250 or Silver staining.

found by Western blot and those of 130 kDa and 160 kDa are predominantly being recognized by asymptomatic patients' antibodies. It may be possible for these patients to possess a greater amount of antibodies against these antigens, so they have not developed any symptoms. Other studies must be carried out to establish if these antigens can be used as markers for protection of development of pathology or in order to produce a vaccine.

Proteins of high molecular weight from epimastigotes are not abundant when sonicated, so we tried to enrich the fraction with other extraction methods. Detergents' structure provides them with amphiphatic characteristics that allow them to aggregate in polar media forming micelles. In the cellular membrane, integral proteins and lipids are anchored on the lipid layer, but in detergent solutions, micelles' hydrophilic regions associate with proteins, extracting them

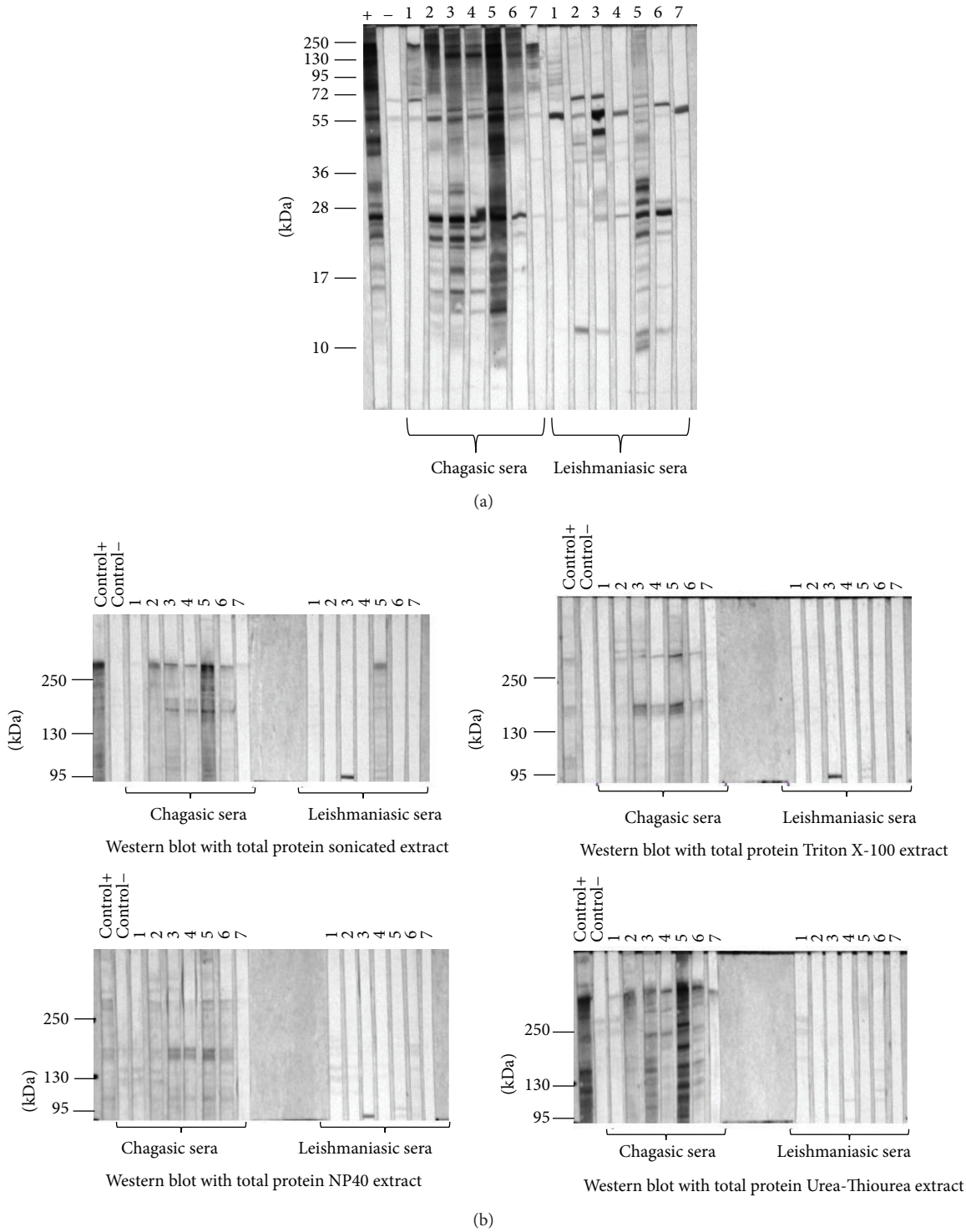

Figure 4: Western blot analysis of chagasic and leishmaniasic patients' sera using different extracts as antigens. (a) SDS-PAGE of sonicated extract was performed in 12% polyacrylamide gel and then transferred into a nitrocellulose membrane for Western blot analysis of the serum samples (dilution 1 : 500) and peroxide-conjugated anti-human IgG (1 : 10000). 100% of the leishmaniasic samples exhibit cross-reaction. (b) SDS-PAGE of sonicated, Triton X-100, NP40, and urea-thiourea extracts was performed in 6% polyacrilamide gel and then transferred into a nitrocellulose membrane for Western blot analysis of the serum samples (1 : 750) and the peroxide-conjugated anti-human IgG (1 : 10000). Unspecific proteins recognized by the negative control were not considered for the final analysis.

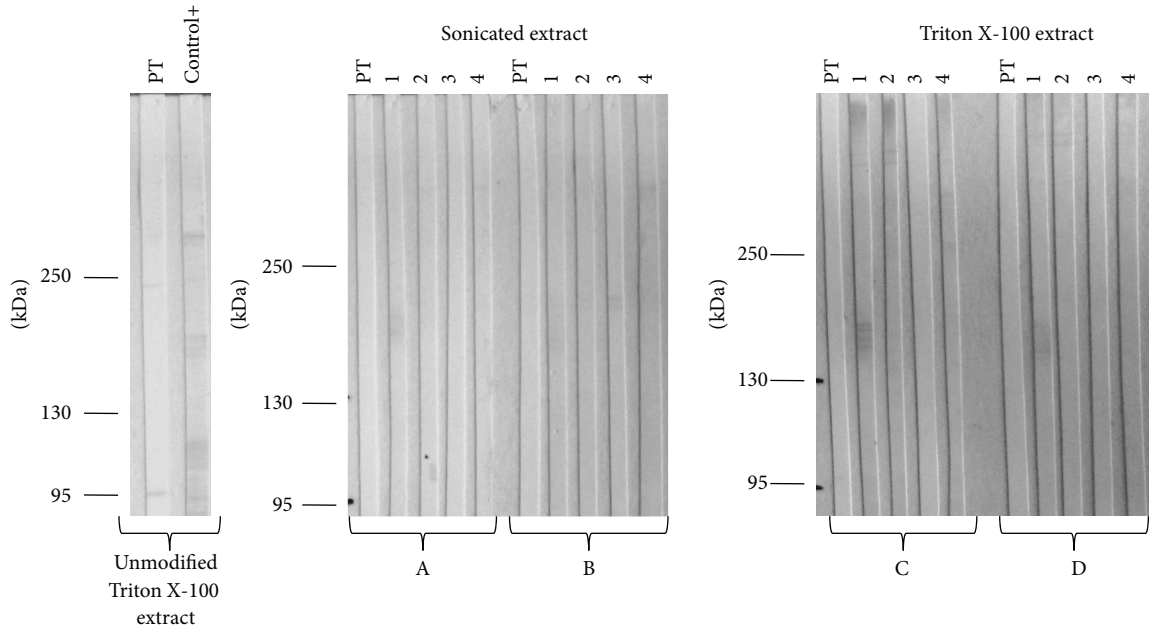

Figure 5: Western blot analysis of chagasic and leishmaniasic patients' sera using deglycosylated extracts. Enzymatic deglycosylation was performed with (A, C) *O*-glycosidase and $\alpha(2\rightarrow 3,6,8,9)$-neuraminidase; (B, D) $\beta(1\rightarrow 4)$-galactosidase and β-N-acetylglucosaminidase. 1 and 2 correspond to chagasic patients' sera samples and 3 and 4 correspond to leishmaniasic patients' sera samples (dilution 1 : 750) and peroxide-conjugated anti-human IgG (1 : 10000). Lectin PT was used as a positive control for carbohydrate recognition. A positive serum sample was also used as a positive control for antigenic recognition.

from the membrane [10]. The ionic detergents, Triton X-100 and NP40, as well as the chaotropic agents, urea-thiourea, were used to produce three distinct protein extracts to be compared with the epimastigotes sonicated protein extract. Few differences were observed between them by SDS-PAGE. But when we used high molecular weight proteins fraction of each protein extract for Western blot analysis of chagasic and leishmaniasic patients, a reduced cross-reaction was found in most of the cases. Nevertheless high molecular weight proteins obtained by Triton X-100 extraction used as antigens in Western blot showed 100% sensitivity and increased specificity to 100%, abolishing false positives obtained when evaluating leishmaniasic patients' sera with sonicated total extract.

This fraction of high molecular weight proteins is predominantly being recognized by chagasic patients' antibodies. However many authors have described the great amount of glycoconjugates present in *T. cruzi* surface [11], which is covered with a rich coat of glycoconjugates, making them well-suited for protection and for the establishment of a persistent infection. Out of these glycoconjugates, mucins are the major components of the surface of *T. cruzi,* anchored to the outer phospholipid layer of the plasma membrane by glycosylphosphatidylinositol (GPI). Mucins have a threonine, serine, and proline rich sequence and their function changes according to the parasite stage. Trypomastigotes and amastigotes mucins are very similar; they have a protective role against proteases and participate in the adhesion and invasion of cells by trypomastigotes. tGPI-mucins (trypomastigotes-mucins) bear terminal Gal(α1,3)Gal epitopes that are a main target of antibody responses in chagasic patients. It has been found that, in the surface of epimastigotes of *T. cruzi* phylogenetic group I (such as Querétaro strain used in this study), it is possible to find the presence of galactofuranose. As humans do not produce glycoconjugates that contain this carbohydrate in particular, a strong immune response to it is induced [5].

Glycan content was determined in the high molecular weight region of the extracts. Lectins are carbohydrate binding proteins and can be used to discriminate and analyze the glycan structures of glycoproteins. The lectin blotting technique detects glycoproteins separated by SDS-PAGE and transferred to nitrocellulose membranes. Lectins of Con A, WGA, and Jacalin were those that bound to a greater number of glycan structures. In high molecular weight proteins of Qro strain, the most abundant carbohydrates were αMan, αGlc, GlcNAc, and αGal. According to Atwood III et al., 2006, *T. cruzi* epimastigotes' high molecular weight proteins of organelle and plasma membrane/cytoplasmic fraction were also found to be αMan rich [12]. In other studies, it has been found that αMan is present as well in lower molecular weight (<90 kDa) antigenic proteins of epimastigotes of *T. cruzi* extracts [7].

When proteins were sonicated, a larger amount of glycoproteins whose molecular weight matches molecular weights of antigens recognized by patients' antibodies were obtained, in contrast to the other extraction methods performed in this study. Sonication process lyses cellular membrane in a mechanic way, cleaving the plasma membrane and releasing proteins. In this extract, the high molecular weight fraction

contains predominantly αMan and αGal glycoproteins. In a fewer amount, two glycoproteins with GlcNAc and NeuNAc were observed. In the same fraction, three glycoproteins with sialic acid residues of 250, 110, and 100 kDa were recognized by lectin of MAA. Triton X-100 extraction allowed obtaining glycoproteins with αMan, αGlc, GlcNAc, NeuNac, and sialic acid. In contrast, NP40 extraction showed only glycan binding to Con A and when extracting proteins with urea-thiourea, only two glycoproteins bound to Con A and Jacalin, respectively.

L. mexicana promastigotes sonicated extract was also analyzed by Lectin blot. There was found a fewer amount of glycoproteins bound to Con A. According to the molecular weight corresponding to the glycoproteins found, these might be present in the *T. cruzi* extract as well (190, 130, and 112 kDa). But glycoproteins of 210, 200, and 112 kDa with GalNAc and βGal recognized by lectin of PT in *L. mexicana* extract were not present in the *T. cruzi* extract. The differences found in the carbohydrate content in high molecular weight proteins of both parasites might be crucial for the differences in the antigenicity of the fraction.

Since glycans can act as antigenic determinants, it was sought whether these structures are being recognized by chagasic and leishmaniasic patients' antibodies. Like Harth et al., 1992, it was found that some glycan structures of antigenic glycoproteins are essential for the antigen-antibody binding [13]. Using an enzymatic deglycosylation kit, glycans were removed from the glycoconjugates in the extract. Western blot analysis demonstrated that both chagasic and leishmaniasic antibodies failed to recognize antigens in the nitrocellulose membrane, so sensitivity of the test was lost. Interestingly, antigenicity may not be established by carbohydrates in other parasites' proteins. *Taenia solium* whole oncosphere antigens were deglycosylated *in situ* and used in Western blot assays. In that case, antigenic reactivity was not reduced [14].

5. Conclusions

In conclusion, high molecular weight proteins extracted with Triton X-100 turn out to be specific of *T. cruzi* when used as antigens in Western blot, since 100% sensitivity and specificity were accomplished. It is important to use the high molecular weight protein fraction in other serological techniques, such ELISA and DOT-ELISA used for diagnosis, and determine a possible increase in the specificity.

It is also essential to highlight the glycans participation as antigenic determinants. When carbohydrates are removed by enzymatic deglycosylation, the antigen recognition is lost and so the sensitivity of Western blot test. It will be interesting to study the immune response of Mexican patients to the glycan content of antigenic mixtures, since the trypanosomatid parasites are rich in this kind of molecules.

Ethical Approval

The study was approved by the Ethics Committee of the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Somatostatin Negatively Regulates Parasite Burden and Granulomatous Responses in Cysticercosis

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Cysticercosis is an infection of tissues with the larval cysts of the cestode, *Taenia solium*. While live parasites elicit little or no inflammation, dying parasites initiate a granulomatous reaction presenting as painful muscle nodules or seizures when cysts are located in the brain. We previously showed in the *T. crassiceps* murine model of cysticercosis that substance P (SP), a neuropeptide, was detected in early granulomas and was responsible for promoting granuloma formation, while somatostatin (SOM), another neuropeptide and immunomodulatory hormone, was detected in late granulomas; SOM's contribution to granuloma formation was not examined. In the current studies, we used somatostatin knockout ($SOM^{-/-}$) mice to examine the hypothesis that SOM downmodulates granulomatous inflammation in cysticercosis, thereby promoting parasite growth. Our results demonstrated that parasite burden was reduced 5.9-fold in $SOM^{-/-}$ mice compared to WT mice ($P < 0.05$). This reduction in parasite burden in $SOM^{-/-}$ mice was accompanied by a 95% increase in size of their granulomas ($P < 0.05$), which contained a 1.5-fold increase in levels of IFN-γ and a 26-fold decrease in levels of IL-1β ($P < 0.05$ for both) compared to granulomas from WT mice. Thus, SOM regulates both parasite burden and granulomatous inflammation perhaps through modulating granuloma production of IFN-γ and IL-1β.

1. Introduction

Cysticercosis is an infection of tissues with larval cysts of the cestode *Taenia solium*. The disease is spread mainly via the fecal-oral route and is contracted by ingestion of food and water that is contaminated with *T. solium* eggs. Following ingestion, the eggs penetrate the intestinal lumen and migrate preferentially to the muscles and brain, where they form cysts that can survive for years. While alive, cysts elicit no or minimal inflammation. On the other hand, dying parasites initiate a granulomatous reaction through pathways that are incompletely understood and manifest clinically as painful muscles nodules or seizures when the cysts are located in the brain.

Somatostatin is a neuropeptide and immunomodulatory hormone produced predominantly by macrophages that binds to receptors expressed on the surface of lymphocytes [1–5] and other cells. Somatostatin produced by macrophages within granulomatous inflammation associated with schistosomiasis downmodulates inflammatory responses in that disease [1, 6]. Treatment of *Schistosoma*-infected mice with octreotide, a somatostatin analogue, reduces granuloma size by 60% and decreases antigen-induced IFN-γ release by macrophages [6]. In addition, somatostatin inhibits production of TNF-α, IL-6, IL-10, and IFN-γ in other *in vivo* and *in vitro* inflammatory settings. For example, somatostatin administration reduced levels of TNF-α and IL-6 in the serum of patients with thyroid eye disease and in the serum

of rats following lipopolysaccharide-induced septic shock [7–11]. Somatostatin also reduced levels of TNF-α, IL-6, and IL-1β produced by LPS-activated monocytes, as well as levels of IL-6, IL-10, and IFN-γ produced by PBMC isolated from systemic lupus erythematous patients [7–11].

Murine *Taenia crassiceps* infection of mice is widely used to model *T. solium* infection in man [12–16]. Using this model, we previously demonstrated that substance P, a neuropeptide, is produced early within granulomas elicited by dying parasites [17], while somatostatin is produced in more mature granulomas [18]. We also demonstrated that the Th1 cytokines, IFN-γ and IL-2, were detected in early granulomas, while Th2 cytokines, IL-4 and IL-10, were detected in more mature granulomas [19]. In addition, we showed that substance P knockout mice infected with *T. crassiceps* produced smaller granulomas than infected WT mice [17] strongly suggesting that substance P is one of the drivers of granuloma formation. The factors that downmodulate granulomatous inflammation, however, were not examined in these studies and remain ill-defined.

Granuloma formation by the host in response to chronic infectious agents is thought to be essential for limiting and eventually clearing infection. In schistosomiasis, antigens released by live eggs initiate granuloma development. As the eggs die and are absorbed, granulomas resolve leaving fibrotic plaques [20]. In cysticercosis, cysts that are dying initiate a Th1 response leading to granulomatous inflammation which help to clear the cyst. Evidence that Th1 responses help clear infection while Th2 response opposes this effect is provided by the findings that IFN-γ or IL-2 administration to *T. crassiceps*-infected mice results in reduced parasite numbers, while IL-10 administration mice increased parasite burden [21]. However, there are no studies that establish the correlation between extent of granulomatous inflammation, *per se*, and parasite load in cysticercosis. We hypothesized that the somatostatin is one of the factors that downmodulates granulomatous inflammation and that, by doing so, it negatively regulates parasite burden in murine cysticercosis.

To examine this hypothesis, we infected somatostatin-deficient mice with *T. crassiceps* and determined their parasite burden and granulomatous responses and compared them to the parasite burden and granulomatous responses observed in infected wild type (WT) mice. The results demonstrate that somatostatin-deficient mice had a markedly diminished parasite burden and a more robust granulomatous response. Not surprisingly, the granulomas of somatostatin-deficient mice produced higher levels of the Th1 cytokine, IFN-γ, compared to granulomas from WT mice. Thus, somatostatin is a negative regulator of granulomatous inflammation in *T. crassiceps* infection and its deficiency leads to decreased parasite burden.

2. Material and Methods

2.1. Mice. All studies with mice were approved by the Institutional Animal Care and Use Committee of Baylor College of Medicine (IACUC protocol no. AN209). Use of all animals involved in this project was carried out according to the provisions of the Animal Welfare Act, PHS Animal Welfare Policy, the principals of the NIH Guide for the Care and Use of Laboratory Animals, and the policies and procedures of Baylor College of Medicine. All possible steps were taken to avoid animal suffering at every stage of the experiments.

Six-week-old WT C57BL/6 mice were purchased from Jackson Laboratories. Homozygous somatostatin-deficient ($SOM^{-/-}$) mice were the kind gift from Drs. David Elliott and Joel Weinstock, University of Iowa, and were generated, as described [22]. Briefly, a mutated *Smst* gene allele with deletion of promoter sequences and the first coding exon was generated by homologous recombination in embryonic stem cells. The somatostatin null allele contains a neo-resistance cassette, but it does not have an expressed reporter gene. Germline chimeric mice were derived by injection of C57BL/6J blastocysts with correctly targeted E14 embryonic stem cells derived from substrain 129P2/Ola mice. F_1 heterozygous mice were obtained by mating chimeric males with C57BL/6J females. Subsequently, F_2 (C57, 129) mice were obtained by mating F_1 males with females, and the three expected somatostatin genotypes were obtained in normal Mendelian proportions. To reduce genetic background variability that is inherent in the original F_2 (C57, 129) mutant strain, the somatostatin null allele was backcrossed for five successive generations onto the C57BL/6J inbred strain to produce N_5 incipient-congenic mice. Homozygous somatostatin knockout mice were healthy and fertile and did not display physical or behavioral abnormalities.

2.2. Murine Cysticercosis Model. WT and $SOM^{-/-}$ female mice were intraperitoneally infected with 10 cysts of the ORF strain of *T. crassiceps*, as described [16, 17]. Three months following infection, mice were sacrificed, their peritoneal cavity opened, and the contents (cysts and granulomas) harvested by washing the peritoneal cavity with HBSS. The washings were placed into a petridish and the cysts and granulomas were enumerated. Granulomas were flash-frozen in liquid nitrogen, weighed, and homogenized in ice-cold PBS containing protease inhibitor, aprotinin (500 KIU/mL, Sigma), followed by centrifugation at 16,000 g at 4 degrees C. Total protein in the supernatant was quantified using the Bradford method (cat no. 500-0006, Bio-Rad, Hercules, CA). IL-2, IFN-γ, IL-4, IL-10, IL-1β, IL-6, and TNF-α protein levels were determined by sandwich ELISA assays (R&D Systems, San Diego, California) as per the manufacturer's instructions; results are expressed as pg cytokine/mg total protein.

2.3. Statistical Analyses. Data presented are mean ± SEM or SD of a minimum of 2 experiments, as indicated. Statistical differences were determined using the Mann-Whitney test.

3. Results

3.1. Somatostatin Deficiency Results in Decreased Parasite Load and Increased Granuloma Size. To examine the contribution of somatostatin to parasite burden in cysticercosis, we infected WT mice and somatostatin knockout ($SOM^{-/-}$) mice intraperitoneally with 10 cysts of the ORF strain of *T. crassiceps*, as described [16, 17]. Three months following

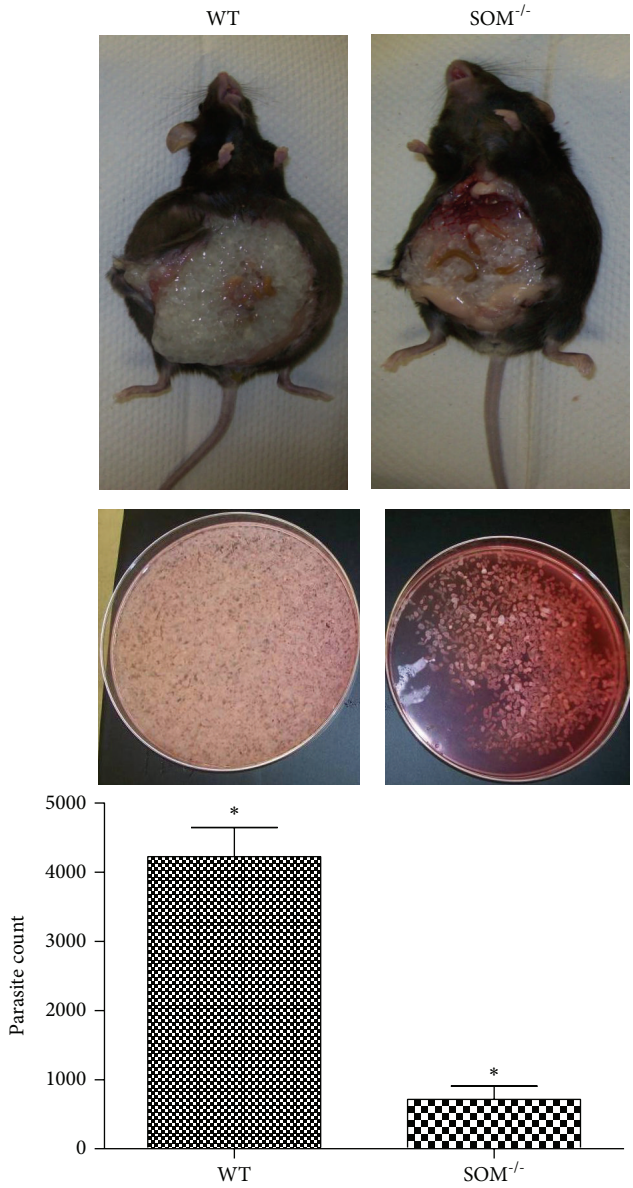

Figure 1: Effect of somatostatin deficiency on parasite load. Representative photographs of infected mice, WT (left side) and SOM$^{-/-}$ (right side), are shown in the top panels following laparotomy to reveal intraperitoneal cysts. Representative photographs of cysts harvested from the peritoneal cavity are shown for each mouse in the middle panels. The bottom panel shows mean ± SD of the number of cysts harvested from WT mice ($n = 6$) and SOM$^{-/-}$ ($n = 6$); the asterisk (∗) indicates that the cyst numbers were different ($P < 0.05$).

infection, mice were sacrificed, their peritoneal cavity was opened via laparotomy, and the peritoneal contents were harvested by washing with HBSS (Figure 1). The washings were placed into a Petri dish and the cysts enumerated. The number of cysts in the peritoneum of SOM$^{-/-}$ mice (714±79) was reduced by 83% compared to WT mice (4,222 ± 173; $P < 0.05$).

To examine the contribution of somatostatin to granuloma formation in cysticercosis, we determined the number

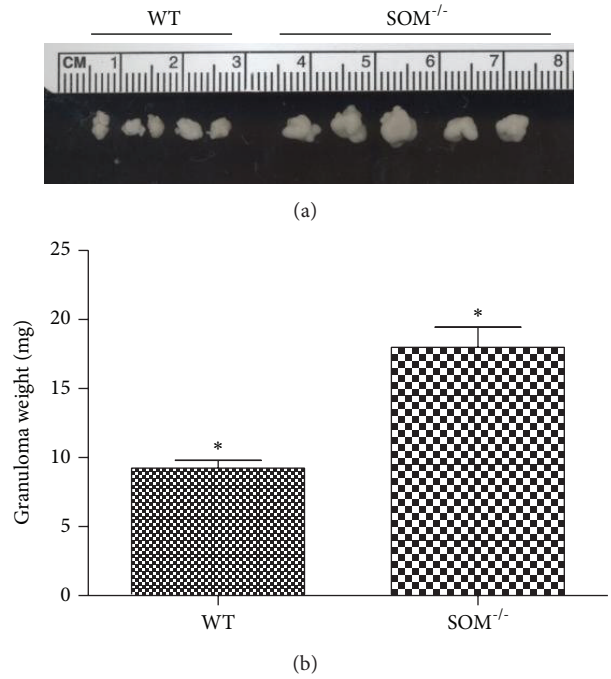

Figure 2: Effect of somatostatin deficiency on size of *T. crassiceps*-induced granulomas. (a) Photograph of representative granulomas removed from infected WT and SOM$^{-/-}$ mice. (b) Weights of granulomas (mean ± SD) obtained from the peritoneal cavity of infected wild type ($n = 6$) and SOM$^{-/-}$ mice ($n = 6$); the asterisk (∗) indicates that the granuloma sizes were different ($P < 0.05$).

and size of granulomas within the peritoneal cavity of *T. crassiceps*-infected WT mice and SOM$^{-/-}$ mice. While there were no differences in the number of granulomas, the size of granuloma determined from their weight (Figure 2) was increased by 95% in SOM$^{-/-}$ mice (17.9 ± 0.6 mg) compared to granulomas obtained from WT mice (9.2 ± 0.2 mg; $P < 0.05$).

3.2. Effect of Somatostatin Deletion on Cytokine Levels in T. crassiceps-Induced Granulomas. Since Th1 cytokines, IFN-γ and IL-2, are known to contribute to granuloma formation, we examined their levels within granulomas from the two groups of mice. Levels of IFN-γ in the granulomas derived from SOM$^{-/-}$ mice (219±32 pg/mg total protein; Figure 3(a)) were increased 1.5-fold relative to levels in granulomas of WT mice (142 ± 21 pg/mg; $P < 0.05$); however, levels of IL-2 in granulomas of SOM$^{-/-}$ mice (548 ± 91 pg/mg total protein) were not different from levels in granulomas of WT mice (714 ± 255 pg/mg; $P > 0.05$). These results suggest that IFN-γ but not IL-2 is contributing to increased granuloma size in SOM$^{-/-}$ mice.

Since Th2 cytokines, IL-4 and IL-10, are known to downmodulate granuloma formations, we also measured their levels in granulomas from the two groups of mice. Somewhat surprisingly, levels of IL-4 in granulomas from SOM$^{-/-}$ mice (1,084 ± 130 pg/mg total protein; Figure 3(b))

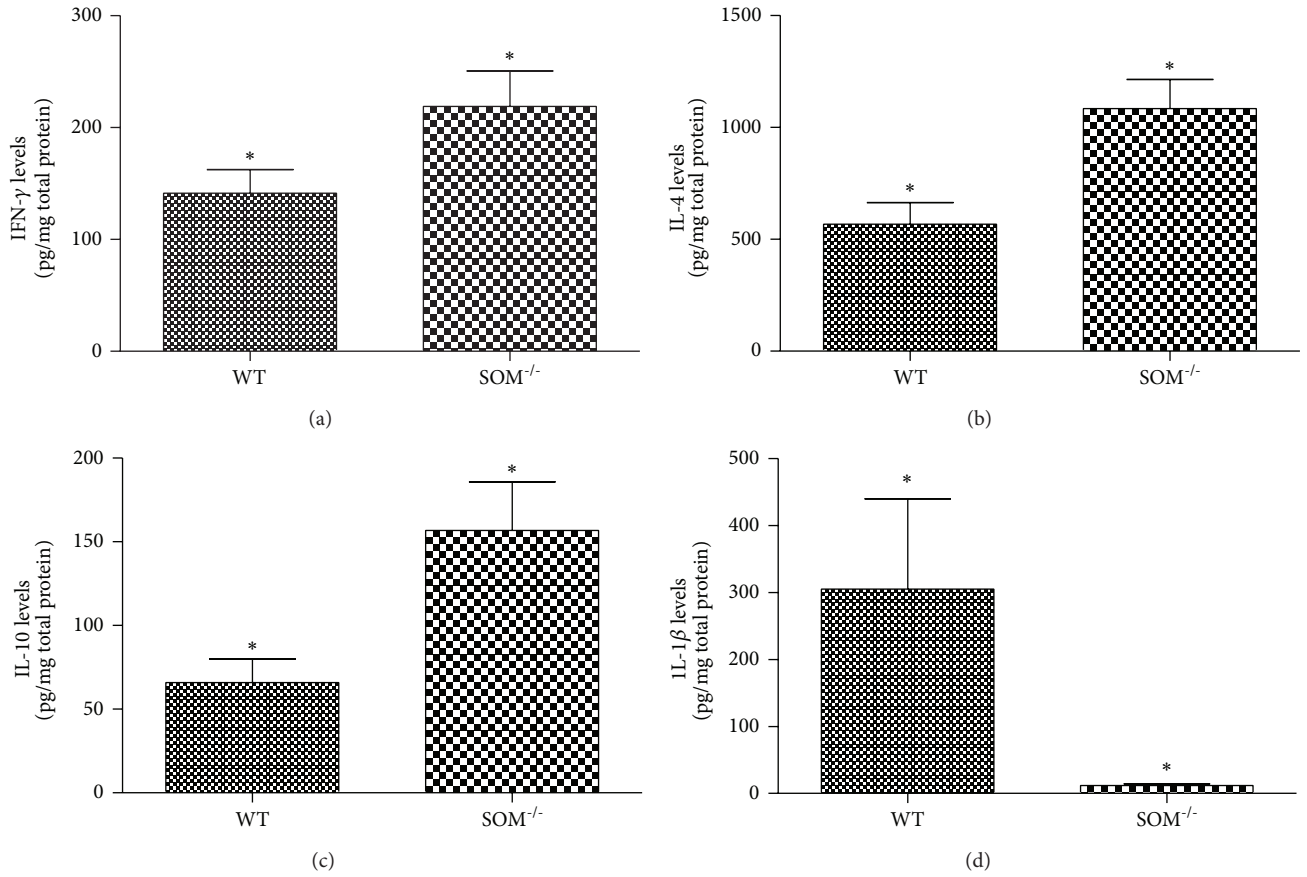

Figure 3: Effect of somatostatin deficiency on levels of cytokines in *T. crassiceps*-induced granulomas. Levels of IFN-γ (a), IL-4 (b), IL-10 (c), and IL-1β (d) normalized to total protein within peritoneal granulomas obtained from *Taenia crassiceps*-infected WT mice ($n = 6$–8) or SOM$^{-/-}$ mice ($n = 6$–8). Data presented are mean ± SEM; the asterisk ($*$) indicates that the cytokine levels were different ($P < 0.05$).

were increased compared to levels in granulomas from WT mice (568 ± 97 pg/mg; $P < 0.05$). The same was true for IL-10 (Figure 3(c)); levels of IL-10 in the granulomas from SOM$^{-/-}$ mice (157 ± 29 pg/mg total protein) were increased compared to levels in granulomas from WT mice (66 ± 15 pg/mg; $P < 0.05$). Thus, the increased granulomatous response seen in SOM$^{-/-}$ mice was not due to reduced levels of the Th2 cytokines, IL-4 and IL-10.

Since the proinflammatory cytokines, IL-1β, IL-6, and TNF-α, contribute to granulomatous inflammation in tuberculosis and other granulomatous diseases, we also examined their levels in granulomas from the two groups of mice. Surprisingly, IL-1β levels in the granulomas derived from SOM$^{-/-}$ mice (12 ± 3 pg/mg total protein; Figure 3(d)) were markedly decreased compared to levels in granuloma from WT mice (305 ± 135 pg/mg; $P < 0.05$); there were no differences in either IL-6 levels (82 ± 22 pg/mg total protein) or TNF-α levels (365 ± 48 pg/mg total protein) in granulomas derived from the SOM$^{-/-}$ mice compared to WT mice (189 ± 76 pg/mg and 502 ± 117, resp.; $P > 0.05$ for both). Thus, it is unlikely that increase in these proinflammatory cytokines contributed to the increase in granuloma size observed in SOM$^{-/-}$ mice.

4. Discussion

In the current studies, we addressed the role of somatostatin in granulomatous inflammation associated with cysticercosis using a murine model of *Taenia crassiceps* infection [13–16]. In cysticercosis, it is thought that granulomas are formed in response to dying parasites to hasten their elimination from the tissues; once the parasite debri is cleared from the tissue, the granuloma resolves. The molecules responsible for downmodulation of granulomatous inflammation after parasite elimination are not known. Based on our earlier studies showing somatostatin detection in more mature granulomas [18] as well as other studies in schistosomiasis showing that somatostatin plays an immunomodulatory role, we hypothesized that somatostatin may be responsible for resolution of granulomatous inflammation in cysticercosis. Our finding of increased granuloma size in SOM$^{-/-}$ mice supports this hypothesis.

Granulomatous inflammation, while being beneficial through removal of the cyst remnants, clearly can be detrimental causing seizures when inflammation occurs in brain parenchyma. Our findings of decreased cyst burden accompanying increased granulomatous inflammation in SOM$^{-/-}$ mice, however, indicate that there is an additional

benefit of granulomatous inflammation, that is, controlling the infection within the peritoneum of infected mice.

We demonstrated that levels of IFN-γ were increased in granulomas from infected SOM$^{-/-}$ mice compared to WT mice. Previous studies of *T. crassiceps* infection demonstrated that mice receiving IFN-γ had lower parasite levels, while mice receiving anti-IFN-γ antibody had larger parasite burdens [21]. Also, IFN-γ has been demonstrated to contribute to decreased burden of another parasite, *Toxoplasma gondii*. Pretreatment with IFN-γ resulted in a 65% reduction of growth of the parasite within astrocytes [23, 24]. Thus, the increased levels of IFN-γ within granulomas may have contributed to the decreased cyst numbers observed in *T. crassiceps*-infected SOM$^{-/-}$ mice.

Based on our findings, another cytokine that may be involved in reduction of parasite burden in *T. crassiceps*-infected SOM$^{-/-}$ mice is IL-1β. Studies have shown that IL-1β stimulates the growth of *Toxoplasma gondii* in astrocytes [25]. We demonstrated that the level of IL-1β was significantly reduced in granulomas from infected SOM$^{-/-}$ mice which correlated with reduced parasite burden. We hypothesize that the same effect observed in *T. gondii* infection may be occurring in *T. crassiceps* infection; IL-1β may stimulate growth of cysts in the peritoneum of infected WT mice and its reduction in the granulomas of SOM$^{-/-}$ mice impairs cyst growth in the peritoneum of these animals.

The finding of reduced IL-1β in the absence of somatostatin is not completely unexpected. Earlier studies examining the ability of somatostatin to modulate production of IL-1β, along with TNF-α and/or IL-6, are conflicting. Some studies showed that somatostatin stimulates the production of IL-1β, TNF-α, and/or IL-6 by human blood cells [26, 27], as well as the expression of IL-1β in articular tissues of rats with ongoing adjuvant-induced arthritis [28]. However, other studies showed that somatostatin decreased secretion of one or more of these cytokines [7, 9, 28–31].

The Th2 cytokine, IL-10, has been shown in earlier studies to induce a significant increase in parasite load [21]. Therefore, our results showing increased IL-10 in the somatostatin knockout mice in which the parasite burden is lower were unexpected. We speculate that the effects of IFN-γ and IL-1β on inhibition of parasite burden likely outweigh the possible stimulatory effects of IL-10.

Our findings have potential implications for treatment and prevention of the detrimental effects of granulomatous inflammation induced as a result of antihelminth treatment in patients with neurocysticercosis and viable cysts. Current options for management of patients with viable cysts include antihelminth treatment along with corticosteroid administration aimed at reducing inflammation. However, corticosteroids can have severe side effects and cannot be used in patients with concurrent latent tuberculosis, strongyloidiasis, and optical cysticercosis. Our finding that somatostatin downmodulates inflammatory responses suggests the possibility of using somatostatin analogues, instead of corticosteroids, as an immunomodulator in these patients to downmodulate granulomatous inflammation in neurocysticercosis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Mitra Khumbatta and Bahrom Firozgary contributed equally to this work.

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Research Article

The Trend in Distribution of Q223R Mutation of Leptin Receptor Gene in Amoebic Liver Abscess Patients from North India: A Prospective Study

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Host genetic susceptibility is an important risk factor in infectious diseases. We explored the distribution of Q223R mutation in leptin receptor gene of amoebic liver abscess (ALA) patients of North India. A total of 55 ALA samples along with 102 controls were subjected to PCR-RFLP analysis. The frequency of allele "G" (coding for arginine) was in general high in Indian population irrespective of the disease. Our results of Fisher exact test shows that heterozygous mutant (QQ versus QR, $P = 0.049$) and homozygous mutant (QQ versus RR, $P = 0.004$) were significantly associated with amoebic liver abscess when compared with homozygous wild (QQ).

1. Introduction

The protozoan parasite *Entamoeba histolytica* is estimated to cause 100 million infections and 100,000 deaths worldwide annually and malnutrition is known to increase susceptibility to infection [1]. Amebiasis is more common in malnourished children, a state that afflicts approximately one-third of children in the developing world [2]. The most common manifestation of *E. histolytica* infection in India is either diarrhea with ulcerative lesion in caecum and colon (intestinal amebiasis) or amoebic liver abscess (ALA). Invasive trophozoites gain access to liver via hepatic portal vein and cause amoebic liver abscess due to necrotic lysis of the liver tissue. It varies in size and number. Necrotic lesions may be single or multiple and may occur in left or right lobe of liver. The common symptoms of ALA are fever, pain in the right hypochondrium, and liver tenderness [3, 4]. Recently a point mutation (Q223R, rs1137101, A to G) in leptin receptor gene at 223aa position has been found to be associated with the susceptibility to *E. histolytica* infection and disease outcome in Bangladesh [5]. It was observed that the children with allele for arginine (223R) were almost 4 times more susceptible to infection compared to those homozygous for the ancestral glutamine allele (223Q). In terms of nucleotides, Q (glutamine) is encoded by A (adenine) whereas R (arginine) is encoded by G (guanine). Leptin is a hormone/cytokine produced largely by adipocytes and to some extent by the stomach, skeleton muscles, and placenta [6]. Leptin plays several important roles in human physiology. It acts through the leptin receptor (LEP-R), a single-transmembrane-domain receptor of the cytokine receptor family which connects nutrition and immunity. In addition to regulating neuroendocrine function, energy homeostasis, haematopoiesis, and angiogenesis, leptin is an important modulator of both the innate and adaptive immune systems [7]. It has been earlier stated that malnutrition in children aggravates the propensity of amoebiasis [8]. Therefore, we proposed to study the segregation of alleles A and G of LEPR gene responsible for Q223R mutation in North Indian population and if it has any association with ALA.

2. Methodology

2.1. Biological Samples. The study patients were recruited from the Department of Gastroenterology, All India Institute

of Medical Sciences, New Delhi, India, after necessary ethical clearances were obtained for the study. All the participating patients gave written consent for the study. ALA pus samples were collected from patients by the attending physician and necessary precautions were taken during pus aspiration from liver, so as to avoid any contamination. The pus aspiration from liver was carried out in those patients who did not respond to chemotherapy and where aspiration was unavoidable for case management. The demographic and clinical details of the patients are represented in Table 1. The samples were transported at 4°C within two hours after collection and stored at –20°C until processed. Blood samples from 102 individuals without any enteric or liver disease were included in the study as controls. The blood samples were collected by venipuncture in vacutainer tubes (BD NJ, USA) containing anticoagulant K_2EDTA solution from individuals visiting the hospital.

2.2. DNA Extraction from Whole Blood and ALA Pus. DNA from blood samples was extractedusing standard protocol [9]. Briefly blood (stored in ACD or EDTA) was resuspended in 15 mL polypropylene centrifugation tubes with 3 mL of nuclei lysis buffer (10 mM Tris-HCl, 400 mM NaCl, and 2 mM Na_2EDTA, pH 8.2). The cell lysates were digested overnight at 37°C with 0.2 mL of 10% SDS and 0.5 mL of a proteinase K solution (1 mg proteinase K in 1% SDS and 2 mM Na_2EDTA). After digestion was complete, 1 mL of saturated NaCl (approximately 6 M) was added to each tube and shaken vigorously for 15 seconds, followed by centrifugation at 2500 rpm for 15 minutes. The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA was transferred to another 15 mL polypropylene tube. Exactly 2 volumes of absolute ethanol were added and the tubes were inverted gently several times until the DNA precipitated. The precipitated DNA strands were removed with a plastic spatula or pipette and transferred to a 1.5 mL microcentrifuge tube containing 100–200 pl TE buffer (10 mM Tris-HCl and 0.2 mM Na_2EDTA, pH 7.5). The DNA was allowed to dissolve for 2 hours at 37°C before quantification. Genomic DNA from liver abscess pus samples for PCR was isolated using QIAamp DNA stool kit using manufacturer guidelines.

2.3. Primer Designing. E. histolytica specific primers were designed after Srivastava et al. from SINE2 [10]. EhSINE2 is highly abundant non-LTR, nonautonomous retrotransposon in *E. histolytica* genome [11]. Some of SINE2 copies show internal deletion, due to which the amplicon revealed size variation and multiple bands were observed near 350 bp. The nucleotide sequence of primer is as follows: F 5′-GTCAGAGACACCACATGAA-3′ and R 5′-CGAGACCCCTTAAAGAAACCC-3′ [10]. A set of PCR primers was designed to amplify the fragment of leptin receptor gene spanning the exon 6 locus of the gene carrying Q223R mutation. Primer sequences are F 5′-CCTGCTTTAAAAGCCTATCCAG-3′ and R 5′-AGTGTTAAGCAAAGTGAGATAAGC-3′. Primers sequences were bioinformatically analysed to ensure specificity using BLAST Programme of NCBI [12].

Table 1: Demographic and clinical details of amoebic liver abscess patients ($n = 55$).

(1) Sex: *n* male (%)/female (%)	48 (87.2)/7 (12.7)
(2) Age at diagnosis: mean (SD)	43 (13.9)
15–30: *n* (%)	9 (16.12)
31–above: *n* (%)	46 (83.87)
(3) Duration of disease (range)	7–20 days
(4) Alcoholic: *n* (%)	
Yes	36 (65.45)
No	16 (29.0)
Ex	3 (5.45)
(5) Location of abscess: *n* (%)	
Right lobe of liver	51 (92.70)
Left lobe of liver	4 (7.3)
(6) No. of abscesses (single/multiple): *n* (%)	41/14 (75/25)
(7) Whether first aspiration (yes/no): *n* (%)	55/0 (100/0)
(8) Drugs for treatment	Metronidazole and ciprofloxacin
(9) Ethnicity	Aryan
(10) Location	North India

2.4. PCR-RFLP. A total of 55 ALA samples along with 102 controls were subjected to PCR-RFLP analysis. DNA was amplified using leptin receptor specific primers. PCR was performed in a touch gene (Nugen Scientific, USA) machine. Thin walled 0.2 mL tubes were used for amplification. A typical PCR reaction (20 μL) included 7.8 μL of autoclaved milliQ water, 2 μL of 10X PCR buffer with $MgCl_2$ (containing 750 mM Tris-HCl (pH 8.8 at 25°C), 200 mM $(NH_4)_2SO_4$, 0.1% Tween-20, 1.5 mM $MgCl_2$), 2 μL of dNTP mix (containing 2 mM of each dNTP), 2 μL (20 pmol) of each primer forward as well as reverse, and 0.2 μL of TaqDNA polymerase (5 U/μL, MBI Fermentas, USA) and 2.0 μL of template DNA. The amplification conditions were one cycle of 94°C for 5 min followed by 30 cycles of 94°C for 30 s, annealing 55°C for 1 min, extension at 72°C for 30 sec, and finally one cycle of 72°C for 10 min and finally held at to 4°C. Volume of template DNA used (2.0 μL; ~50 ng) worked fine for PCR amplification. The sample containing all reagents except the template DNA was treated as the negative control. The size and integrity of the products were checked by electrophoresis. 10 μL of the PCR product was run on a 0.8–1.2% agarose gel at 5 V/cm for an appropriate time period. Restriction enzyme BseNI was used to digest the PCR amplified product of 386 bp and the fragments generated upon digestion are represented in Figure 1. Restriction enzyme BseNI digests only when the sequence reads nucleotide A at the locus. Thus digestion of 386 bp PCR product yielded three bands of 221 + 146 + 19 bp in case of homozygous (AA, assuming A as wild allele) wild and two bands of 367 + 19 bp in case of homozygous mutant (GG) (Figure 1). As expected, the digestion of heterozygous mutant yielded four bands of 367 + 221 + 146 + 19 bp as shown in a representative gel. All bands except 19 bp were visible on

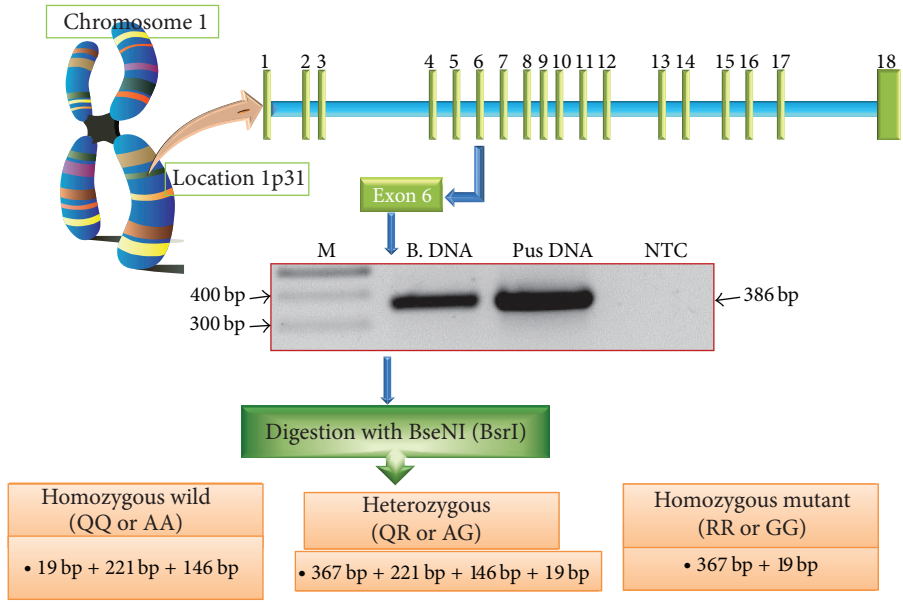


Figure 1: Schematic representation of the methodology followed for detection of Q223R mutation in leptin receptor gene. Digestion of PCR amplified product of 386 bp by restriction enzyme BseNI (BsrI) yields three bands of 221 bp + 146 bp + 19 bp in homozygous wild, four bands of 367 bp + 221 bp + 146 bp + 19 bp in heterozygous, and two bands of 367 bp + 19 bp in homozygous mutant. Allele "A" codes for glutamine and allele "G" codes for arginine in leptin receptor gene. Lane M = 100 bp Marker, lane B. DNA = human blood genomic DNA, lane pus DNA = ALA (amoebic liver abscess) pus DNA, and NTC = no template control.

1.5% agarose gel. Sequencing of mutated fragment confirmed the presence of mutation in Indian population (Figures 2(a) and 2(b)).

2.5. Statistical Analysis. Data was evaluated by SPSS software version 12 using standard contingency χ^2 tests or Fisher's exact test for calculating the differences in genotype frequency between cases and controls. A two-tailed P value <0.05 was considered significant. Multiple comparisons were done using one way ANOVA based on the conservative Bonferroni correction. The significance level of $\alpha = 0.05$ was chosen for all sets.

3. Results and Discussion

Out of 55 collected samples, 54 samples gave positive result with PCR conducted with *E. histolytica* specific primers accounting for 98% efficiency in diagnosis. We assessed the association of the SNP with a number of different diseases related outcomes and for possible confounding variables. Genotype and allele frequencies for SNP rs1137101 (Q223R) in *LEPR* gene of ALA cases were stratified by phenotypic subgroups and represented in Table 2. Genotype and phenotype profiling of ALA patients studied here revealed that gender, age, and alcoholism are other important risk factors for amoebic liver abscess. Frequency of allele G was calculated and is represented in Figure 3(a). We did not observe significant difference in allele frequency of "G" among control and ALA patients. However, the distribution of genotype frequency followed the following pattern AA < GG < AG in control and GG < AA < AG in ALA patients of North India (Figure 3(b)). Fisher exact test was performed to check the association of mutation Q223R with ALA using SPSS version 12 software. Our results showed that heterozygous mutant (QQ versus QR, $P = 0.049$) and homozygous mutant (QQ versus RR, $P = 0.004$) were significantly associated with amoebic liver abscess when compared with homozygous wild (QQ) (Table 3). Mutation Q223R in leptin receptor gene is very important as it increases the susceptibility of *E. histolytica* infection in malnourished children. Malnutrition represents a significant health problem in the developing

Table 2: Genotype and allele frequencies for SNP rs1137101 (Q223R) in *LEPR* gene of ALA cases stratified by phenotypic subgroups (n = 55).

SNP	SNP rs1137101 (Q223R) in *LEPR* gene			
Genotype	AA (15)	AG (31)	GG (9)	Total (55)
(1) Sex				
Male	10	30	8	48
Female	5	1	1	7
(2) Age at diagnosis (Yr)				
15–30	5	4	0	9
31–above	10	27	9	46
(3) Alcoholic				
Yes	11	19	6	36
No	4	10	2	16
Ex	0	2	1	3
(4) No. of abscesses				
Single	8	27	6	41
Multiple	7	4	3	14

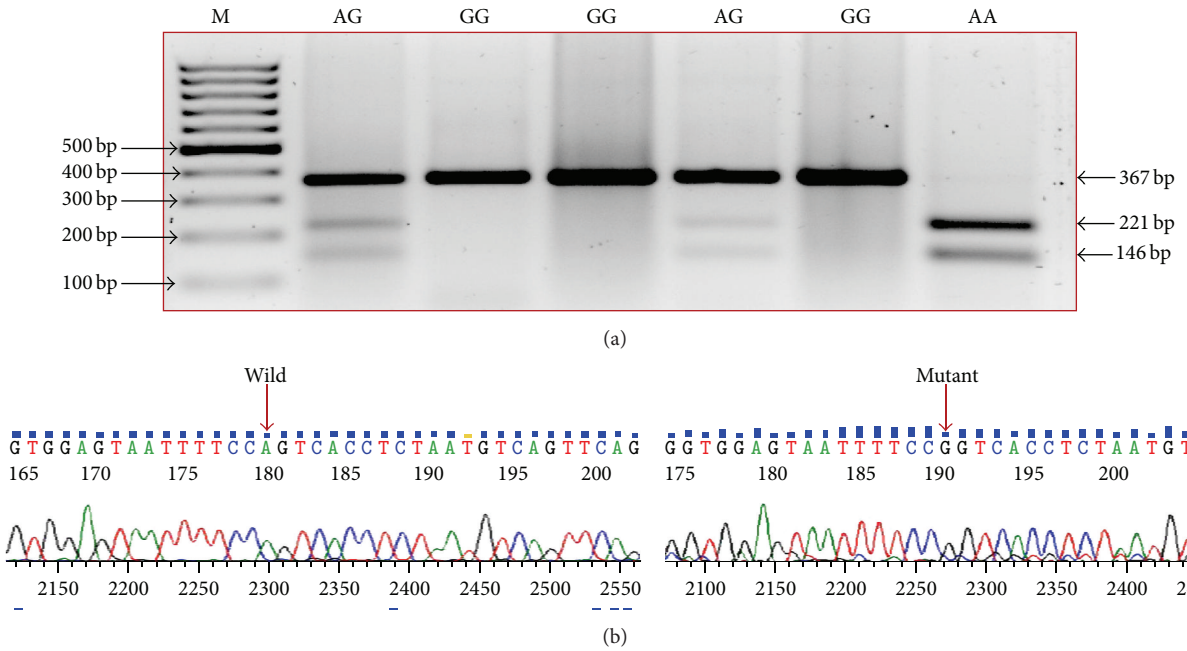
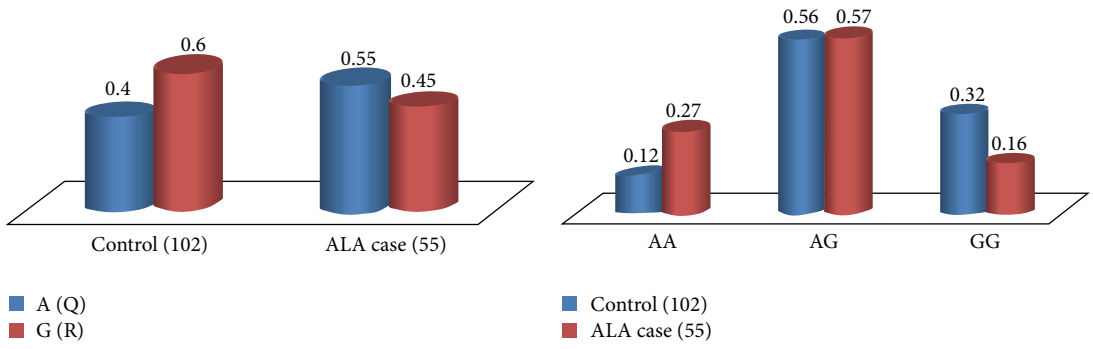


Figure 2: (a) PCR-RFLP analysis of Q223R SNP (rs1137101) from leptin receptor gene. Lane M = 100 bp Marker, lane AG, lane GG, and lane AA represent restriction enzyme digested PCR product of ALA pus DNA of different genotypes. After digestion with BseNI restriction enzyme, wild type AA is visible as two bands 221 bp, 146 bp and the third band of 19 bp is invisible due to smaller size. Heterozygous AG is visible as three bands 367 bp, 221 bp, 146 bp and 19 bp (invisible) whereas homozygous mutant GG is visible as bands of 221 bp, 146 bp and 19 bp (invisible). (b) Sequencing results of wild "A" and mutant "G" are detected by PCR-RFLP confirming the presence of SNP in DNA of ALA patients.



(a) Allele frequency for RS1137101 (Q223R)

(b) Genotype frequency of RS1137101 (Q223R) in LEPR gene

Figure 3: Allele and genotype frequencies of SNP Q223R (rs1137101) in *LEPR* gene in amoebic liver abscess patients and control samples of North India. (a) Distribution of allele frequency. Allele "A" codes for glutamine whereas allele "G" codes for arginine amino acid in *LEPR*. (b) Distribution of genotype frequency.

world including India and growing body of evidence has indicated an epidemiological connection between susceptibility to infection and malnutrition. The leptin levels in malnourished children have been reported to be lower than the well-nourished ones with a concomitant suppression of inflammatory responses [13, 14]. Two recent studies had explored the link between malnutrition, leptin signaling, and susceptibility to amebic infection. The first study by Duggal et al. involved the prospective observation of a cohort of 185 Bangladeshi children by household visits every other day over a period of nine years. During this study period, 90 percent of the children enrolled had at least one bout of *E. histolytica* infection. The children were also tested for polymorphisms in their leptin and leptin receptor genes. They found that mutation Q223R increased susceptibility to intestinal infection by *E. histolytica* depending on the presence of allele "G" in homozygous or heterozygous state [5].

The second study by Guo et al. showed that mice lacking the functional leptin receptor developed devastating mucosal destruction after *E. histolytica* infection [15]. Leptin-mediated resistance to amebiasis is via its action on intestinal

Table 3: Association of leptin receptor gene (*LEPR*) polymorphism at Q223R locus with amoebic liver abscess. ALA case ($n = 55$), Control ($n = 102$), Q (glutamine) = A (wild), and R (arginine) = G (mutant). P value = or <0.05 was considered significant. *Refers to significant P values.

rs1137101	N = ALA (control)	OR	95% CI	P value
QQ versus QR	15 (12) : 31 (57)	0.435	0.181–1.045	0.049*
QQ versus RR	15 (12) : 9 (33)	0.218	0.076–0.629	0.004*
QR versus RR	31 (57) : 9 (33)	0.501	0.213–1.182	0.08

epithelium rather than hematopoietic cells or the brain and requires leptin receptor signaling through STAT3 [15]. The in vitro studies have shown that the Q223R polymorphism in leptin receptor attenuates leptin-dependent STAT3 activation to that of the wild-type (WT) receptor and it is the leptin regulation of host apoptotic genes TRIB1 and suppressor of cytokine signaling 3 (SOCS3) via STAT3 which is responsible for protection [16]. A recent study in *E. histolytica* infected mice (223R mice compared to Q223 mice) has shown that the majority of leptin-linked differentially regulated genes were involved in apoptosis, cellular proliferation, or recruitment of hematopoietic cells. The differential regulation of these genes suggests that the Q223R polymorphism attenuates the ability of LEPR stimulation to protect cells against amebic killing and/or apoptosis [17]. Similarly our study also shows that most of the ALA patients had "AG" genotype and allele "G" is associated with ALA patients. The presence of allele "G" is an important risk factor in Indian population.

4. Conclusion

Our study concludes that the mutation Q223R is associated with susceptibility to *E. histolytica* infection in North Indian population but large population based studies are needed to confirm our observation in Indian population. The frequency of allele "G" is higher in Indian population than that of allele "A." The worldwide distribution of allele "G" in Q223R mutation shows that it is more prevalent in Asian and African subcontinents whereas allele "A" is more predominant in European population.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Differential Expression and Immunolocalization of Antioxidant Enzymes in *Entamoeba histolytica* Isolates during Metronidazole Stress

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Entamoeba histolytica infections are endemic in the Indian subcontinent. Five to eight percent of urban population residing under poor sanitary conditions suffers from *Entamoeba* infections. Metronidazole is the most widely prescribed drug used for amoebiasis. In order to understand the impact of metronidazole stress on the parasite, we evaluated the expression of two antioxidant enzymes, peroxiredoxin and FeSOD, in *Entamoeba histolytica* isolates during metronidazole stress. The results reveal that, under metronidazole stress, the mRNA expression levels of these enzymes did not undergo any significant change. Interestingly, immunolocalization studies with antibodies targeting peroxiredoxin indicate differential localization of the protein in the cell during metronidazole stress. In normal conditions, all the *Entamoeba* isolates exhibit presence of peroxiredoxin in the nucleus as well as in the membrane; however with metronidazole stress the protein localized mostly to the membrane. The change in the localization pattern was more pronounced when the cells were subjected to short term metronidazole stress compared to cells adapted to metronidazole. The protein localization to the cell membrane could be the stress response mechanism in these isolates. Colocalization pattern of peroxiredoxin with CaBp1, a cytosolic protein, revealed that the membrane and nuclear localization was specific to peroxiredoxin during metronidazole stress.

1. Introduction

Entamoeba histolytica, an enteric protozoan, is a well-established causative agent of amoebic dysentery. It has a high potential for invading and destroying human tissue. The parasite lives in the human gut in an environment of reduced oxygen pressure, and during tissue invasion it gets exposed to reactive oxygen species such as superoxide radical anions (O_2^-) and hydrogen peroxide (H_2O_2). The microaerophilic *E. histolytica* overcomes the elevated levels of oxygen and its derivatives during tissue invasion with its antioxidant enzymes. The antioxidant enzymes in *E. histolytica* are an iron containing superoxide dismutase (Fe-SOD), thioredoxin reductase (flavin reductase), and peroxiredoxin -a thiol-specific 29 kDa surface antigen, which has both peroxidase and antioxidant activity [1]. Peroxiredoxin has also been referred to as Eh 29, the 29 kDa thiol dependent peroxidase of *E. histolytica* [2]. Peroxiredoxins of *Entamoeba* have been shown to play a role in virulence by helping the parasite to survive host immune response. It plays a role in combating reactive oxygen species (ROS) and reactive nitrogen species (RNS) attack by inflammatory host cells. Peroxiredoxins can degrade hydrogen peroxide—the primary lethal oxygen derivative in *Entamoeba* effectively [3]. Studies have shown that a highly virulent strain of *Entamoeba* is less susceptible to H_2O_2 compared to an attenuated strain which has lost its virulence due to prolonged culturing [4, 5]. It was seen that inhibition of Eh29 gene expression led to the effective decrease in cytopathic and cytotoxic activities in *E. histolytica* trophozoites. Sizes of liver abscesses were smaller in hamsters inoculated with an Eh29 downregulated trophozoites compared to the normal HM-1 : IMSS, suggesting that peroxiredoxin of *E. histolytica* has a role in survival of trophozoites in the presence of ROS and

thereby in the pathogenesis of amoebiasis [2]. *E. histolytica* peroxiredoxin is induced by a high oxygen environment [6] and is also induced by Trichostatin A, a drug that increases the resistance to oxidative stress in the parasite [7].

E. histolytica peroxiredoxin is a galNAc lectin associated protein. It has been postulated that during host parasite interaction the lectin recruits peroxiredoxin to the host parasite surface, a mechanism by which the parasite protects itself during tissue adherence and invasion from oxidative attacks from activated host phagocytic and epithelial cells [8]. Nonvirulent *E. histolytica* was found to be more susceptible to *in vitro* oxygen challenge compared to virulent strain *E. histolytica*. In case of the virulent *E. histolytica,* resistance to oxygen challenge is due to greater ability to reduce O_2^- and hydrogen peroxide as well as pyruvate ferredoxin oxidoreductase (PFOR) reactivation [9]. Peroxiredoxin expression was shown to be higher in HM-1 : IMSS, a virulent strain compared to a less virulent Rahman strain while SOD was present at a higher level in Rahman in comparison to HM-1 : IMSS [10]. It has been reported that the pathogenic *E. histolytica* contains as much as 50 times higher levels of peroxiredoxin, compared to *Entamoeba dispar*—a morphologically similar but noninvasive species [1]. Peroxiredoxin has been shown to increase by 2.1-fold in one hour high-oxygen-exposed trophozoites compared to controls [6].

Metronidazole is a synthetic 5 nitroimidazole and is used to treat infections by anaerobic protozoans like *Entamoeba* and *Giardia*. It is the single most widely prescribed drug for amoebiasis. The presence of metabolic pathways of low redox potential in *Entamoeba* and other anaerobic protozoans contributes to the selective toxicity of metronidazole to the protozoan leaving the human host unaffected. Indiscriminate use of antiamoebic drugs has led to an increase in the MIC of these drugs [11]. The enzyme Ferredoxin activates metronidazole inside the *Entamoeba* cell by reducing the nitro group of the drug into a nitroso free radical, which is cytotoxic to the cell. Thioredoxin reductase has also been reported to activate metronidazole in *Entamoeba* [12]. During the reoxidation of the active drug inside the parasite, a reactive oxygen species is generated. This reactive oxygen species is detoxified by iron containing superoxide dismutase (FeSOD) to hydrogen peroxide and oxygen. Subsequently, peroxiredoxin scavenges the hydrogen peroxide and converts it to water [13].

In vitro Fe-SOD activity was shown to be three times more in *Entamoeba histolytica* (HTH-56 : MUTM) strain resistant to 10 μM metronidazole [14]. Laboratory induced resistance to 40 μM metronidazole in *E. histolytica* (HM-1 : IMSS) was also associated with an increase in the expression of Peroxiredoxin (2.9 fold) and FeSOD (5 fold) [15].

Tazreiter et al. (2008) assessed the response of the amoeba to 50 μM of metronidazole using a microarray, qRT-PCR, and two-dimensional gel electrophoresis. The results indicated only a modest increase in mRNA levels of peroxiredoxin and iron containing superoxide dismutase. The increase in mRNA expression was however not reciprocated at the protein level [16]. Schlosser et al. (2013) have reported that metronidazole treatment of *E. histolytica* reduces the activity of important oxidative stress regulatory enzymes including SOD and peroxiredoxin [17].

Since metronidazole is indiscriminately used in India, there is a possibility of development of metronidazole resistance in the *Entamoeba* isolates from India. The major aim of this work was to understand the behavior of the antioxidant enzymes during metronidazole stress in standard axenised laboratory strain HM-1 : IMSS versus clinical isolates of *Entamoeba histolytica*, from New Delhi, India, and Dhaka, Bangladesh. The expression of two genes, peroxiredoxin and Fe-SOD, involved in metronidazole inactivation and in oxidative stress response was studied in the above isolates. Immunolocalization of peroxiredoxin in the isolates was compared. Studying the behavior of these antioxidant enzymes in clinical isolates from endemic locations like Delhi and Dhaka when challenged with metronidazole will help in understanding their differential response to the drug.

2. Materials and Methods

2.1. Entamoeba Strains and Culture. E. histolytica strain HM-1 : IMSS cells were maintained and grown in TYI-S-33 medium supplemented with 15% adult bovine serum, 1 X Diamond's vitamin mix, and antibiotics (0.3 units/mL penicillin and 0.25 mg/mL streptomycin) at 35.5°C. The cells were subcultured twice a week. Clinical isolate MS96 3382 cells from Dhaka, Bangladesh, were grown and maintained in LYI-S-2 medium supplemented as was done for TYI-S-33. MS96 3382 cells were subcultured every 48 hours. Medium was prepared as described by Clark and Diamond, 2002 [18].

2.2. Isolation and Maintenance of Patient Isolates of E. histolytica in Xenic Culture. Clinical isolate 654 was from a patient sample from Safdarjung hospital, New Delhi, while MS96 3382 (henceforth referred as MS96) was isolated from an urban slum in Dhaka. They were isolated from stool samples and maintained in Robinson's BRS medium with added *Escherichia coli* [18, 19] and subcultured thrice a week.

2.3. DNA Isolation and Identification of E. histolytica. Cells from xenic cultures were pelleted at 600 g at 4°C and stored in 70% ethanol at −20°C for DNA isolation. DNA was isolated from xenic cultures using QIA Amp DNA minikit for isolation of genomic DNA (Qiagen catalog no. 51366). Strains were identified as *E. histolytica* or *E. dispar* based on the PCR amplification using primers specific for *E. histolytica* and *E. dispar* [20].

2.4. HM-1 : IMSS Strain Adapted to 20 μM Metronidazole. HM-1 : IMSS cells growing in mid log phase under microaerophilic conditions were exposed to low concentration of metronidazole (5 μM) for 24 hours and the surviving cells were transferred to fresh medium without the drug and allowed to grow until mid-log phase. The procedure was repeated with the same concentration of drug thrice before higher drug concentrations were used following the protocol of Wassmann et al. 1999 [15]. HM-1 : IMSS cells were adapted initially to 10 μM metronidazole, then to 15 μM

metronidazole, and finally to 20 μM of metronidazole over a period of 12 months. An attempt to increase the concentration beyond was unsuccessful as the surviving cells could not proliferate. These cells, adapted to 20 μM metronidazole, were henceforth referred to as 20 μM A and were subsequently maintained in 20 μM metronidazole. The percent cell survival in metronidazole after 72 hours was estimated using trypan blue. Cell counting was done using a haemocytometer.

2.5. Short Term Metronidazole Stress to Xenic and Axenic Cells. In case of axenic cells (HM-1 : IMSS) and MS 96 (AX), the cells were grown till the log phase in 2 × 50 mL flasks in TYIS33 and LYI S-3 medium, respectively. The medium was then decanted and fresh medium with 20 μM metronidazole was added. The cells were then incubated for 24 hours with the drug at 35.5°C. These metronidazole treated cells will be henceforth referred to as 20 μM S and MS96 (AX) 20 μM, respectively. The treated cells were harvested for RNA isolation and immunofluorescence studies. Trizol reagent was added to the cell pellet and mixed thoroughly by pipetting and then pellet was stored at −80°C for RNA isolation. For immunofluorescence microscopy, the harvested cells were washed in PBS buffer and fixed in cold methanol for at least 20 minutes at −20°C.

In case of xenic culture eight tubes (5 mL Bijou bottles) each of cells MS96 (X) and 654 were grown in Robinson's medium till log phase (24 hours); then they were harvested by centrifugation at 600 g for 5 minutes at 4°C and cells (approximately 50,000 cells per bottle) were resuspended in 3 mL fresh BRS having concentration of 25 μM of metronidazole in case of MS96 (X) and 50 μM in case of clinical isolate 654. This was overlaid on a new saline agar slant in 5 mL Bijou bottles. The medium was completed and bottles were incubated at 37°C for 24 hours and then harvested by centrifuging at 600 g for 5 minutes in RNAase free falcon tubes (50 mL). The supernatant was carefully decanted. The harvested cells were stored for RNA isolation and immunofluorescence microscopy as described in case of axenic cells. Cell survival after metronidazole treatment was counted using haemocytometer. The clinical isolates treated with metronidazole were termed as 654 (50 μM) and MS96 (X) 25 μM, respectively.

2.6. Expression Level of Antioxidant Enzymes Peroxiredoxin and Superoxide Dismutase by Semiquantitative RT-PCR

2.6.1. Isolation of RNA. Total RNA was isolated from normal and treated *E. histolytica* trophozoites incubated for time periods ranging from 0 h to 24 h using Trizol reagent (Invitrogen) following the manufacturer's protocol. The isolated RNA was treated with DNase (Roche) according to the manufacturer's protocol.

2.6.2. Semiquantitative RT-PCR. Reverse transcription was performed using 5 μg of DNase treated RNA from normal cells and stress induced *E. histolytica* cells using a random Hexamer from Promega and MMLV RT enzyme following manufacturers protocol.

The synthesized cDNA was amplified using set of peroxiredoxin and FeSOD forward and reverse primers. The sequence of *Entamoeba* peroxiredoxin has been cloned and reported earlier by Torian et al. (1990) and Bruchhaus and Tannich (1993) [21, 22]. Primer sequences and accession numbers of peroxiredoxin and FeSOD are as shown in Table 1. The isoforms of entamoeba peroxiredoxins to which our peroxiredoxin primers set bind were checked using Eupath Db (http://amoebadb.org/amoeba/). The primers selected for this study amplified eleven isoforms of peroxiredoxin. 18S rRNA was used for normalization. PCR amplification of the target gene and 18S rRNA was carried out in the same tube simultaneously. In all the reactions, initial denaturation was carried out at 94°C for 5 min, targeted genes were amplified by 30 amplification cycles, annealing was done for 1 minute at 50°C for peroxiredoxin and 18S rRNA at 48°C for FeSOD, and an extension was done at 72°C for 1 min followed by a final extension at 72°C for 5 min. The products were run on 1.2% agarose gel, stained with ethidium bromide, and finally documented and quantified using the Alpha Imager Gel Documentation System. In case of axenic cultures a nontemplate control was used while in case of xenic cultures, cDNA was prepared from total RNA, isolated from uninoculated xenic culture medium containing only bacteria, and was used as a blank.

2.6.3. Spot Densitometry. The bands obtained after electrophoresis were evaluated using spot densitometry with Alpha Ease FC software. Densitometric value of peroxiredoxin and Fe-SOD band area of each cell line were expressed as percent of 18S rRNA band density. The experiment was repeated with at least three sets of RNA on all *Entamoeba* isolates studied.

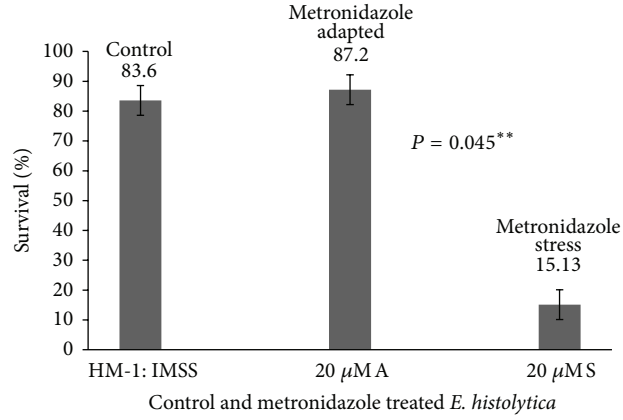
2.6.4. Statistical Analysis. Mean, standard deviation, and paired *t*-test for the control set and treated groups were carried out in case of normal and metronidazole stress conditions. ANOVA test was employed for comparing the expression of antioxidant enzymes between different isolates.

2.7. Immunofluorescence Microscopy. Cells were harvested in the log phase and then washed with PBS buffer, fixed in cold methanol for 20 m at (−20°C), washed twice with PBS, and permeabilized with 0.1% triton-X-100 for 5 min at room temperature, washed, and blocked with 3% BSA at R.T for 60 min. After blocking, cells were washed thrice with PBS and incubated for 45 min at RT with monoclonal antiperoxiredoxin antibody generated in mouse at 1 : 250 dilution, washed with PBS, and incubated for 30 min with anti-mouse secondary antibody tagged with Alexa-448 in 1 : 500 dilution along with nuclear strain Hoechst 33342 (2.5 μg/mL).

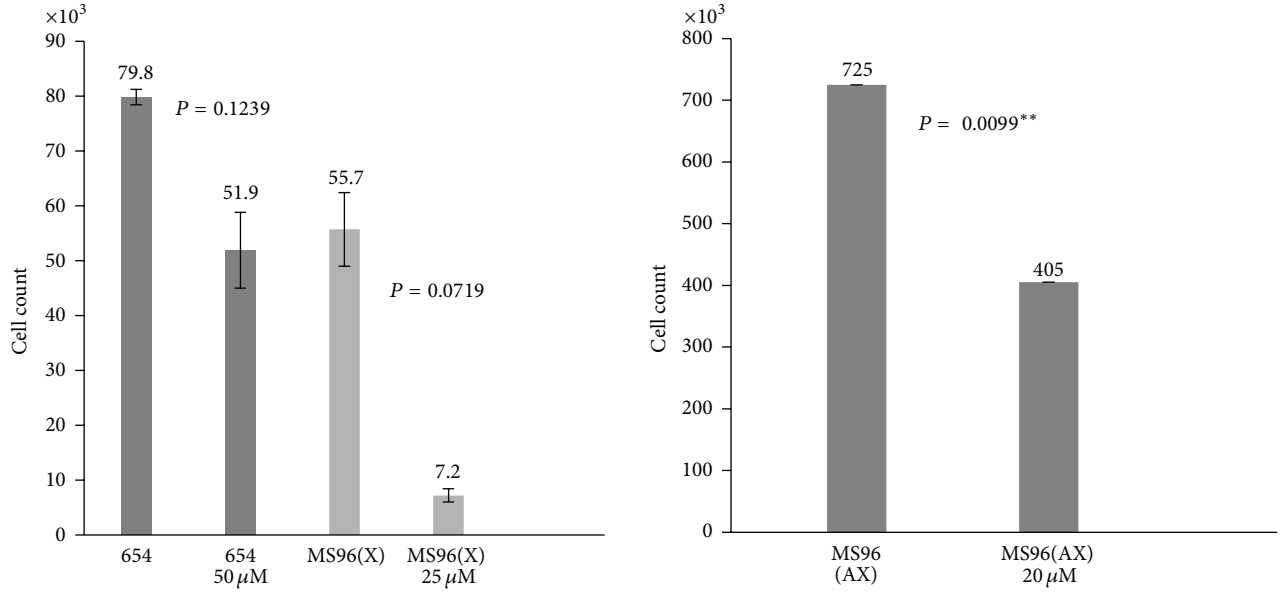
Colocalization of peroxiredoxin with another cytoplasmic protein CaBp1 was also performed [23]. Blocked cells were incubated with antiperoxiredoxin antibody generated in mouse together with a monoclonal anti-CaBp1 antibody generated in rabbits (1 : 250). The cells were washed with PBS and incubated for 30 m with anti-mouse secondary antibody

Table 1: Description of primers used for RT_PCR.

Enzyme	Primer composition	Tm	Amplicon size
Eh peroxiredoxin (Prx)	F 5′ AAA TCA ATT GTG AAG TTA TTG G 3′ R 5′ TCC TAC TCC TCC TTT ACT TTT A 3′	53.6°C 56.8°C	100 bp
Fe SOD Accession number (XM_643735.2)	F 5′ ACA ATT ACC TTA TGC TTA TAA 3′ R 5′ TCC ACA TCC ACA CAT ACA AT 3′	52°C 54°C	240 bp
Entamoeba histolytica 18S ribosomal RNA gene	F 5′ TCA GCC TTG TGA CCA TAC TC 3′ R 5′ AAG ACG ATC AGA TAC CGT CG 3′	61.7°C 68.9°C	200 bp



(a)



(b)

Figure 1: (a) Percent cell survival in HM-1 : IMSS after treatment with metronidazole. Percent cell survival after treatment with 20 μM metronidazole for 72 hours was estimated using trypan blue. Cell survival during metronidazole shock (20 μM S) was significantly low compared to adapted cells (20 μM A) and untreated HM-1 : IMSS cells. $P = 0.045$. (b) Cell count in clinical isolates of *E. histolytica* after metronidazole treatment. Cell counts in clinical isolates were carried out 24 h after treatment with metronidazole. Each pair of columns shows the untreated and treated cells of each strain. *P* values were calculated using paired *t*-test.

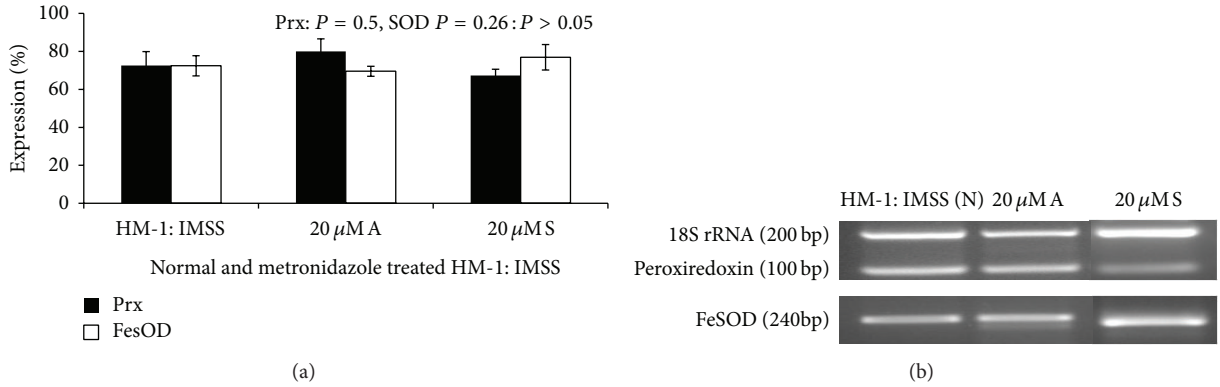


Figure 2: (a) Expression of peroxiredoxin and FeSOD in HM-1 : IMSS cells during Metronidazole stress. The densitometric data from semiquantitative RT-PCR analysis of peroxiredoxin (Prx) and FeSOD during metronidazole stress in HM-1 : IMSS are represented graphically. Data are mean ± SD of three independent experiments. 18S rRNA PCR was used as an internal control. Densitometric values were expressed as % after normalizing with 18S rRNA. $P > 0.05$. (b) Representative gel images.

tagged with Alexa-488, and anti-rabbit secondary antibody tagged with Alexa 555 in 1 : 500 dilution and Hoechst 33342 (2.5 μg/mL). Colocalization studies with fibrillarin using polyclonal anti-rabbit anti-fibrillarin antibody were also used to confirm the nucleolar localization of peroxiredoxin in the cells according to the method of Jhingan et al. 2009 [24]. Trophozoites were then washed thrice with PBS and mounted on glass slide in 25% glycerol with antibleach p-phenylenediamine (Sigma). All steps were done in 1.5 mL microfuge tubes by pelleting at 4°C, 500 g for 5 min. Phase contrast and fluorescent images were taken using a Zeiss Axio Imager M1 microscope (Germany) with a 100x magnification.

3. Results

3.1. Effect of Metronidazole on the Growth of Different Strains of E. histolytica. Cell survival of HM-1 : IMSS cells and 20 μM A cells (adapted to 20 μM metronidazole) was assessed by counting percent cell survival after 72 h. Cell survival was significantly higher both in 20 μM A cells as well as untreated HM-1 : IMSS cells in comparison to the HM-1 : IMSS cells given a 20 μM metronidazole shock for 72 h (20 μM S) (Figure 1(a)). This shows that the 20 μM A cells were able to survive and multiply constantly in the presence of the 20 μM metronidazole while the 20 μM S cells could not. The growth of clinical isolates in the presence of metronidazole was assessed by cell count after 24 hours of treatment.The concentration of metronidazole chosen for each clinical isolate was based on plate tests done to calculate MIC according to the method of Upcroft and Upcroft, 2001 [25]. MIC values were found to be 20 μM, 25 μM, and 50 μM for MS96 (AX), MS96 (X), and 654, respectively. Cell counts in the treated population were lower compared to untreated cells in all the three clinical isolates (Figure 1(b)). In case of axenic isolate MS96 (AX) the reduction in cell count was significant ($P = 0.0099$).

3.2. Metronidazole Stress Responses in HM-1 : IMSS Cells by RT PCR. The expression of both the antioxidant enzymes peroxiredoxin and SOD did not change significantly either in 20 μM S or 20 μM A when compared to untreated cells as measured by RT PCR ($P > 0.05$) (Figures 2(a) and 2(b)).

3.3. Immunolocalization of Peroxiredoxin in HM-1 : IMSS Cells. For studying the localization of peroxiredoxin, we took an average of forty cells per group. The enzyme localized principally to two cellular compartments (nucleus and membrane) in more than 50% of untreated cells. In order to confirm the nuclear localization of peroxiredoxin, we used two stains, one specific for nucleus (Hoechst) and anti-fibrillarin antibody to stain the nucleolus. In 15 to 20% cells the peroxiredoxin protein was localized to the membrane and in less than 5% cells to the nucleus only. In order to see whether membrane localization was characteristic of all cytoplasmic proteins, colocalization of CaBp1 along with peroxiredoxin was studied in these cells. It was seen that CaBp1 localized in the cytoplasm in 70% cells while peroxiredoxin localized to nucleus and membrane. In 30% cells CaBp1 showed membrane localization and it was exclusively at the site of phagocytic cup. Peroxiredoxin was seen to localize to specific pockets of the membrane rather than the whole membrane in 25% of the cells (Figure 3, panel (1)).

Peroxiredoxin localization in cells adapted to grow in metronidazole (20 μM A) was similar to that of untreated cells with both nuclear and membrane localization (Figure 3, panels (6) and (7)). Nuclear localization of peroxiredoxin protein was further confirmed by the nucleolus staining with fibrillarin.

During (20 μM S) metronidazole shock given over a period of 6 hours, 80% of the cells showed membrane and nuclear localization of peroxiredoxin and 20% cells showed only membrane localization while CaBp1 localized to the cytoplasm only even during shock (Figure 3, panel (2)). However, after 12 h of metronidazole shock, 70% cells showed membrane localization and 15% showed membrane and nuclear localization while 15% showed only nuclear localization. CaBp1 was localized in the cytoplasm and in some cells only at the site of phagocytosis (Figure 3, panel (3)).

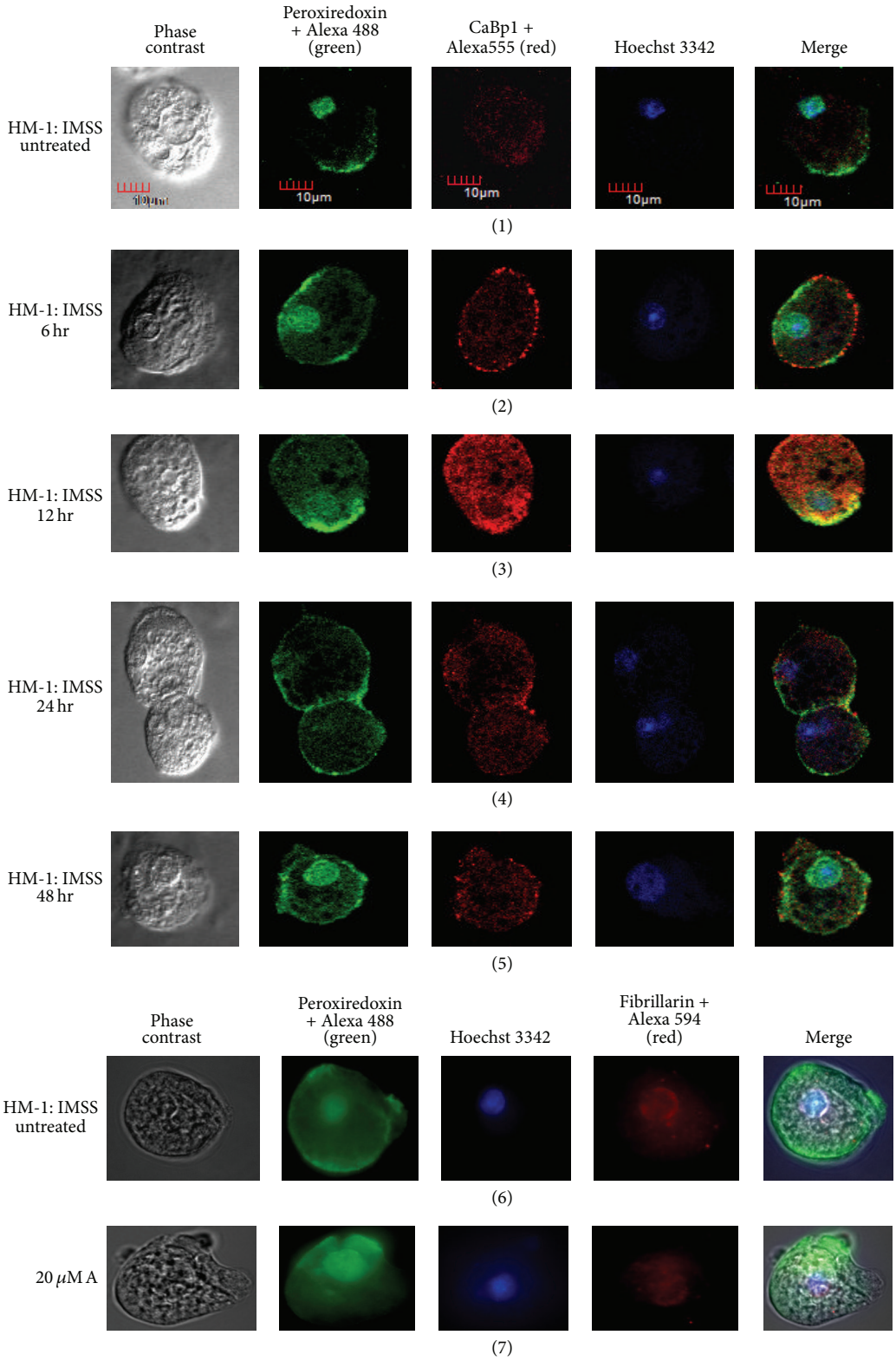


Figure 3: Immunolocalization of peroxiredoxin in *E. histolytica* strain HM-1 : IMSS during metronidazole stress. The images from left to right in each panel show phase contrast, peroxiredoxin stained by Alexa 488 (green), CaBp-1 stained by Alexa-555, Hoechst 3342 for nuclear staining, and the merged image. Peroxiredoxin was present in the membrane and the nucleus in the untreated cells (panel (1)), during short term metronidazole shock the peroxiredoxin localized to the membrane with loss of nuclear staining within 24 hours (panels (2), (3), and (4)) and then reverted back to the normal position after 48 hours (panel (5)). CaBp1 localized to the cytoplasm. Panels (6) and (7) show untreated cells and cells adapted to metronidazole (20 μM A), respectively. Peroxiredoxin localized to nucleus and membrane in both cases. The fourth panel from left shows fibrillarin stained with Alexa 594 to mark the nucleolus (panels (6) and (7)).

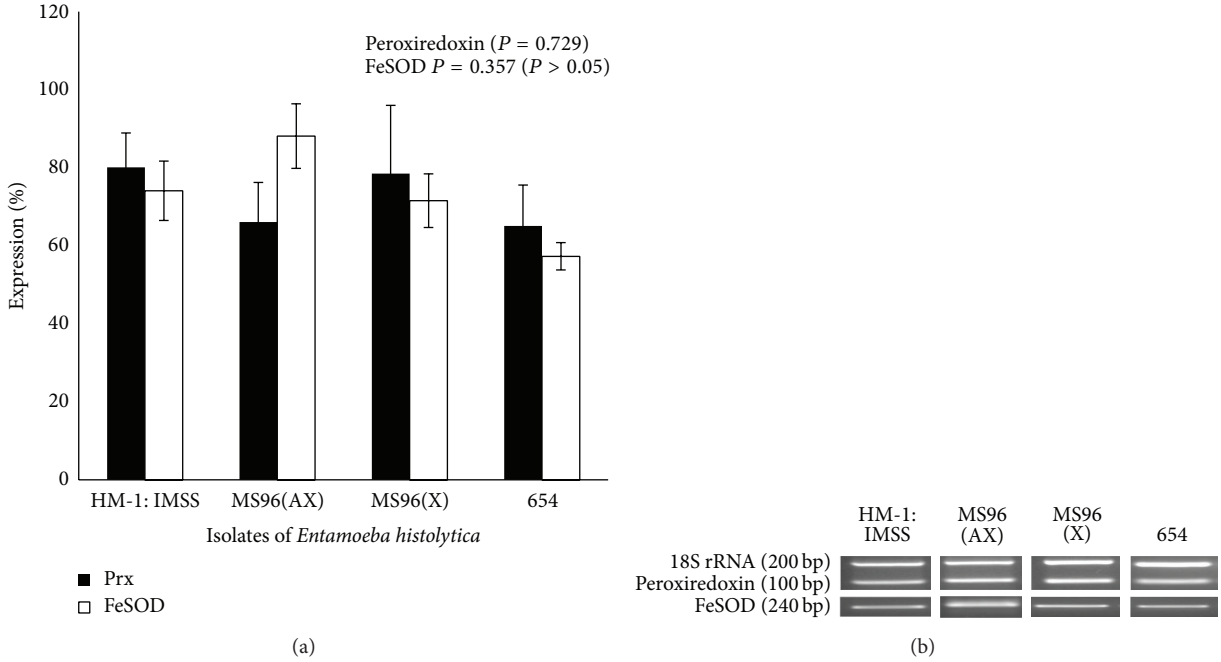


Figure 4: (a) Expression of peroxiredoxin and FeSOD in different isolates of *E. histolytica*. Graphical representation of densitometric data from semiquantitative RT-PCR analysis of Peroxiredoxin and FeSOD in *E. histolytica* trophozoites. No significant difference was seen in the expression levels of the two enzymes between the different isolates ($P > 0.05$). (b) Representative gel images.

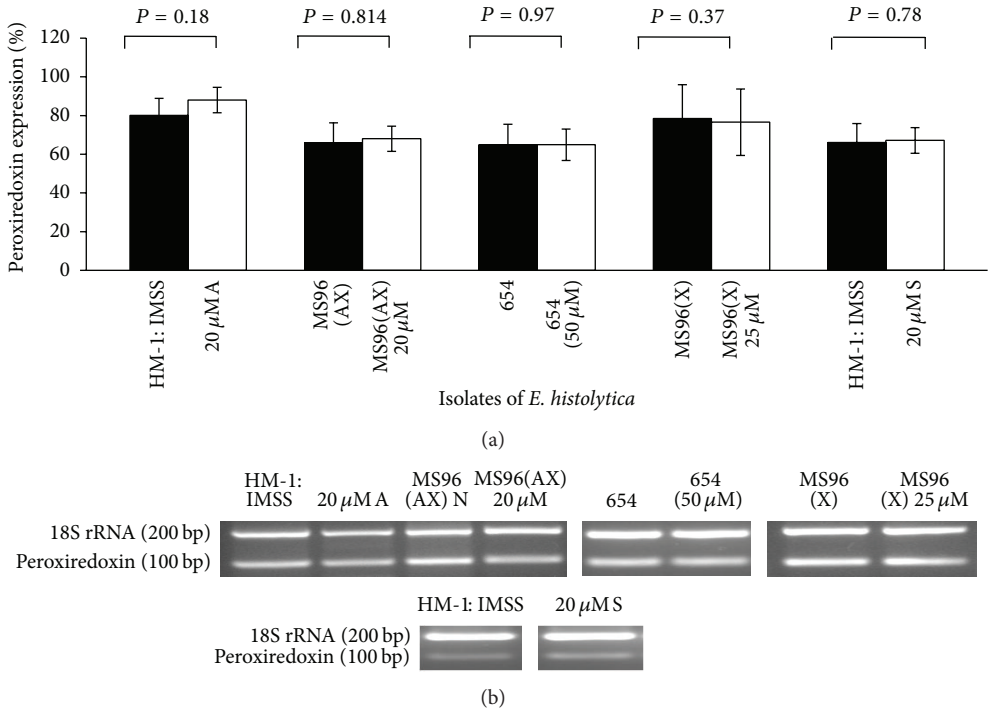


Figure 5: (a) Expression of peroxiredoxin in different isolates of *E. histolytica* during metronidazole stress. Graphical representation of densitometric data from semiquantitative RT-PCR analysis of peroxiredoxin in different isolates of *E. histolytica* in conditions of metronidazole stress. RT-PCR details as described in Figure 2. Each pair of columns shows the untreated and treated cells of isolates. Data are mean ± S.E for at least three independent experiments. $P > 0.05$. (b) Representative gel images.

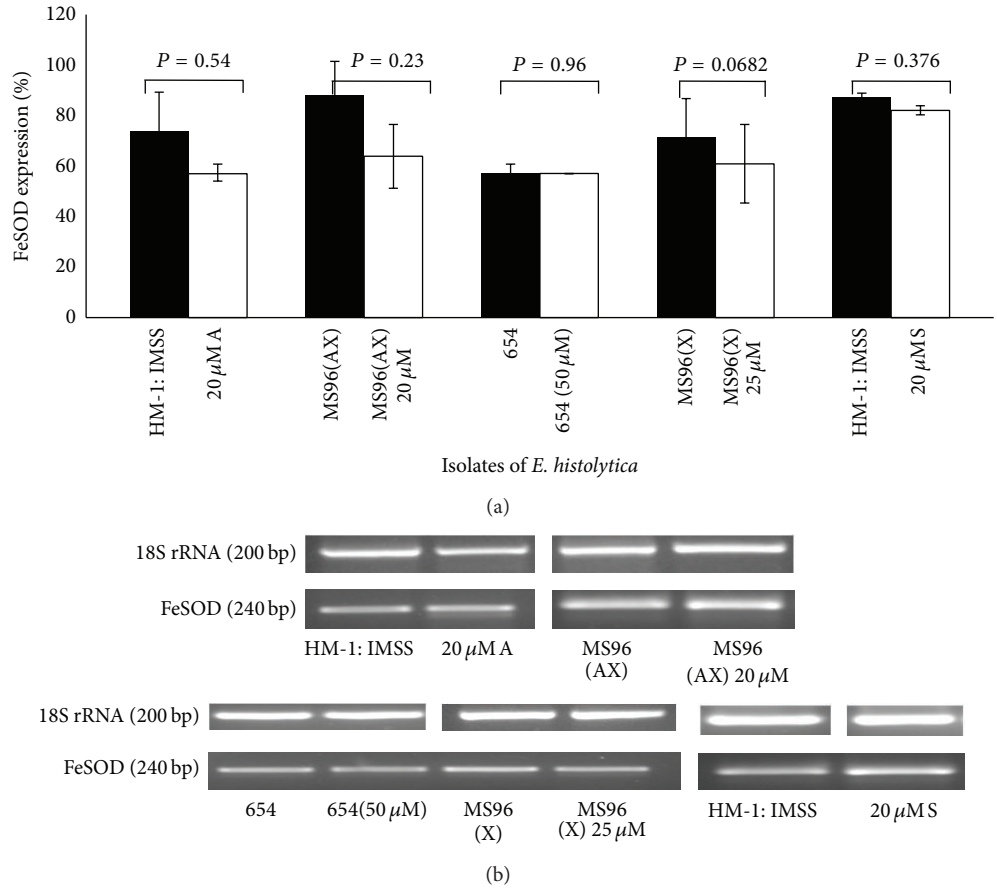


Figure 6: (a) Expression of FeSOD in different isolates of *E. histolytica* during metronidazole stress. Graphical representation of densitometric data from semiquantitative RT-PCR analysis of FeSOD in different isolates of *E. histolytica* in conditions of metronidazole stress. RT-PCR details are as described in Figure 2. Each pair of columns shows the untreated and treated cells of a strain. Data are mean ± S.E for at least three independent experiments. $P > 0.05$. (b) Representative gel images.

After 24 h, in 87% of cells the peroxiredoxin localized only to the membrane without any nuclear localization while the rest of the cells showed both membrane and nuclear localization (Figure 3, panel (4)). After 48 h of shock, the localization of peroxiredoxin in cells tends to revert back to normal condition showing both membrane and nuclear localization together (Figure 3, panel (5)). Our observation shows that the introduction of metronidazole triggered a flux of peroxiredoxin to the membrane from the nucleus within 24 h of stress which reversed after a period of 48 h.

The localization to the nucleus and membrane was specific to peroxiredoxin because on colocalization with CaBp1 in standard HM-1 : IMSS cells, peroxiredoxin localized mostly to the membrane and to the nucleus in contrast to CaBp1 localization, which was cytoplasmic and localized to the membrane (only during phagocytosis) but never to the nucleus as reported earlier [23].

3.4. Expression of Peroxiredoxin and FeSOD in Clinical Isolates. In untreated conditions, peroxiredoxin and FeSOD expression levels did not show any significant change either in the clinical isolates maintained in xenic conditions or MS96 (AX) maintained in axenic conditions, compared to HM-1 : IMSS. One-way ANOVA was used to analyze the data (Figure 4).

When clinical isolates were subjected to different concentrations of metronidazole shock (20, 25, and 50 μm for MS96 (AX), MS96 (X), and 654, resp.) no significant changes were observed in the expression level. However MS96 (X) exhibited consistent decrease in expression of FeSOD gene but did not attain a significant value when data was analyzed using paired *t*-test (Figures 5 and 6).

3.5. Immunolocalization of Peroxiredoxin in Clinical Isolates. In axenically grown clinical isolate MS96, peroxiredoxin localized to pockets of the membrane and also to the nucleus in untreated cells (Figure 7, panel (1)). When the cells were given 20 μM metronidazole shock for 24 h, peroxiredoxin localized to the periphery of cytoplasmic membrane with loss of nuclear staining in more than 80% of the cells (Figure 7, panel (2)). After 48 h of shock, all the cells reverted back to normal condition showing both nuclear and membrane localization of peroxiredoxin (Figure 7, panel (3)).

In case of xenic isolate MS96 and 654 the protein localized both to the nucleus and membrane in untreated cells but during metronidazole shock, it localized only to

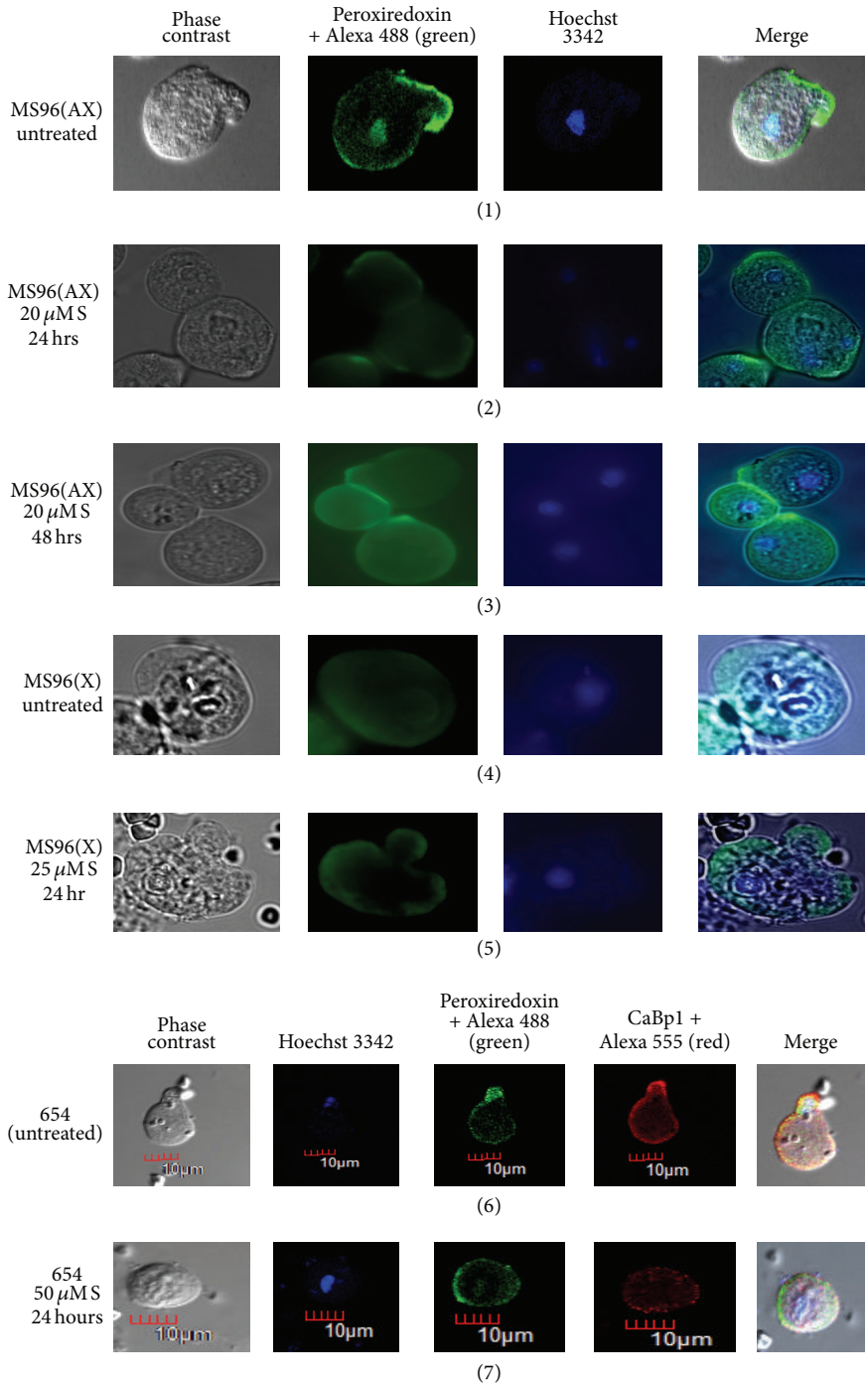


Figure 7: Immunolocalization of peroxiredoxin in clinical isolates of *E. histolytica* given short term metronidazole stress. The images from left to right in each panel show phase contrast, peroxiredoxin stained by Alexa 488, Hoechst 3342 for nuclear staining, and the merged image. In normal conditions Peroxiredoxin localized to the membrane and to the nucleus in axenic clinical isolate MS96 (AX) (panel (1)). After 24 and 48 hours of metronidazole stress peroxiredoxin localized to the membrane with loss of nuclear staining (panels (2) and (3)). Xenic isolates MS96 (X) and 654 showed loss of nuclear staining with metronidazole shock (panels (4) to (7)). Panels (6) and (7) show cytoplasmic localization of CaBp1 during the stress.

the membrane (Figure 7, panels (4) to (7)). Our colocalization study using CaBp1 in xenic isolate 654 confirmed similar results as observed in the axenic isolates (Figure 7, panels (6) and (7)).

4. Discussion

Our results have shown that the expression of both the antioxidant genes did not change significantly during

metronidazole stress conditions either in HM-1 : IMSS strain or in clinical isolates. The reports dealing with the changes in the expression of antioxidant enzymes during metronidazole stress are not conclusive [14, 15]. According to the recent report by Schlosser et al. (2013) a reduction in Peroxiredoxin and SOD enzyme activity is explained to be linked to the Thioredoxin reductase/Thioredoxin system. Reduced thioredoxin donates the electron required for peroxiredoxin to detoxify H_2O_2. It was explained that adduct formation between thioredoxin reductase (TrxR) and reduced metronidazole affects the thioredoxin reductase activity of TrxR leading to a decreased activity of peroxiredoxin [17]. Other reports also reveal similar behavior of antioxidant enzymes (FeSOD and peroxiredoxin) in *E. histolytica* which were not modulated in different stress conditions [26, 27].

Earlier studies on the localization of peroxiredoxin in *Entamoeba* revealed membrane localization of peroxiredoxin mostly in formalin fixed cells that were pretreated with the antibody against peroxiredoxin [1, 21]. Tachibana et al.1990 and Cheng et al. 2004 [28, 29] demonstrated that this protein was localized both in the nucleus and cytoplasm using a polyclonal antibody. However, our results demonstrate nuclear and membrane localization of peroxiredoxin using monoclonal antibodies against peroxiredoxin on *Entamoeba histolytica* cells fixed in methanol and permeabilized by Triton X100. The nuclear localization of peroxiredoxin was confirmed by Hoechst and Fibrillarin staining of the nucleus. Localization of peroxiredoxin in the nucleus along with membrane in untreated cells is reasonable if the protection of DNA from oxidative stress is mediated by peroxiredoxin.

Our results however clearly reveal a substantial change in localization pattern of peroxiredoxin during metronidazole stress indicating its recruitment to the surface. This response was observed both in standard laboratory strain HM-1 : IMSS as well as in clinical isolates. The localization of CaBp1, another cytoplasmic protein, was exclusively in the cytoplasm and never to the nucleus unlike peroxiredoxin. CaBp1 however localized to the membrane only during phagocytosis as reported earlier [23]. Thus it can be concluded that the protein peroxiredoxin behaved differently from this cytosolic protein CaBp1. During metronidazole stress, peroxiredoxin was localized mostly to specific pockets of the membrane; however, these pockets are still not defined at present.

When we adapted the HM-1 : IMSS cells to 20 μM metronidazole for a period of one year, we observed similar behavior of the cells as untreated HM-1 : IMSS showing the localization of peroxiredoxin both in the nucleus and the membrane. Thus we can infer that the relocalization of the enzyme to the membrane is an immediate and not a long term response.

Our results revealed that the Indian isolate could tolerate higher concentration of the drug compared to standard strains. Our study further concentrated on the expression of the antioxidant enzymes in the clinical isolates of *Entamoeba* when exposed to short term or long term metronidazole stress. Changes in the localization pattern of the antioxidant enzyme peroxiredoxin observed in our isolates during metronidazole shock indicated the protective role of this antioxidant enzyme towards the drug. This supported our hypothesis about how the clinical isolates show differential tolerance to the drug encountered in the host. We further conclude that the change in localization of the antioxidant enzyme is however not reciprocated by an increased mRNA expression of the protein. To our knowledge, this is for the first time, the expression of antioxidant genes, peroxiredoxin, and super oxide dismutase and the immunolocalization pattern of peroxiredoxin has been demonstrated in clinical isolates of *Entamoeba histolytica*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Erythrophagocytosis in *Entamoeba histolytica* and *Entamoeba dispar*: A Comparative Study

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Entamoeba histolytica is the causative agent of human intestinal and liver amebiasis. The extraordinary phagocytic activity of *E. histolytica* trophozoites has been accepted as one of the virulence mechanisms responsible for their invasive capacity. The recognition of the noninvasive *Entamoeba dispar* as a different species has raised the question as to whether the lack of pathogenic potential of this ameba correlates with a limited phagocytic capacity. We have therefore compared the process of erythrophagocytosis in both species by means of light and video microscopy, hemoglobin measurement, and the estimation of reactive oxygen species (ROS). In the present study, we confirmed that *E. dispar* has lower erythrophagocytic capacity. We also observed by video microscopy a new event of erythrocyte opsonization-like in both species, being more characteristic in *E. histolytica*. Moreover, *E. dispar* showed a lower capacity to produce ROS compared with the invasive species and also showed a large population of amoebae that did not engulf any erythrocyte over time. Our results demonstrate that *E. histolytica* has a higher phagocytic capacity than *E. dispar*, including a higher rate of production of ROS in the course of ingesting red blood cells.

1. Introduction

Entamoeba histolytica, an enteric parasite capable of invading intestinal mucosa and spreading to other organs, mainly the liver, is a significant source of morbidity and mortality in developing countries [1, 2]. The motile form of the parasite, the trophozoite, usually lives as a harmless commensal in the lumen of the large intestine where it multiplies and differentiates into a cyst, the resistance form, responsible for transmission of the infection. Occasionally, trophozoites would invade the intestinal mucosa and produce dysentery or *amoeba* and spread to other organs [3]. The existence of two different species of *Entamoeba*, initially proposed by Brumpt in 1925, was approved at the XIII Seminar on Amebiasis, held in Mexico City [4]. At present, unequivocal evidence for the existence of two morphologically similar and closely related species of *Entamoeba* in humans has been substantiated by immunological, genetic and molecular studies. As a result, *E. histolytica*, the invasive organism was formally redescribed to separate it from the noninvasive, and more common, *Entamoeba dispar* [5].

E. histolytica invasion starts when trophozoites residing in the colon deplete the mucus, interact with enterocytes, dismantle cell junctions, and lyse host cells [6], whereas *E. dispar* does not break down the mucus barrier or cause epithelial cell damage when in contact with cells on human colonic explants [7].

Although erythrophagocytosis has been proposed as a qualitative pathogenicity indicator rather than a quantitative virulence indicator [8], this process is widely considered as

one of the most prominent characteristics of *E. histolytica* virulence [9–11].

Biochemical changes accompanying the process of endocytosis include increases of oxygen and glucose consumption, the activity of the pentose or hexose monophosphate cycle, and hydrogen peroxide production. Together, these changes constitute the respiratory burst [12]. Once phagocytosis occurs, the respiratory burst is carried out as part of the metabolic processes to remove endocytosed material.

Having the opportunity to compare invasive versus noninvasive parasites, we decided to analyze comparatively the erythrophagocytosis process to determine possible differences between these two species of *Entamoeba*. The erythrophagocytic process was registered with light microscopy by means of the Novikoff et al. [13] staining to quantify the amount of ingested erythrocytes and to determine whether the erythrophagocytic capacity of each species correlates with the surface area of each amoeba as determined by light microscopic measurements. We have also used spectrophotometry to analyze the amount of ingested hemoglobin. To corroborate the percentage of nonphagocytic populations, video microscopy, flow cytometry, and confocal microscopy were used. Moreover, the extent of the respiratory burst, using NBT, was measured for each amoebic species.

2. Materials and Methods

2.1. Cells. Amoebas were cultured in borosilicate glass tubes under axenic conditions. *E. histolytica* trophozoites HM1-IMSS species were grown to logarithmic phase (72 h) in TYI-S-33 medium at 36°C [14] and *E. dispar* trophozoites in YI-S culture medium [15] for 72 h at 36°C. Both culture media contained 10% bovine serum and a vitamin mixture. Parasites were harvested by chilling the culture tubes at 4°C in a water-ice bath for 10 min and then they were centrifuged at 900 ×g for 5 min. Type B human erythrocytes (Rh+) were freshly obtained in Alsever's solution (Sigma Aldrich Company, UK) and washed 3 times in the same solution to remove white blood cells. The erythrocytes were counted and used in a 1 : 100 (trophozoites : erythrocytes) ratio in quantitative erythrophagocytosis assays and 1 : 5 ratio for video microscopy analysis of erythrophagocytosis. Yeasts of the genus *Candida albicans* species CAI4 generated from SC5314 (Clinical Systemic Isolate) [16] were routinely maintained in YPD medium [17]. Minimal defined medium consisted of 2% glucose supplemented with yeast nitrogen base (DIFCO). After that, *Candida albicans* were washed 2 times and harvested in phosphate buffered saline solution (PBS) and centrifuged at 900 ×g for 5 min and finally counted and used at a 1 : 100 (trophozoite : yeast) ratio in phagocytosis assays to measure the respiratory burst.

2.2. Erythrophagocytosis. *E. histolytica* and *E. dispar* trophozoites were washed in TYI-S-33 and YI-S without bovine serum, respectively. To establish the interaction, erythrocytes were added, and the interaction was carried out for 5, 10, and 15 min at 37°C without bovine serum, using a 1 : 100 amoeba-erythrocyte ratio for quantitative studies and 1 : 5 amoeba-erythrocyte ratio for video microscopy studies. Analysis was done with the AxioVision SE64 software with images obtained with a Zeiss Axiophot photomicroscope.

2.3. Quantitative Erythrophagocytosis, Study, and Correlation of Ingestion/Area. For quantitative experiments, 900 μL (5×10^5 amoebas) was incubated with 100 μL (5×10^7 erythrocytes) at different times, at 37°C. At the end of the incubation time, amoebas were resuspended in 1 mL of distilled water to lyse free erythrocytes and stop erythrophagocytosis. Trophozoites of both species can resist the osmotic shock without alterations of the plasma membrane permeability. Cells were centrifuged at 900 g/min for 5 min and pellets were fixed with 2.5% glutaraldehyde in PBS. Phagocytosed erythrocytes were visualized by phase contrast microscopy with the alkaline benzidine method as described [13]. Amoebas were incubated for 30 min at 37°C in 2 mL of 3,3-diaminobenzidine (Sigma) at a concentration of 2 mg/mL, 0.2% H_2O_2 in 0.05 M 2-amino-2-methyl-propanediol-HC1 (Merck-Schuchardt) at pH of 9.7. After washing with PBS, the number of erythrocytes present in the cytoplasm of 100 amoebas, in each time, was counted in triplicate. Considering that *Entamoeba* strains do not contain peroxisomes [18], cytoplasmic components positive to benzidine were considered only as ingested erythrocytes. Correlation tests regarding ingestion/area were conducted by measuring the surface area of each amoeba and the number of erythrocytes phagocytosed, using the AxioVision software SE64.

2.4. Indirect Determination of Erythrophagocytosis (Quantification of Hemoglobin). For a precise analysis of erythrophagocytosis, a quantitative determination of hemoglobin was done. Nonfixed trophozoites were washed with Turk's solution to eliminate noningested erythrocytes; trophozoites were pelleted and lysed with 1 mL of formic acid and the amount of hemoglobin was measured by spectrophotometric analysis at 400 nm.

2.5. Video Microscopy. Amoebas (1.25×10^5) were placed on coverslips resuspended in 100 μL of the respective medium and then erythrocytes (6.25×10^5) were added in 5 μL of Alsever's solution, maintaining slide temperature at 37°C. Micrographs and video micrographs sequences were taken with a Zeiss Axiophot microscope.

2.6. Fluorescent Labeling of Cells. To avoid manipulation of erythrocytes, 15 μL of whole blood were resuspended in Alsever's solution (300 μL) and labeled with 2.5 μL of Sytox 9 through incubation for 1 h at room temperature under constant stirring. After that, cells were washed twice at 600 ×g for 10 min and finally erythrocytes were resuspended in 1.5 mL Alsever's solution.

2.7. Flow Cytometry and Confocal Microscopy. The existence of a nonphagocytic population in both species was quantitative and qualitatively determined by flow cytometry and confocal microscopy respectively. By flow cytometry the number

of amoebas that had ingested stained erythrocytes was evaluated at different times, as mentioned. Once the time had elapsed, cells were centrifuged at 900 ×g for 10 min and then fixed with freshly prepared 4% (v/v) paraformaldehyde, for 1 h. After that, cell suspensions were washed 4 times with PBS and then read in a flow cytometer FACS-Calibur (Becton Dickinson). Confocal microscopy was used to distinguish ingested erythrocytes from free erythrocytes and to show amoebas that had not phagocytosed. Coverslips were mounted with Vectashield (Vector Laboratories; Ontario, Canada) and analyzed by confocal microscopy in an LSM700 microscope (Carl Zeiss Microimagin GmbH, Carl Zeiss, Germany).

2.8. Reduction of Nitroblue Tetrazolium (NBT) to Assess the Phagocytic Function. Amoebas (3.5×10^5/400 μL) previously adhered for 15 min to coverslips were incubated with yeast (3.5×10^7/100 μL) for 30, 60, and 120 min by the addition of 500 μL of NBT (1 mg/mL in sterile PBS) [19]. At the end of interaction, the reaction was stopped by adding 1 mL of 70% methanol dissolved in PBS for 10 min and washing once with PBS to remove excess of methanol; immediately after, 0.5% safranin dissolved in water was added and incubated for 1 min. The excess was removed with several washes until the sample was slightly stained. Coverslips were mounted, and yeast that had been reduced was counted using a phase contrast microscope. Counts were done taking 100 amoebas randomly, in triplicate, for each processed sample, and color changes that had occurred inside of cells were counted, taking as a positive value at least 1 single reduced yeast, and amoebas that did not show a color change in their cytoplasm were taken as a negative value. This method was performed in triplicate with only 2 possible variants (positive and negative) whereby only positive and negative cells where counted in each time.

2.9. Statistical Analysis. In the data obtained concerning the number of phagocytosed erythrocytes, absorbance of hemoglobin, and amoebas that reduced NBT when exposed to pathogenic yeast at different times with each species of *Entamoeba*, the following descriptive measures were calculated: average, minimum, and maximum standard deviation, quartiles (Q1, Q2, Q3), and coefficient of variation (Data not shown). Likewise, box and whisker diagrams were developed to further describe the phenomena occurring in the process of erythrophagocytosis and phagocytosis. To determine whether significant differences were present in the number of ingested erythrocytes, absorbance of hemoglobin, and amoeba showing NBT reduction, factorial analysis of variance was applied (ANOVA) with the transformed data of these variables (data were transformed using the Box-Cox technique because the Anderson-Darling test indicated that data were not normal). Once the ANOVA test was performed to establish which conditions showed differences, the Tukey's test was applied to measure the difference in the mean values between groups. These statistical analyses were carried out using MiniTab software version 16.0.

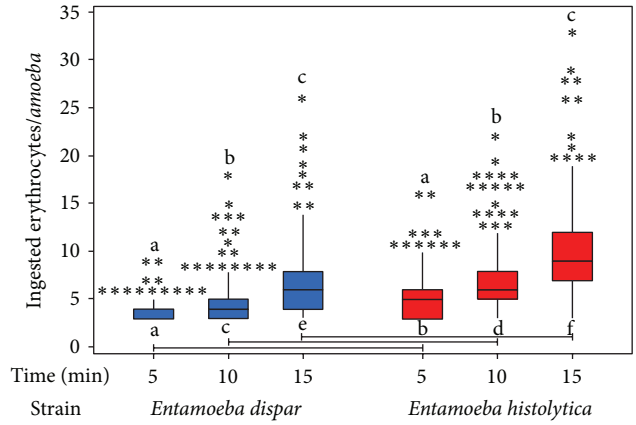


FIGURE 1: Erythrocyte uptake performed with *E. dispar* (blue) and *E. histolytica* (red) by light microscopy. The experiment was repeated three times independently in triplicates. The statistical comparisons showed significant differences in the erythrophagocytic capacity between species and among times ($P < 0.001$; different letters on top). Moreover, species compared with their reciprocal times also showed a significant difference ($P < 0.001$; bottom letters: a with b, c with d, and e with f) (*). Outliers.

3. Results

A comparative analysis of the phagocytic capacity of *E. histolytica* and *E. dispar* may help to understand the mechanisms involved in the virulence of *E. histolytica*. To date limited information exists about the erythrophagocytic capacity of *E. dispar*. Here we demonstrate basic differences in the phagocytic process between these two parasites.

3.1. Comparative Analysis of the Erythrophagocytic Capacity between E. dispar and E. histolytica

3.1.1. Determination of the Number of Ingested Erythrocytes. As a first step in the analysis of erythrophagocytosis, the number of ingested erythrocytes per amoeba was determined after 5, 10, and 15 min of interaction. As shown in Figure 1, this is a time-dependent process for both *E. dispar* and *E. histolytica*, with a clear increase in the number of ingested erythrocytes with longer times of interaction. ANOVA test between species and time variables was carried out showing that these variables, "species" and "time," showed a significant difference ($P < 0.001$). With Tukey's test, each variable with respect to all other variables of "species" and "time" was compared. Results show that, for each reciprocal combination, the P value was less than 0.001 ($P < 0.001$). This confirms significant differences among all combinations (Figure 1).

3.1.2. Determination of Hemoglobin Content in Trophozoites. To corroborate the results obtained by direct assays, indirect measurements were carried out by spectrophotometry to determine the absorbance produced by the hemoglobin contained in erythrocytes ingested by 5×10^5 trophozoites of each species at previously described times. Therefore, it was possible to obtain additional and more accurate information

about the erythrophagocytosis process. In addition, ANOVA between different variables (species and time) was carried out to determine if differences found in absorbance between *E. histolytica* and *E. dispar* were significant.

As expected, there were clear differences in the erythrophagocytic capacity of *E. dispar* versus *E. histolytica* ($P < 0.001$); however, in contrast to results found when counting erythrocytes, hemoglobin determination did not show significant differences among time in the same species. With Tukey's test, each variable with respect to all other "species" and "time" variables were compared. Results show that, for each reciprocal combination, the P value was less than 0.001 ($P < 0.001$) (Figure 2).

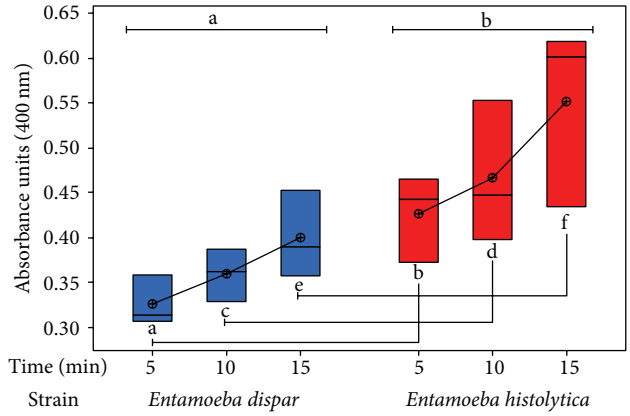


Figure 2: Hemoglobin content in *E. dispar* (blue) and *E. histolytica* (red) after erythrophagocytosis, determined by spectrophotometry. The experiment was repeated three times independently in triplicates. Quantification of erythrophagocytosis measured by ingested hemoglobin indicated no significant differences among times (letters on top, connected by the same line segment; a and b). A significant difference between species compared with their reciprocal times ($P < 0.001$; bottom letters connected by the same line segment) was observed.

3.1.3. Correlation between Trophozoites' Surface Area and Ingested Erythrocytes as a Function of Time. To analyze whether the amoeba area could be a factor related with a higher rate of erythrophagocytosis, a dispersion analysis was performed. Results showed a relative, time-dependent association between amoeba area and number of phagocytosed erythrocytes, with "r" values of 0.5, 0.60, and 0.63 for *E. dispar* at 5, 10, and 15 min respectively, and with "r" values of 0.56, 0.59, and 0.70 for *E. histolytica* at 5, 10, and 15 min, respectively. Therefore, there was a direct relationship between the average surface area of the amoeba and the number of ingested red cells, with a significant difference ($P < 0.001$) between the two species; furthermore, this ratio increased with time. Although the same trend is observed for both species, the correlation values of *E. histolytica* were higher (Figure 3).

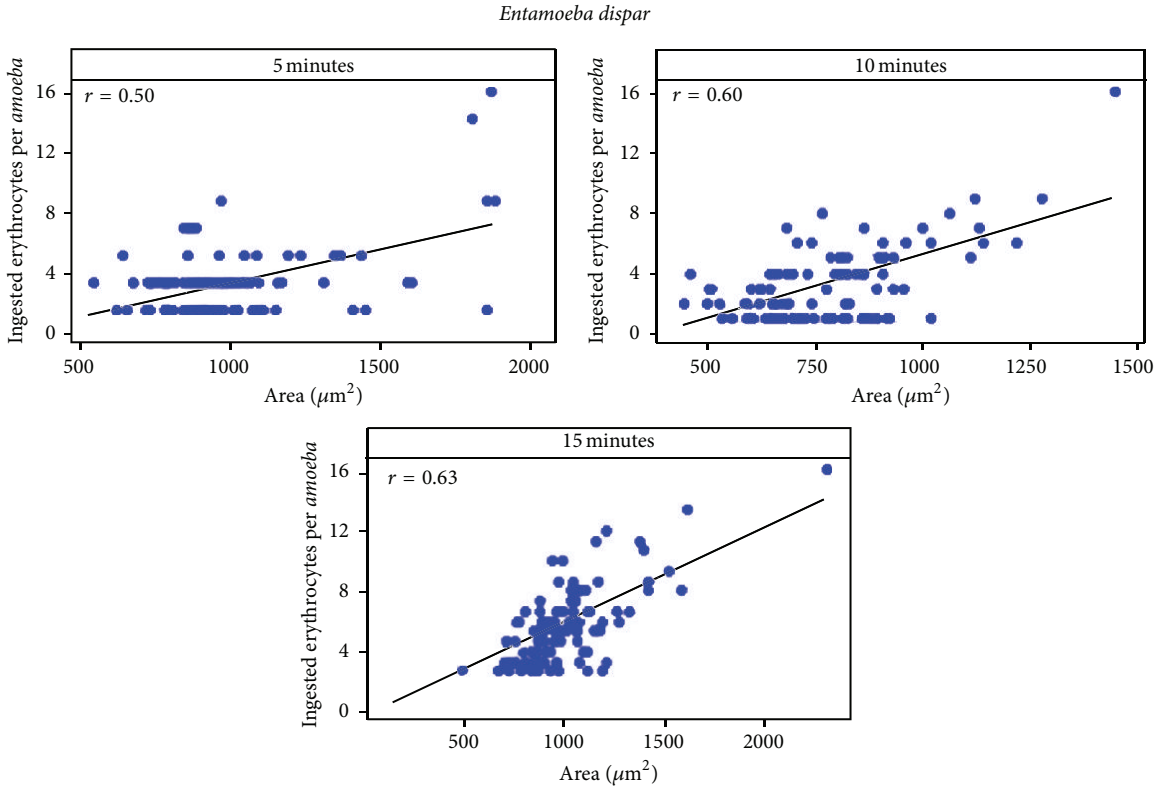
3.2. Comparative Analysis of the Erythrophagocytic Process between E. dispar and E. histolytica

3.2.1. Opsonization-Like Event during Erythrophagocytosis. Erythrophagocytosis by *E. histolytica* has been a widely studied mechanism [8, 9, 11, 20–22]. However, with the aid of video microscopy, a new characteristic of this process was observed. Before describing this event, it is necessary to mention that the adhesion process between *E. dispar* and erythrocytes is rather weak. Apparently, amoebas have an opsonization—like mechanism which consisted in the fact that erythrocytes that had had a previous contact with amoebas were clearly more susceptible to be bound and/or be ingested by other trophozoites. This event was observed with both species, though with clear differences between them. Figure 4(a)(A) shows how an *E. dispar* trophozoite (green) has adhered to a group of erythrocytes (red) on the caudal pole; as mentioned before, probably due to the weakness of this binding, this group of erythrocytes is released from the amoeba (Figure 4(a)(B–D)); however, these erythrocytes were attracted to another amoeba (blue) (Figure 4(a)(E)), despite having more erythrocytes surrounding it. This amoeba (blue) seems to show some tropism for those erythrocytes that had been previously adhered to the green amoeba; subsequently, the amoeba (blue) adheres to the same group of erythrocytes and continues its mobility. However, this group of red blood cells becomes totally detached from the trophozoite (Figure 4(a)(F–I)).

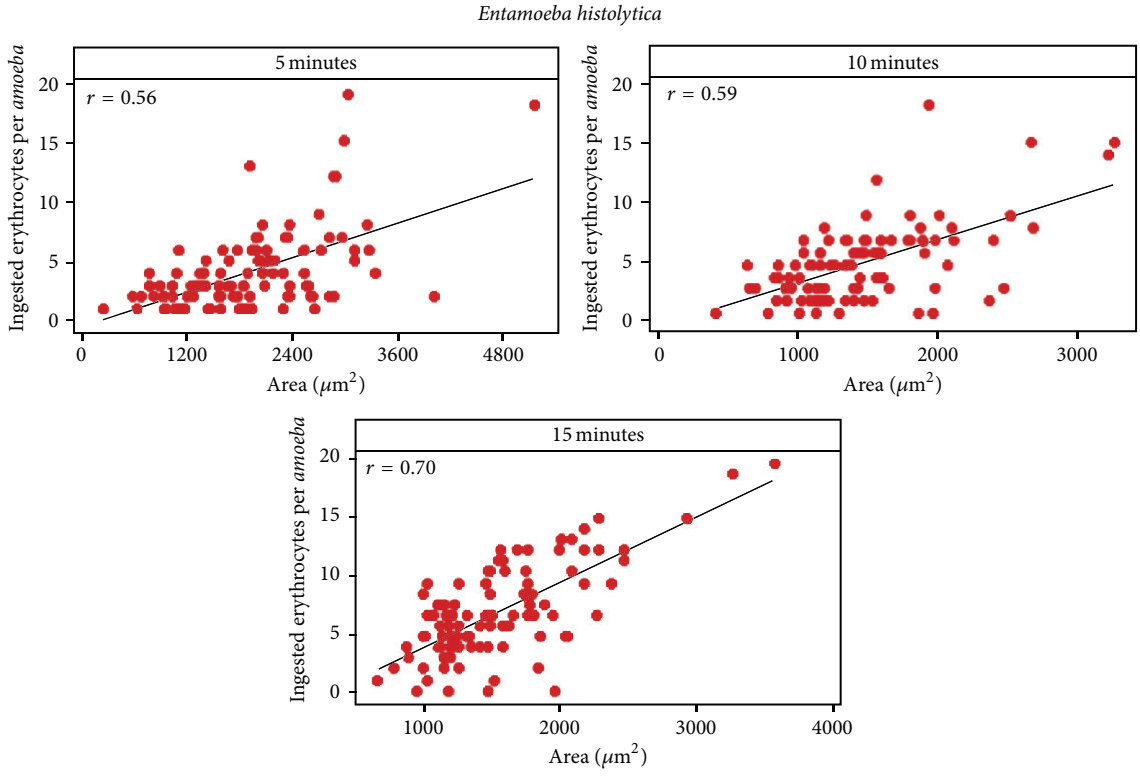
E. histolytica also presented this phenomenon, although the attachment strength was higher than that observed with *E. dispar* due to the apparent morphological distortion and stress (tension) generated in erythrocytes. Figure 4(b)(A) shows an amoeba (green) with a large number of adhered erythrocytes in the caudal pole that can be tracked all the way until it encounters another amoeba (blue) (Figure 4(b)(B–D)); this second amoeba (Figure 4(b)(D)) had the ability to impressively adhere to the same group of erythrocytes that are being carried by the first amoeba and to generate a competition force to acquire the group of red cells (Figure 4(b)(E–H)); finally a break of this agglomerate occurs, and both amoebas capture a portion of the initial erythrocyte group (Figure 4(b)(I)).

3.2.2. Nonphagocytic Population of Amoebas. Having evidence by video microscopy about the low adhesion capacity of *E. dispar* and observing that there were a notable number of nonphagocytic cells, we decided to determine the percentage of the nonphagocytic population for each species. Results showed that, after 20 to 30 min interaction of *E. dispar* with erythrocytes, (Figure 5(a)(A–I)) only a small number of amoebas with a limited number of internalized erythrocytes (red dots) were observed. In contrast, *E. histolytica* trophozoites, with only 5 min of interaction with erythrocytes (Figure 5(b)(A–I)) showed not only a large number of red blood cells in their cytoplasm (red dots) but also a very small number of amoebas that did not have ingested erythrocytes.

Having demonstrated by video microscopy that a nonphagocytic population exists, flow cytometry assays were performed to quantitate this phenomenon. As expected for



(a)



(b)

Figure 3: Scatter diagrams showing correlations between area and the number of ingested erythrocytes with respect to interaction time. There is a direct and significant relation ($P < 0.001$) for both strains, meaning that the larger is the area of the amoeba, these engulf more erythrocytes. This ratio increases as time passes. The same trend for both strains was observed; however, correlations were higher for *E. histolytica* ("*r*" value).

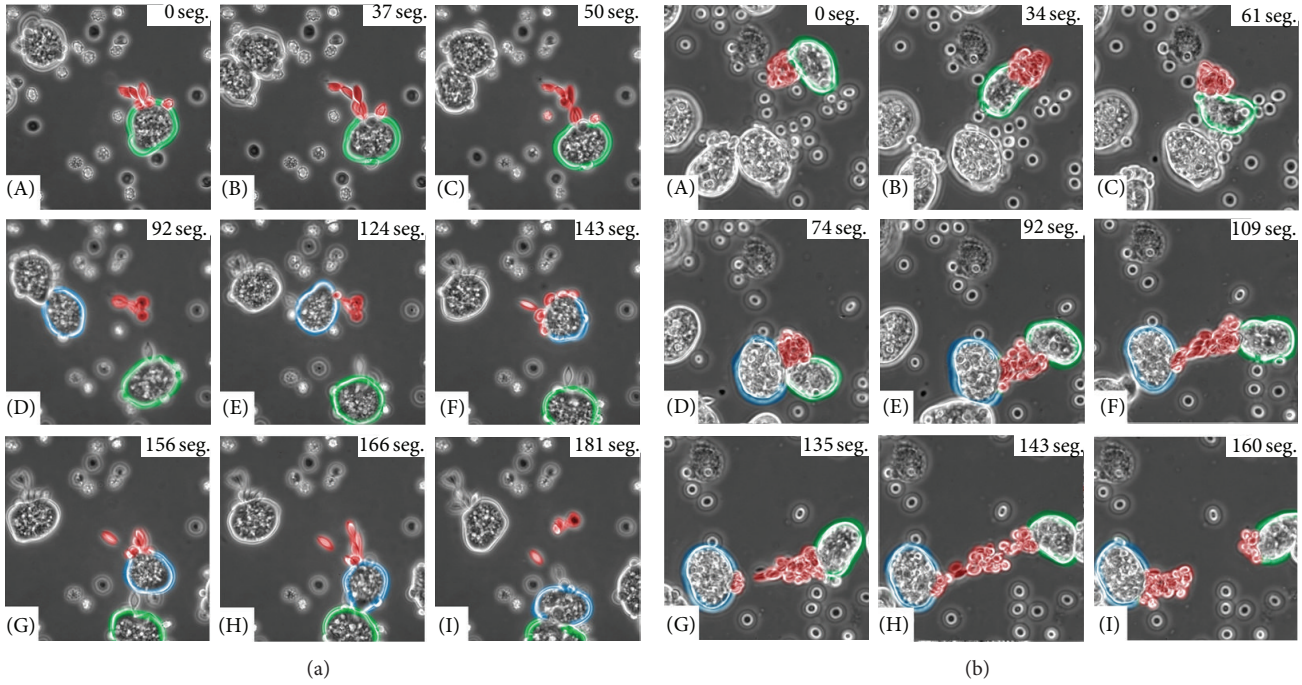


Figure 4: Real time video microscopy showing the opsonization-like event. (a) *E. dispar*: Figure 4(a)(A) shows a trophozoite (green) with a group of bound erythrocytes (red); then it can be seen how these erythrocytes completely detach from this amoeba (Figure 4(a)(B–D)), probably due to a low affinity binding. A new trophozoite (blue) appears in the scene (Figure 4(a)(D)) ready to bind the erythrocytes that had been bound to another amoeba (Figure 4(a)(E and F)). Once again, these erythrocytes will detach from this blue amoeba (Figure 4(a)(G–I)). (b) *E. histolytica*: Figure 4(b)(A) shows a trophozoite (green) which binds erythrocytes (red) sending them to the caudal pole until it encounters another trophozoite (blue) (Figure 4(b)(B–D)); after both amoebas bound to the same group of erythrocytes, it is appreciated how they "fight" to keep the erythrocytes clump (Figure 4(b)(E–H)). Finally, Figure 4(b)(I) shows how every amoeba keeps a portion of the erythrocytes. Numbers shown in the upper right corner of each image correspond to time in seconds.

control assays, only 0.08% from 20,000 events of nonstained erythrocytes, showed some autofluorescence. In contrast, Sytox-stained erythrocytes showed that 99.87% of the cells were positive for Sytox fluorescence; thus Sytox-positive erythrocytes were then used for interaction experiments (data not shown). Figure 6(a) shows the summary of these experiments where there was a significant difference ($P < 0.001$) between species; however, there were no differences in the times measured. This confirms that nonphagocytic population remains constant regardless of the time. It is important to mention that a viability test, with Trypan blue, was performed on trophozoites populations; they always had a 97% or higher viability (data not shown).

The previous results obtained by flow cytometry were confirmed by confocal microscopy. Figure 6(b) shows the two species, at 15 min of interaction, and illustrates how the number of ingested erythrocytes by *E. dispar* is smaller than that in *E. histolytica*; also it shows an *E. dispar* trophozoite without any ingested erythrocytes (Figure 6(b), left side, arrowhead); as expected in the *E. histolytica* field, all trophozoites have ingested fluorescent erythrocytes (Figure 6(b), right side).

3.3. Comparative Analysis of ROS Production during Erythrophagocytosis by E. dispar and E. histolytica. The redox capability of each species by the NBT technique was analyzed by light microscopy. The presence or the absence of blue formazan in yeast *Candida albicans* inside amoebas was determined in 100 randomly chosen amoebas. Results regarding the variable "species" on the ability to produce reactive oxygen species and the variable "time" required to produce them showed a significant difference ($P < 0.001$). With Tukey's test, each variable with respect to all other variables "species" and "time" was compared. Results show that, for each reciprocal combination, the P value was less than 0.001 ($P < 0.001$); however, at 1 and 2 h no significant differences between times for each of the species were found because the maximum value of the respiratory burst occurs during the first hour [23] (Figure 7). Even though there was no significant difference, if we consider the slope, we can see that the number of amoebas that reduce NBT increase with the time much more rapidly in the case of *E. histolytica* in comparison with *E. dispar*.

4. Discussion

Phagocytosis is a central feature in the pathogenesis of invasive amebiasis, still an important public health problem [24]. Considering that not all *Entamoeba* species have the same degree of virulence, understanding of the various pathogenic mechanisms is an overriding goal. The unequivocal evidence

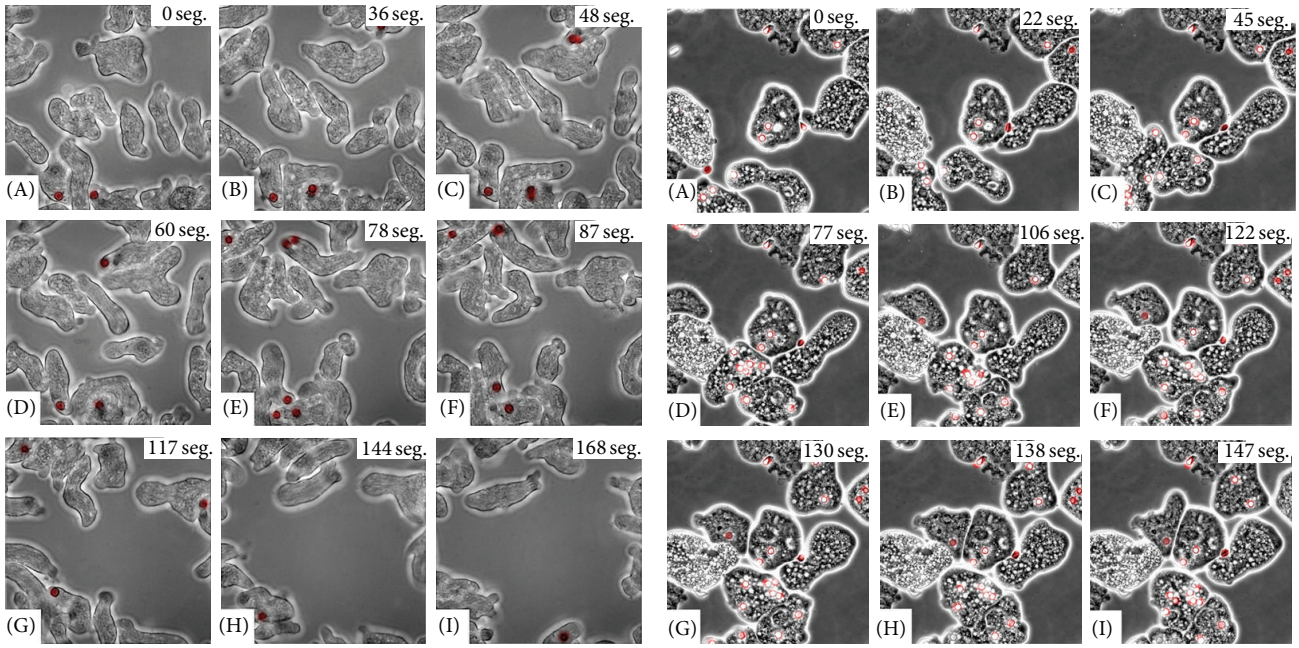


FIGURE 5: Major nonphagocytic subpopulation of *E. dispar* versus a major phagocytic subpopulation of *E. histolytica*. Images taken from a video microscopy followup of the erythrocytes-trophozoites interaction showing the existence of a nonphagocytic subpopulation of *E. dispar* (most of the cells are nonphagocytic). Only a few cells contain erythrocytes (red dots) inside their cytoplasm even after 20 min interaction ((a)(A–I)). On the contrary, images taken from the video microscopy followup of *E. histolytica* trophozoites show that nearly 90% or higher of the population have ingested erythrocytes (red dots), after only 5 min of interaction ((b)(A–I)). Numbers shown in the upper right corner of each image correspond to time in seconds starting after 20 min incubation for *E. dispar* and 5 min incubation for *E. histolytica*.

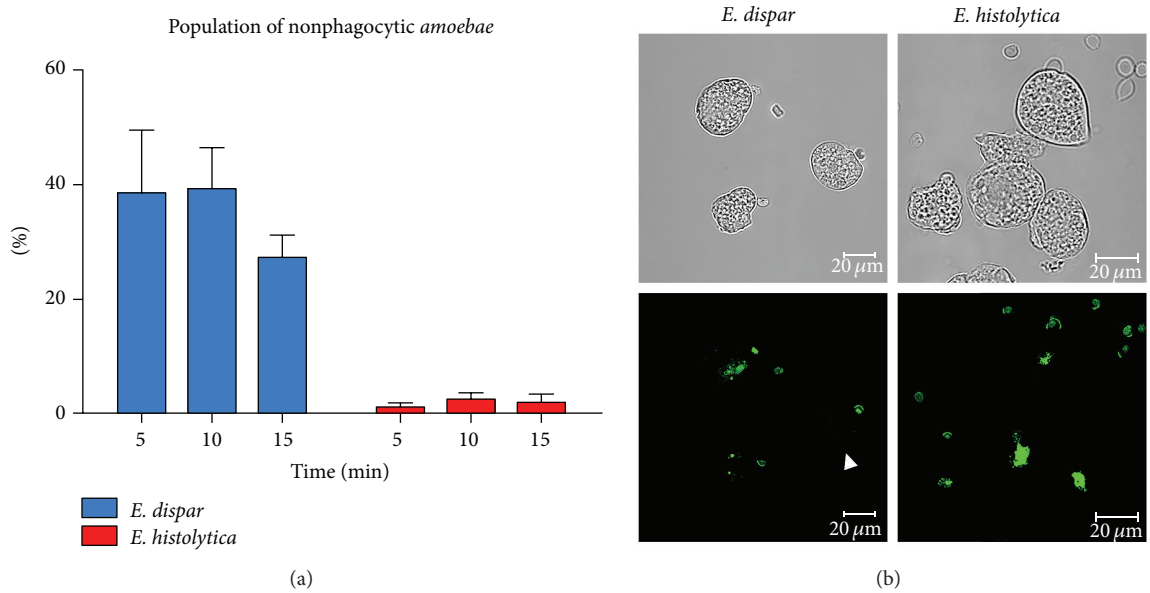


FIGURE 6: Quantitation and confocal analysis of the nonphagocytic and phagocytic subpopulations of *E. dispar* and *E. histolytica*. Graph showing the percentage of nonphagocytic amoebas after different times of interaction for *E. dispar* (blue) and *E. histolytica* (red) (a). Experiments were done in triplicate for each of the analyzed times. (b) shows representative images of phagocytic and nonphagocytic subpopulations for *E. dispar* (left side) and *E. histolytica* (right side). Upper panels, light microscopy; lower panels, confocal images. Arrow head: a nonphagocytic trophozoite.

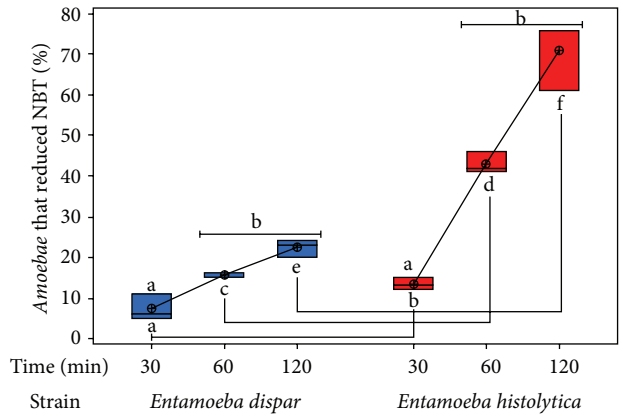


FIGURE 7: Evaluation of the difference in the oxide-reduction ability between *E. dispar* and *E. histolytica* using the NBT reduction assay. The experiment was repeated three times independently in triplicates. Results describing the oxide-reduction ability of both strains, measured by reduction of NBT, indicate that after 60 min incubation, there is not a significant change in the amount of formazan produced (letters on top connected by the same line segment); however, between 30 and 60 min there is a significant difference appreciated in both species (letters on top). Moreover, species compared with their reciprocal times also showed a significant difference ($P < 0.001$) (different letters on bottom connected by the same line segment).

for the existence of *E. dispar*, an amoeba closely related to *E. histolytica*, but not invasive, opens the way to study differences and similarities shared by the two species of amoebas that colonize the human intestine [25–28].

In this work, we have addressed the study of erythrophagocytosis from three different approaches, quantitative, qualitative, and biochemical, allowing presenting a comprehensive panorama of this phenomenon. We therefore were able to demonstrate the existence of a nonphagocytic subpopulation in each species, much larger, however, in *E. dispar* (40% in *E. dispar* versus 5% in *E. histolytica*). Moreover, a new event, similar to the two species, was the opsonization-like process of erythrocytes; this event was also stronger in *E. histolytica* than in *E. dispar*. *E. histolytica* has a larger capacity to induce phosphatidylserine exposure in the erythrocytes surface while *E. dispar* exhibits a deficient adhesion process with red blood cells and also a very poor induction of phosphatidylserine exposure, resulting in a less efficient phagocytosis [20]. The importance of phosphatidylserine exposure for *E. histolytica* engulfment of host cells has been suggested by the work of Bailey et al., [22], who previously demonstrated that liposomes containing phosphatidylserine or synthetic negatively charged phospholipids, dicetyl phosphate, stimulate *E. histolytica* actin polymerization, a necessary event for efficient phagocytosis.

4.1. Erythrophagocytosis. This process is highly asynchronous with many variations that generate high standard deviations; furthermore, when values are analyzed in this way, it is not easy to find statistically significant differences. For this reason, statistical analysis of this study was performed using box and whisker plots; this allowed us to collect information about individual amoebas and not to treat them as a joint population. This gave us the opportunity to identify particular events that usually are not detectable or distinguishable by other techniques, such as the presence of *E. histolytica* amoebas that engulfed 20 or even uncountable erythrocytes; this could be due to the presence of amoebas that ingested erythrocytes more rapidly or to the presence of a subpopulation of larger size as shown by the graphs of scattering values of area versus number of phagocytosed erythrocytes. In the case of *E. dispar*, the average population area ranges from 500 to 1200 μm^2, and very few amoebas fall out of range while the average for *E. histolytica* population falls in the range of 1000 to 2000 μm^2 with many more individual amoebas being bigger than 3000 μm^2.

The spectrophotometric analysis of hemoglobin content, a more sensitive technique used as a complementary approach for erythrophagocytosis evaluation, was useful because samples were treated as total populations and individual variations were overcomed. This might explain why in the graph showing results of hemoglobin content, significant differences among times of interaction were not found within species.

Going back to results obtained by Trissl et al. [9] and comparing their results with ours in terms of the number of ingested cells by the parasites obtained from an asymptomatic carrier, with those obtained in this work, it is clear that their values were similar to those obtained by us with *E. dispar*.

On the subject of the data obtained by flow cytometry, confocal microscopy, and video microscopy, we were able to record a population of nonphagocytic amoebas for each species and their behavior over time, supporting the results obtained by Sateriale et al. [29] who were able to separate amebic subpopulations of *E. histolytica* with higher and lower rates of phagocytosis. When microarrays tests were applied, these authors found that highly phagocytic amoeba showed overexpression of at least 121 genes with respect to nonphagocytic amoeba; therefore, additional studies with *E. dispar* will be necessary to differentiate sub- and overexpressed genes in this species. This confirms the heterogeneity of the amoebic populations that might show different virulence even within the same population. Nowadays, the availability of the genomic sequences of *E. histolytica* and *E. dispar* would allow the analysis of the genetic divergence and differential gene expression between these two species and those genes and molecules associated with the erythrophagocytosis process (adherence, movement, endocytosis, etc.), to identify virulence mechanisms present in the virulent species and absent or not expressed in the nonvirulent parasites [30].

4.2. Respiratory Burst. In mammals, the production of reactive oxygen species during phagocytosis has been widely studied and has been associated with the killing capacity of immune cells to destroy microorganisms [31]. In *E. histolytica* there are only a few reports [32, 33] that describe a NBT reduction activity that implies that oxidoreduction activities are essential virulence components. It is known that *E. histolytica* is a highly phagocytic parasite and with respect

to *E. dispar* we have shown that these amoebas can also phagocytose erythrocytes, however at a lower proportion; consequently we decided to analyze if the phagocytic process in both *Entamoeba* species would trigger an oxidative burst, when feeding amoebas with pathogenic yeasts *Candida albicans*. Results showed that as low as 30% of *E. dispar* trophozoites were able to reduce NBT, in comparison with *E. histolytica* where as much as 80% of the cells reduced NBT. The conversion of NBT to formazan by the oxidative metabolism of *E. histolytica* and *E. dispar* was used as a viability indicator, to measure their survival after challenge with various antiamoebic drugs [34, 35]. However, to date there are no studies that associate the production of oxygen reactive species during phagocytosis by *Entamoeba* species. On the contrary, the ability of trophozoites to neutralize reactive oxygen species has been characterized and proposed as a virulence mechanism or as a mechanism that provides the trophozoite with a major ability to survive to immune cells attack [36]. Therefore, the results here presented are consistent with the fact that *E. dispar*, being considered a noninvasive amoeba, displays a smaller range of ROS production with respect to *E. histolytica*.

Recent studies have suggested that the generation of ROS and the presence of NADPH oxidase are necessary in cancer cells for the formation of specialized structures called invadosomes (mechanosensory adhesive modules that consist of a dense core filamentous actin surrounded by a ring of adhesion molecules able to infiltrate tissue under physiological and pathological conditions) that allow for a more efficient tissue invasion process [37–40]. In this regard, the possible existence of invadosome-like structures in *E. histolytica* and its lack in *E. dispar* is worth pursuing.

5. Conclusions

We performed a comparative study of erythrophagocytosis between *E. dispar*, a noninvasive amoeba, and *E. histolytica*, a highly virulent and invasive parasite. Results demonstrate that the phenomenon is present in both *Entamoeba* species and that there are significant differences between the two amoebas. Both, phagocytosis and the ability to produce reactive oxygen species, are clearly more pronounced in *E. histolytica* in comparison to *E. dispar*.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Evaluation of Antibody Response to Various Developmental Stage Specific Somatic Antigens of *Paramphistomum epiclitum* in Goats

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Electrophoretic analysis of various developmental stage specific somatic antigens of *Paramphistomum epiclitum* (Digenea: Paramphistomidae), namely, metacercariae (McAg), immature intestinal flukes (ImIAg), immature ruminal flukes (ImRAg), and adult flukes (AAg), was done by native polyacrylamide gel electrophoresis. Result revealed presence of 3 (range 15.2–40.3 kDa), 13 (9.3–121.2 kDa), 14 (9.3–169.3 kDa), and 15 (8.0–169.3 kDa) polypeptides in McAg, ImIAg, ImRAg, and AAg, respectively. With an aim to identify a suitable immunodiagnostic antigen for early diagnosis of amphistomosis, the IgG antibody response to various developmental stage antigens in goats experimentally infected with metacercariae of *P. epiclitum* was evaluated by ELISA. The highest OD values were recorded with ImIAg which ranged between 0.23 and 0.55 with a significant increase from the 2nd week till 8th week of infection with a peak at 6th week. The analysis of statistical significance using a one-way analysis of variance with multiple pair wise comparisons revealed that IgG response was significantly higher with all antigens ($P < 0.01$) except McAg ($P > 0.05$) with a maximum mean difference of 0.1838 in comparison to control with ImIAg, thus, indicating that ImIAg which could be further exploited for its potential is a candidate for immunodiagnostic antigen for early diagnosis of amphistomosis.

1. Introduction

Paramphistomum epiclitum (Digenea: Paramphistomidae) is a gastric trematode affecting ruminants with a wide range of geographic distribution and is prevalent in several states of India [1–4]. The life cycle is indirect and sexually mature fluke in the rumen releases the eggs along with faeces which hatch in water into ciliated miracidia. The miracidia then enters the body of an intermediate host (snail), in which it develops to produce cercariae and encyst to become metacercariae on aquatic plants or other suitable substrata. Upon ingestion of viable metacercariae once inside the duodenum and jejunum, their cysts are removed, they penetrate the intestinal wall by actively destroying the mucosa and then migrate to the rumen, where they grow into adult [5]. The adult stages generally have low pathogenicity while the migrating immature ones cause severe pathology and even kill the host in heavy infections as they are attached to the wall of the small intestine causing hemorrhagic inflammation, characterized by focal infiltration of macrophages and lymphocytes in the lamina propria [6]. The injury caused in ruminants severely affects production, since these parasites cause a lower feed conversion, a loss of weight, and/or a decrease in milk production, responsible for severe economic losses [7].

The diagnosis of most of the helminth diseases is done through the demonstration of its eggs in the faeces of the infected animal. However, in case of amphistomosis as the infected animals exhibit clinical symptoms much before passing of eggs in the faeces (disease syndrome is caused by immature stages and has a relatively long prepatent period), coproscopic analyses often results in misdiagnosis and could not be used in early diagnosis [8]. Thus, immunological diagnosis can prove to be an important tool for early diagnosis of amphistomosis which is essential for prompt treatment

before irreparable damage to the rumen and small intestine occurs [9]. Research on several parasitic trematode infections has shown that the immune system is stimulated to produce specific antibodies against the parasites and its detection by means of indirect enzyme-linked immunoassays can prove to be an effective tool for early disease diagnosis [10–12].

Regarding immunodiagnosis of amphistomosis limited work has been done utilizing either adult worm somatic antigen [13–17], excretory/secretory antigen [11, 18] or coproantigen [12] as antigen. It is also well known that helminth parasites during the course of development undergo antigenic polymorphism which induces drastic alterations in immune response, so use of these different developmental stage antigens is very important in immunodiagnosis. Further, as life cycle of *P. epiclitum* involves various developmental stages, therefore, the antigens derived from these stages may exhibit different immune response in the host. Hence, study of immune response against the various developmental stage antigens would be helpful in identification of sensitive immunodiagnostic antigen for early diagnosis of prepatent amphistomosis. In the present study, antigens derived from different developmental stages of the parasite, namely, metacercariae, immature intestinal, immature ruminal, and adult ruminal flukes, have been used to evaluate the IgG response in goats experimentally infected with *P. epiclitum*.

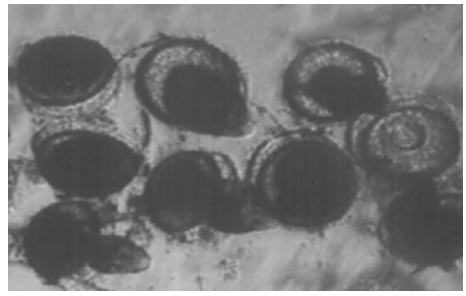

Figure 1: *In vitro* excystment of metacercariae of *P. epiclitum*.

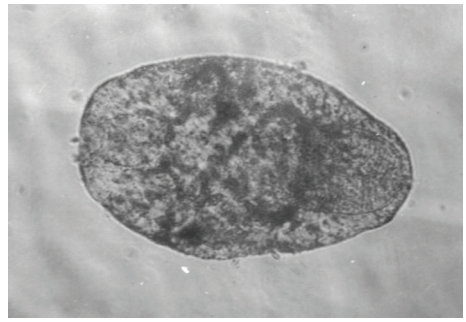

Figure 2: Juvenile fluke of *P. epiclitum*.

2. Materials and Methods

2.1. Collection of Metacercariae. *Indoplanorbis exustus* snails were collected from ponds of villages nearby Indian Veterinary Research Institute, Izatnagar, India, during the monsoon and post-monsoon seasons, maintained in the laboratory in glass troughs and fed fresh spinach leaves. Snails were screened individually for *P. epiclitum* infection by exposure to artificial light (40-watt candescent bulb) which caused emergence of cercariae within an hour. Infected snails were sorted out and cercariae emerging out from them encysted as metacercariae on yellow polythene sheets [19]. The metacercariae were stored in triple distilled water at room temperature (25–28°C) till further use for antigen preparation and setting up of experimental infection in goats. Before use, the viability of metacercariae was determined on the basis of motility of juveniles within the cyst as observed under stereoscopic microscope and *in vitro* excystment of viable metacercariae as per the method described by Jyoti et al. [20] (Figure 1). Briefly, 100 metacercariae were taken in a small petridish and 5 mL N/20 HCl and equal volume of solution containing 0.8% NaCl and 1% NaOH was added and incubated at 44°C for 10 min. Then, L-cysteine HCl @ 4 mg/mL was added and kept at room temperature for 30 min. Later, 10 mL of 2% solution of bile salts was added and incubated at 44°C for 10–12 h. The freshly excysted juvenile flukes were maintained in Ringer's Locke solution at room temperature (Figure 2).

2.2. Collection of Parasite. Adult and immature stages of *P. epiclitum* were collected from rumen and small intestine from the gastrointestinal tracts of goats obtained from local abattoir. The parasites were collected in 0.01 M phosphate buffered saline, pH 7.2 (PBS), thoroughly washed with PBS and stored at −20°C for antigen preparation. The identification of the parasite was confirmed after preparing permanent slides using standard keys [21] (Figures 3 and 4). Briefly, the flukes were placed between two glass slides, flattened, and tied with a piece of thread and then placed in 70% alcohol for 24 hours. The flukes were dislodged from the slides and subjected to overnight Borax Carmine staining, followed by destaining in 2% acid alcohol, graded dehydration, clearing in clove oil, and mounting in DPX (distyrene plasticizer and xylene).

2.3. Preparation of Lifecycle Stage Specific Somatic Antigens of P. epiclitum. Somatic antigens derived from various developmental stages, namely, metacercariae (McAg), immature intestinal flukes (ImIAg), immature ruminal flukes (ImRAg), and adult flukes (AAg), were prepared as previously described by Bennett et al. [22] and Guobadia and Fagbemi [23] with slight modifications.

Briefly, for preparation of McAg approximately 10,000 metacercariae were washed with 0.01 M PBS, homogenized, sonicated for six cycles at 16 μ peak for 10 sec with 30 sec interval at 4°C, and centrifuged at 11,750 ×g for 30 min at 4°C and supernatant was collected. The supernatant was filtered using 0.22 μm syringe filter (Millipore, Billerica, MA, USA); a cocktail of protease inhibitors (Sigma-Aldrich, St. Louis, IL, USA) was added and stored at −20°C.

Similarly, immature *P. epiclitum* flukes obtained from the small intestine and rumen of goats were utilized for preparation as ImIAg and ImRAg, respectively. Briefly, the immature flukes were homogenized in 0.01 M PBS in a Teflon coated

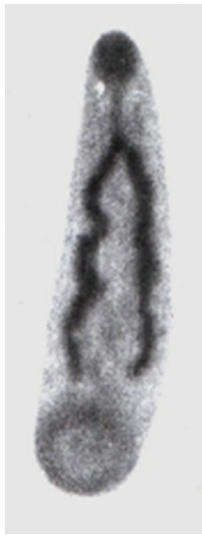

Figure 3: Immature *P. epiclitum*.

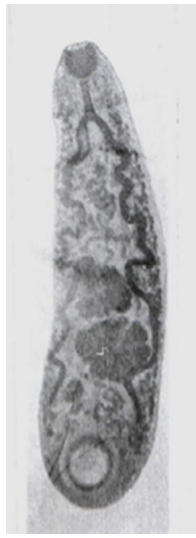

Figure 4: Mature *P. epiclitum*.

homogenizer at 4°C, sonicated for five cycles at 8 μ peak for 2 min with 1 min interval at 4°C, and centrifuged at 11,750 ×g for 1 hr at 4°C. Supernatant was collected and pooled, filtered using 0.22 μm syringe filter (Millipore, Billerica, MA, USA); a cocktail of protease inhibitors (Sigma-Aldrich, St. Louis, IL, USA) was added and stored at −20°C. Further, adult flukes collected from the rumen of goats were utilized for the preparation of AAg as per the method described above.

2.4. Protein Estimation. Protein concentrations of all the antigens were estimated as per the method of Lowry et al. [24], using bovine albumin fraction V as standard.

2.5. Polyacrylamide Gel Electrophoresis (PAGE). Native PAGE of various lifecycle stage specific somatic antigens was performed in 5–15% gradient resolving and 5% stacking polyacrylamide gels using a discontinuous system as described by Laemmli [25]. For determination of molecular weight a prestained protein marker (14.3–97.4 kDa) (Bangalore Genei, Bengaluru, India) was also subjected to electrophoresis in a vertical electrophoresis system (Bangalore Genei, Bengaluru, India) at 120 V constant voltage. The gels were subjected to Coomassie brilliant blue (CBB) stain for 4 h, followed by destaining and scanned by gel documentation system (Syngene, Frederick, MD, USA).

2.6. Experimental Infection of Goats. Six healthy male goats of about six months of age were procured from the Sheep and Goat Farm, LPM Division, IVRI, Izatnagar. Faecal sample of all animals were examined using sedimentation and floatation techniques to confirm their *P. epiclitum* naive status. Four goats were given a dose of 3,500 viable metacercariae of *P. epiclitum* orally after 12 h of fasting and two were maintained as control. The animal experimentations were conducted in compliance with the ethical considerations and guidelines issued by CPCSEA/Institutional Animal Ethics Committee (IAEC) on laboratory animals.

2.7. Collection of Sera Samples. Blood samples were collected from the jugular vein of all animals from day zero to eight weeks postinfection at weekly interval. The sera were separated, aliquoted in 1.5 mL, and stored at −20°C after adding thiomersal (10 mg/mL) @ 5 μL/mL.

2.8. Enzyme Linked Immunosorbant Assay (ELISA). For the analysis of the humoral IgG response, ELISA was carried out with all four somatic antigens following the method of Njau and Nyindo [26] with slight modifications. Flat-bottomed polystyrene Greiner 96-well plates (Sigma-Aldrich, St. Louis, IL, USA) were coated with 100 μL of antigen per well in carbonate bicarbonate coating buffer (pH 9.6) (protein concentration 10 μg per mL) and incubated at 4°C overnight. Then the plates were washed thrice with PBS containing 0.1% Tween-20 (washing buffer), blocked with 150 μL of 5% skimmed milk in PBS, and incubated at room temperature for 1 h. The washing step was repeated again before adding 100 μL of 1 : 250 dilutions of the primary sera collected from experimentally infected and control animals in PBS (in quadruplet wells) and kept at 37°C for 2 h. Following washing, again 100 μL of 1 : 5000 diluted rabbit anti-goat IgG conjugated with horseradish peroxidase (Bangalore Genei, Bengaluru, India) in PBS was added and incubated for 2 h at 37°C. After washing, the reaction was developed by adding 100 μL of substrate solution containing 40 mg O-phenyldiamine (Sigma-Aldrich, St. Louis, IL, USA) in 100 mL phosphate citrate buffer (pH 5.0) and 40 μL hydrogen peroxide and incubated in dark at room temperature for 30 min. The colour reaction was stopped by the addition of 3 N sulphuric acid and optical density values were read at 492 nm using Titertek multiskan plate reader (Labsystems, Finland). The control wells for primary antibody, secondary antibody, substrate, and antigen were maintained in the plate. The analysis of statistical significance using a one-way analysis of variance (ANOVA) with group multiple comparisons was done using the Tukey's test (GraphPad Prism 4 software).

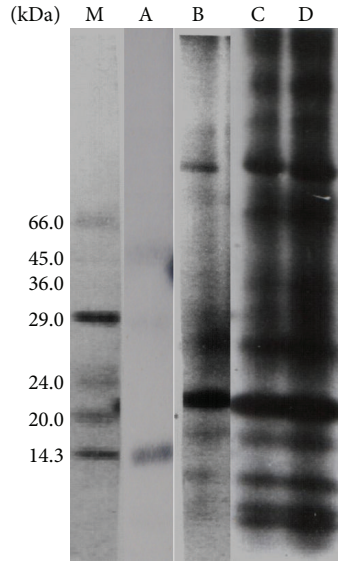

Figure 5: Native-PAGE profile of various stage specific antigens of *P. epiclitum*. Lane M: molecular weight marker; Lane A: metacercarial antigen (McAg); Lane B: immature intestinal fluke antigen (ImIAg); Lane C: immature ruminal fluke antigen (ImRAg); Lane D: adult fluke antigen (AAg).

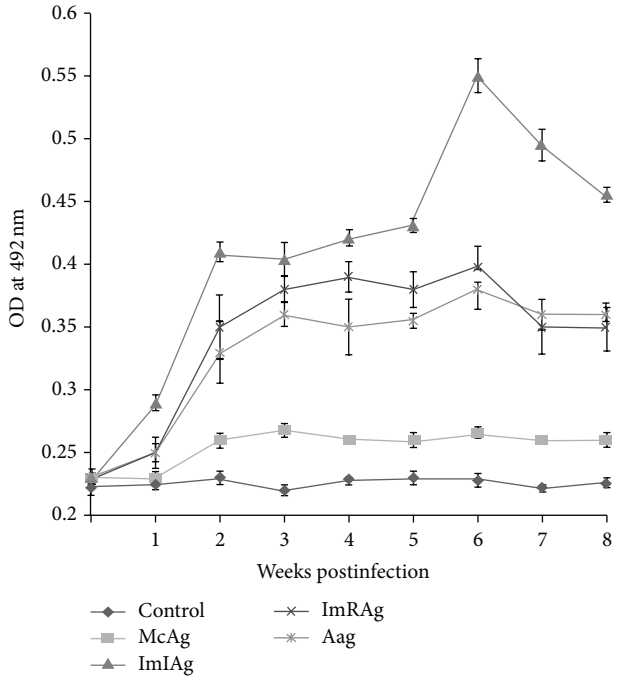

Figure 6: Antibody response by ELISA using various stage specific antigens of *P. epiclitum*.

3. Results

3.1. In Vitro Excystment of Metacercariae. The percent of excystment in metarcercarie stored for 2, 10, 20, and 60 days was 95, 87, 85, and 40%, respectively (Figure 1). The freshly excysted juvenile flukes were maintained in Ringer's Locke solution at room temperature for 5-6 days (Figure 2).

Table 1: Comparison of IgG response against different stage specific antigens of *Paramphistomum epiclitum* in ELISA by Tukey's test (versus noninfected control).

Antigen	*P* value	Mean difference	95% confidence limit
McAg	>0.05	0.02911	−0.04758 to 0.1058
ImIAg	<0.001	0.1838	0.1071 to 0.2605
ImRAg	<0.001	0.1164	0.03975 to 0.1931
AAg	<0.01	0.1026	0.02586 to 0.1792

3.2. Protein Concentration of Antigens. Protein concentrations of various stage specific somatic antigens, that is, McAg, ImIAg, ImRAg, and AAg, were 1.2, 4.6, 16.0, and 13.5 mg/mL, respectively.

3.3. Native PAGE. Electrophoretic analysis of various somatic antigens revealed 3 polypeptides of 40.3, 31.5, and 15.2 kDa in McAg. Further, ImIAg, ImRAg, and AAg showed 13, 14, and 15 polypeptides in range of 9.3–121.2, 9.3–169.3, and 8.0–169.3 kDa, respectively, by native PAGE. The 169.3 kDa polypeptide was found lacking in ImIAg but was present in the other two somatic antigens whereas polypeptide of 8.0 kDa was recorded only in AAg. Among all the antigens 13 polypeptides of mol. wt. 121.2, 103.2, 79.1, 65.0, 43.0, 36.9, 32.7, 26.2, 18.9, 15.4, 11.8, 10.0, and 9.3 kDa were found common (Figure 5).

3.4. ELISA with Various Stage Specific Antigens. The humoral IgG response generated in goats experimentally infected with metacercariae of *P. epiclitum* was monitored through ELISA by using all four stage specific somatic antigens (McAg, ImIAg, ImRAg, and AAg) up to 8 weeks post infection (wpi). A marked variation in antibody response was recorded with various antigens. A negligible antibody response was recorded when McAg was used for ELISA as the OD values in the experimentally infected goat sera were almost similar to noninfected control up to 8 wpi. The OD values recorded with ImIAg antigen in experimental sera samples ranged between 0.23 and 0.55 which was more than double as compared to control. There was a marked increase in the antibody response of infected group from the 2nd week and was maintained till 8th week of infection with a peak at 6th wpi. Similarly, when ImRAg was employed as antigen the OD values ranged between 0.23 and 0.40 with experimental sera and the trend of response was similar to ImIAg. For AAg the OD value ranged between 0.23 and 0.38 with highest values at 6 wpi (Figure 6). Hence, all the antigens except McAg detected a marked IgG response in the experimental sera as early as 2nd week post infection. In ANOVA with multiple pair wise comparisons the IgG response was significantly higher with all antigens ($P < 0.01$) except McAg ($P > 0.05$) with a maximum mean difference of 0.1838 in comparison to control with ImIAg (Table 1).

4. Discussion

Amphistomosis is a neglected ruminant disease causing high morbidity and mortality in tropical and subtropical countries resulting in great economic losses. Prevalence of various species of amphistome parasites among the livestock has been reported from a number of states of India [1–4]. The rumen amphistomes particularly *Gastrothylax crumenifer* and *Paramphistomum epiclitum* have marked seasonality in egg production [27] which makes the task of conventional diagnosis more difficult during routine parasitological investigation involving detection of eggs in faecal samples. Although pioneer work on the life cycle of various amphistomes has been worked out in this country, immunodiagnosis was neglected.

The electrophoretic profile of various stage specific somatic antigens of *P. epiclitum* was studied which showed a difference in the polypeptide profile of various antigens in terms of their differential electrophoretic mobility on native PAGE gel. Although the current study seems to be first of its kind reporting the polypeptide profile of various antigens of *P. epiclitum* of goats but in similar studies SDS-PAGE analysis of crude somatic adult antigen of *P. epiclitum* showed 12 polypeptides in the range of 19.9–85.1 kDa [28] and 14 polypeptides in the range of 14.1–95.5 kDa [15]. Further, the presence of 13 common polypeptides among the various stage specific antigens supports the view that some major polypeptides are conserved during evolution, whereas the variation in the polypeptide profile may be due to genomic heterogeneity [29].

The probability of diagnosis of animals infected with amphistomes is more by detecting the circulating antibodies in the sera samples of infected animals as compared to the functional/structural antigen of the parasite by ELISA [13]. It is interesting to note that studies on immunodiagnosis of amphistomosis have been mostly based on either adult worm somatic antigen [13–17], excretory/secretory antigen [11, 18], or coproantigen [12]. A number of investigators have tried to develop specific serodiagnostic tests for early detection of a related trematode species, *Fasciola*, in animals [10, 30–32], whereas in the current study the antigens were derived from different developmental stages of the parasite, namely, metacercariae, immature intestinal flukes, immature ruminal flukes, and adult ruminal flukes, to evaluate the IgG response in goats experimentally infected with *P. epiclitum*. The polypeptide profile changes recorded may be probably due to the structural changes occurring during development of parasites which could affect the immune response in host, which has also been suggested in case of nematodes [33]. Results show that antigen prepared from immature stages collected from small intestine when employed for ELISA was found to be more immunogenic as evident by the significant increase ($P < 0.001$) in the IgG response and maximum mean difference of IgG in comparison to control group and thus could be a better candidate for early diagnosis of disease. Further, ImRAg and AAg also showed promising results and detected significant increase in IgG levels which could be further exploited for immunodiagnosis, whereas McAg was found to be totally unsuitable for the purpose. Similarly for immunodiagnosis of amphistomosis, adult fluke antigen has been used to detect antibodies at 8 days postinfection (PI) against *P. cervi* [34] and 15 days PI [35] in sheep and goat. Boch et al. [36] detected antibodies against *P. cervi* in calf 8–24 days PI with peak titre of 1 : 512 at 24–50 days PI which persisted up to the stage of egg excretion. Diaz et al. [11] analyzed IgG antibody response against *Calicophoron daubneyi* in cattle utilizing ELISA and found a notable IgG response in naturally infected cattle.

Results of the present study show that developmental stage antigens are very important in the immunodiagnosis of immature amphistomosis in the early stage. Further, identification of immunodominant polypeptides of the most immunogenic (ImIAg), their purification, and large scale validation by use in immunoassays like ELISA need to be carried out with sera collected from field, slaughter house, and experimentally infected animals to work out the sensitivity and specificity. Thus antigens derived from immature stages can lay the possible foundation for development of a serological kit for early diagnosis of amphistomosis in near future.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Exacerbated Skeletal Muscle Inflammation and Calcification in the Acute Phase of Infection by Mexican *Trypanosoma cruzi* DTUI Strain

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A murine model was used to study the histopathological aspects and cytokine expression levels in skeletal muscle provoked by the infection with Mexican TcI strains. BALB/c mice were inoculated with the virulent Querétaro strain and the nonvirulent Ninoa strain. Parasite numbers were counted in blood and skeletal muscle at different times post-infection, and real time-PCR expression levels of the cytokines IL-12, IL-4, IL-10, IFN-γ, and TNF-α were evaluated. In the acute phase of infection, a high parasitic load, both in blood and skeletal muscle, was detected. The histopathological analyses showed an exacerbated inflammation and granulomatous-like infiltrate with the Querétaro strain. Interestingly, extensive calcification areas were observed in the skeletal muscle surrounded by inflammatory infiltrates. TNF-α and IL-10 expression exhibited a significant increase at the peak of infection. In summary, Querétaro strain, a Mexican TcI strain, is virulent enough to induce high inflammation and calcification in skeletal muscle of the hind limbs, which could be related to high expression levels of TNF-α.

1. Introduction

Chagas disease, an important health problem in Latin America, is the manifestation of tissue damage resulting from infection with the hemoflagellate protozoan parasite, *Trypanosoma cruzi*. This parasite constitutes a very heterogenic taxon, allowing the clustering of *T. cruzi* strains into six discrete typing units (DTUs): TcI to TcVI [1]. The DTU most abundant and dispersed in the Americas is TcI, which can be associated with domestic and sylvatic cycles and, in human infection, with cardiomyopathy [2]. Several studies have shown that *T. cruzi* genetic heterogeneity is related to the progression and severity of Chagas disease. In South America, strains belonging to *T. cruzi* II group (currently known as TcII to VI DTUs) have been mainly associated with the domestic/peridomestic cycle and hence with the development of this illness [3]. Although DTUI (TcI) dominate the sylvatic cycle of the Amazonia area, some countries from the northern region of South America, such as Venezuela and Colombia, have demonstrated the predominance of TcI strains in human [4–6]. In Mexico, it has been reported that 98% of the isolated strains belong to TcI and are closely related to each other [7, 8]; the presence of chronic symptomatic evolutions has also been shown [9–11], with 5000 people affected with severe chronic chagasic cardiomyopathy [12], indicating that TcI is dominant in Mexico even in human infections.

T. cruzi can infect many different cell types, but most strains show a preference for growth in muscle tissue forms. Here, the acute infection elicits a variety of immune effectors mechanisms characterized by the participation of major immune cells such as macrophages and T-cells, as well as the production of several proinflammatory (IL-12, TNF-α, and IFN-γ) and regulatory (IL-4, IL-10) cytokines [13–15]. However, these responses not only permit the control of the parasite, but also triggered a strong inflammatory reaction that can result in severe tissue damage. Studies in experimental models of Chagas disease have shown myofibrosis and

myositis as well as degeneration and necrosis of myofibres [16, 17], although the extent and severity of the lesions can vary depending on the animal strain [18] or the *T. cruzi* strain [19].

Severe inflammation has been considered as an important feature of tissue calcification in some clinical conditions. Two mechanisms of calcification are recognized: metastatic calcification (elevated levels of calcium and/or phosphate in serum that produce systemic mineralization) and dystrophic calcification (associated with injury, infection, or rheumatic diseases with normal calcium/phosphate homeostasis) [20, 21]. Dystrophic calcification is also related to cellular death, leading to catabolic enzymes and calcium release. In cases of aortic calcification, tissue mineralization has been observed near to inflammatory infiltrates, and degradation products from apoptosis can promote it [22]. Some studies have shown association of the cytokine tumor necrosis factor alpha (TNF-α) with vascular calcification [23, 24]. This cytokine is present in both acute and chronic inflammation and, in chronic cases, can induce the activation of apoptotic pathways [22].

The different biological and genetic properties of this intracellular parasite contribute to a wide diversity in infectivity, virulence, pathology, and tissue parasitism in a broad range of mammalian hosts [25]. There are a vast number of research papers related to the pathogenesis and histology of *T. cruzi* infections, but most of them used *T. cruzi* II parasites or are conducted in South American countries. Thus, the genetic and biological characterization of circulating TcI strains in Mexico is necessary. In our search for characterization of Mexican strains, we have found biological differences among them, in spite of their genetic closeness [8, 9], and more recently we described the immunological response of mice to virulent and nonvirulent Mexican strains [26, 27].

In the present work, we continue this search by studying the skeletal muscle from mice infected with two Mexican TcI strains, named Ninoa and Querétaro, with regard to parasitism and inflammatory response, and cytokines expression. Our data showed differences in parasitemia and tissular parasitism between these strains. Remarkably, infection with the Querétaro strain triggered an exacerbated inflammatory reaction, which led to skeletal muscle injury and important calcification of the tissue particularly during the acute phase. Besides, the infection with this strain induces higher expression levels of the cytokines TNF-α and IL-10, compared to Ninoa infection, that could be related to the tissue damage observed. *In vitro* studies also demonstrated a higher release of TNF-α by macrophages infected with the Querétaro strain. To our knowledge, this is the first report showing calcification phenomenon in mice skeletal muscle of the hind extremities due to *Trypanosoma cruzi* TcI infection.

2. Materials and Methods

2.1. Parasites. The Mexican *T. cruzi* strains, Querétaro and Ninoa, both belonging to DTU TcI [7, 8] were used for this study. Ninoa (MHOM/MX/1994/Ninoa) strain was isolated from an acute human Chagas disease patient in Oaxaca, Mexico [28]. Querétaro (TBAR/MX/0000/Querétaro) strain was isolated from an insect vector, *Triatoma barberi*, at Querétaro, Mexico. The strains were maintained by serial passage in BALB/c mice; bloodstream trypomastigotes were obtained by cardiac puncture of these animals (collected in heparinized vials) and used for inoculations of the experimental mice.

2.2. Mice and Experimental Groups. Seven- to eight-week-old female BALB/c mice weighing 18–20 g were obtained from our animal facilities (Animal House of the Instituto de Investigaciones Biomédicas, from the Universidad Nacional Autónoma de México, IIB-UNAM). This study was approved by the ethics committee for animal experimentation of the IIB-UNAM. All procedures and experimental protocols were performed according to Biosafety committee of the IIB-UNAM for the use of animals in experimental conditions. Two groups of 12 animals each were intraperitoneally infected with 10^4 blood trypomastigotes in 200 μL of sterile PBS (Querétaro group and Ninoa group); the control group (12 animals) was inoculated with the same volume of PBS. Parasitemia levels were determined by counting the number of parasites present in a 5 μL blood sample collected from the tail vein every third day. Three animals from each experimental group were sacrificed at 1, 15, 21 (acute phase), and 90 days (chronic phase) after inoculation (pi) and the skeletal muscle from the hind leg were obtained, as previous studies have shown it as a target tissue of this parasite. Two independent experiments were carried over (a total of 24 animals per group).

2.3. Histopathological Analysis. Half of the skeletal muscle obtained from each animal, either infected or control mice, were fixed in 4% paraformaldehyde and paraffin-embedded for histopathological analysis. Tissue sections (5 μm thick) were deparaffinized and stained with hematoxylin-eosin (H-E). Five nonconsecutive slides (sections separated by 50 μm) per animal were examined under a light microscope (Optiphot-2, Nikon), and pictures were taken using a Coolpix 4300 camera (Nikon). The proportion of tissue area presenting inflammatory cells was estimated over the total sections of the tissues analyzed, using the inflammation scores described by Sun and Tarleton for skeletal muscle: 0 = normal, 1 = scarce cellular infiltrate, 2 = diffuse infiltrate, 3 = abundant infiltrate, and 4 = granulomatous-like infiltrate [29].

Additionally, sections were treated with the von Kossa stain in order to verify the presence of calcium deposits. Briefly, deparaffinized slides were treated with 1% aqueous silver nitrate solution under UV light for 30 minutes, washed with distilled water, and then treated with a solution of 5% sodium thiosulphate for 5 minutes (to remove the unreacted silver). The slides were counterstained with nuclear fast red for 5 minutes.

2.4. Immunohistochemistry. Sections from the paraffin-embedded tissues were deparaffinized and rehydrated through several ethanol baths (absolute, 96%, 70%, and 50%) and water in order to perform immunohistochemical studies to evaluate tissue parasitism and detect macrophages in the inflammatory infiltrates. Briefly, the hydrated tissues

were blocked with 2% bovine serum albumin in phosphate-buffered saline (BSA/PBS) for 2 h at room temperature in a moist chamber in order to block unspecific unions. Slides were then incubated overnight at 4°C with a biotinylated F4/80 antibody, a macrophage-specific antibody (1 : 50; Caltag Laboratories, CA, USA) and a polyclonal rabbit anti-*T. cruzi* serum (1 : 1000; serum was previously obtained in our laboratory using a total extract from the Querétaro strain). Slides were then washed and incubated for 60 min with a mix of streptavidin-phycoerythrin (SA-PE, 1 : 500; Caltag Laboratories) for the detection of macrophages and a fluorescein-labelled anti-rabbit IgG (anti-IgG-FITC, 1 : 100; Sigma Immuno Chemicals, MO, USA) for the detection of parasites. After washing 3 times with PBS, the slides were counterstained with DAPI 1 : 1000 (Molecular Probes, Oregon, USA) and mounted. Preimmune serum and IgG2b antibody (Caltag Laboratories), the same isotype of the F4/80 antibody, were used as negative controls. The stained slides were visualized using a Zeiss fluorescence microscope. By this technique, the number of parasite nests (tissue parasitism) was counted using the 40x objective in 60 fields per mouse in skeletal muscle. In the same slides, the number of fields with presence of macrophages was counted as well as the number of infiltrated nests.

2.5. Total RNA Extraction and cDNA Synthesis. The other half of the tissues obtained was used to extract total RNA. Isolated skeletal muscles were frozen immediately in dry ice and stored at −70°C until use. RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following the recommendations of the manufacturer. Integrity of RNA was verified by agarose gel electrophoresis.

Target RNA (2 μg) was treated with 2 U of recombinant DNase I (Kit DNA-*free*, Ambion, Inc., USA) in a final volume of 20 μL, as recommended. Five hundred ng of DNase treated-RNA were reverse transcribed using 1.25 U/μL MultiScribe RT, 0.4 U/μL RNase Inhibitor, 2.5 μM oligo $d(T)_{16}$, 0.5 mM dNTPs, 2.75 mM $MgCl_2$, and 1x Taqman RT buffer, in a total volume of 20 μL (TaqMan Reverse Transcription Reagents kit; Applied Biosystems, CA, USA). The reaction proceeded for 10 min at 25°C, followed by 30 min at 48°C, and finally 5 min at 95°C in a PTC-100 Programmable Thermal Controller (MJ Research Inc.). The single-strand cDNA synthesized was stored at −20°C until its use.

2.6. Real-Time PCR Amplification. PCR reactions were performed in the ABI Prism 7000 SDS (Applied Biosystems) using the SYBR Green PCR kit. Each amplification reaction was performed in a final volume of 20 μL, containing 1x SYBR Green PCR Master Mix, 300 nM of each primer (reverse and forward), and 50 ng of single-strand cDNA sample. PCR conditions were as follows: 1 cycle at 95°C for 10 min and 40 cycles at 95°C for 15 sec, 60°C for 1 min. A no-RT control (DNase-RNA treated without being reverse transcribed) for each sample and a negative control (without DNA sample) for each primer pairs were included in each PCR reaction set.

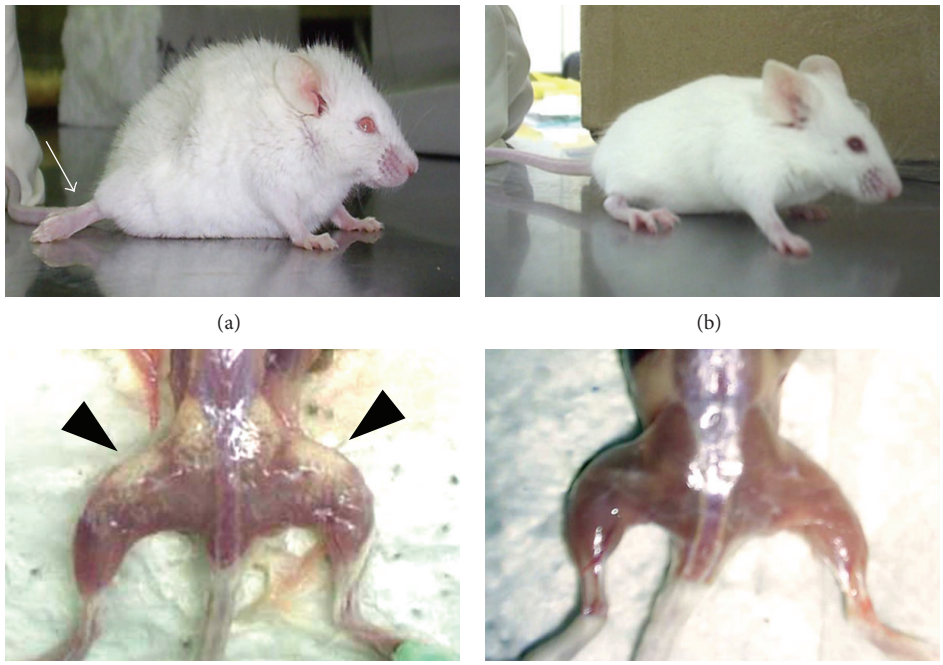
The target genes analyzed included IL-4 (FWD: GCAGAGACTCTTTCGGGCTTT; REV: TCATTCATGGTGCAGCTTATCG), IL-10 (FWD: GGAAGACAATAACTGCACCCACTT; REV: CCGCAGCTCTAGGAGCATGT), IL-12 (FWD: CGTGCTCATGGCTGGTGCAAAG; REV: CACATGTCACTGCCCGAGAGT), IFN-γ (FWD: AATGAACGCTACACACTGCAT; REV: TGGCAGTAACAGCCAGAAACA) and TNF-α (FWD: GGGCAGGTCTACTTTAGAGTCATTG; REV: GGCTGGGTAGAGAATGGATGAA), and the constitutive gene HPRT (hypoxanthine phosphoribosyl transferase; FWD: GAAAGACTTGCTCGAGATGTCA; REV: AGCACACAGAGGGCCACAA) was used to normalized the data. The primers were designed using the Primer Express Applications-based primer design software (Applied Biosystems, CA, USA). Also, the capability of HPRT gene as an internal reference gene for our model was verified (its expression is not affected by *T. cruzi* infection).

In order to obtain the amplification efficiencies for each gene, standard curves were elaborated by using 21 days pi Querétaro-infected-mouse skeletal muscle cDNA serial dilutions (range 1–100 ng). Each dilution was amplified with every primer pair. Ct values were plotted against the logarithm of initial cDNA concentration. Amplification efficiency of each gene was calculated from the linear equations obtained, with the formula: $E = 10^{-1/\text{slope}}$ [30].

Real-time PCR results were analyzed using the Q-Gene software, of free access at http://www.biotechniques.com/ [31]. This program takes into account the amplification efficiencies of the genes to calculate the normalized gene expression of the target gene (with respect to a reference gene) and compares its relative expression to the control group.

2.7. In Vitro Assays. Trypomastigote forms of *T. cruzi* Ninoa and Querétaro strains were obtained from infected mice and maintained in Vero cells cultures grown in D-MEM medium (GIBCO) supplemented with 10% fetal bovine serum (FBS) and incubated at 37°C, 95% relative humidity, and 5% CO_2. Culture supernatants were collected for parasites harvesting and centrifuged at 800 g for 10 min; the pellet was left undisturbed for 30 min, in order to allow the alive trypomastigotes to swim towards the supernatant and eliminate the most of cellular debris. Then, the supernatant was collected and centrifuged at 2000 g for 10 min.

Macrophages J774 (2×10^6 cells per 25 cm^2 cellular culture bottle) were cultured previously in D-MEM supplemented with 10% FBS with or without 100 U/mL IFN-γ at 37°C, 95% relative humidity, and 5% CO_2. After, cells were infected with trypomastigotes at a parasite-to-cell ratio of 7 : 1 for Querétaro strain or 15 : 1 for Ninoa strain, according with previous infection assays performed in our laboratory (data not shown) and incubated for 24 h. Production of cytokines IL-10, IL-12, and TNF-α was measured from supernatant cultures using IL-10 Mouse ELISA kit (ENDOGEN), IL-12 Mouse ELISA kit (ENDOGEN), and TNF-α Mouse ELISA kit (ENDOGEN), following manufacturer's instructions. Duplicate measures from three independent experiments were carried out.



(a) (b) (c) (d)

Figure 1: Macroscopic characteristics of a mouse infected with 10 000 trypomastigotes of Querétaro strain ((a), (c)) and a control mouse ((b), (d)). The Querétaro strain infection provokes back bristling hair and loss of mobility of their posterior extremities ((a); arrow). After sacrifice and removal of the skin, the skeletal muscle of mouse infected with Querétaro strain showed pale areas on the tissue ((c); arrowheads).

2.8. Statistical Analysis. The data are expressed as the arithmetic mean ± standard deviation of six mice for each experimental point from two independent experiments. The differences between the groups were determined by Student's *t*-test (for parasitemia, histological analyses, and *in vitro* assays) or by one-way ANOVA with Bonferroni posttest, considering $P < 0.05$ as statistically significant (for real-time PCR analyses).

3. Results

3.1. Clinical Disease. The animals infected with the Querétaro strain showed back bristling hair, a continuous tremor in their whole body, and loss of mobility of their rear extremities by days 13–15 pi (Figure 1(a)). These manifestations were coincident with the increasing in the parasitic load (Figure 2(a)), but they recovered some mobility of the hind legs by 90 days pi. On the contrary, neither the mice infected with Ninoa strain (not shown) nor the uninfected control mice (Figure 1(b)) showed these characteristics.

An important difference between both infections was the presence of pale areas (chalky white patches) observed only on the skeletal muscle of the posterior extremities from the animals infected with the Querétaro strain (Figure 1(c)), as well as loss of muscular mass in the same area. These pale areas were still seen until 90 days pi.

3.2. Parasitemia. The circulating blood parasites were counted every third day from 5 to 90 days pi. Parasitemia become detectable by day 12 pi and reached the peak by day 21 pi with 4.5×10^6 parasites/mL for the Querétaro infection, being significantly higher than with the Ninoa infection by almost 5-fold increase ($P < 0.05$). Then, the number of parasites decreased until reach undetectable levels by day 35 pi with the Querétaro strain, but during the Ninoa infection the parasites became undetectable until day 60 pi (Figure 2(a)).

3.3. Tissue Parasitism. The parasitic load in skeletal muscle was examined over the course of infection in mice with each strain of *T. cruzi*. The amastigote nests were detected by immunohistochemistry and counted (Figure 2(c)). By day 15 pi the number of nests in Querétaro group was 19.6 ± 7.6, whereas in Ninoa group it was only 0.5 ± 1.2, meaning a 35-fold difference between strains ($P < 0.05$; Figure 2(b)). By day 21 pi, a higher number of amastigote nests was still seen during Querétaro infection (12.9 ± 10.8) in relation to Ninoa infection (3.9 ± 3.4), with a significant 3.3-fold difference between strains ($P < 0.05$). In the chronic phase of infection (90 day pi), tissue parasites were neither detected in Querétaro nor in Ninoa strain infection.

3.4. Inflammatory Infiltrates. Infection with both *T. cruzi* strains induced inflammatory infiltrates in the skeletal muscle which was analyzed in slides stained with H-E; such inflammation could be observed as of 15 days pi (Figures

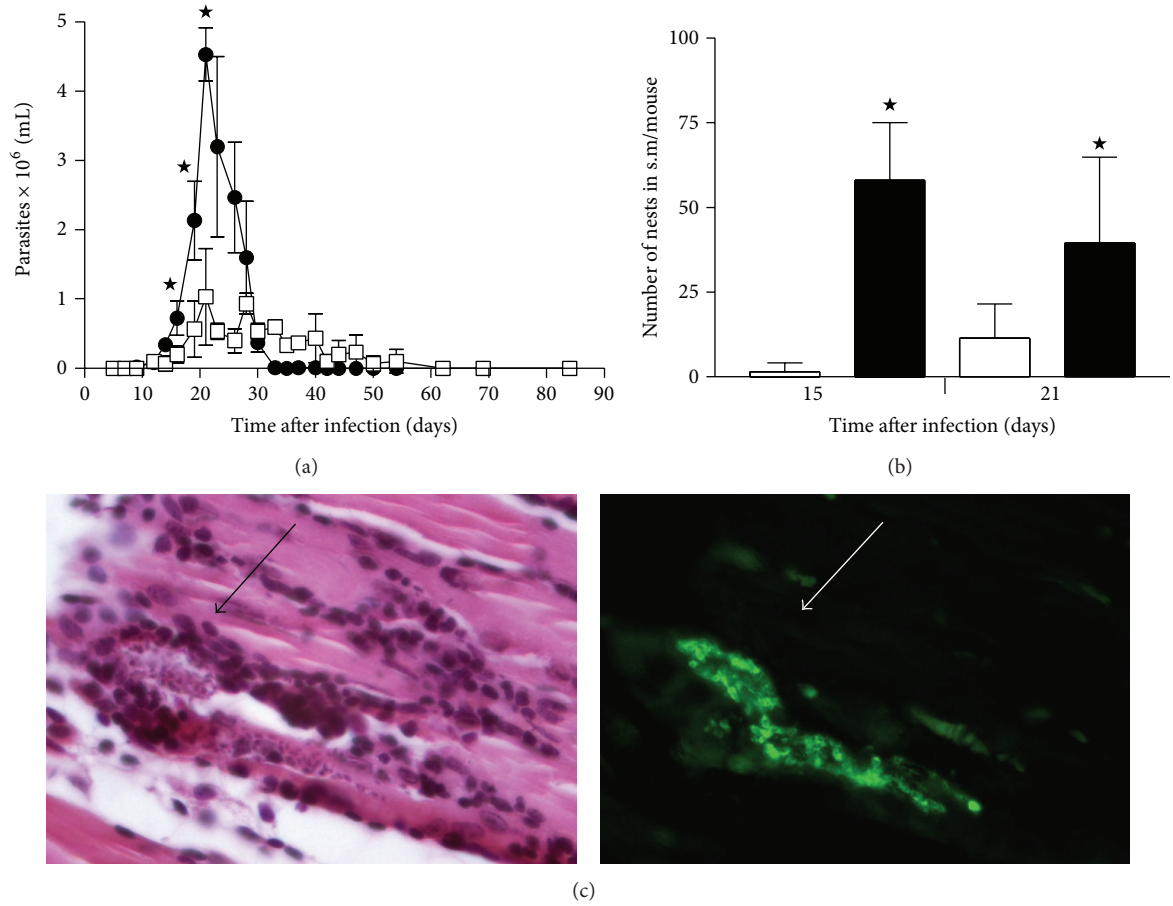


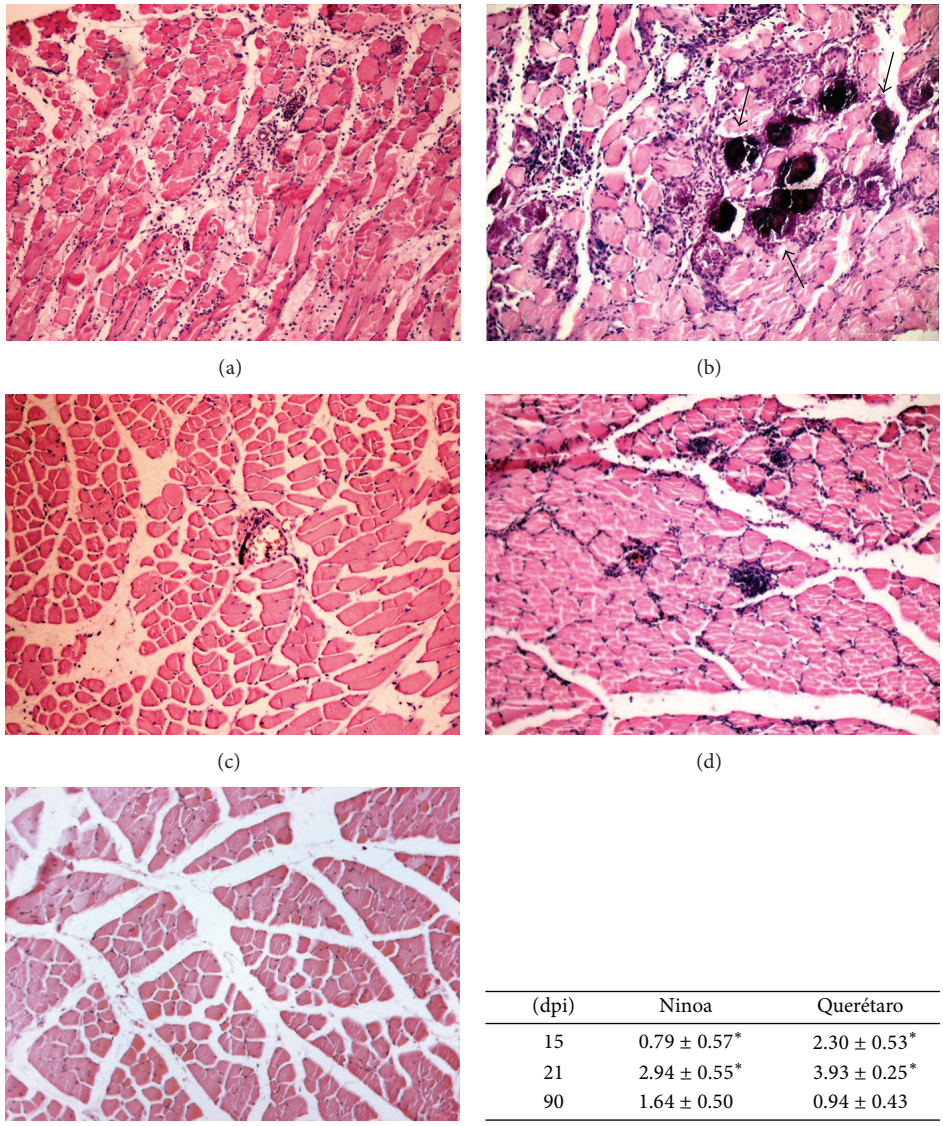
Figure 2: Kinetic of infection of two Mexican *Trypanosoma cruzi* strains in blood and skeletal muscle. (a) Number of blood parasites during the infection with Querétaro (●) or Ninoa (□) strains. Data represent average ± SD of at least three mice by experimental point. A representative graph of two independent experiments is shown. $^{\star}P < 0.05$ with Student's t-test. (b) Number of nests of parasites in skeletal muscle (Querétaro, solid; Ninoa, cleared). Data represent average ± SD of 6 mice from two independent experiments. $^{\star}P < 0.05$ with Student's t-test. (c) Microphotograph of the same field stained with H-E (left) and immunofluorescence (right) of a nest in s.m. infected with Querétaro strain, 21 dpi. Magnification 400x. (s.m. = skeletal muscle).

3(a) and 3(c)). At the peak of infection, the tissue sections obtained from the Querétaro group had intense interstitial inflammatory infiltrate with profuse cells on muscular fibers with granulomatous-like infiltrates, as well as disorganization and myofibers necrosis (Figure 3(b)). In contrast, sections of muscle in Ninoa infected mice presented clusters of inflammatory cells, observed at interstitial spaces, with mild tissue damage (Figure 3(d)). At the chronic phase of infection (90 dpi), the inflammatory infiltrates were scarce in both groups (data not shown).

Infiltrates in Querétaro group at day 15 pi were 2.30 ± 0.53, and 3.93 ± 0.25 for the 21 day pi, whereas for Ninoa infection they were 0.79 ± 0.57 at 15 days pi and 2.94 ± 0.55 at 21 days pi (Figure 3(f); arbitrary units), according to the score described in Material and Methods. These scores represented a statistically significant difference of 3- and 1.3-fold for 15 and 21 days pi, respectively ($P < 0.001$). At 90 days pi, the inflammation diminished and the score was similar for both strains.

The histological analyses showed that most of the inflammatory infiltrates were composed mainly of mononuclear cells. To identify the presence of macrophages, double immunofluorescence techniques to simultaneously stain macrophages and parasites were used. A large number of cells in these infiltrates were macrophages and some of them were associated with amastigote nests (Figures 4(a)–4(c)). Apparently, intracellular amastigotes were also found in macrophages (Figure 4(d)). Moreover, many macrophages appeared closed to calcification areas. At chronic phases, some macrophages were still seen (data not shown).

3.5. Tissue Calcification. An interesting finding was the presence of sites with intense basophilic stain exclusively in skeletal muscle from animals infected with Querétaro strain (arrows, Figure 3(b)). In order to determine the presence of calcium deposits in these basophilic areas, sections were treated with the von Kossa stain, making evident that stained



(dpi)	Ninoa	Querétaro
15	0.79 ± 0.57*	2.30 ± 0.53*
21	2.94 ± 0.55*	3.93 ± 0.25*
90	1.64 ± 0.50	0.94 ± 0.43

(f)

Figure 3: Histopathological analysis of *Trypanosoma cruzi* infection in skeletal muscle. (a) Querétaro, 15 days pi; (b) Querétaro 21 days pi; note the intense basophilic stain areas (arrows); (c) Ninoa, 15 days pi; (d) Ninoa, 21 days pi; (e) Control. Magnification 100x. (f) Inflammation scores (arbitrary units) for Querétaro and Ninoa infections. Data represent average ± SD of 5 nonconsecutive slides per animal of 6 mice in two independent experiments. $^{*}P < 0.001$, with Student's t-test.

areas corresponded to calcification sites, as shown in Figures 4(e)-4(f): the dark brown-stained areas indicate tissue calcification (arrows). More calcification areas were observed during the peak of infection, and these areas can still be seen at 90 days pi, along with regeneration of the muscle, which is seen as holes between fibers indicating the possible substitution of myofibers by adipose tissue (Figure 4(f)). Remarkably, none of these calcification areas were seen on skeletal muscle from Ninoa-infected mice or control mice at any time after infection, as neither H-E nor von Kossa staining revealed any calcinosis area (data not shown).

3.6. Cytokines Profile in Skeletal Muscle. To assess the expression levels of some cytokines following *T. cruzi* infection, real-time PCRs were used to quantify the mRNA expression profiles of IL-4, IL-10, IL-12, IFN-γ, and TNF-α within the skeletal muscle at days 1, 15, 21, and 90 pi. In general, the overall expression of each cytokine in both infections was similar with detectable transcripts present at day 15 pi, peaking at day 21 and decreasing by day 90 (Figure 5). Only the IL-10 and TNF-α transcripts showed a significant difference ($P < 0.05$) between both strains at the peak of infection (Figures 5(d) and 5(e), resp.), being higher with the Querétaro strain but decreasing dramatically by 90 days pi.

In *in vitro* assays, a higher production of TNF-α was induced in macrophages infected with the Querétaro strain, even without previous activation with IFN-γ (Figure 5(f)).

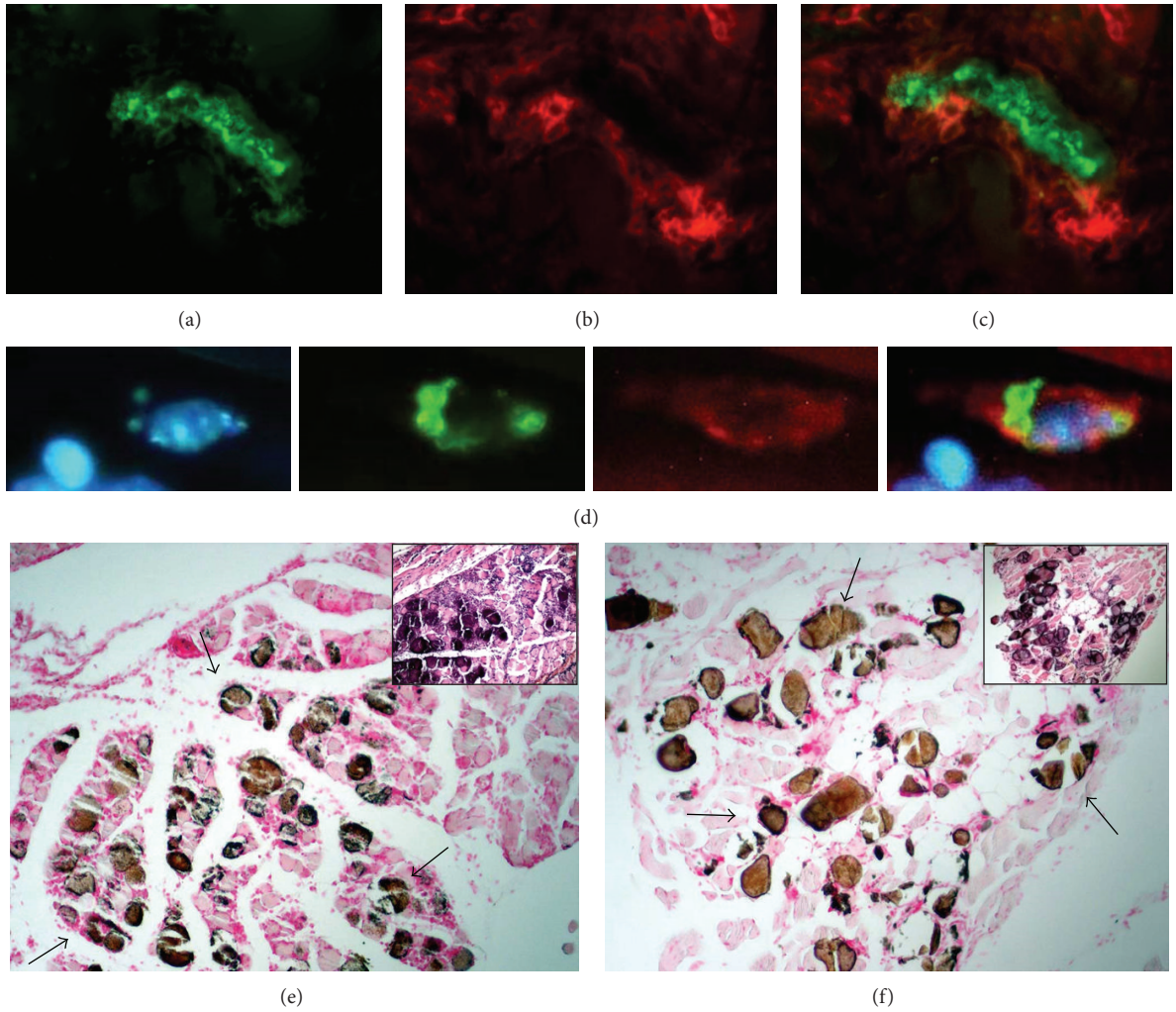


Figure 4: Macrophages and calcification areas in skeletal muscle due to Querétaro strain infection. (a) Immunofluorescence of a large amastigote nest in skeletal muscle of mice infected with Querétaro strain, 21 days pi. (b) Same field showing immunofluorescence of macrophages surrounding the nest. (c) Merge of (a) and (b). Magnification 400x. (d) Microphotographs of amastigotes (green) inside a macrophage (red); counterstained with DAPI. Extreme right: merge. Zoom of a magnification 400x. (e) Calcification areas stained in brown (arrows), 21 days pi. (f) Calcification areas (arrows) at 90 days pi. The inserts show calcification areas stained with H-E. Magnification 100x.

4. Discussion

In previous studies, our research group has shown the important presence of *T. cruzi* TcI strains in Mexico [7, 8]. However, the published information on biological and histopathological characteristics of the Mexican TcI strains is scarce.

In this study, we analyzed the histopathological damage of skeletal muscle from mice caused by the infection with the Mexican TcI strains, Querétaro and Ninoa. Animals infected with Querétaro strain presented back bristling hair, a continuous tremor in their whole body, and stiffness of the hind limbs, manifestations that are not shown by Ninoa-infected mice. In a previous study, looking for a molecular diagnosis method to study tropism and growth kinetics of *T. cruzi* in a murine model, similar characteristics in mice infected with another Mexican isolation (JALGO, obtained from Triatominae feces collected at Jalisco, Mexico) were reported, and the animals succumbed to the infection; however, the genetic group of this strain was not reported [32].

As previously described [26, 27], the magnitude of the parasitemia at the pick during the Querétaro infection was higher than with the Ninoa infection. The number of parasites detected in blood correlated with the number of amastigote nests detected in the skeletal muscle, being significantly higher in animals infected with the Querétaro strain. The detection of tissular parasites was made by immunohistochemistry, facilitating the localization of amastigote nests, mainly of small ones or those covered by inflammatory cells. These results together indicate that Querétaro strain has a higher ability to invade and replicate into the host organism than Ninoa strain.

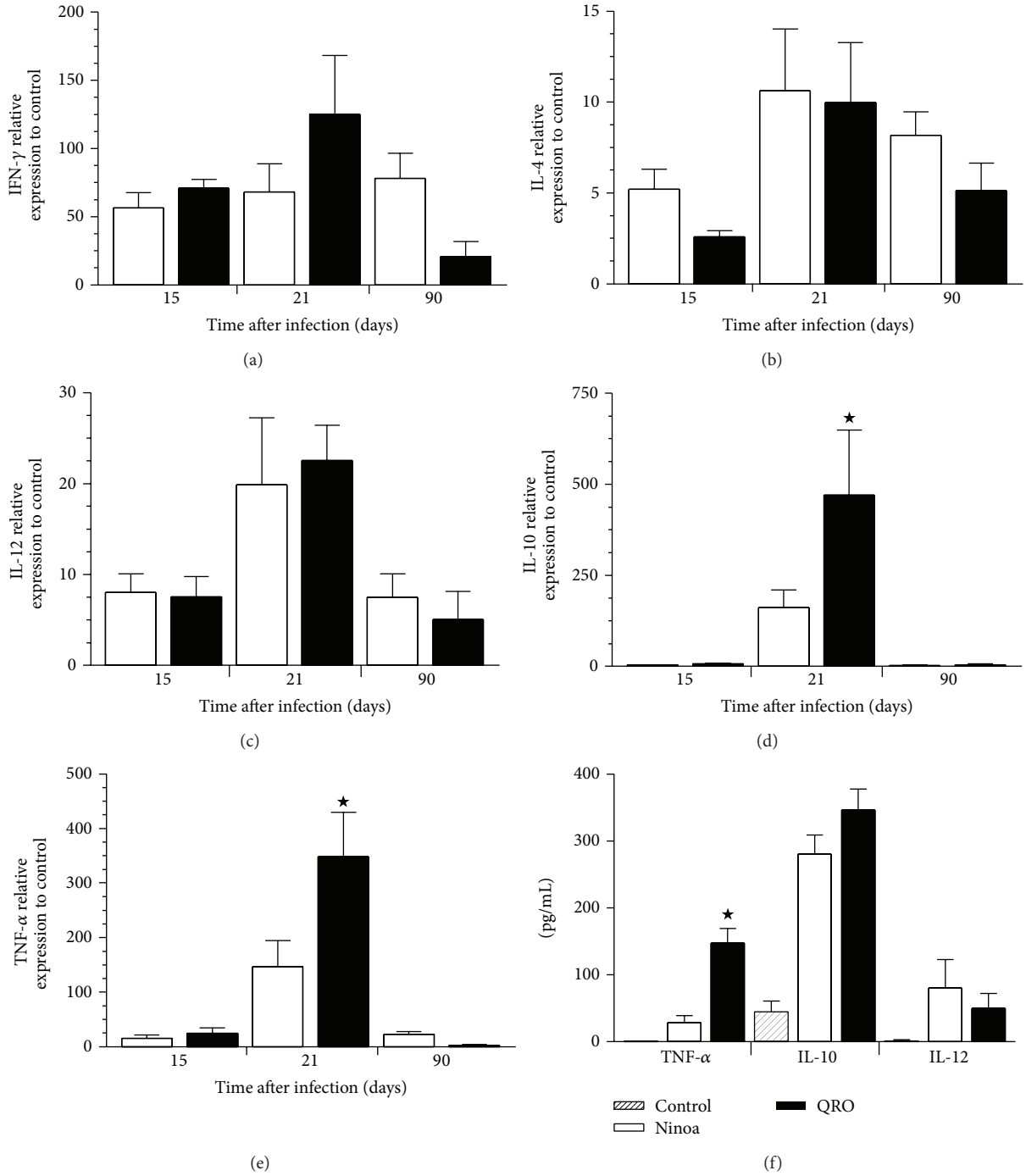


Figure 5: Cytokines determination in skeletal muscle from mice infected with two Mexican *Trypanosoma cruzi* strains (Ninoa, cleared bars; Querétaro, solid bars; Control, stripped bars). mRNA expression levels were quantified by qPCR: (a) IFN-γ, (b) IL-4, (c) IL-12, (d) IL-10, and (e) TNF-α. Data are expressed as average ± SEM of 6 mice per experimental group from 2 independent experiments. $^{\star}P < 0.05$ with ANOVA test and Bonferroni posttest. (f) Cytokine concentration (pg/mL) of *in vitro* infections of macrophages J774 with each TcI strain. Data are presented as average ± SD of double measures from three independent experiments. $^{\star}P < 0.05$ with Student's t-test.

T. cruzi infection provoked an inflammatory phenomenon mainly composed of mononuclear cells. An increase in the intensity and extension of the inflammatory infiltrates was observed, and the peak of inflammation correlated with the peak of parasitic load, at 21 days pi. Querétaro strain induced severe focal and diffuse inflammatory foci with necrosis of the tissue and granulomatous-like infiltrates, whereas, in comparison, Ninoa strain provoked a moderate inflammation. These results support the previous observation that the characteristics of each strain promote differences

in evasion of the host immune response that could be responsible for higher parasitic loads and, in consequence, more intense *in situ* inflammatory reactions, as has been suggested by Garzon and collaborators [33]. In contrast, at the chronic phase of infection, where no parasites were detected, inflammatory foci have diminished, indicating that the host immune response has controlled the infection.

The analysis also revealed an interesting finding of *T. cruzi* pathogenesis: the presence of calcium deposits in the areas with intense basophilic stain, exclusively observed during the Querétaro infection. These areas corresponded with the pale zones macroscopically observed (shown in Figure 1) from where the samples were taken. The chalky white patches of the zone reminded an illness of the cattle known as white muscle disease, which is consequence of tissue calcification, and is also associated with stiffness of the hind limbs [34]. The deposited calcium contributes to the white color of the affected musculature. In this study, the presence of calcium deposits was verified by dark brown-stained areas after treating slides with the von Kossa staining. Tissue calcification can be the result of tissular damage involving death cell or tissular proteins denaturalization that allow the precipitation of calcium salts (dystrophic calcification). This sort of calcification can also be associated with unspecific lesions of degenerative or necrotic type. For example, in animal models of calcification induced by cardiotoxin injection, it has been suggested that calcification is preceded by death cell of the injured tissue, with disruption of the skeletal architecture [21]. In our model of infection, calcification seems to be coincidental with the exacerbated inflammation, indicating a relationship between the efforts to control the infection and the development of the lesion. Thus, the extensive and necrotic damage observed could be the reason of the calcinosis produced with the Querétaro strain infection. Considering the damage that calcification produce in muscular tissue, the paralysis of posterior extremities that Querétaro infected mice showed could be a consequence of the calcinosis process, perhaps due to the destruction of muscular architecture. There are some reports that mention this paralysis [32, 35], but it has been only recently that Ramirez-Archila and coworkers [36] confirmed that contractile properties of skeletal muscle are impaired during infection with TcI parasites, and these properties remained attenuated as a consequence of the replacement of the muscular fibers by fibrous tissue and fat. The same authors found calcifications in the skeletal muscle studied, but they studied the rectus abdominis muscle. A recent study reports the presence of skeletal muscle fibers undergoing calcification and necrosis as a consequence of *T. cruzi* II infection in WT and Daf1-deficient mice [37]. There is another report that superficially mentions muscular calcification as a consequence of *T. cruzi* infection, but that study was achieved using also a genotype II strain [38]. To our knowledge, the present work is the first report of tissue calcification in skeletal muscle from the posterior extremities induced by a *T. cruzi* TcI infection.

An important effector cell involved in this parasite control is the macrophage. Immunofluorescence techniques showed abundant presence of macrophages surrounding amastigote nests as well as areas with calcification and tissular damage. The presence of macrophages has been demonstrated in inflammation areas closest to mineralized tissue in calcific aortic valves in humans [39]. Besides, it has been reported that macrophages can enhance osteogenic signals elicited by vascular smooth muscle cells, thus playing a significant role in the formation of plaque calcification [40]. Although the incidence of macrophages in the inflammatory infiltrates was higher, other lymphocytic cellular types were present. A previous report indicated the presence of macrophages, plasmatic cells, and some eosinophils at the infiltrates in skeletal muscle of mouse infected with Ninoa strain [28]. In the present study, it was not possible to determine the exact nature of other cellular types because of the technique selected. It is known that CD4- and CD8-antigens are labile and is recommended to use freeze-tissue sections for their detection [29]. More studies are needed, in order to obtain a more complete image of the cells controlling the parasite replication in skeletal muscle during a *T. cruzi* I infection.

We then became interested in the cytokines expressed in infected skeletal muscle, since the cytokine profile during an infection can be crucial for its outcome. A balance between proinflammatory and regulatory cytokines is necessary in order to control the infection [41]. Hence, the expression of proinflammatory and regulatory cytokines was evaluated by real-time PCR. An important finding was the elevated levels of TNF-α mRNA during the acute phase of infection with Querétaro strain. It has been shown that macrophages in inflammatory infiltrates of calcific aortic valves express TNF-α [42] and that calcification process may be regulated by mechanisms involving the presence of this cytokine [43]. The significant increment in TNF-α levels could be associated with the histological findings of this work, particularly those of calcification. Besides, elevated concentrations of TNF-α were also detected when macrophages *in vitro* were infected with the virulent strain. Interestingly, the infection alone was capable of activating the macrophages J774 and inducing the production and release of this cytokine.

Therefore, the high expression of TNF-α could be part of the mechanisms involved in the calcinosis process and the severe tissular damage observed in the skeletal muscle during Querétaro infection. It has been reported that an exacerbated inflammatory response in this sort of infection, involving proinflammatory cytokines such as IL-12, IFN-γ and, particularly, TNF-α, contribute not only to control the infection but also to damage the host tissue. For example, TNF-α presence has been shown in necrotic areas in spleen from *T. cruzi* infected mice [44]. Accordingly, the intense inflammatory reaction observed in Querétaro infected animals could have caused a necrotic damage of the muscle ending in its calcification.

In the present study it was also notable the upregulation of IL-10, a cytokine that possess a downregulatory activity on the development and effector functions of cell-mediated immunity. Previous studies have shown that infection of IL-$10^{-/-}$ mice with the protozoan parasite *T. cruzi* resulted in a reduced parasitemia but caused increased mortality [45, 46],

possibly indicating a more systemic immune response which, if not regulated by IL-10, results in a toxic overproduction of proinflammatory cytokines, like TNF-α [47]. In this way, the increased levels of IL-10 found in *T. cruzi*-infected skeletal muscle may be a necessary event for controlling the strong cell-mediated immunity elicited by this parasite.

In conclusion, the most relevant finding of the present work was the development of calcification in the skeletal muscle of hind legs, exclusively with Querétaro strain and particularly during the acute phase of infection, even though both strains studied belong to DTU I. This calcification event may probably be a consequence of the exacerbated inflammatory process and the consequent disruption of the tissue elicited by this infection and could be related to a significant increment in TNF-α levels.

Ethical Approval

The study was approved by the Ethics Committee of the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Crosstalk among *Taenia crassiceps* (ORF Strain) Cysts Regulates Their Rates of Budding by Ways of Soluble and Contact Signals Exchanged between Them

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Herein we report that *in vitro* experiments with different initial parasite densities (1, 5, and 10 cysts per mL of culture medium) show that cysts at densities of 10 and 5 grow faster than those at 1, and that they release into the culture medium factors which increase the budding rates of the slower lower-density ones. Close contact among the incubated cysts also favors budding, thus suggesting the participation of surface sensors of parasite crowding. Thus, contact signals, together with the release of soluble growth factors, could endow cysts with the capacity to sense and regulate their numbers inside their habitat in relation to their population density.

1. Introduction

Taenia crassiceps is a cestode parasite which naturally affects canines and murines as definitive and intermediary hosts, respectively [1]. When *T. crassiceps* metacestodes are experimentally placed inside the peritoneal cavity of receptor mice, the cysts rapidly initiate their asexual reproduction through the daily generation of numerous visible and easily accountable buds [2]. Once parasites harbor in the peritoneal cavity of susceptible mice (i.e., Balb C/AnN), their numbers grow exponentially until reaching maximal parasite loads in the order of a couple of thousands per infected mouse, which together occupy as much volume as the mouse itself, some 3-4 months after infection, without causing apparent illness to their host.

Many host and parasite factors influence the early rates of parasite growth, such as genetic background, sex, and immune status of the host, as well as the original strain of the parasite, but rarely if ever does the intensity of infection rise above the levels mentioned.

While studying the effects of the genetic background, sex, and immunological status of mice, as well as of the time elapsed after intraperitoneal infection with *T. crassiceps* ORF cysts, we noticed that parasite loads and antibody response of individual mice varied considerably within the same strain and sex of infected mice even when the mice were infected with an equal number of apparently identical cysts collected from the same donor female mouse [3]. Discarding technical error as a comparatively negligible source of variance in murine cysticercosis experiments [4], large part of the individual host response variation in murine cysticercosis could also be attributed to the parasites inside it. This alternative source of variation was not considered before because no major genetic differences among individual cysts were expected as they derive from a single strain of parasites (ORF) and also because budding is an asexual form of parasite reproduction with reduced chances of genetic recombination as a source of diversity [5].

So, we set out to test the hypothesis that a significant source of variation lies in the reproductive capacity of each of the apparently identical cysts contained in the inoculums, each possibly having different budding capacity at the time of their inoculation, associated perhaps with their degree of individual age and differentiation. Accordingly, *in vitro* budding rates of 1, 5, or 10 cysts per mL of culture medium were registered microscopically at different days after culture, together with a visual record of their appearance, motility, and physical relation with each other. The *in vitro*

experiments demonstrated that increasing parasite densities increased their rate of budding, as if the faster cysts recruited the laggard ones into faster budding [6]. Herein we report that budding rate regulatory signals interchanged between parasites are behind such recruitment.

2. Material and Methods

2.1. Parasite Collection. The cysts employed in *in vitro* experiments were collected from different single donor BalbC/AnN female mice that had been infected i.p. 2 months before or more to develop massive parasite loads [7]. The donor mice were killed by etherization in accordance with our institute's ethical procedure for experimental animals treatment (http://www.biomedicas.unam.mx/_administracion/reglamentos_formatos/archivos_pdf/reglamentoBioterio.pdf) and, immediately afterwards, their peritoneal cavities were incised to release cysts into a Petri dish containing phosphate buffered saline (PBS) and 100 μg/mL penicillin/streptomycin at room temperature. Typically, a significant fraction of the harvested cysts (~10–20%) corresponded to a subpopulation of tiny (0.1–0.3 mm) nonbudding, motile, and transparent cysts, from which groups of 1, 5, or 10 cysts were selected to perform the study.

2.2. Parasite Cultures. Nonbudding cysts were microscopically selected and were cultured in RPMI 1640 medium at 37°C for 10 days.

2.3. Testing for Released Growth Factors. The culture media (1 mL) from cultures of cysts at a density of 5 or 10 cysts per mL were transferred daily, for 10 days, to wells which contained single cysts. The number of buds produced by singly cultured cysts was counted and compared with that of the control group of individually cultured cysts which received only daily fresh RPMI 1640 medium.

Transwell chambers (Costar) with a 5 μm pore membrane were used to carry out a variant of the experiment of medium transfer. In this case, 1, 5, or 10 cysts were placed in the following combinations: 1/5, 5/1, 1/10, and 10/1 (upper chamber/lower chamber). The chambered cysts were cultured for 10 days and the number of buds on each of the individual cysts was microscopically counted. The medium was replaced by fresh medium on a daily basis.

2.4. Testing Effects of Parasite Contact upon Parasite Growth. Five or 10 nonbudding cysts were forcefully put in contact by placing them in the bottom of conical polypropylene 50 mL Corning tubes cut in their conical tip for this purpose and then glued with silicone onto the flat bottom of a well of a 6-well culture plate filled with 1 mL of RPMI 1640 medium and then cultured. In a parallel experiment, 5 or 10 cysts were individually placed inside the widest part of a 1000 μL pipette tip cut and attached to the flat bottom of a well of a 6-well culture plate with silicone so that there was one parasite in each device to prevent contact between cysts. The well was then covered with culture medium so that the cysts shared the same medium without being in contact. The crowded and the individually placed collections of cysts were cultured for 10 days and the numbers of buds produced in both conditions were microscopically counted. The medium was replaced by fresh medium every third day.

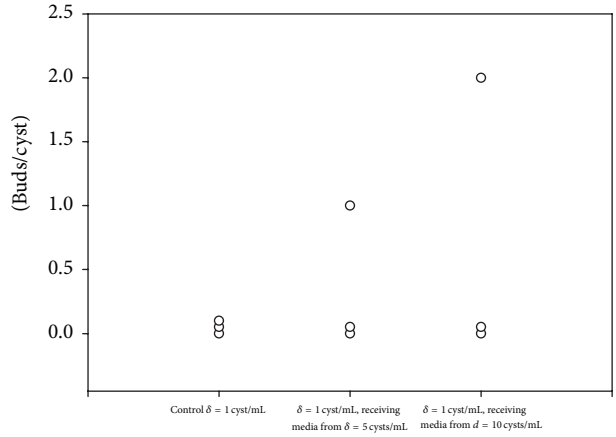

Figure 1: Effect of soluble factors on the budding of cysticerci. One microscopically nonbudding cysticercus/mL was cultured for over 10 days in fresh culture media; culture media from 5 cysticerci/mL or from 10 cysticerci/mL changed on a daily basis. The number of buds generated by the single cysticerci in each culture condition was counted at the 10th day. Experiments were performed by triplicate. Data represent the number of buds/cyst in each cysticercus and are plotted as individual value. Representative experiment is shown.

2.5. Statistical Analysis. Student t-test was used for data statistical analysis.

3. Results

When cysts were collected from different donor mice, it became evident there was considerable variation in their rates of budding between donor mice, a variation which prompted us not to mix parasites harvested from various mice in subsequent experiments but use the cysts harvested from the same mouse in experiments designed to identify the nature of the budding rate regulatory signals.

To ascertain if the signals from the parasite are secreted, culture medium from rapidly budding cysts (d = 5 or 10 cyst/mL) was transferred to cysts whose budding rate was low (density = 1/mL). Figure 1 shows that culture medium from rapidly budding cysts (d = 5 or 10 cyst/mL) significantly promoted the number of buds/cyst in cysts cultured at density = 1/mL from 0.0 to 0.3 (d = 5) and 0.6 (d = 10). Also, when using the transwell system to evaluate buds/cyst when transmembranly cocultured with 5 or 10 cysts, it was found that, indeed, single cysticerci bud more effectively from 0.0 to 1.1 and 0.8 when cocultured with 5 and 10 cysts, respectively (Figure 2).

To explore if contact between the cysts was of consequence for the release of such a regulatory growth factor, we designed a simple device to force contact between the cysts and another to prevent it. Figure 3 shows that, at the same density, the cysts bud more rapidly and efficiently when they

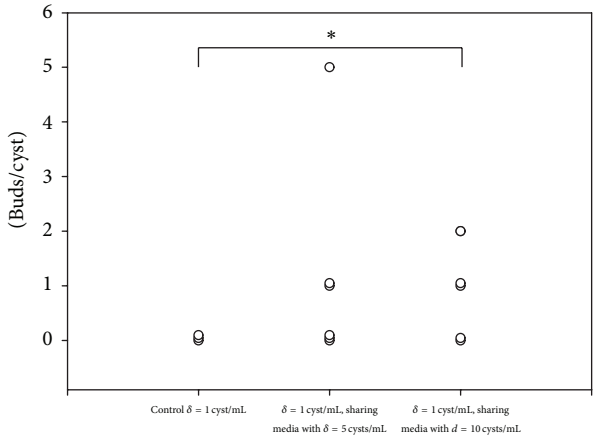

Figure 2: Single microscopically nonbudding cysticercus was cultured for over 10 days in a transwell system with 5 cysticerci or with 10 cysticerci on the other side of the membrane, in 1 mL of culture media. The number of buds generated by the single cysticerci in each culture condition was counted at the 10th day. Experiments were performed by triplicate. Data represent the number of buds/cyst in each cysticercus and are plotted as individual value. Representative experiment is shown. The asterisk on top of the figure represents a significant difference between the selected groups ($P < 0.05$, 2-tailed Student's t-test).

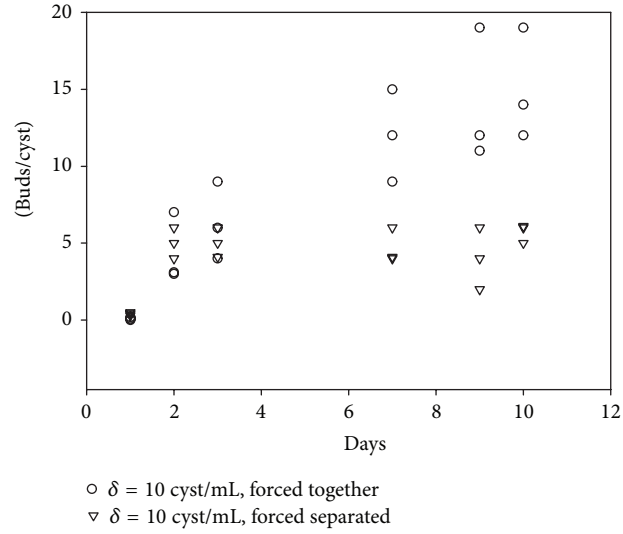

Figure 3: Effect of contact on the budding of cysticerci. Ten microscopically nonbudding cysticerci were cultured over 10 days in 1 mL of culture media. Cysticerci were forced to contact each other by culturing them on a conical-bottomed well or were forced to be separated. The number of buds generated in each culture condition was counted on days 1, 2, 3, 7, 9, and 10. Experiments were performed by triplicate. Data represent the number of buds/cyst in each cysticercus and are plotted as individual value. Representative experiment is shown. The asterisk on top of the figure represents a significant difference between the selected groups ($P < 0.05$, 2-tailed Student's t-test).

are in contact than when they are not. Parasite contact seems to be a determining factor in a rapid budding response.

4. Discussion

We had previously noted that, in *in vitro* experiments, the density at which the cysts were incubated affected their rate of budding [6]. We now present evidence that there is a kind of communication between cysts in the form of a secretable molecule with an effect similar to a growth factor that enhances the rate (number of buds/cyst) of budding of cysts *in vitro*. We do not know if this factor is synthesized *de novo* by the cyst or it is a host factor absorbed by the cyst and eventually released. We favor the notion that it is synthesized by the cyst because the experiments last for several days and imply daily change of culture medium with fresh one. This argument is reinforced by the fact that the budding rate is further increased by placing the cysts in contact, probably through increased synthesis or increased release of this growth factor. Additionally, the fact that cysts contact enhances budding recalls the mechanisms like ligand-receptor interaction might be involved in the process as it happens in other systems [8]. The crowding cysts at density = 10 per se promote the highest budding efficiency which indicates that crowding is a powerful factor controlling the population of cysts even more than with hormone supplementation[6]. For that reason we assume that the phenol red in the medium has, if any, a very little effect over the budding.

We do not know the kind of signaling pathway involved in this phenomenon in *T. crassiceps*. It is known that families of genes important for signaling and development evolved before the divergence of the lineages of sponges and eumetazoan and are, therefore, in all animal lineages. [9]. The similarity of these systems between different phyla allows us to assume that the secretion of this growth factor *in vivo* can influence different host cells from organized neuroimmunoendocrinological networks [10, 11]. Likewise does the liver parasite *Opisthorchis viverrini*, which secretes mitogenic factors that can induce proliferation of host cells leading to a cholangiocarcinoma [12].

In conclusion, the *in vitro* studies presented here show that *T. crassiceps* secretes factors that affect the rate of budding of cysts and that these factors may change depending upon the host, time of infection, or contact between parasites.

Conflict of Interests

The authors declare that they have no conflict of interests.

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