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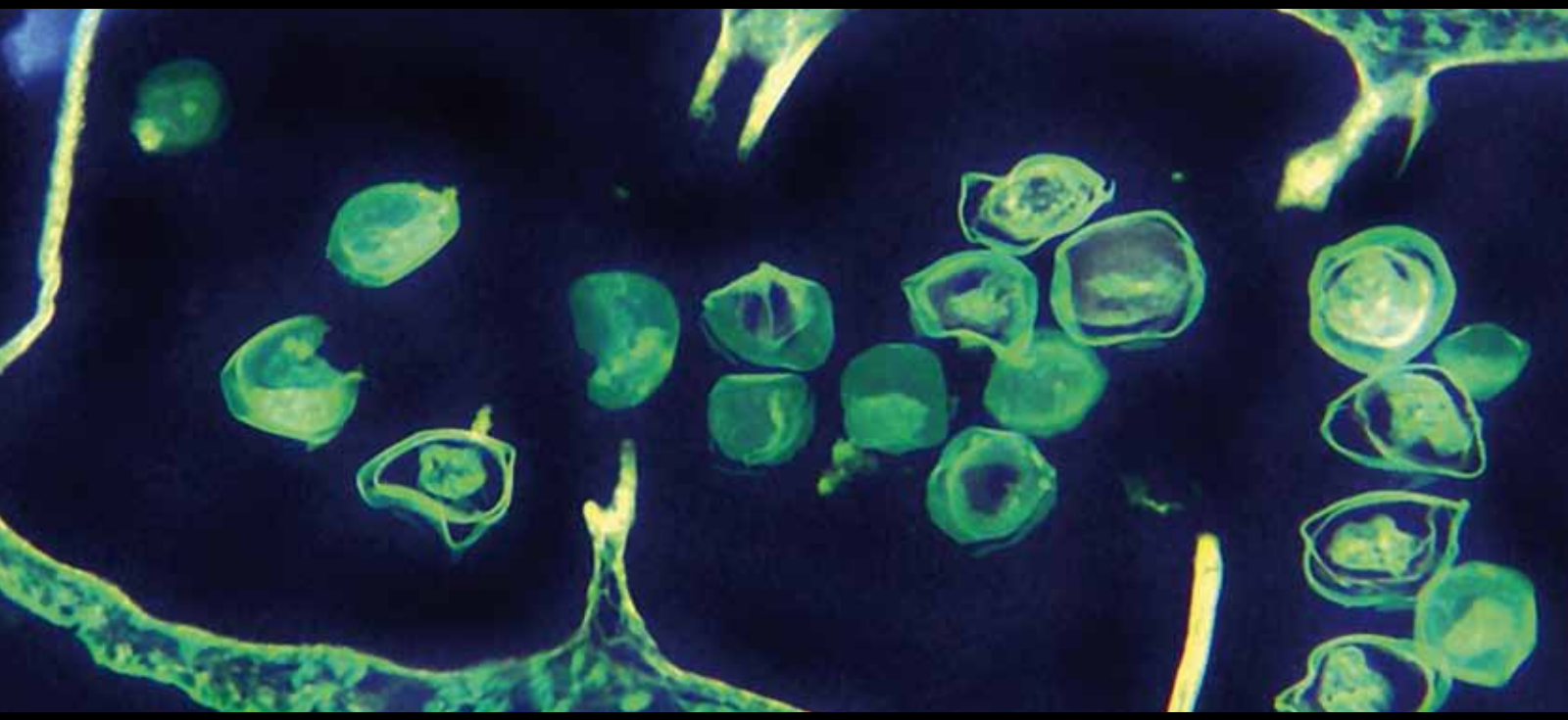
Immunity to Protozoan Parasites

Guest Editors: Marcela F. Lopes, Dario S. Zamboni, Hugo D. Lujan, and Mauricio M. Rodrigues



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Editorial Board

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Editorial

Immunity to Protozoan Parasites

Marcela F. Lopes,[1] Dario S. Zamboni,[2] Hugo D. Lujan,[3] and Mauricio M. Rodrigues[4]

[1] *Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, 21941-902 Rio de Janeiro, RJ, Brazil*
[2] *Departamento de Biologia Celular Molecular e Bioagentes Patogenicos, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, 14049-900 Ribeirão Preto, SP, Brazil*
[3] *Laboratorio de Bioquímica y Biología Molecular, Facultad de Medicina, Universidad Católica de Córdoba, X5004ASK Cordoba, Argentina*
[4] *CINTERGEN, Escola Paulista de Medicina, UNIFESP, 04044-010 São Paulo, SP, Brazil*

Correspondence should be addressed to Marcela F. Lopes, marcelal@biof.ufrj.br

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Protozoan parasites cause several diseases, such as Malaria, Leishmaniasis, and Trypanosomiasis, hampering human development worldwide. Many protozoa cause infections that often follow chronic courses, owing to coevolution between parasites and host immune system. The survival and transmission of pathogenic protozoa depends on their ability to evade or subvert host's innate and adaptive immune responses. A great challenge to research in immunology and parasitology is the development of strategies that favor immunity against protozoan parasites and prevent their evasion, chronic, or recurrent infections and associated pathologies. This special issue includes original papers and reviews that summarize current advances in our understanding on the mechanisms of immunity to protozoan parasites in humans and experimental animal models.

The discovery of pattern recognition receptors (PRRs) devoted to detection of pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) fostered a vibrant research in the past decade, identifying new receptors, new ligands on pathogens, and key signaling pathways inducing innate immunity [1–3]. Therefore, it is not surprising that about 70% of the papers published here deal with early steps of immune responses to protozoan infections, including the role of parasite products, host receptors, molecular mechanisms, and effector cells of innate immune system.

Four papers of this special issue discuss molecules present at initial encounter between hosts and protozoan parasites plus the secretions of their insect vectors. Macrophage migration inhibitory factor (MIF, "*Macrophage migration inhibitory factor in protozoan infections*") is a host inflammatory cytokine with protective or pathogenic actions in distinct protozoan infections. Interestingly, parasites also express their own MIFs, a relevant finding that deserves further investigation. During transmission of *Trypanosoma cruzi*, lysophosphatidylcholine (LPC, "*Lysophosphatidylcholine: a novel modulator of Trypanosoma cruzi transmission*") is introduced with vector's secretions and recruits host cells, promoting infection. Apoptotic cells also express LPC among other "find me" and "eat me" signals that favor their clearance by professional phagocytes. As described elsewhere, interactions between apoptotic cells and *T. cruzi*-infected macrophages induce intracellular parasite replication [4]. Similarly, *Leishmania amazonensis* parasites express phosphatidylserine (PS, "*Subversion of immunity by Leishmania amazonensis parasites: possible role of phosphatidylserine as a main regulator*"), mimicking apoptotic cells to inhibit host cell activation and exacerbate infection. In "*New insights on the inflammatory role of Lutzomyia longipalpis saliva in Leishmaniasis*", the authors discuss how immunization against vector's saliva components might help development of a new approach to prevent Leishmaniasis. Further research will be required to define target antigens involved in distinct biological activities of vector's saliva for novel vaccines against *Leishmania spp*. infections.

Another four papers review the role of Toll-like receptors (TLRs) on immunity to parasitic infections, their ligands,

and how this knowledge can be translated into PAMP-based adjuvants for novel vaccine strategies. Authors present what we have learnt from experiments performed in mice defective in one or multiple TLRs or key signaling molecules to gain insight into their role on immunity triggered by protozoan PAMPs, with emphasis on *T. cruzi* and *Leishmania* infections. Other PRRs, such as Nod-like receptors (NLR), only recently came into spotlight in immunity to protozoan parasites [5]. Lack of putative PRRs not only affects innate immunity, but also the development of certain adaptive immune responses, presumably due to their role on antigen-presenting dendritic cells (DCs). This is particularly relevant for new vaccine approaches aiming at inducing memory T cells against intracellular protozoan infections (*The immune response to Trypanosoma cruzi: role of toll-like receptors and perspectives for vaccine development*). Whereas protozoan PAMPs activate innate immunity through TLR-induced signaling pathways, other molecules, such as lipophosphoglycan (LPG, "*Innate immune activation and subversion of mammalian functions by Leishmania lipophosphoglycan*"), subvert the immune responses, allowing parasite escape (*Toll-like receptors in Leishmania infections: guardians or promoters*). Furthermore, detection of protozoan PAMPs by immune and target tissue cells can induce protective, regulatory, or even pathogenic reactions upon infection (*Nonimmune cells contribute to crosstalk between immune cells and inflammatory mediators in the innate response to Trypanosoma cruzi infection*). How responses to stress occur in pathogenic protozoa and host tissues has been a topic of increasing interest for development of drugs to treat parasitic diseases [6].

There are also three papers that address the major effector cells in innate immunity to protozoan parasites: neutrophils, macrophages, dendritic cells, and their arsenal of effector molecules. Lately, monocytes were also imputed as major effector and regulatory players against protozoan parasites [7, 8]. ETosis was recently described in neutrophils as a death process leading to extrusion of antimicrobial DNA traps, which can also be triggered by and act on protozoan parasites (*ETosis: a microbicidal mechanism beyond cell death*). In "*Reactive oxygen species and nitric oxide in cutaneous Leishmaniasis*", the authors describe effector molecules expressed by phagocytes and subsets of macrophages, with protective or regulatory roles in protozoan infections. In "*The role of vitamin D and vitamin D receptor in immunity to Leishmania major infection*", the authors take advantage of deficiency in vitamin D receptor to show that vitamin D controls susceptibility to *Leishmania major* infection in resistant, but not susceptible mice. Vitamin D receptor signaling seems to inhibit the ability of DCs both as effector cells and as promoters of Th1-protective adaptive immunity.

In four papers of this issue, the authors approach the role of adaptive immunity in both protection and pathogenesis of protozoan infections. "*Thymus atrophy and double positive escape are common features in infectious diseases*" and "*B cell response during protozoan parasite infections*" discuss how protozoan parasites negatively affect development and modulate functions of T and B lymphocytes in cellular and humoral responses to infections. In "*Evidence for T cell help in the IgG response against tandemly repetitive Trypanosoma cruzi B13 protein in chronic Chagas disease patients*", authors investigated how the cooperation between T and B cells result in humoral responses to a parasite antigen in chronic chagasic patients. In "*Comparison of protective immune responses to apicomplexan parasites*", the researchers focus on the immune responses to apicomplexan protozoa, including *Plasmodium*, *Toxoplasma*, and *Cryptosporidium*. They also discuss issues such as antigenic variation that precludes development of conventional or transmission vaccines against these infections. Recently, disruption of the mechanism of antigenic variation in the protozoan *Giardia lamblia*, which is regulated posttranscriptionally by an RNA interference-like mechanism [9], allowed the formulation of an effective vaccine against this intestinal parasite [10].

In another paper, the researchers propose a mathematical model to predict Malaria transmission in endemic areas under drug pressure, taking into account induction of relapses by *Plasmodium vivax* infection, which confer the ability to boost adaptive immunity and prevent clinical Malaria.

Hopefully, the strong body of information available in this special issue will facilitate research on new mechanisms of immunity as well as the development of novel vaccine and immunotherapeutic tools against parasitic diseases, which are some of the most deadly human infections.

Marcela F. Lopes
Dario S. Zamboni
Hugo D. Lujan
Mauricio M. Rodrigues

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Review Article

Reactive Oxygen Species and Nitric Oxide in Cutaneous Leishmaniasis

Maria Fátima Horta,[1] Bárbara Pinheiro Mendes,[1] Eric Henrique Roma,[1] Fátima Soares Motta Noronha,[2] Juan Pereira Macêdo,[1] Luciana Souza Oliveira,[1] Myrian Morato Duarte,[2] and Leda Quercia Vieira[1,3]

[1] *Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte, MG, Brazil*
[2] *Departamento de Microbiologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, 31270-901 Belo Horizonte, MG, Brazil*
[3] *Núcleo de Pesquisas em Ciências Biológicas (NUPEB), Instituto de Ciências Biológicas e Exatas, Universidade Federal de Ouro Preto, Morro do Cruzeiro, 35400-000 Ouro Preto, MG, Brazil*

Correspondence should be addressed to Leda Quercia Vieira, lqvieira@icb.ufmg.br

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Cutaneous leishmaniasis affects millions of people around the world. Several species of *Leishmania* infect mouse strains, and murine models closely reproduce the cutaneous lesions caused by the parasite in humans. Mouse models have enabled studies on the pathogenesis and effector mechanisms of host resistance to infection. Here, we review the role of nitric oxide (NO), reactive oxygen species (ROS), and peroxynitrite ($ONOO^-$) in the control of parasites by macrophages, which are both the host cells and the effector cells. We also discuss the role of neutrophil-derived oxygen and nitrogen reactive species during infection with *Leishmania*. We emphasize the role of these cells in the outcome of leishmaniasis early after infection, before the adaptive T_h-cell immune response.

1. Introduction

More than 20 *Leishmania* species cause leishmaniasis in people with different genetic backgrounds and general states of health. Further, the diversity of clinical manifestations, epidemiology, and immunopathology makes leishmaniasis a complex disease to study. Clinical manifestations include ulcerative skin lesions, destructive mucosal inflammation, and disseminated visceral infection (kala azar). Morbidity includes disfigurement and disability. However, some features are shared by all forms of infection by these protozoan parasites: parasitism is persistent, tissue macrophages are the main parasitized cell, and the host immune response defines the outcome of the disease [1].

Cutaneous leishmaniasis is caused by several species of the genus *Leishmania*, including *L. major, L. tropica, L. aethiopica, L. mexicana, L. braziliensis, L. guyanensis, L. panamensis, L. peruviana*, and *L. amazonensis*. The *Leishmania* genus is divided in two subgenera, *Leishmania* and *Viannia*. In the subgenus *Leishmania, L. amazonensis*, *L. mexicana* (complex *L. mexicana*), and *L. major* (complex *L. major*) are by far the most studied species that cause cutaneous leishmaniasis. The subgenus *Viannia* comprises two important species that cause cutaneous leishmaniasis, *L. guyanensis* (complex *L. guyanensis*) and *L. braziliensis* (complex *L. braziliensis*) [2, 3].

The promastigote stage of the parasite lives in the gut of sandflies (*Phlebotomus* in the Old World and *Lutzomyia* in the New World) [4]. In the insect gut, *Leishmania* promastigotes develop into metacyclic (infective) forms and enter the vertebrate host when female sandflies take a blood meal. In the vertebrate host, phagocytic cells ingest the metacyclic promastigotes that, inside the phagolysosome, differentiate into the amastigote form and replicate. The

amastigotes rupture the macrophage and proceed to infect other macrophages in the tissue, and, if unchecked by the immune system, they will replicate indefinitely. The parasites rely on macrophages for successful replication, although they can also be taken up by neutrophils [5, 6] and dendritic cells [7]. *Leishmania* do not enter cells actively; thus, they are macrophage obligatory parasites, and the mechanism of entrance is accepted to be phagocytosis [7]. The exit of parasites from the macrophage is less clear. It is becoming apparent that the release of intracellular pathogens is not simply a consequence of a physical or metabolic burden imposed on the host cell, but rather of particular exit strategies governed by the microorganisms (reviewed in [8]). In *Leishmania*, parasite-derived pore-forming cytolysins, which we call leishporin, may be involved [8–13]. The life cycle of *Leishmania* is complete when sandflies feed on infected hosts, ingesting infected cells.

Although the immune response induced by infection with *Leishmania* has been the subject of many investigations, the mechanisms that underlie host resistance and pathogenesis in leishmaniasis are not entirely understood. During the late 80s and early 90s, the discovery of two distinct subpopulations of CD4+ T helper cells based on their cytokine production, Th1 and Th2 [14], finally explained resistance and susceptibility to *L. major* in the murine model. The resistance of C57BL/6 and the susceptibility of BALB/c mice were shown to be the result of the development of a Th1 or Th2 response, respectively. IFN-γ produced by Th1 cells induces the expression of inducible nitric oxide synthase (iNOS or NOS2) by macrophages. This enzyme catalyzes the oxidation of the guanidino nitrogen of L-arginine to produce nitric oxide (NO), which kills the parasite. In contrast, the Th2 response not only activates macrophages to produce arginase (by the action of IL-4, IL-13, and IL-10), which competes with iNOS for the same substrate, but also inhibits the ability to produce NO [15–19]. For some time, Th1 cells and NO were thought to be the sole protagonists of mouse resistance to leishmaniasis, until other reports (referred below) showed that the polarization of the response to Th1 or to Th2 does not explain host resistance or susceptibility to all species of *Leishmania* and does not occur in all host/parasite combinations. Hence, infection with *L. amazonensis* is an example of the still controversial nature of protective immunity in mice. The disease caused in C57BL/6 mice by *L. amazonensis*, for instance, appears to depend on Th1 cells [20], and lesions in C3HeB/FeJ mice do not heal after induction of a Th1 response during chronic infection [21]. However, Th1 cells help mice control *L. amazonensis* infection established by promastigotes, but not by amastigotes [22], and a Th1 response elicited by *L. major* confers resistance in C3HeB/FeJ and C57BL/6 mice to *L. amazonensis* challenge [23, 24]. Likewise, the lack of resistance of C57BL/10 to *L. amazonensis* infection [25] and of BALB/c to *L. mexicana* [26] does not correlate with the presence of a typical Th2 response, suggesting that susceptibility to these species of *Leishmania* is due to a failure to mount a Th1 response, rather than the presence of a Th2 response. Conversely, the resistance of BALB/c to *L. braziliensis* appears to be due to the absence of a Th2 response rather than to the presence of a Th1 response [27]. The inconsistency of the pattern protection/Th1 and pathogenesis/Th2 to all species of *Leishmania* was recently reviewed [28].

Indeed, except for a few references [29–31], innate immunity has largely been overlooked with respect to the mechanism of host resistance to *Leishmania* infection. Dendritic cells, macrophages, and neutrophils, along with their early-produced cytokines and reactive nitrogen and oxygen species, have not been spotlighted as effector cells during the initial stages of infection. Even the leishmanicidal competence of macrophages has mostly been described as a T-cell-dependent event, even though inducers of NO are available very early after infection, namely, type 1 interferons (IFN-α and IFN-β) and type 2 (IFN-γ) interferons. While IFNs-α and -β have been shown to be secreted by macrophages [32], IFN-γ is produced by NK cells [16, 30, 33, 34] and possibly by γ/δ T cells [35], NKT cells [35], or even macrophages [36, 37], although the latter is still controversial [38]. More recently, however, innate immunity effector cells have been suggested to be coparticipants in the maintenance or elimination of the parasites, acting in the early stages of infection in the absence of a T_h-cell response.

In this paper, we highlight the participation of both NO and reactive oxygen species (ROS) in the resistance and pathogenesis of cutaneous leishmaniasis. We first address the fate of promastigotes in the initial phase of the infection, discussing the role of these leishmanicidal molecules in eliminating part of the parasite burden while the adaptive response is still absent (innate immunity). We also discuss the role of these molecules at later phases of the disease, when T_h cells are available (adaptive immunity). In both circumstances, we emphasize the differences among the various *Leishmania* species and mouse strains. The mechanisms that *Leishmania* utilize to evade killing by NO and ROS have been the subject of a recent review and will not be discussed here [39].

2. ROS and NO

Neutrophils and macrophages produce ROS in response to phagocytosis and ligands of pattern recognition receptors (PRRs). The patterns recognized by PRRs can be either of pathogenic origin (pathogen-associated molecular patterns (PAMPs)) or induced by danger patterns (damage-associated molecular patterns (DAMPs)) that signal tissue damage, which are generally hidden from PRRs, such as ATP [40–42]. Moreover, endothelial activation can also induce ROS production by neutrophils [43]. In response to these signals, nicotinamide adenine dinucleotide phosphate- (NADPH-) dependent phagocyte oxidase (Nox2, also known as phox or gp91^{phox}) is assembled, and superoxide is produced from molecular oxygen [44, 45]. Superoxide may be dismutated into hydrogen peroxide, which can, in turn, generate hydroxyl radicals and other ROS. Macrophages produce ROS in higher quantities than neutrophils [43, 46, 47].

NO is also produced by neutrophils and macrophages in response to IFN-γ and a second signal provided by a

PAMP ligand or TNF-α. iNOS expression is induced by these signals. iNOS promotes the oxidation of the guanidino nitrogen of L-arginine, resulting in the production of NO and citrulline [47].

In activated macrophages, superoxide and NO are produced in nearly equimolar quantities and generate peroxynitrite ($ONOO^-$), a free radical that is also highly toxic to pathogens [48].

3. First Encounters—The Neutrophils

As early as 30 seconds after exposure of C57BL/6 mice to *L. major* through the bite of infected sandflies or needle inoculation of promastigotes, the injected area is infiltrated by neutrophils, which has been elegantly visualized by two-photon intravital microscopy [49]. Recruited neutrophils readily phagocytose promastigotes, which remain viable, although it is not known to what extent parasites are taken up or survive. In fact, it has been reported that during the first 24 h, most parasites are localized extracellularly and can be taken up later by macrophages [49]. The above report showed that parasites taken up by the early neutrophil migration are kept alive inside these cells and do not suffer from oxidative stress. However, another study showed that at later time points, neutrophils might play a role in parasite attrition [50], and, within 2 days, parasites inside neutrophils show a wide variation in their morphology from healthy to completely destroyed forms [50]. Killing of intracellular parasites has been identified by severe signs of damage, such as aggregated cytoplasm and extended vacuolization or complete lysis [50], indicating that neutrophils can act as parasite killers within the first few days of infection. Neutrophils act through an array of microbicidal mechanisms, of which the ability to produce NO [51] and ROS [52] are the most studied in leishmaniasis. Indeed, *L. major* has been shown to induce NO production by mouse neutrophils *in vitro* [53] and to stimulate the respiratory burst in mouse [54], rabbit [55], and human [56] neutrophils. Another study, however, showed that *L. major* failed to induce a respiratory burst in human neutrophils, and *L. major*-containing phagosomes did not colocalize with granules involved in superoxide production [57]. However, work by Peters et al. [49] has very eloquently shown that there is no oxidative stress within the first hours of infection.

Inflammatory neutrophils harvested from BALB/c mice four hours after i.p. infection with *L. major* harbor more parasites than C57BL/6 cells, which, in turn, produce considerably higher amounts of NO than BALB/c in response to *L. major* and IFN-γ [53]. In agreement with these data, we have shown that neutrophils from uninfected C57BL/6 mice express much more iNOS and produce more NO than cells from BALB/c mice when stimulated with IFN-γ *in vitro*, indicating that the ability of these cells to be activated to produce NO is inherent to each strain. These data suggest that NO produced by neutrophils may help to control infection with *L. major* in very early disease stages. *In vitro*, however, iNOS expression and NO production can be inhibited in neutrophils from both mouse strains by live, but not dead, promastigotes of *L. major* (our unpublished results).

In BALB/c mice, an iron-induced oxidative burst appears to prevent the growth of *L. major*, protecting the animals from developing the typical large lesions. This oxidative burst has mainly been attributed to neutrophils [58, 59]. However, C57BL/6 resistance and BALB/c susceptibility inversely correlate with the ability of their neutrophils to generate ROS since BALB/c neutrophils produce more ROS than C57BL/6 neutrophils when stimulated with phorbol myristate acetate (PMA). *L. major* has also been shown to inhibit a PMA-induced respiratory burst in neutrophils from both strains of mice (our unpublished results).

Interestingly, the rapid recruitment of neutrophils to *L. major*-induced lesions was previously reported to follow different kinetics in susceptible BALB/c and resistant C57BL/6 mice, which might account for these opposite outcomes. In susceptible mice, almost 100% of the initial cellular infiltrate is composed of neutrophils, half of which is replaced by mononuclear phagocytes in 2-3 days. Neutrophils comprise the other half of the cellular infiltrate for at least 12 days after infection. In contrast, in resistant mice, only about 60% of the initial cellular infiltrate is composed of neutrophils, and the number of these cells drastically decreases to only 1-2% at later time points. In resistant mice, mononuclear phagocytes predominate at later time points, comprising more than 70–80% of the cells [49]. Notably, infection with *L. major* also results in the differentiation of distinct neutrophil populations in BALB/c and C57BL/6 mice. The parasite induces CD49d expression in BALB/c, but not in C57BL/6, neutrophils. The levels of Toll-like receptor (TLR) 2, TLR7, and TLR9 mRNA are significantly higher in C57BL/6 cells than in BALB/c cells. Moreover, C57BL/6, but not BALB/c, neutrophils secrete biologically active IL-12p70 and IL-10. BALB/c neutrophils instead transcribe and secrete high levels of IL-12p40, which forms homodimers with inhibitory activity. In C57BL/6 mice, neutrophils may constitute one of the earliest sources of IL-12, while in BALB/c mice, secretion of IL-12p40 may contribute to impaired early IL-12 signaling [53]. Furthermore, C57BL/6 neutrophils were found to release 2-3-fold more elastase than BALB/c cells, which contributes to parasite killing through activation of TLR4 [60]. These distinct neutrophil phenotypes may thus influence both the early resistance or susceptibility and the development of an *L. major*-specific immune response. The role of these different populations of neutrophils on resistance to parasites through reactive nitrogen and oxygen species production deserves further investigation.

Recently, the interaction of neutrophils and macrophages has been investigated *in vitro* (reviewed in [5]). Dead neutrophils from C57BL/6 mice can activate infected macrophages to kill *L. major*. In this system, activation is mediated by the induction of TNF-α by neutrophil elastase, but NO is not involved in parasite killing. Rather, superoxide is partially responsible for parasite killing, as evidenced by the partial inhibition of this effect when catalase was added to this *in vitro* system [60, 61]. The same results were obtained with dead human neutrophils and *L. amazonensis*-infected human macrophages [62]. In another study, live murine

neutrophils induced killing of *L. braziliensis*, but not *L. major*, by infected macrophages. Superoxide production was detected in this system, and killing of parasites was inhibited by *N*-acetylcysteine, a superoxide scavenger. Killing of *L. braziliensis* by macrophages cocultured with live neutrophils was also independent of NO [63]. Neutrophil-induced killing of *L. amazonensis* by macrophages from resistant and susceptible mouse strains was also described and is mediated by neutrophil elastase, TNF-α, and platelet-activating factor (PAF), but not by NO or reactive oxygen species [64].

In response to pathogens, neutrophils may release the so-called neutrophil extracellular traps (NETs), which are fibrous nets composed of decondensed chromatin, histones, and granule antimicrobial proteins that trap and kill microbes extracellularly [65, 66]. NETs extruded by human neutrophils cultured *in vitro* were shown to kill *L. amazonensis, L. major*, and *L. chagasi*. These NETs were found in lesions from patients. Killing of parasites was found to be mediated mainly by histones [67]. Importantly, NET formation is defective in patients suffering from chronic granulomatous disease, who lack Nox2 activity [68]. In fact, reactive oxygen species are required to initiate NETs. Oxidative stress ruptures neutrophil elastase and mieloperoxidase-containing granules, and neutrophil elastase binds to chromatin and cleaves histones, a reaction that is further enhanced by mieloperoxidase, independent of its enzymatic activity. This enzyme promotes chromatin decondensation, which culminates in NET release due to cellular rupture [69]. The molecular mechanism linking ROS production to chromatin decondensation and binding to antimicrobial proteins is still unknown.

Although several *in vivo* studies have addressed the role of neutrophils during infection with *L. major*, their function in resistance to the parasite is not totally understood and is still a subject of debate. Due to the heterogeneous models used to study the role of neutrophils in experimental leishmaniasis, it is still unknown whether these cells have a protective or pathogenic role. Like other immune responses in murine models, the neutrophil function appears to depend on the species and even the strain of *Leishmania* and the genetic background of mice used as host (thoroughly reviewed in [70]). Hence, even less clear is the *in vivo* role of reactive oxygen and nitrogen species from neutrophils in *Leishmania* resistance or pathology caused by the parasites. However, *in vitro* evidence suggests that ROS from neutrophils are involved in killing of the parasite, suggesting that ROS may be important for resistance to parasites early in infection.

4. Latecomers—The Macrophages

Like neutrophils, macrophages are microbicidal cells that are able to produce NO and ROS [47]. Paradoxically, these cells are also the long-term host cell for *Leishmania*. In experimental leishmaniasis, macrophages are as crucial for parasite survival as for its elimination [71]. The role played by these cells depends on the type of activation and the vulnerability of the parasite to the effector mechanisms.

The mechanism by which macrophages are responsible for resistance to *Leishmania* was first characterized by *in vitro* experiments using murine macrophages infected with *L. major*. In this model, killing of parasites is dependent on the activation of macrophages by IFN-γ and a second signal that triggers TNF-α. This signal is given by amastigotes, promastigotes, or parasite-derived glycoinositolphospholipids (GIPLs) and lipophosphoglycan (LPG), but not by killed cells or cellular lysates. Once these two signals are present, iNOS is induced and NO is produced [72–74]. The clear role of NO in killing *L. major* was established by pharmacological inhibition of the production of NO *in vitro* and by the observation of a higher susceptibility of iNOS knockout mice to infections with *L. major* [16, 74–76]. It was further confirmed by the inability of macrophages from iNOS knockout mice to be activated and kill *L. major* by IFN-γ [77]. Hence, NO clearly has a crucial role in killing of *L. major* by IFN-γ-activated macrophages.

During *L. amazonensis* infection, IFN-γ and TNF-α are not produced at high levels as in *L. major* infection [25, 78]. Therefore, infection of *L. major*-resistant mice with *L. amazonensis* leads to chronic lesions and inefficient control of parasites at the site of infection. IFN-γ-activated macrophages from CBA/J mice infected with either *L. major* or *L. amazonensis* are able to kill the former, but not the latter. When very high concentrations of NO were generated *in vitro*, axenic *L. amazonensis* amastigotes succumbed. In addition, macrophages infected with *L. amazonensis* produce less TNF-α when compared to those infected with *L. major* [79]. However, macrophages infected with either *L. major* or *L. amazonensis* produce similar levels of NO (measured as nitrite in culture supernatants) and express similar levels of iNOS message when activated with IFN-γ [79]. Corroborating these data, we found lower levels of TNF (α and β were measured collectively) from *L. amazonensis*-infected macrophages from C57BL/10 mice than from *L. major*-infected macrophages (Figure 1(a)). In addition, two days after infection in the hind footpad, popliteal lymph node cells from C3H/HeN, C57BL/10 (mouse strains resistant to *L. major*), and BALB/c mice produced more TNF *ex vivo* when infected with *L. major* than with *L. amazonensis* (Figure 1(b)). Interestingly, *L. amazonensis*-infected CBA/J macrophages also produce less reactive oxygen species than *L. major*-infected cells [79], which could be, in part, responsible for the different abilities of macrophages to kill these two species of *Leishmania*. The mechanism by which *L. amazonensis* resists killing remains unknown.

Even more intriguing is the observation that low doses of IFN-γ actually promote amastigote growth within macrophages [22]. In accordance with this observation, at later stages of infection, increased amounts of NO were found in the more susceptible BALB/c mice than in C57BL/6 mice infected with *L. amazonensis* as lesions progressed and parasites expanded because C57BL/6 mice partially control lesions and parasite growth [80].

IFN-γ-activated macrophages represent the host-parasite interaction in which T cells are already producing a large amount of this cytokine. During the first 2 days after infection with *L. major*, nearly all macrophages recruited to

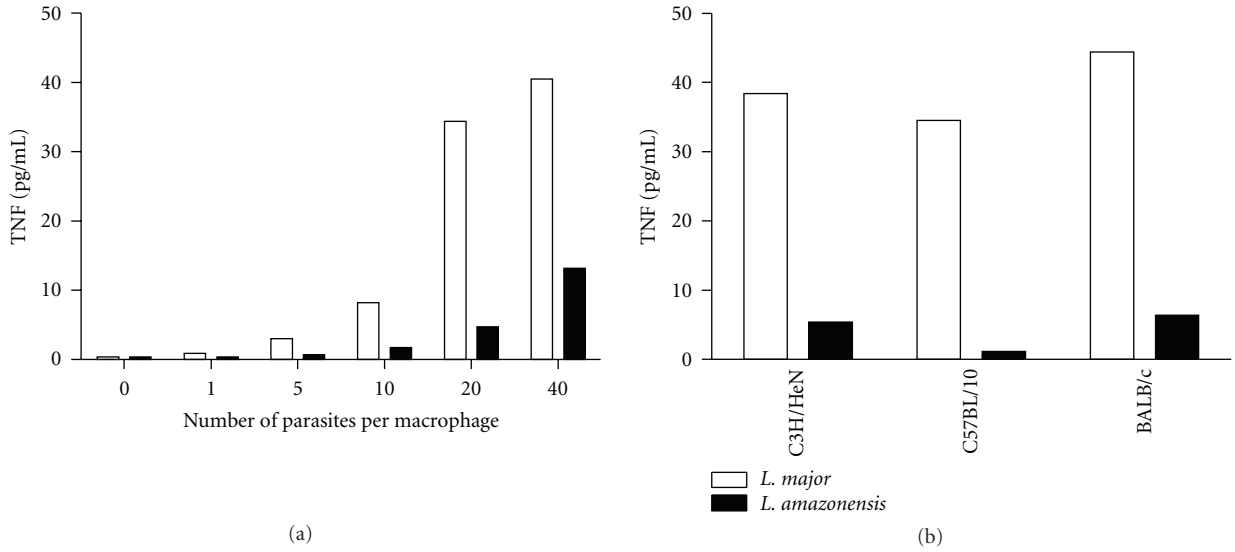

Figure 1: Infection with *L. major* induces more TNF than infection with *L. amazonensis*. (a) TNF production by inflammatory macrophages from C57BL/10, mice infected *in vitro* with *L. major* or *L. amazonensis*. (b) Production of TNF *ex vivo* by lymph node cells from C3H/HeN, C57BL/10 and BALB/c mice infected with *L. major* or *L. amazonensis*, 2 days after infection. A biological assay that does not distinguish between TNF-α or TNF-β was used in these experiments. These are representative experiments of more than five performed experiments (L. Q. Vieira and P. Scott, unpublished).

the site of infection contain phagocytosed parasites, both in C57BL/6 and in BALB/c mice. However, the percentage of cells (mostly neutrophils and mononuclear phagocytes) containing intact parasites in BALB/c mice is higher than that in C57BL/6 cells (mostly mononuclear cells), and the elimination of parasites from the site of infection is higher in resistant mice [50]. This suggests that parasites may also be killed by tissue mononuclear cells well before the onset of a T-cell response. Whether this killing is mediated by reactive oxygen and nitrogen species remains unknown.

Isolated macrophages from C57BL/6 mice produce more NO than macrophages from susceptible strains when stimulated with IFN-γ [81–84], TNF-α [81, 85], or LPS [83, 85–89]. This is an interesting but poorly explored aspect of the murine models of resistance/susceptibility to microbial infections, which is clearly independent of the development of an adaptive Th1 or Th2 response. Mills et al. [90] systematically tested this observation and generalized it to other strains of mice. They showed that macrophages from strains that are typical Th1 responders (termed M-1) or typical Th2 responders (termed M-2) differ qualitatively in their ability to be activated, as measured by their arginine metabolic programs. M-2 macrophages from BALB/c mice (prototypes of Th2 responders) stimulated with a particular concentration of LPS not only produce little or no NO, but increase arginine metabolism to ornithine. In contrast, M-1 cells from C57BL/6 mice (prototypes of Th1 responders) generate a strong NO and citrulline response and appear to decrease their production of ornithine.

We investigated the molecular basis of the differential production of NO by macrophages from mice with resistant or susceptible phenotypes to *L. major* by *in vitro* stimulation with IFN-γ and LPS. We have shown that M-1 macrophages show a remarkably strong expression of the enzyme iNOS upon stimulation when compared to M-2 cells [84]. The accumulation of iNOS mRNA is also higher in M-1 cells. Interestingly, however, we found that the accumulation of the iNOS protein is more dramatic than the accumulation of iNOS mRNA. The accumulation of both iNOS mRNA and protein is not a consequence of a higher stability of the molecule. The data showed that iNOS gene expression is differentially regulated in M-1 and M-2 macrophages and suggested that it is transcribed and translated at different rates in these two types of cells [84]. Recent results from our group indicate that the higher iNOS expression in M-1 macrophages may be multifactorial and may be regulated by higher levels of TNF-α, IL-12, and IFN-β (unpublished data).

The intrinsic differential sensitivity to IFN-γ and LPS of M-1 or M-2 cells has led to two important observations regarding the *in vivo* infection.

(1) Small amounts of IFN-γ (from NK, NKT, or γ/δ T cells) or other pathogen-derived inducers may induce M-1, but not M-2 cells, to kill the pathogen through NO, before T cells differentiate into the IFN-γ-Th1 subpopulation. In fact, larger numbers of *L. major* are found in iNOS-deficient macrophages than in wild-type macrophages 72 hours after infection, indicating that some NO is produced by macrophages that have not been activated with IFN-γ and that NO, even if not detectable, exerts some control of parasite growth [75, 77]. Further evidence of a NO-dependent T_h-cell-independent mechanism was obtained when resting human macrophages were infected with NO-susceptible and NO-resistant *L. amazonensis* and *L. braziliensis* isolates and selected *in vitro* with increasing concentrations of $NaNO_2$: NO-resistant parasites grew better in resting macrophages than the NO-susceptible isolates [91].

(2) Activated M-1 and M-2 cells can distinctly affect subsequent production of Th1-dominant or Th2-dominant cytokines (IFN-γ or TGF-β1, resp.), positioning macrophages as key performers in directing the Th1 or Th2 outcome. M-1 and M-2 macrophages differentially influence the Th lymphocyte response, and how macrophages are stimulated determines the route that Th responses will take [90]. These observations indicate that macrophages may contribute to the outcome of an immune response through mechanisms other than by acting as established NO-producing cells and that their role in determining the resistant/susceptible phenotype in mice may be significant. M-1 macrophages not only can mount an early (innate) resistance, but also can consolidate the status of resistance by favoring a Th1 adaptive response.

In addition to NO, ROS are considered to be a major macrophage effector mechanism induced by IFN-γ to control infections. Upon bacteria or other pathogen engulfment by a phagocytic cell, ROS are rapidly produced by NADPH oxidase, an enzymatic complex comprised of membrane bound ($p22^{phox}$ and $gp91^{phox}$) and cytosolic ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac-1/2) proteins [45, 92], which may be assembled after TLR stimulation by bacterial products via MyD88-dependent p38 MAPK activation [93].

Macrophages [54, 76] and neutrophils [54] produce ROS in response to *Leishmania in vitro*. Killing of *L. major* by IFN-γ-activated macrophages is dependent on NO production, but not on the production of superoxide or peroxynitrite [76]. Lesions in Nox2 knockout mice [94] (Nox2 mice are genetically deficient in the NADPH-dependent phagocyte oxidase. These mice were originally described as a model for chronic granulomatous disease and are more susceptible to bacterial infection, and neither neutrophils nor macrophages present respiratory burst oxidase activity [94].) infected with *L. major* are similar to those in wild-type C57BL6 mice. Nox2 knockout mice control *L. major* at the site of infection at early time points, but display an unexpected reactivation of *L. major* infection after long periods of observation (more than 200 days of infection). Further, they show deficient control of parasite replication in draining lymph nodes and spleens, suggesting that Nox2 is important for the control of *L. major in vivo* at later times of infection by preventing visceralization [54]. The participation of ROS in killing of *L. amazonensis* by mouse [95, 96] or human [97] macrophages has been reported. Our preliminary data suggest that macrophages from Nox2 knockout mice behave similarly to macrophages from wild-type mice when infected with *L. amazonensis*. Moreover, similar to infection with *L. major*, Nox2 knockout mice control parasites at the site of infection as well as wild-type mice (Figure 2). Surprisingly, at earlier times of infection, lesions are larger in Nox2 knockout mice, and, at later times of infection, they become smaller than in wild-type mice (Figure 2(a)). This indicates that the differences in Ros activity on macrophage behavior at different stages of infection may be due to differences in the inflammatory infiltrate. The contradictions between the *in vitro* evidence for a role for ROS in resistance to *L. amazonensis* and *in vivo* data remain to be explained.

Although BALB/c mice are the prototype model of susceptibility to most species of *Leishmania* (such as *L. major* and *L. amazonensis*), *L. braziliensis* [27, 98] and *L. guyanensis* [99] do not cause large skin lesions in this mouse strain. Our studies using *L. guyanensis* have shown that BALB/c mice develop minor or no lesions, do not enable parasite replication, and do not die of the infection. In addition, *L. guyanensis* [99] and *L. braziliensis* [100], unlike *L. amazonensis*, fail to survive within nonactivated peritoneal macrophages *in vitro*. *In vitro* infection of BALB/c macrophages with *L. guyanensis* does not activate the production of NO; instead, it activates a respiratory burst that is exceptionally higher than that activated by infection with *L. amazonensis*. We have further shown that the production of ROS is responsible for the elimination of *L. guyanensis* by macrophages. We have also shown that *L. guyanensis* amastigotes die inside BALB/c macrophages through an apoptosis-like process mediated by parasite-induced ROS [99]. These findings demonstrate an important killing mechanism of *L. guyanensis* amastigotes. ROS are probably involved in resistance to infection with this species because mice that are unable to activate the respiratory burst by the regular administration of apocynin, an inhibitor of NADPH oxidase, do not control the infection as in untreated animals (our preliminary results). Together, our results suggest that the elimination of *L. guyanensis in vivo* may occur in early infection due to ROS production, before the development of an adaptive T_h response.

There is evidence that peroxynitrite ($ONOO^-$) is not involved in the killing of *L. major* [54, 76], but the role of this important oxidant has not been thoroughly explored. In contrast, the production of nitric oxide and $ONOO^-$ has been shown during infection with *L. amazonensis* in BALB/c (more susceptible to infection) and C57BL/6 mice (more resistant to infection). The production of nitric oxide *in vivo* was detected as the nitrosyl hemoglobin complex by electron paramagnetic resonance analysis of nitrosyl hemoglobin in blood drawn from mice and in infected footpads at several time points, and $ONOO^-$ formation was inferred from immunodetection of nitrotyrosine [101, 102]. C57BL/6 mice presented higher levels of nitrosyl complexes than BALB/c mice at 6 weeks of infection, at which point lesions became chronic in this partially resistant mouse strain. Nitrosyl complexes increased in BALB/c mice, which was dependent on lesion size. iNOS and nitrotyrosine-containing complexes colocalize in lesion macrophages from both mouse strains, and the most probable agent of protein nitration is $ONOO^-$ [102]. Peroxynitrite killed *L. amazonensis* axenic amastigotes *in vitro* more efficiently than nitric oxide [102]. The authors proposed that in the susceptible mouse strain, $ONOO^-$ is involved in tissue damage. It is possible that the delayed production of $ONOO^-$ impairs the capacity of BALB/c mice to control *L. amazonensis*. Treatment of C57BL/6 mice with Tempol, a stable cyclic nitroxide radical that protects cells from damage due to oxidative stress, promoted larger lesions, parasite growth, and lower levels of nitric oxide products and nitrotyrosine [103]. Albeit transient, this effect of Tempol provides further evidence that $ONOO^-$ is involved in the control of *L. amazonensis in vivo*.

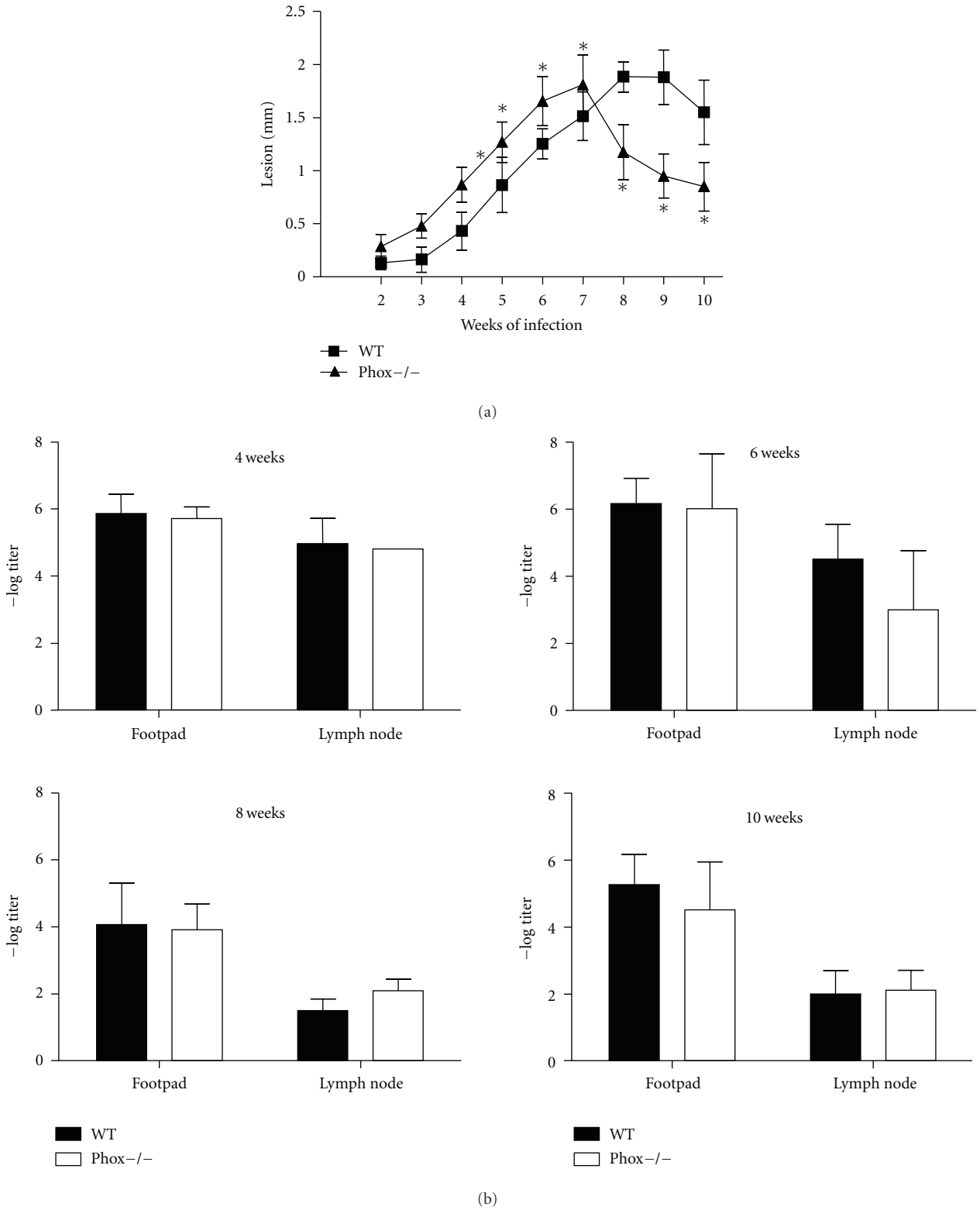

Figure 2: Course of infection with *L. amazonensis* in wild-type C57BL/6 and Nox2 knockout mice (a) and parasite quantitation using a limiting dilution analysis (b). *indicates statistical difference by Student's *t* test, $P < 0.05$ (E. H. Roma and J. P. Macedo, unpublished).

5. Concluding Remarks

The role of reactive oxygen and nitrogen species in killing of *Leishmania* has been the subject of many studies, but there is still much that is not understood. The following questions remain: why do some species of parasites resist oxidative stress? Why do cells that can kill parasites with reactive species harbor live parasites? Is there some attrition when parasites enter neutrophils and macrophages? What is the role of peroxinitrite? What is the reason for the differences in the oxidative responses among different species of parasites? What is the role of reactive oxygen and nitrogen species in the inflammatory response? Collective efforts to fully comprehend the mechanisms that produce disease upon infection with *Leishmania* and the strategies hosts employ to avoid them have been made. However, leishmaniasis persists without safe treatments or effective vaccines. Perhaps the recent attention paid to components of the innate immune system might help to unravel this complex parasite-host relationship.

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Review Article

Toll-Like Receptors in *Leishmania* Infections: Guardians or Promoters?

Marilia S. Faria, Flavia C. G. Reis, and Ana Paula C. A. Lima

Instituto de Biofisica Carlos Chagas Filho, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro, Bloco G, CCS, Ilha do Fundão, Cidade Universitária, Rio de Janeiro 21941-902, RJ, Brazil

Correspondence should be addressed to Ana Paula C. A. Lima, anapaula@biof.ufrj.br

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Protozoa of the genus *Leishmania* cause a wide variety of pathologies ranging from self-healing skin lesions to visceral damage, depending on the parasite species. The outcome of infection depends on the quality of the adaptive immune response, which is determined by parasite factors and the host genetic background. Innate responses, resulting in the generation of mediators with anti-leishmanial activity, contribute to parasite control and help the development of efficient adaptive responses. Among those, the potential contribution of members of the Toll-like receptors (TLRs) family in the control of *Leishmania* infections started to be investigated about a decade ago. Although most studies appoint a protective role for TLRs, there is growing evidence that in some cases, TLRs facilitate infection. This review highlights recent advances in TLR function during *Leishmania* infections and discusses their potential role in restraining parasite growth versus yielding disease.

1. Introduction

Infections with parasitic protozoa have been a long-term health issue in the tropical and subtropical regions of the globe. Among those, diseases caused by infections with microbes of the *Leishmania* genus are one of the most widespread infirmities. *Leishmania* parasites are transmitted to the vertebrate host by the sand fly bite that injects the infective metacyclic forms under the skin. The flagellated parasites are rapidly engulfed by phagocytic cells either resident or recruited to the wound site (i.e., neutrophils, macrophages and dendritic cells), reviewed in [1]. While the passage through neutrophils is believed to be transient, serving as a temporary safe hideaway, the parasites are able to establish productive infections in macrophages, where they differentiate to amastigotes and replicate inside the parasitophorous vacuole. The pathology resulting from the infection is related to the parasite species that can either induce cutaneous (i.e., *L. major*, *L. mexicana, L. guyanensis*), mucocutaneous (i.e., *L. amazonensis*, *L. braziliensis*), or visceral leishmaniasis (*L. donovani*, *L. chagasi*). The outcomes of infections are complex, depending not only on the parasite species but also on the immune status of the host. As an example, although *L. amazonensis* is primarily associated with cutaneous manifestations, it can also provoke mucocutaneous and/or diffuse cutaneous leishmaniasis. Infections with *L. panamensis* or *L. braziliensis* can generate a persistent hyperinflammatory response, where a mixed T-helper 1 (Th1)/T-helper 2 (Th2) response is observed, leading to nonresolving lesions in humans [2, 3]. *L. guyanensis* can also migrate away from the primary infection site, generating distal secondary lesions similar to those observed with *L. braziliensis* [4].

Cutaneous leishmaniasis comprises self-healing skin ulcers in immunocompetent individuals, however, parasite persistence remains, helping to maintain protective immunity [5]. Mucocutaneous leishmaniasis involves parasitic dissemination to the nasopharyngeal area, leading to destructive secondary lesions. It is characterized by a persistent inflammatory response associated with increased expression of proinflammatory mediators that are crucial for the recruitment of cells to the site of infection [6]. About 5 to 10% of individuals asymptomatic or with resolved cutaneous lesions may develop mucocutaneous lesions [7, 8]. Although the development of a type-1 immune response is crucial for

the control of parasites, unresolved inflammation can derive from lack of an appropriate modulation of those responses in the case of tegumentary leishmaniasis (reviewed in [9]). On the other hand, the progression of visceral leishmaniasis is often accompanied by a decay in the type-1 response [10]. In this scenario, increasing immunosuppression contributes to disease progression not only due to the presence of immunoregulatory cytokines but also because of partial destruction of lymphoid tissues [11, 12].

The mouse model for cutaneous leishmaniasis caused by *L. major* has been largely explored over the years, providing a solid body of data defining the immunological mechanisms involved in mounting innate and adaptive protective responses (reviewed in [13]). However, those findings cannot be readily extrapolated for models of infections by other *Leishmania* species, rendering it indispensable to investigate the parameters of immunological responses for each combination of parasite species, host cell, and/or animal model for the disease. Although adaptive immunity is essential for the resolution of infection, there is growing evidence that innate mechanisms make an important contribution to the antiparasitic defenses.

Innate responses develop after the initial sensing of invading microbes, leading to the production of effector molecules that contribute to contain initial infection and to mount the subsequent adaptive immune response [14]. The early immune reaction against *L. major*, *L. braziliensis,* and *L. infantum* during experimental infections has been analyzed in detail, revealing, for example, differential susceptibility to nitric oxide (NO) and reactive oxygen species (ROS) in phagocytes located at distinct organs. While the production of NO is required for the leishmanicidal activity against *L. major* [15] and *L. braziliensis* [16] in the skin of infected mice, it is dispensable in the spleen and mildly important in the lymph node. Although NO was believed to be important to the control of *L. donovani* in the liver and spleen of mice [17], it was later established that neither iNOS nor NADPH oxidase (phox) is essential to restrict parasite replication in the liver [18, 19]. The relative importance of such mediators has been recently covered by Liese and coworkers [14] and will not be explored in this review.

The potential contribution of Toll-like receptors in fighting parasitic infections has gained attention in the last decade. We will cover the main findings regarding the interplay between Toll-mediated responses and *Leishmania* infections.

2. Toll-Like Receptors (TLRs) and *Leishmania*

Toll-like receptors (TLRs) are hallmarks of cellular receptors that recognize pathogen-associated molecules and participate in innate responses to infections (reviewed in [10]). There are currently 13 mammalian TLRs described, while TLRs 1–9 are functionally conserved between humans and mice, TLR10 seems to be functional in humans but divergent at the C-terminus in the mouse, rendering it inoperant. TLR11 is functional in the mouse but truncated in humans. The conserved TLRs can be divided into extracellular: TLR1-2, TLR4-6, and TLR11 [20] or intracellular: TLR3, TLR7-9 and TLR13 [21], and those receptors recognize specific groups of ligands either at the cell surface or in the endosomal compartment, respectively [22]. Each TLR detects distinct sets of molecules from viruses, bacteria, fungi, and parasites, and upon binding, they recruit different adaptor proteins such as MyD88 or TRIF [22]. TLRs initiate innate responses in a variety of ways, leading to the production of inflammatory cytokines by macrophages and different subtypes of dendritic cells (DCs) and of type I interferons (IFN) by inflammatory monocytes, macrophages, and DCs [22]. Neutrophils also express the majority of TLR family members and several coreceptors but lack intracellular TLR3 and TLR7 (reviewed in [23]). In those cells, TLR activation often leads to the generation of reactive oxygen species (ROS), cytokine production, increased cellular survival, receptor expression, and phagocytosis [24].

The microbial molecules recognized by TLRs are conserved polymers, such as bacterial lipopolysaccharides (LPSs), peptidoglycans, unmethylated bacterial DNA, and double-strand viral RNA, among others. Since protozoans lack most of these structures, TLRs must recognize other groups of molecules in order to sense those microbes. The recognition of *Trypanosoma cruzi* tGPI (glicosylphosphatidylinositol anchor) by TLR2 [35] and of glycoinositolphospholipids by TLR4 [25], of *Plasmodium* hemozoin by TLR9 [26], and of the profilin-like molecule of *Toxoplasma gondii* by TLR11 [30] are such examples.

The phagocytosis of *Leishmania* by macrophages, contrary to the observed with other pathogens, is marked by the absence of many proinflammatory cytokines [27]. Furthermore, infected macrophages become unresponsive to subsequent challenges with the TLR4 ligand LPS, a feature that is associated with parasite phosphoglycans [28, 36]. Since TLR recognition is often associated with the production of proinflammatory cytokines and with the generation of additional effector molecules, it is unquestionably important to determine the implications of TLR activation during *Leishmania* infections. A few *Leishmania*-derived molecules have been reported to activate TLRs, and the majority of the studies to date focused on the activation of TLR2, TLR4, and TLR9.

2.1. TLR2 and MyD88. Initial experiments suggesting that *Leishmania* induces TLR-mediated responses came from studies using cells lacking the adaptor molecule MyD88 [29]. Those analyses showed that *L. major* activates the promoter region of IL-1α, but not of IL-6, IL-8, or IL-10, through MyD88-dependent pathway in macrophages [29]. It was then reported that MyD88-dependent pathways are required for the development of the protective IL-12-mediated Th1 response against the *L. major* in C57BL6 resistant mice, since MyD88$^{-/-}$mice infected with *L. major* developed a nonprotective Th2 response [37]. The animals had enlarged nonhealing lesions and low IL-12 plasma levels suggesting that TLR-mediated responses were important to develop effective antiparasite immunity. At the same time, it was revealed that the higher susceptibility of MyD88$^{-/-}$ mice to

L. major infections correlated with elevated levels of IL-4, despite the lack of ulcerating lesions [38]. *L. donovani*, *L. braziliensis*, *L. major,* and *L. mexicana* induce the maturation of DCs *in vivo* and the observation that DC maturation was attenuated in MyD88$^{-/-}$ mice infected with *L. donovani* implied the involvement of TLRs in DC maturation and T-cell priming [39]. Although the studies using MyD88-deficient mice suggested that TLRs influence the adaptive immune response during *Leishmania* infections, they did not offer undisputable evidence for the participation of TLRs, mainly because MyD88 can also function as an adaptor protein to the IL-1 receptor [40]. Furthermore, there are pathways downstream of TLR3 and TLR4 which are independent of MyD88, such as the activation of TLR4 through IRF3 leading to the production of type-1 interferons [41]. Therefore, it became crucial to prove the direct involvement of TLRs for the responses observed in the infection models described above.

The direct activation of TLR2 by *Leishmania* components was subsequently reported. Purified *L. major* lipophosphoglycan (LPG) induced the upregulation and stimulation of TLR2 on human NK cells, with additional enhancement of TNF-α and IFN-γ [42]. LPGs of *L. major*, *L. mexicana*, *L. aethiopica*, and *L. tropica* were defined as TLR2 ligands in studies using murine macrophages, although the stimulation with *L. tropica* LPG was only marginal [34]. At this point, the possibility that species- or strain-specific structural differences in the phosphoglycan chain could contribute to receptor recognition or activation was raised. Those studies revealed that the induction of TNF-α synthesis by *L. major* LPG required the presence of the lipid anchor and of functional MyD88. Intriguingly, LPG also induced the expression of suppressors of the cytokine signaling family proteins SOCS-1 and SOCS-3 [34]. The finding that negative modulators of cytokine synthesis were also induced by LPG indicated that TLR2 stimulation by *L. major* can lead to both positive and negative inflammatory signals. The fact that SOCS1 itself directly downmodulates TLR4 signaling pathways [43] illustrates how the initial stimulation of TLR2 by *L. major* can ultimately lead to the attenuation of further TLR responses.

More recently, it was shown that LPG stimulates cytokine production by human peripheral blood mononuclear cells via TLR2 as well [31]. Although cellular LPG isolated from promastigotes is structurally similar to soluble LPG present in culture supernatants, they differ in the average number of phosphorylated oligosaccharide repeat units and in glycan composition [32]. In the above-mentioned study, while both forms of LPG induced the production of ROS in a TLR-2-dependent manner, Th1-promoting cytokines were induced solely by soluble LPG, leading to the proposal that the first encounter and recognition of *L. major* by membrane-derived LPG after interaction with TLR2 provides a cytokine milieu for consequent Th2 differentiation [31]. Later on, the same authors provided evidence that the induction of NO production by macrophage cell lines was achieved with both LPG forms, and was dependent on TLR2, but not on TLR4 signaling [44]. More recently, synthetic oligosaccharides based on the LPG structure were shown to induce IL-12 and Th1 responses *in vivo* through TLR2, corroborating that TLR2 stimulation by *Leishmania* LPG potentiates the inflammatory responses in mice [33]. Those findings assign a protective role for TLR2 and MyD88 during *L. major* infection, in particular, TLR2 seems required to mount an effective Th1 response (Figure 1, left pannel).

The consequences of TLR2 activation to the control of leishmaniasis was further verified using the TLR2 ligand, arabinosylated lipoarabinomannan (Ara-LAM), in a Balb/c model of visceral leishmaniasis [45]. Ara-LAM induced the expression of TLR2 in macrophages infected with *L. donovani in vitro*, which was accompanied by the production of NO and of proinflammatory cytokines. Mice pre-treated with Ara-LAM and subsequently challenged with *L. donovani* showed around 80% reduction of infection in the liver and the spleen, paralleled by a strong Th1 response. However, one should take care at interpreting those results since Ara-LAM could exert its effect *in vivo* through additional mechanisms besides TLR2 activation.

The parasite can also modulate the expression of TLRs, interfering with their availability and/or the subsequent quality of the responses mediated by those receptors. For example, two antigens isolated from *L. donovani* amastigotes were shown to upregulate TLR2 expression in RAW264 macrophages [49]. The 65 and 98 kDa proteins induced elevated levels of MAPK p38, as well as its phosphorylation in relation to that of ERK1/2, leading the authors to conclude that they induced the activation of TLR signaling proteins. However, one should interpret those conclusions with caution, specific controls for the activation of TLR2 (i.e., neutralizing antibodies or cells derived from knock-out mice) were lacking in this study. Furthermore, the contribution of minor nonproteinaceous contaminants in the preparations cannot be excluded considering that the antigens were isolated by elution from SDS-PAGE gels and were not further purified. In another study, while verifying the expression of TLRs during the infection of Balb/c mice with *L. chagasi*, attempts were made to correlate upregulation of TLR expression with a potential role for those receptors in generating inflammatory versus anti-inflammatory responses [50]. The authors observed upregulation of TLR2 and TLR4 from day 1 to day 28 postinfection, and increased expression correlated with higher mRNA levels for TNFα, IL-17, IL-10, and TGFβ during early infection. On the other hand, there was an inverse correlation between the expression of TLR2-4 and that of IL-12 or IFN-γ, and parasite load, leading to the proposal that those TLRs are involved in the recognition of the parasite during visceral leishmaniasis. However, while most of those studies evaluated the induction of cytokine mRNA and of mRNA for TLRs, they did not provide direct evidence that TLRs are required for the induction of cytokine synthesis. Increased expression of TLRs might have occurred as a consequence of the inflammatory environment in the spleen, making it difficult to evaluate to what extent the activation of TLRs is implicated in those findings.

L. panamensis was found to induce upregulation of TLR1, TLR2, TLR3, and TLR4 in human primary macrophages [51]. The activities of TLR4 and TLR3 correlated with

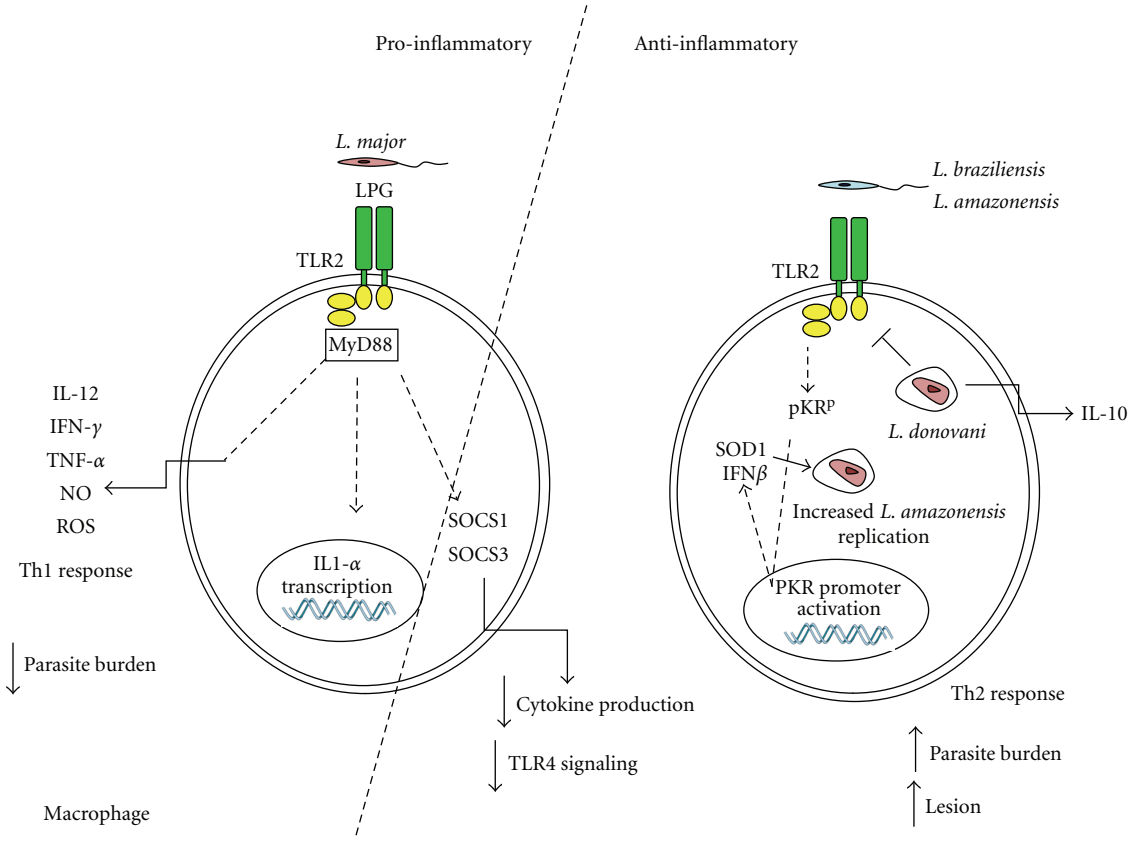

Figure 1: Model for the activation of TLR2 by *Leishmania sp.*, leading to a proinflammatory (left), or anti-inflammatory arm (centre and right). In early studies, *L. major* stimulated the transcription of IL1α through a MyD88-dependent pathway, but not the IL1α production at the protein level, suggesting the triggering of anti-inflammatory stimuli negatively controlling IL1-α production [22]. LPG or additional undefined TLR2 ligands promote the synthesis of cytokines, nitric oxide (NO), and reactive oxygen species (ROS) that are related to parasite killing and to the development of a protective Th1 response, through MyD-88-dependent pathways [22–29]. *L. major* LPG also induces the production of suppressors of the cytokine signaling family proteins SOCS-1 and SOCS-3 [26], whose activities are associated with diminished cytokine production and prevention of TLR4 signaling [30]. Lack of TLR2 increased the resistance to infections with *L. braziliensis* [31] and *L. amazonensis* [32] decreased lesion formation and parasite burdens, suggesting that TLR2 is required for disease promotion. In RAW macrophages, the infection with *L. amazonensis* promotes the phosphorylation of PKR and the activation of the PKR promoter and enhances the synthesis of both PKR and of type 1-IFNs, and those events require TLR2 [33]. The levels of SOD1 expression are elevated in association with PKR activation and IFNβ production, resulting in increased parasite replication [33]. *L. donovani* downmodulates TLR2 responses in macrophages by inhibiting MAPp38 kinase, leading to IL10 production [34]. The dashed lines indicate that intermediate steps of the pathways are either not identified or not represented in the figure.

TNF-α secretion and the leishmanicidal activity of those macrophages [51]. A global analysis of gene expression in IFN-γ-treated THP-1 macrophages infected with *L. major* showed that genes related to TLR signaling are also differentially expressed upon infection [46], revealing how the parasite could interfere with the capacity of the host cell to respond via TLR-mediated routes. *L. major* can also induce the expression of TLR2, TLR7, and TLR9 in polymorphic nuclear cells (PMNs) from C57BL6 mice [52], evidencing that infection provokes alterations in the levels of several TLRs in multiple cells of the innate system.

Additional parasite molecules were implicated in TLR2 activation. The *L. infantum* protein related to the silent information regulator 2 (SIR2) family leads to the proliferation of activated B lymphocytes, causing increased expression of major histocompatibility complex (MHC) II and the costimulatory molecules CD40 and CD86, in a TLR2-dependent fashion [53]. The maturation of DCs induced by SIR2, accompanied by the secretion of IL12 and TNF, was also dependent on TLR2, while the activation of macrophages still took place in the absence of TLR2.

Other studies suggest that *Leishmania* downregulates TLR2-mediated responses and attempted to dissect some of the downstream molecular pathways involved therein. The infection of human THP1-derived macrophages by *L. donovani in vitro* suppresses TLR2 and TLR4-stimulated IL-12 release, with an increase in IL-10 production, through parasite-dependent contact [54]. Parasites were shown to negatively modulate the TLR2-stimulated signaling pathway by suppressing MAPKp38 phosphorylation and activating extracellular regulated kinase (ERK)1/2 phosphorylation [54]. It was also reported that *L. donovani*, *L. mexicana*,

and *L. major* exploit the macrophage tyrosine phosphatase SHP-1 to inactivate kinases involved in TLR signaling [55]. The infection of bone-marrow-derived macrophages *in vitro* leads to IRAK inactivation, impairing LPS-mediated activation as well as macrophage function [55].

Studies using TLR2-deficient mice revealed a somewhat contrasting scenarium (Figure 1, right pannel). TLR2 was necessary for the development of lesions in mice infected with *L. braziliensis* [56]. While MyD88$^{-/-}$ mice developed larger and prolonged lesions compared to those in control mice, the lack of TLR2 resulted in enhanced DC activation and increased IL-12 production after infection. *L. braziliensis*-infected TLR2-deficient DCs were more competent in priming naïve CD4 T cells *in vitro*, correlating with increased IFN-γ production *in vivo* and enhanced resistance to infection. More recently, it was reported that TLR2-deficient mice are also less susceptible to the infection with *L. amazonensis* than the wild-type C57BL6 counterpart, as evidenced by lower parasite burden and reduced recruitment of inflammatory cells in the first weeks of infection [57]. At the same time, it was described that the activation of the protein kinase PKR during the infection of macrophages by *L. amazonensis* is crucial for parasite survival [58]. Finally, The engagement of PKR and the subsequent production of type-1 IFNs and of superoxide dismutase (SOD-1) were necessary for effective parasite growth and dependent on TLR2, indicating that this TLR is required for the successful infection of macrophages by *L. amazonensis* [47]. Those findings suggest that TLR2 plays a role in facilitating the establishment of the disease, depending on the *Leishmania* species in question.

2.2. TLR4. Besides the direct activation of TLRs by parasite molecules, the engagement of those receptors indirectly by nonparasite ligands during phagocytosis could influence the outcome of infection. Studies using TLR4$^{-/-}$ mice indicated that this receptor plays a protective role in *L. major* infections [48]. The authors observed diminished parasite load at the skin lesions of infected mice at the initial stages of infection, that is, 24 h, and found increased parasite survival in host cells from TLR4-deficient mice, which correlated with a higher activity of arginase [48]. The same group subsequently showed that the lack of TLR4 results in increased parasite growth during both the innate and adaptive phase of the immune response and in delayed healing of the cutaneous lesions [59]. However, TLR4 does not seem important to define the range of chemokines produced in the skin or in the draining lymph nodes of mice infected with *L. major* [60]. In agreement with a protective role for TLR4, it was described that the lack of SLAM, a cell surface receptor of macrophages that regulates TLR4-transduced signals, increased the susceptibility of mice to *L. major* infections [61]. Importantly, this phenotype was attributed to defective macrophage function, suggesting that the activation of host cell TLR4 contributes to the control of parasite growth *in vivo*. The first potential TLR4 ligand present in *Leishmania* was described a few years ago in *L. pifanoi* amastigotes and consists of a proteoglycolipid complex (P8) composed of a cysteine and serine metalloprotease, host-derived ApoE, and four glycolipids [62, 63]. Stimulation of macrophages with P8 or its isolated glycolipids provoked the synthesis of a range of proinflammatory cytokines, including IL1β and TNFα and the responses to intact P8 were dependent on TLR4, MD2, CD14, and MyD88 [62] (Figure 2, left). However, the exact molecular entity serving as the TLR4 ligand in P8 still remains to be determined. More recently, TLR4 was implicated in the mechanism underlying the inhibition of IL-12 production in LPS-treated macrophages infected with *L. mexicana*. Metacyclic promastigotes were found to greatly increase the phosphorylation of the three major MAP kinases, ERK, p38, and JNK, in a manner dependent on TLR4 but not on TLR2 [64]. Parasites prolonged the induction of iNOS or COX-2 expression in LPS-stimulated macrophages, enhanced PGE2 and NO production, and increased the expression of arginase-1. The induction of iNOS, COX-2, and arginase-1 were also dependent on TLR4, supporting the hypothesis that this TLR plays an anti-inflammatory role in macrophages during *L. mexicana* infections, ultimately preventing IL-12 production.

On the other hand, the anti-leishmanial role of TLR4 might be exerted by multiple downstream effector molecules. For example, macrophages of the mouse strain SPRE/Ei, which is resistant to LPS, have normal MyD88-mediated signaling pathways but are defective in the production of type-1 IFNs [65]. Those mice were found to be highly susceptible to *L. major*, a feature that was attributed to the poor induction of IFNβ-dependent genes. These observations suggest that type-1 IFNs pose as effectors that, through a paracrine/autocrine loop, contribute to parasite control downstream of TLR4 stimuli. The cross-talk between TLR4 and additional receptors should also be taken into consideration when viewing anti-leishmanial properties attributed to this TLR. For example, in macrophages, the stimulation of the glucocorticoid receptor by progesterone downmodulates TLR4-induced NO and IL-12 production and reduces the killing of *L. donovani* by activated macrophages [66] Likewise, complement-derived C5a negatively regulates TLR4-induced IL-12, IL-23, and IL-27 in macrophages, leading to decreased Th1 responses *in vivo* [67]. It was found that Th1- enhanced immunity in C5a-receptor$^{-/-}$ mice confers protection against an *L. major* challenge [67], exemplifying how other receptors of innate immunity might affect susceptibility to leishmania infections through the modulation of TLR responses.

The engagement of TLRs during the interaction between different cells of the innate system can also dictate the fate of parasites in infected macrophages. It was reported that apoptotic neutrophils from C57B6 mice induce the production of TNF-α by *L. major*-infected macrophages *in vitro*, leading to parasite elimination [68]. Later on, the activation of macrophage TLR4 by the neutrophil-derived serine protease, neutrophil elastase (NE), was proposed as the underlying mechanism leading to parasite death [69] (Figure 2, scheme c). More recently, the phagocytosis of apoptotic neutrophils by bone-marrow-derived macrophages in a proinflammatory context *in vitro* was found to induce a stable M2b phenotype, rendering those macrophages more permissive to

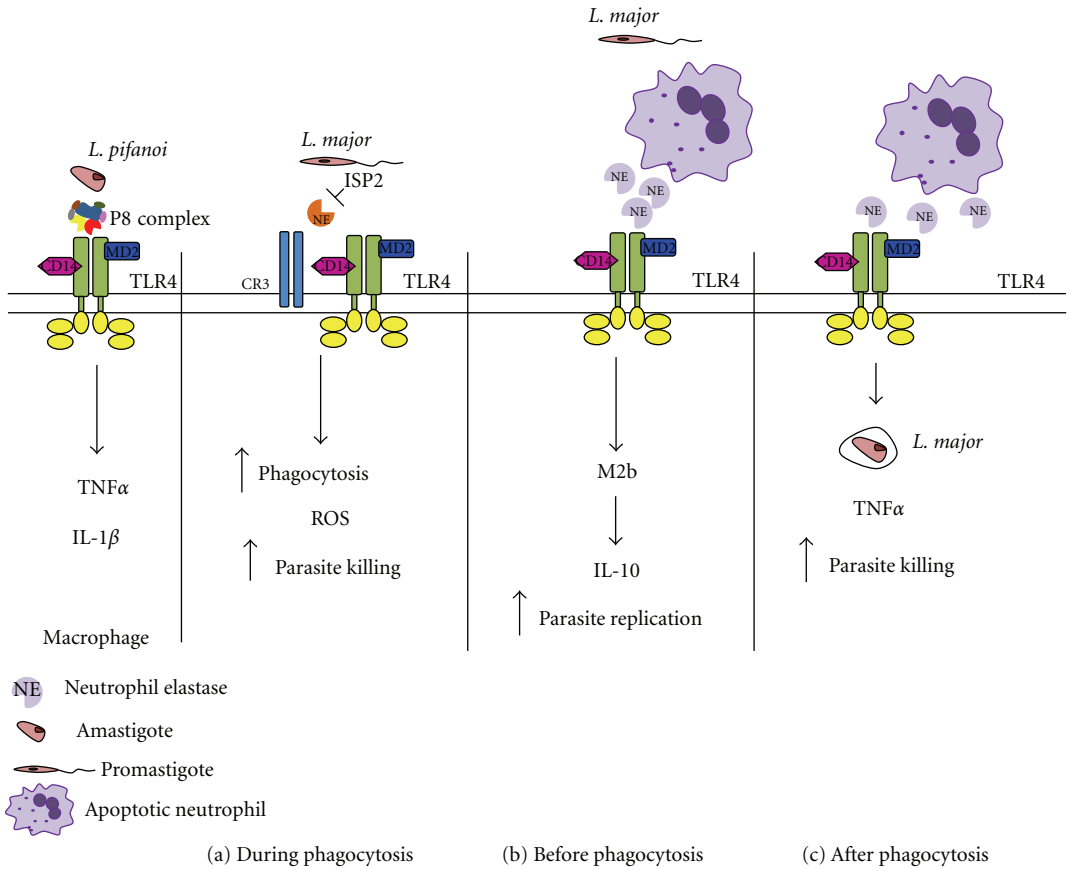

Figure 2: Model for the activation of TLR4 in different settings of *Leishmania* infections. *L. pifanoi* amastigote-derived P8 glycolipid complex stimulates TLR4 in macrophages in an MD2, CD14, and MyD88-dependent manner, leading to the production of proinflammatory cytokines (left panel) [46]. Microenvironments during the activation of TLR4 in macrophages of C57BL6 mice by neutrophil elastase (NE): in (a), during the phagocytosis of *L. major* promastigotes, CR3, TLR4, and NE at the surface of macrophages increase parasite uptake but lead to ROS production and to partial parasite elimination within 24 h [47], the control of NE activity by parasite ISP2 prevents TLR4 activation and protects parasite from intracellular killing [47, 48]; in (b), macrophages remove apoptotic neutrophils by phagocytosis and acquire a M2b phenotype, leading to IL10 production and increased permissiveness to parasite growth; in (c), macrophages are infected by *L. major* and subsequently interact with apoptotic neutrophils, resulting in the activation of TLR4 by NE and in the production of TNF that promotes parasite killing.

the replication of *L. major* [70]. The induction of the M2b phenotype, correlating with higher IL10 levels and a Th2-type response, was dependent on TLR4 and NE activities, suggesting that the engagement of TLR4 might be beneficial to the parasite at later stages of infection (Figure 2, scheme b). Indeed, rechallenge of those macrophages with LPS promoted parasite growth and the treatment of C57B6 mice with apoptotic neutrophils 3 days before infection increased parasite burdens, demonstrating how TLR4-mediated signals can contribute to create permissive niches for parasite replication at later stages [58].

We have recently described that NE present at the surface of murine macrophages can also activate TLR4 during the phagocytosis of *L. major*, without requirement of neutrophils [71]. Wild-type *L. major* is able to control NE activity, at least in part, through its endogenous protease inhibitor structurally similar to bacterial ecotins, named ISP2 (inhibitor of serine peptidases) [72]. Parasites lacking ISP2 promptly engage macrophage TLR4 due to uncontrolled NE activity [71] (Figure 2, scheme a). Furthermore, we found cooperativity between TLR4 and CD11b, a subunit of the complement type 3 receptor (CR3), in facilitating the uptake of *L. major* by macrophages. TLR4 activation led to the production of ROS, provoking partial elimination of intracellular parasites a few hours after internalization [71]. We found that the cooperativity between CD11b and TLR4 to enhance the phagocytosis of parasites was strictly dependent on the activity of NE, setting another example of how multiple components of innate immunity can act concertedly to improve the defense against invading parasites. The ability of *Leishmania* to promote the cross-talk between those receptors might exacerbate TLR4 responses in light of the finding that optimal signaling through TLR4 requires multiple cell surface associated molecules such as CD11b and HSP90 [73]. We observed that early engagement of TLR4 can influence how macrophages respond to *L. major* in at least two fronts: (i) first, by promoting enhanced phagocytosis and early killing of promastigotes (i.e., 24 hrs), and (ii) secondly,

by restraining the subsequent growth of amastigotes in the following 2–4 days. Those observations suggest that signals mediated through TLR4 at the parasitophorous vacuole could affect the microenvironment surrounding the parasite, controlling sustained intracellular growth.

Recently, a screen of TLR4 mutations in patients with cutaneous leishmaniasis revealed that certain genotypes were largely favoured in individuals with chronic or acute disease, as compared to asymptomatic donors or noninfected individuals, leading to the proposal that TLR4 polymorphism may lead to increased susceptibility or severity of the disease [74]. Although the consequences of TLR activation in macrophages as a result of *Leishmania* infections are being widely investigated, we are still lacking information on how TLRs might affect the interaction of the parasites with neutrophils. The mobilization of TLRs in neutrophils, in particular that of TLR4, is known to induce a wide range of responses. Of interest, IRAK4 is crucial for the exocytosis of neutrophil secretory granules induced by TLR4 [75]. In view of the findings that NE affects the outcome of parasite growth in macrophages [69–71], the stimulation of TLR4 in neutrophils could regulate the levels of secreted NE, influencing parasite burden in macrophages.

2.3. TLR9 and TLR3. Studies aiming at improving the therapy for *L. major* infections using recombinant IL-18 pointed to a potential involvement of the CpG-TLR9 pathway in the protective antiparasite response. While promoting rIL18 expression in infected mice through gene gun delivery, the authors observed a synergistic role of the plasmid vector with rIL18, which was dependent of CpG motifs [76]. The induction of proinflammatory cytokines, in particular of IL-12, by antigen-presenting cells was found to be dependent on TLR9 activation by CpG, indicating that TLR9 stimulation could lead to protective immunity against the parasite. In attempting to define which TLRs were involved in the increased susceptibility of MyD88-deficient mice to *Leishmania* infections, Liese and coworkers showed that TLR9 was required to induce IL-12 in bone-marrow-derived DCs by either *L. major* or its DNA [77]. Furthermore, they observed that TLR9$^{-/-}$ mice exhibited more severe skin lesions and higher parasite burdens as compared to C57B6 controls, coupled to a transient increase in IL-4, IL-13, and arginase. Even though there was an increase in Th2 cytokines, they did not observe alterations in the levels of IFNγ in the draining lymph node, concluding that deficiency in TLR9 does not influence the quality of the Th1 response [77]. The same group reported that plasmacytoid DCs responded to *L. infantum* by secreting IFNs α and β and IL-12 in a TLR9-dependent manner and that NK-induced cytotoxicity was abolished in TLR9$^{-/-}$mice [78]. Those results were the first to link TLR9, IL-12, and DCs to the activation of NK cells during visceral leishmaniasis. The protective role of NK cells in murine leishmaniasis is now proven to be strictly dependent on IL-12 secreted by myeloid DCs in response to *Leishmania* via TLR9 (reviewed in [14]).

The involvement of TLR9 in controlling *L. major* infection was further investigated in the following studies. Lesion progression and parasite burden were higher in TLR9$^{-/-}$ mice as compared to C57BL/6 controls, which was concomitant with a transient inhibition of a Th1 response [79]. The authors showed that the activation of bone-marrow-derived DCs, or of DCs isolated from the spleen, by *L. major* or by parasite DNA, followed by IFN-γ production by CD4 T cells, was abolished by treatment with DNAse or by alkalinization of endosomal compartments with chloroquine, supporting a role for TLR9 in DC activation by the parasite [79] (Figure 3, left panel). Those findings caused some controversy as to the impact of TLR9 deficiency in attenuating the Th1 response [80]. Since in both studies there were delays in the healing of skin lesions in TLR9$^{-/-}$ mice, it is feasible that the Th1 response is at least partially compromised in those mice.

The recognition of *Leishmania* by TLR3 in endosomal compartments was brought up a few years ago. Experiments using RNA interference provided evidence that in IFN-γ primed macrophages, TLR3 is required for the production of NO and TNF-α induced by infection with *L. donovani*, besides contributing to parasite phagocytosis [81] (Figure 3, right panel). Since *Leishmania* does not contain double-stranded RNA, the standard ligand for TLR3, the origin of the stimulatory factor of TLR3 remained an open question. More recently, an elegant work from Ives and coworkers shed light on this matter, providing evidence that RNA from viruses present in *L. guyanensis* serve as a source of TLR agonists, promoting inflammatory cytokines and chemokines [82] (Figure 3, right panel). The elevated cytokine production was dependent on the TLR3-TRIF pathway, and augmented via MyD88-transduced signals. Intriguingly, those responses rendered mice more susceptible to infection, as observed by increased swelling and parasite burden, together with more pronounced metastasis. Those findings add to the growing list of examples where the subversion of TLR responses by *Leishmania* serves to promote infection instead of playing a protective role.

Additional factors can further influence TLR activation indirectly, affecting the outcome of adaptive immunity. For example, it was found that dyslipidemia inhibits TLR-induced production of IL-12, IL-6, and TNF-α, as well as upregulation of costimulatory molecules by CD8α^- DCs, *in vivo*, leading to impaired Th1 and enhanced Th2 responses. Such dysfunction compromised host resistance to *L. major*, revealing that a dyslipidemic microenvironment can interfere with DC responses to *Leishmania* through TLRs [83].

2.4. TLRs Stimulation in Vaccination and Immunotherapy. The use of TLR activators as adjuvants in formulations to improve the efficacy of experimental vaccination against *Leishmania* has also been largely explored. Even before the knowledge that TLR stimulation could play a role in the defense against *Leishmania*, CpG oligodeoxynucleotides (ODNs) were tested as an adjuvant in the immunization of mice with *L. major*-soluble antigen aiming at inducing a protective Th1 response, leading to improved survival [84]. This observation supported the proposal that TLR agonists could serve as cost-effective alternatives in vaccine

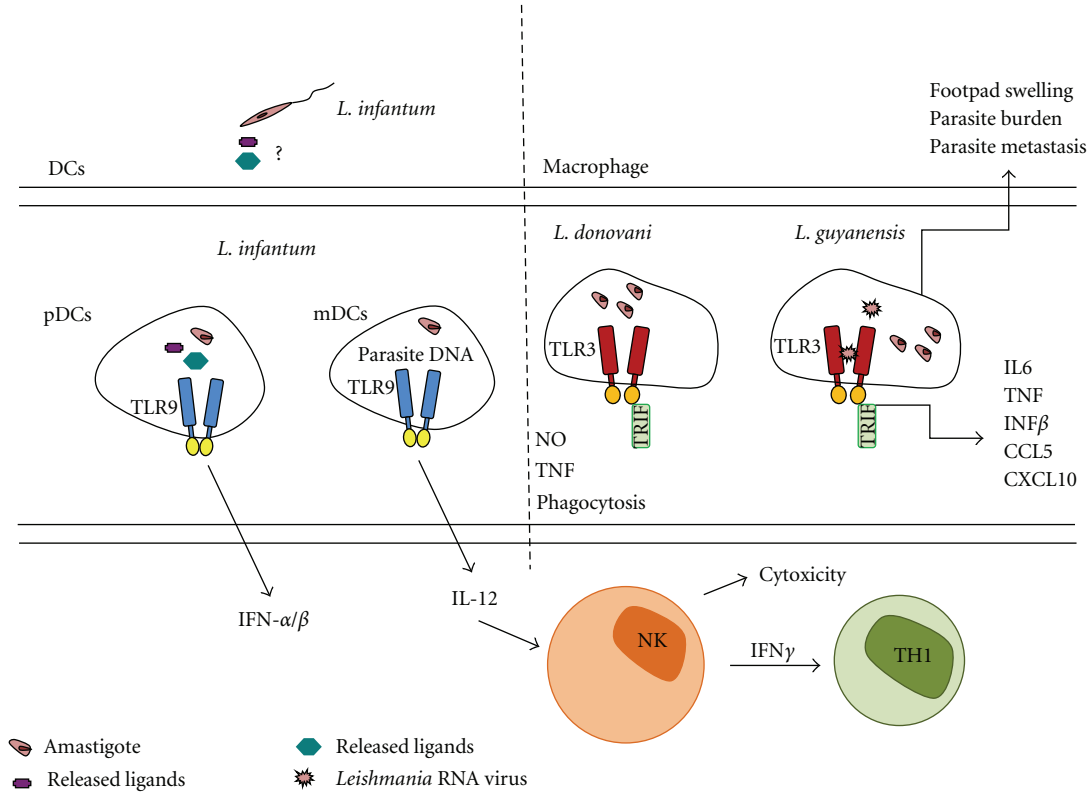

Figure 3: Proposed models for the activation of intracellular TLR9 and TLR3 by *Leishmania*. TLR9 of plasmocytoid DCs (pDCs) can be activated by putative TLR9 ligands secreted by *L. infantum* promastigotes and taken up by endocytosis or in endosomal compartments. TLR9 in myeloid DCs (mDCs) can be activated by *L. infantum* DNA after parasite phagocytosis and destruction in the endosomal compartment, leading to the activation of NK cells and protective Th1 responses [4, 63, 64]. TLR3 is required for the production of TNF, NO and the phagocytosis of *L. donovani* by macrophages, suggesting a protective role for infection [67]. Intracellular TLR3 is activated in a TRIF-dependent pathway in macrophages infected with metastatic *L. guyanensis*, by double-stranded RNA from the *Leishmania* virus LRV1 [68]. This leads to the production of proinflammatory cytokines and chemokines, as well as type1-IFNs. Despite the inflammatory response, TLR3 is required for the effective development of footpad swelling, for significant parasite burden and its dissemination in infected hamsters, suggesting that TLR3 activation facilitates disease pathology by parasites of the *Leishmania Viannia* subgenus.

formulations against leishmaniasis. Along those lines, the formulation of multisubunit recombinant vaccines with the TLR4 agonist, monophosphoryl lipid A, elicited protective immunity against *L. major* challenge in Balb/c mice, an approach that could serve as an alternative to the use of recombinant IL12 [85]. An analogue of lipid A was subsequently used as a TLR4 agonist in experimental vaccination with *L. amazonensis* antigens and was proven beneficial for both immunoprophylaxis and immunotherapy, associated mainly with increased levels of IL12 and IFNγ [86]. CpG (ODN) were throughoutly tested as adjuvants in experimental vaccinations to induce protective responses to *L. donovani* [87, 88], or to *L. major* in Balb/c mice, when coencapsulated with antigens in liposomes [89]. CpG also protected Balb/c mice immunized with ribosomal proteins against a second challenge with *L. major*, being effective in providing long-term immunity [90, 91]. Finally, Cpg ODN improved the clinical outcome of immunized rhesus macaques infected with *L. major*, supporting the hypothesis that TLR9 stimulation might improve the efficacy of vaccination against cutaneous leishmaniasis [92]. Using a combined approach of costimulation of different TLRs, the immunogenicity of recombinant antigens from *L. donovani* (iron superoxide dismutase B1 and peroxidoxin 4) was greatly improved by immunization in conjunction with TLR9 or TLR4 agonists, resulting in a Th1-type response in Balb/c mice [93]. The use of ligands for TLR7 and/or TLR8 (imiquimod and the related R848 compound) in the immunization of Balb/c mice with crude *L. major* antigen induced a Th1 response and was protective against subsequent challenges [94].

More recently, the formulation of a defined polyprotein anti-*Leishmania* vaccine candidate in conjunction with TLR4 or TLR9 agonists was evaluated as an immunotherapeutical treatment in a mouse model of cutaneous leishmaniasis [95]. A strong effective T cell response was observed during disease, followed by cured lesions and reduced parasite burden upon immunization with the combination of both agonists, suggesting that TLR synergy may serve as a tool for the treatment of parasite infection. TLR2 stimulation was also proposed as an alternative to revert the loss of CD8 function in patients with diffuse cutaneous leishmaniasis [96]. It was found that, in patients infected with *L. mexicana*

who develop diffuse lesions, the reduced cytotoxicity and proliferation of CD8 cells was typical of cellular exhaustion and could be restored *in vitro* by stimulation with TLR2 agonists, including *Leishmania* LPG [96]. The design of CD8 activators based on TLR2 activation could be beneficial in reverting the observed in chronic patients with diffuse lesions. More recently, a more sophisticated vaccination approach was attempted in order to promote protective responses against *L. panamensis* in a murine model of chronic disease [97]. The strategy of prime-boost, consisting of the priming using a single antigen and heterologous DNA, was combined with the addition of a TLR2/1 agonist (PAM3CSK4) as an adjuvant, leading to an effective protection against *L. pananemensis* infections [97]. Furthermore, they provided evidence that TLR2 stimulation during priming is essential for the elevation of CD4 and CD8 memory T cell responses and reduction of IL-13 and IL-10 levels, which were required for protection. Those findings were a great step forward in the design of simple and cost-effective vaccines against *Leishmania* (*Viannia*) species that are refractory to commonly used strategies to other *Leishmania* species. It also offers an alternative for DNA-based vaccination schemes in cases where the CD8 T cell activation is crucial.

3. Concluding Remarks

The activation of several members of the TLR family of receptors is observed during the infections of isolated cells or in experimental infections of animals with different *Leishmania* species. The consequences of such activation are complex and depend on the nature of the TLR, the cell type, the parasite species, and the timing in which those events occur. Although studies using TLR-deficient animals offered the initial picture of how those receptors can act in the protection or promotion of disease, the underlying molecular mechanisms are still obscure. In particular, it is indispensable to identify the parasite factors that contribute to TLR activation and/or negatively regulate their responses in the individual cell types and to evaluate to what extent TLR activation in each of those cells contributes to pathology. While most of the earlier studies focused on the requirement of TLRs for cytokine synthesis and for the development of adaptive immunity, the studies on if and how those receptors contribute to the biogenesis of the parasitophorous vacuole and to the control of parasite intracellular growth are still largely missing. TLR activation has emerged as a promising alternative to improve the efficacy of vaccination with parasite antigens and may also act synergistically with other components during immunotherapy. However, a more detailed study of the consequences of such strategies must be addressed before the combined activation of TLRs can be explored in the future in the treatment of patients with more severe and diffuse lesions.

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Review Article

ETosis: A Microbicidal Mechanism beyond Cell Death

Anderson B. Guimarães-Costa,[1] Michelle T. C. Nascimento,[1] Amanda B. Wardini,[2] Lucia H. Pinto-da-Silva,[3] and Elvira M. Saraiva[1]

[1] *Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro (UFRJ), 21941-901 Rio de Janeiro, RJ, Brazil*
[2] *Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro (UFRJ), 21941-901 Rio de Janeiro, RJ, Brazil*
[3] *Instituto de Veterinária, Universidade Federal Rural do Rio de Janeiro (UFRRJ), 23890-000 Seropedica, RJ, Brazil*

Correspondence should be addressed to Elvira M. Saraiva, esaraiva@micro.ufrj.br

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Netosis is a recently described type of neutrophil death occurring with the release to the extracellular milieu of a lattice composed of DNA associated with histones and granular and cytoplasmic proteins. These webs, initially named neutrophil extracellular traps (NETs), ensnare and kill microorganisms. Similarly, other cell types, such as eosinophils, mast cells, and macrophages, can also dye by this mechanism; thus, it was renamed as ETosis, meaning death with release of extracellular traps (ETs). Here, we review the mechanism of NETosis/etosis, emphasizing its role in diseases caused by protozoan parasites, fungi, and viruses.

1. Introduction

Upon inflammation, neutrophils are the first cells to be recruited to the inflammatory site, in a process orchestrated by chemokines, a series of attractive molecules produced locally. Neutrophils then migrate to the inflamed site where they contain and eliminate microorganisms using three basic strategies: phagocytosis, with ingestion and killing of microorganisms inside special compartments of the cell, degranulation, which consists in the extravasation of the granules content to the extracellular milieu, and by a new antimicrobial mechanism named netosis that also occurs in the extracellular milieu, when DNA associated to proteins is expelled from the cell [1–3].

Neutrophils are the most abundant leukocytes in the blood and constitute the first line of host defense against invading pathogens. These cells are also known as polymorphonuclears (PMNs) or granulocytes, since they have a segmented nucleus with lobules linked by nuclear filaments and hold large numbers of three different types of granules. These granules were classified according to their protein content as primary, azurophil, or peroxidase-positive granules because of their high myeloperoxidase (MPO) loading. Besides MPO, these granules possess cathepsin G, defensins, elastase, and proteinase 3, among many others proteins. MPO production stops between promyelocyte and myelocyte transition stages during maturation at the bone marrow, and then, the next granules formed are all peroxidase negative. The secondary granules contain collagenase, gelatinase, lactoferrin, and sialidase, and the tertiary granules enclose gelatinase, β2-microglobulin, and others. In addition, secretory vesicles are also present in the neutrophil cytoplasm; however, the protein content of these vesicles has not been completely elucidated [4, 5].

2. NETosis Occurs with Extrusion of Neutrophil Extracellular Traps (NETs)

In a seminal work, Brinkmann and colleagues in 2004 described that upon activation by phorbol myristate acetate (PMA), lipopolysaccharide (LPS), interleukin 8 (IL-8), or by Gram-positive and -negative bacteria, neutrophils release their chromatin to the extracellular medium, associated with different proteins, forming the so-called NETs. Currently, there is an increasing list of synthetic and physiological molecules, as well as microorganisms and their products, which can activate neutrophils to release NETs (Table 1).

Table 1: Microorganisms or molecules able to trigger extracellular traps release.

Activators	Cells	Reference
Activated endothelial cell	Neutrophil	[6]
Antiribonucleoprotein IgG	SLE Neutrophil	[7]
Aspergillus fumigatus (conidia or hyphae)	Neutrophil	[8, 9]
Autoantibodies (anti-LL-37/anti-HNP)	SLE Neutrophil	[10]
Calcium	HL-60 lineage, neutrophil	[11, 12]
Candida albicans (hyphae or yeast)	Neutrophil	[13]
Cryptococcus gattii	Neutrophil	[14]
Cryptococcus neoformans	Neutrophil	[15]
Eimeria bovis	Neutrophil	[16]
Enterococcus faecalis	Neutrophils	[17]
Equine spermatozoa	Neutrophil	[18]
Escherichia coli	Neutrophil, monocyte	[17, 19–21]
Glucose oxidase	Neutrophil	[22]
GM-CSF + C5a	Neutrophil	[23, 24]
GM-CSF + LPS	Neutrophil	[24]
Haemophilus influenzae	Neutrophil	[25, 26]
Helicobacter pylori	Neutrophil	[27]
Hydrogen peroxide	Neutrophil, mast cell, chicken heterophil	[22, 28, 29]
Interferon (IFN)-α+ C5a	Neutrophil	[23]
IFN-γ + C5a	Neutrophil, eosinophil	[23, 30]
IFN-γ + C5a, LPS or eotaxin	Eosinophil	[30]
Interleukin 5 + LPS/C5a/eotaxin	Eosinophil	[30]
Interleukin 8	Neutrophil	[1, 31]
Interleukin 23 and IL-1β	Mast cells	[32]
Klebsiella pneumoniae	Neutrophil (tissue)	[33]
Lactococcus lactis	Neutrophil	[34]
Leishmania amazonensis (promastigotes/amastigotes/lipophosphoglycan)	Neutrophil	[35]
Leishmania amazonensis, L. donovani, L. major, L. chagasi (promastigotes)	Neutrophil	[35–37]
Lipopolysaccharide (LPS)	Neutrophil	[1]
Listeria monocytogenes	Neutrophil	[20, 38]
Mannheimia haemolytica and leukotoxin	Neutrophil	[39]
M1 protein/M1 protein-fibrinogen complex	Neutrophil, mast cell	[40, 41]
Mycobacterium tuberculosis, M. canettii	Neutrophil	[42]
Nitric oxide	Neutrophil	[43, 44]
Panton-Valentine leukocidin, autolysin, and lipase	Neutrophil	[45]
Phorbol myristate acetate (PMA)	Neutrophil, mast cell, chicken heterophil	[1, 12, 28, 29]
PMA + ionomycin	Neutrophil	[17]
Platelet-activating factor	Neutrophil	[27]
Platelet TLR-4	Neutrophil	[46]
Pseudomonas aeruginosa	Mast cell	[28]
Serratia marcescens	Neutrophil	[17]
Shigella flexneri	Neutrophil (tissue)	[1]
Staphylococcus aureus	Neutrophil, mast cell	[22, 28, 45]
Statins	Neutrophil, monocytes/macrophages	[47]
Streptococcus (Group A–GAS)/Pilus	Neutrophil, mast cell	[34, 40, 48]
Streptococcus dysgalactiae	Neutrophil	[17]
Streptococcus pneumoniae	Neutrophil (tissue)	[49]
Streptococcus pyogenes	Mast cell	[28]
Syncytiotrophoblast microparticles	Neutrophil	[31]

Table 1: Continued.

Activators	Cells	Reference
TNF-α	HL-60 lineage	[11]
TNF-α + ANCA IgG	Neutrophil	[50]
Yeast particulate B-glucan	Neutrophil	[51]
Yersinia enterocolitica	Neutrophil	[52]
δ-Toxin from *Staphylococcus epidermidis*	Neutrophil	[53]

Initially, NET composition was described as being formed by decondensed chromatin scaffolds associated to proteins of the different types of neutrophil granules [1]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase subunits, human peptidoglycan protein short-S, and pentraxin (PTX)-3 were also detected in association with NETs [20, 54, 55]. Later, using proteomic analysis, besides histones and granule proteins, cytosolic and cytoskeleton proteins, catalase and glycolytic enzymes were found linked to the NETs [15]. This proteomic study evidenced that among the NET-associated proteins, histones were the most abundant, followed by the neutrophil elastase. Intriguingly, NADPH oxidase, PTX3, and cathelicidin LL-37 previously detected in NETs by immunofluorescence staining were not found associated to NETs by proteomic analysis [15]. These discrepancies could be due to a loose association of these missing proteins to the NETs, which could have been lost by the NET processing for the proteomic analysis. Recently, IL-17 was found associated to NETs in psoriasis skin biopsies evidenced with specific antibodies by immunofluorescence staining [32].

3. NETosis Mechanism

Although very little is known about the mechanism of NET release, some morphological features are easily observed during netosis (Figure 1). Thus, after stimulation, neutrophil nuclei lose segregation of eu- and heterochromatin, its characteristic lobular form vanishes, and the nuclear membrane swells up, fragmenting into vesicles. This is followed by the granules' membranes disruption, which allows the mixing of nuclear, cytoplasmic, and granular contents. Subsequently, the plasma membrane is permeabilized, allowing NET release to the extracellular milieu [22]. NET structure is composed of smooth strands of 15–17 nm diameter decorated with globular domains ranging from 25 to 50 nm [1, 15].

Netosis is a new type of cell death, different from necrosis and apoptosis [22]. Under netosis, there is neither DNA fragmentation nor phosphatidylserine exposure in the outer membrane leaflet, hallmarks of these latter forms of cell death, respectively. The intact nuclear envelope differentiates necrosis from netosis, and NET-DNA was not detected in the culture supernatants of apoptotic or necrotic neutrophils [22]. Moreover, netosis seems to be independent of caspases and RIP-1 kinases, since pretreatment of neutrophils with zVAD-fmk or necrostatin-1 did not affect netosis completion [15, 56]. All these features differentiate netosis from apoptosis and necrosis. However, it was recently shown that netosis induced by PMA was dependent on a simultaneous activation of both autophagy and superoxide production, and neither mechanism isolated was capable to induce netosis [56]. Thus, inhibition of autophagy prevents chromatin decondensation and netosis, without affecting superoxide production, demonstrating that NADPH oxidase activity is necessary, but insufficient to, alone, trigger netosis. Furthermore, induction of autophagy in neutrophils from chronic granulomatous disease patients, which possess a defective NADPH oxidase, thus, unable to generate reactive oxygen species (ROS), was not sufficient to promote chromatin decondensation [56].

In addition, netosis seems to occur by a mechanism independent of neutrophil granule exocytosis, since Rab27a-deficient neutrophils, which are deficient in exocytosis, are able to release NETs [20].

Presently, the great majority of stimuli described to induce netosis are dependent on ROS production by the multienzyme complex NADPH oxidase. Drugs that inhibit NADPH enzyme, hence, inhibit NET release [22, 44, 57]. Moreover, neutrophils from patients with chronic granulomatous disease are incapable of forming NETs [22, 58, 59].

However, a novel netosis process was recently described for the bacterium *Staphylococcus aureus*, occurring independently of ROS and neutrophil lysis [45]. In this new mechanism of netosis, that occurs very rapidly, vesicles containing DNA sprout from the nuclear membrane are extruded intact to the extracellular environment, where they break and release their chromatin content that traps and kills bacteria. This more rapid mechanism would precede the ROS-dependent NET release [45]. Similarly, ROS generation is not sufficient to rescue netosis from patients suffering from syndrome of neonatal neutrophil dysfunction, a disease associated with sepsis and other severe infections [57]. Another ROS-independent NET release was reported for *Leishmania donovani* [37].

It has been demonstrated at the molecular level that chromatin decondensation, a pivotal event during netosis, occurs with elastase migration from the primary granules to the neutrophil nuclei, where this enzyme partially degrades histones [33]. As follow, myeloperoxidase also migrate to the nucleus, synergizing with elastase for the chromatin decondensation by a still unknown mechanism, which is independent of its enzymatic activity. Importantly, neutrophils from myeloperoxidase-deficient patients are unable to release NET [60]. Treatment of neutrophils with specific neutrophil-elastase inhibitors abrogates NET formation, and purified neutrophil elastase was able to digest histones

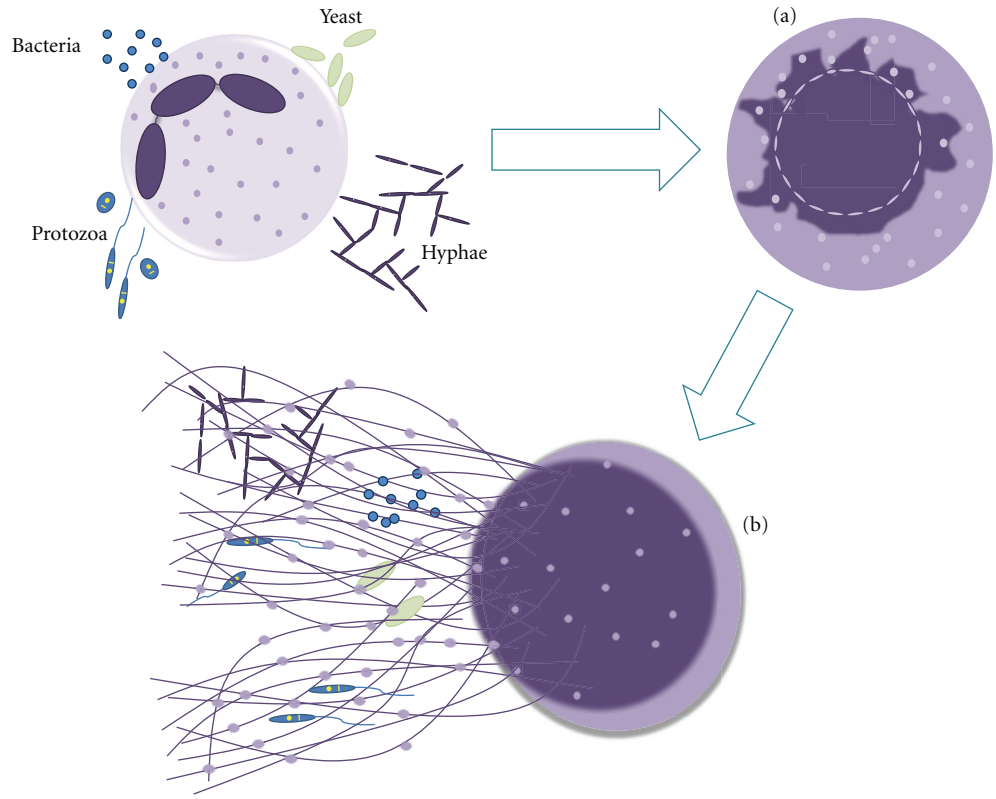

Figure 1: Mechanism of Neutrophil Extracellular Traps release. Neutrophils are stimulated by contact with bacteria, protozoan, fungi (yeast and hyphae forms) or their products (not shown), leading to: (a) ultrastructural alterations of nuclear shape with chromatin decondensation, swollen and fragmentation of the nuclear membrane, which allow the association of granules and cytoplasmic proteins with the chromatin, and (b) release of extracellular structures consisting of a DNA-backbone, decorated with histones, neutrophil granular and cytoplasmatic proteins (NETs), which ensnare and kill microorganisms.

and promote chromatin decondensation in isolated nuclei. Moreover, mice knockout for the elastase-encoding gene did not produce NET in a model of pulmonary infection by *Klebsiella pneumoniae* [33].

Another important event involved in chromatin decondensation is histone hypercitrullination, a reaction catalyzed by peptidyl arginine deiminase 4 (PAD4), in which histones' arginines are converted to citrullines by deimination. Hypercitrullinated histones were detected in NETs, but not in apoptotic neutrophils [11, 61]. It has also been demonstrated that mice knockout for PAD4 enzyme are unable to form NETs upon activation by different stimuli, being deficient in bacterial killing by these traps [11, 62, 63]. Infection of $PAD^{+/+}$ and $PAD^{-/-}$ mice with group A *Streptococcus* confirmed that $PAD^{-/-}$ animals were more susceptible to infection, presenting more lesions than $PAD^{+/+}$. Moreover, neutrophils purified from $PAD^{-/-}$ animals did not release NETs when stimulated by lipopolysaccharide or oxygen peroxide as their wild-type counterparts [62].

Hitherto, we know that upon protein kinase C activation by PMA or by diacylglycerol (DAG) analogs, Raf-MEK-ERK pathway is required for NET formation and also that this signaling pathway is upstream of NADPH oxidase activation, since diphenylene iodonium (DPI) did not abolish phosphorilation of ERK [27]. Moreover, the antiapoptotic protein myeloid cell leukemia (Mcl)-1 is overexpressed in PMA-activated neutrophils, a pathway required for PMA/DAG/*Helicobacter pylori* NET induction [27].

The participation of Rac2, an isoform of Rac small GTPases, in NET induction by PMA or LPS-stimulated mice neutrophils has also been evidenced [44]. Rac2 null mice have negligible NET production in comparison to Rac1 null and wild-type counterparts, showing that Rac2 isoform is required for NET release. Rac2 mutants are unable of producing NETs due to a lack of ROS production, which is rescued by the addition of hydrogen peroxide to neutrophils. Since Rac has been shown to regulate nitric oxidase synthase (NOS), the role of nitric oxide (NO) on NET production by wild-type and Rac2 mutant mice was investigated. Indeed, L-NAME, an NOS inhibitor, reduced NET release induced by PMA in mice neutrophils [44]. Confirming the role of nitric oxide on NET production, 7-NI, another NOS inhibitor, also decreased NET release induced by PMA in human macrophages [43]. Intriguingly, treatment with SNAP, an NO donor, did not induce NET release either in wild-type or Rac2 null neutrophils. However, increased NET formation was observed by SNAP treatment of PMA-activated wild-type neutrophils, suggesting that ROS production by NADPH oxidase may be required for NET induction by NO [44]. These discrepancies could be due

to the source of the neutrophils used in each study: SNAP induced netosis in human blood neutrophils [43] but not in mice bone marrow neutrophils [44].

The participation of NO in NET induction was also demonstrated using SNAP and SNP, two NO donors [43]. Human neutrophils treated with these compounds release NET in a dose- and time-dependent manner, which was inhibited by N-acetyl cysteine, an ROS scavenger. SNAP-induced NET was abolished by DPI, an NADPH oxidase inhibitor, as well as by 4-aminobenzoic acid hydrazide (ABAH), a myeloperoxidase inhibitor, suggesting that NET induction by NO occurs with increasing free radicals generation [43].

4. Functions of Neutrophil Extracellular Traps

NETs grab microbes avoiding their dissemination through the organism, and also offering a high local concentration of antimicrobial proteins. Bactericidal activity of NET-associated histones has been proved against *Shigella flexneri*, *Salmonella typhimurium, Salmonella enterica, Staphylococcus aureus, Streptococcus pyogenes,* and *Bacillus anthracis* [1, 64]; however, it remains to be determined whether the bactericidal activity of histones is modulated by its hypercitrullination. In addition, the cytosolic calprotectin protein complex (S100A8/A9) associated to NETs kills *Candida albicans* and *Cryptococcus neoformans* [15].

Net-associated histones and calprotectin have been considered as bactericidal and fungicide, respectively, but many other NET-linked components are also endowed with microbicidal properties, at least in their free forms [1, 65, 66]. These molecules include cathelicidin LL-37, defensins, bacterial permeability-increasing protein (BPI), lactoferrin, myeloperoxidase, proteinase 3, and elastase [3, 4, 67]. Furthermore, these components could act synergistically as shown for two of them, the peptidoglycan recognition protein-S and lysozyme, which colocalize in NETs [67].

Even though resistant to NET-mediated killing, group A *Streptococcus, S. pneumoniae*, *Mycobacterium tuberculosis,* and *Haemophilus influenza* are caught by NETs, suggesting that this property could also have an important role for the host immune response [25, 26, 42].

Presently, it is unknown how so diverse and different microorganisms are ensnared by NETs. Many of the NETs constituents are highly cationic, and it is likely that NETs can bind negatively charged surfaces, while NET-specific recognition sites for microorganisms could not be excluded [68].

Albeit NETs are toxic for microorganisms, some microbes are able to escape the NET-mediated killing. To name a few of these strategies, the capsule expression and M1 protein of group A *Streptococcus* are important to NET resistance [69]. In addition, endonucleases expressed by *Staphylococcus*, *Streptococcus pneumonia,* and group A *Streptococcus* enhance bacterium survival by digesting NET-DNA scaffold [34, 49, 70–72]. Importantly, since DNA constitutes the NET backbone, its digestion with DNase rescues microorganisms from NET-mediated killing [1, 13, 35]. NET-escape mechanisms as well as NET role on autoimmune diseases are reviewed elsewhere [13, 73–77].

5. Cells Able to Release Extracellular Traps (ETosis)

Netosis was first described in neutrophils, thus the origin of its name. However, other cells such as eosinophils and mast cells also release extracellular traps (ETs) composed by DNA and antimicrobial proteins [28, 30]. Monocytes and macrophages were also shown to release extracellular traps, but to a lesser extent when compared to neutrophils, and the microbicidal activity of these ETs has not yet been determined [21, 78]. Since it seems to be a more general mechanism, shared by different cell types, the release of intracellular DNA to the extracellular milieu was renamed as ETosis, meaning death with release of DNA extracellular traps. Interestingly, eosinophils release their mitochondrial DNA, in a death-independent way [30]. These same authors reported that GM-CSF-primed neutrophils released mitochondrial DNA associated with granule proteins, forming neutrophil extracellular traps in response to LPS or complement C5a fragment [24].

Besides occurring in different cell types, ETosis seems to be a well-conserved mechanism, since release of ETs was reported for neutrophils or related cells in many different organisms, such as ox, horse, fish, mouse, and cat neutrophils, as well as chicken heterophils [12, 17–19, 29, 36, 38, 39, 44]. Although classical ETs were not observed in *Galleria mellonella*, DNA derived from oenocytoid cells participates in the haemolymph coagulation, an important mechanism for microbes killing in insects [79].

Interestingly, ETs were also evidenced in plants and seem to play an important role on root tip defense against fungal infections, implying a similar behavior between plant root and animal cells, which extrude ETs in a defense mechanism important for plant growth and survival [80, 81].

Because it was first described in neutrophils, which are easy cells to work with, the majority of the data available analyze netosis aspects, mainly when the studies were related to infectious diseases, although it has an important role in autoimmune diseases such as systemic lupus erythematosus and vasculitis [75–77].

6. NETs and Protozoa

Neutrophils are rapidly recruited to infection sites during infections with many protozoa and microbial pathogens, pointing out the importance of these phagocytes in the immune response to these infectious agents. *Leishmania* particularly meets neutrophils in the very beginning of the infection process, since these protozoan parasites are inoculated by the sand fly vector in a pool of blood. Thus, based on the interaction between *Leishmania* and neutrophils, we investigate the capacity of this trypanosomatid to induce netosis, to better understand the first steps of the *Leishmania* infection [35]. Initially, we detected that both forms of the parasite, promastigotes and amastigotes,

were able to induce NET extrusion in human neutrophils and were caught by the resulting meshes (Figure 2; see Supplementary video in supplementary material available online at doi:10.1155/2012/929743). In addition, we demonstrated that the glycoconjugate lipophosphoglycan, which is expressed on the promastigote cell surface, induced neutrophils to release NET in a dose-dependent manner. NETs were toxic for the promastigotes, an effect mediated by the histones present in the lattices, whose toxicity to the parasite was neutralized by antihistone antibodies [35]. The ability of histones to kill *Leishmania* was further confirmed by parasite death upon its exposure to histones purified from calf thymus (Figure 3). NETs occurrence goes further beyond experimental findings since the presence of NETs in lesion biopsies of patients with active cutaneous leishmaniasis was also shown [35].

Leishmania sensitivity to histone-mediated toxicity also includes promastigotes of *L. mexicana, L. braziliensis, L. major, and L. amazonensis*, although the death mechanism mediated by these proteins was still unknown [82]. Interestingly, amastigotes from *L. mexicana* and *L. amazonensis* were resistant to the histone-mediated toxicity [82]. NET induction was also shown for *L. donovani* promastigotes, but, contrary to the former findings, the authors reported that both *L. donovani* and *L. major* promastigotes were resistant to the NET-mediated killing [37].

Apicomplexans such as *Plasmodium*, the causative agent of malaria, and *Eimeria,* the causative agent of eimeriosis in cattle, comprise some of the most life-threatening protozoan parasites. Although a direct NET induction by *Plasmodium* has not been described yet, there is one report showing NETs in the blood of *Plasmodium falciparum*-infected children, showing infected erythrocytes and trophozoites attached to structures identified as NET by DNA staining [83].

Behrendt and coworkers [16] reported that sporozoites of *Eimeria bovis* induce NET extrusion by calf neutrophils, and this traps snare sporozoites. Although a NET-lethal effect on *Eimeria* sporozoites was not demonstrated, the parasite infectivity to bovine primary endothelial cells was decreased in comparison to untreated parasites. Thus, arrest of *Eimeria* sporozoites by NETs may prevent host cell invasion required for this parasite replication.

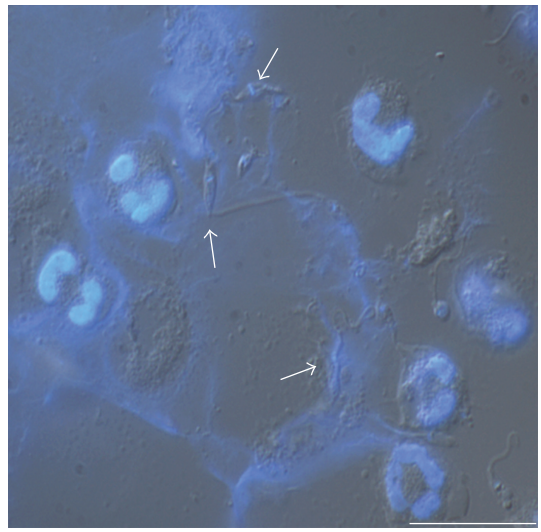

Figure 2: Immunostaining of NETs induced by *Leishmania*. Naïve neutrophils were incubated with promastigotes (1 : 5 ratio) for 1 h at 35°C. Cells were fixed and stained with DAPI and shown merged with differential interference contrast image. Arrows point to NET-ensnared promastigotes (Bars: 20 μm).

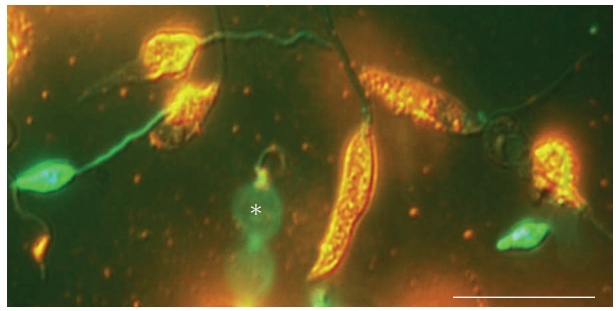

Figure 3: Histone toxicity to promastigotes. Promastigotes were incubated with purified histone for 30 min and stained by the live/dead method. Dead promastigotes stained in yellow/orange and live promastigotes in green. Differential interference contrast image merged with the fluorescence staining of the same cells. (*) The beating of a live promastigote flagellum. Bars, 20 μm.

7. NETs and Fungi

Fungal pathogens cause a wide and increasing number of severe infections with high mortality rates, mainly in immunocompromised individuals. The phenomenon of NET release was observed in experimental models of fungal infections using *Candida albicans, Cryptococcus neoformans, Aspergillus nidulans, A. fumigatus*, and *Cryptococcus gattii* [8, 13, 15, 58, 84].

C. albicans is the major cause of mycoses in humans, which can range from mild superficial infection of the skin to severe disseminated systemic disease. *In vitro* studies have shown that hyphae and yeast forms of *C. albicans* induce and are trapped and killed by NETs released from human neutrophils, as well as *C. neoformans* [13, 15]. Calprotectins, a calcium-binding cytoplasmic heterodimeric protein complex, were shown as the major antifungal component associated to the NETs, *in vitro* and *in vivo*. Calprotectin chelates essential metal ions such as Zn^{2+} and Mn^{2+} resulting in reduced *C. albicans* growth in subcutaneous and pulmonary infection of mice [15, 85]. Moreover, immunodepletion of calprotectin abolished the growth inhibitory activity of NETs *in vitro*, and calprotectin-deficient mice were unable to clear *C. albicans* infection [15].

The role of calprotectin against *Aspergillus* was also established in calprotectin knockout mice, which lost the ability to control the fungal infection [86]. On the contrary, an *in vitro* study using *A. fumigatus* showed that although resting conidia and germinal tube forms are able to induce NETs, the webs did not kill the fungus. The control of swollen conidia germination is achieved by phagocytosis,

although NET inhibits polar growth of germ tube forms in a calprotectin-dependent manner [9].

In a human case of an invasive pulmonary aspergillosis caused by *A. nidulans* and refractory to therapy, the role of NET formation was pointed out through a successful restoration of the immune response against this fungus by gene therapy, in an 8.5-years-old boy with chronic granulomatous disease. Restoring NADPH oxidase expression after $gp91^{phox}$ gene transfer, neutrophils defense against the conidia and hyphae forms of *A. nidulans* was reestablished in the treated patient [58]. This study unequivocally proved the role of ROS production for NET induction, at least for fungi.

Another study showed that, in a mice model of *A. fumigatus*-lung infection, NETs arise in 3-4 hours after immigrating neutrophils reach the infectious focus. Moreover, high amount of NETs against hyphae forms were observed in comparison to conidia. The presence of hydrophobin RodA, the major component of resting conidia surface, reduced NET formation. This fungal protein was identified as being an important factor to protect conidia from recognition [87], and now it seems to be an important factor to prevent triggering of NET extrusion; however, the molecular mechanism behind prevention of NET formation remains to be elucidated [88].

The encapsulated yeast *C. gatti,* is the agent of one-tenth to one-third of pulmonary cryptococcosis and meningitis worldwise. This species is distinguished from *C. neoformans* by its occurrence in trees, rather than pigeon droppings. Special diagnostic tools used to differentiate these species include the cigar-shaped yeast morphology in the host cerebrospinal fluid, agglutinating serotype, creatine assimilation, and elongated rod-shaped basidiospores [14, 84]. A study analyzing neutrophils, interaction with *C. gatti* showed an extensively NET formation *in vitro*, but without correlation with fungal killing. Moreover, comparison studies demonstrate that *C. gatti,* expressing extracellular fibrils were more resistant to neutrophil killing than capsulate mutants [14].

Whether NETs really have a role in the control of fungal infections needs to be better explored, thence it is not the only mechanism, although it means an important tool to detain infection.

8. NETs and Viruses

Although presently there are no data about virus capacity to directly induce NET release, the role of netosis in viral infections has been addressed for feline leukemia virus (FeLV) and influenza A virus infections [38, 63, 89].

Our group described that netosis of cat neutrophils could be modulated by the feline leukemia virus (FeLV) infection [36]. In fact, neutrophils from FeLV (−) and from asymptomatic FeLV (+) cats release NETs when stimulated with PMA or *Leishmania*. However, neutrophils from FeLV (+) symptomatic cats spontaneously release high quantities of NETs in comparison to either the neutrophils from FeLV (−) or from asymptomatic FeLV (+) cats. On the other hand, neutrophils from FeLV (+) symptomatic cats do not respond to NET-releasing stimuli. Our data suggest that netosis could be related to disease status, at least in FeLV-feline infection, and that this feature could be used as a diagnostic tool [36].

In a recent study, NET was induced in a murine model of influenza A virus (A/Puerto Rico/8/34 H1N1) pneumonitis and correlated to lesions in the alveoli and bronchioles, leading to complications of acute respiratory distress syndrome [89]. Also analyzing infection with influenza A virus (A/WSN/33/H1N1 strain) in NET-impaired mice (PAD4 knocked out) and in wild-type counterparts, NET induction was found in the bronchoalveolar lavage (BAL) of the wild-type mice, but not in the $PAD4^{-/-}$ mice [63]. Interestingly, neutrophils obtained from lungs of wild-type mice release NET upon contact with influenza A-infected alveolar epithelial cells. However, no differences in morbidity and mortality were observed in both mice strains, ruling out the hypothesis that NET could mediate viral clearance in this model of acute infection [63].

9. *In Vivo* Detection of ETosis

The physiologic role of ETs can be supported by its abundance in sites of infected and noninfected models of inflammation. Accordingly, etosis has been reported in spontaneous human appendicitis [1], experimental dysentery induced by *Shigella* in rabbits [1], infections by *Streptococcus* [90], pneumonia by *Streptococcus pneumonia* [90], blood of children infected by *Plasmodium falciparum* [83], periodontitis [91], infections by *Aspergillus nidulans* [58] and by *Eimeria bovis* [16], as well as in pulmonary *Aspergillus fumigatus* infections [8], and cutaneous lesions of leishmaniasis [35]. This phenomenon was also demonstrated to be relevant in human preeclampsia [31], in small vessels vasculitis [50], in systemic lupus erythematosus [7, 50, 77], on thrombosis [92], and in psoriasis [32].

10. Closing Remarks

Although etosis was recently described [1], the literature concerning this new form of cell-mediated killing is growing quickly. Even though many advances have been achieved recently, the molecular mechanism underlying NET release is far from being understood. Different works have pointed out that only around 30% of the neutrophils stimulated by different stimuli dye by netosis. Many different microorganisms and molecules induce NET release, but it is unknown if netosis is triggered by common or different signaling pathways. Besides, many of the stimuli that induce NET release were formerly also described as inducers of other neutrophil functions, such as phagocytosis or chemotaxis. Thus, it is important to determine how and why some neutrophils, facing a parasite for instance, activate the netosis process, while others in the same population phagocytose the parasite. Neutrophils were short-lived cells and, presently, no markers of subpopulations are available to test if the different outcomes reflect distinct neutrophil subpopulations. Also, the maturing status of these cells could contribute for these differences, or the cytokine milieu as has been reported for polarized tumor-associated neutrophils [93].

Anyhow, netosis, or etosis in general, must be a strictly regulated process since it is a death mechanism, which released components, could participate either in antimicrobial defense and/or harm host tissues, and induce autoimmunity, where these structures are gaining increased relevance.

ETosis is proving to be a critical mechanism of host defense, offering new potential for disease control and defining new targets for intervening on infectious and also autoimmune diseases, besides grant novel tools for diagnosis [94] and/or prognosis [95].

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Review Article

Innate Immune Activation and Subversion of Mammalian Functions by *Leishmania* Lipophosphoglycan

Luis H. Franco,[1] Stephen M. Beverley,[2] and Dario S. Zamboni[1]

[1] *Department of Cell Biology, School of Medicine of Ribeirão Preto, University of São Paulo, FMRP/USP, 14049-900, Ribeirão Preto, SP, Brazil*
[2] *Department of Molecular Microbiology, Washington University School of Medicine, 660 S. Euclid Avenue, St. Louis, MO 63110, USA*

Correspondence should be addressed to Dario S. Zamboni, dszamboni@fmrp.usp.br

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Leishmania promastigotes express several prominent glycoconjugates, either secreted or anchored to the parasite surface. Of these lipophosphoglycan (LPG) is the most abundant, and along with other phosphoglycan-bearing molecules, plays important roles in parasite infectivity and pathogenesis in both the sand fly and the mammalian host. Besides its contribution for parasite survival in the sand fly vector, LPG is important for modulation the host immune responses to favor the establishment of mammalian infection. This review will summarize the current knowledge regarding the role of LPG in *Leishmania* infectivity, focusing on the interaction of LPG and innate immune cells and in the subversion of mammalian functions by this molecule.

1. Introduction: *Leishmania* and Lipophosphoglycan

Leishmaniasis is caused by infection with protozoan parasites of the Trypanosomatid genus *Leishmania*. The disease is endemic in several regions, including west Asia, Africa, and South America. In humans, several disease manifestations have been observed, ranging from self-healing cutaneous lesions to progressive and fatal systemic infection [1]. Leishmaniasis is transmitted by the bite of phlebotomine sand flies and in most parts of the world is a zoonosis, although in some areas direct human-fly-human transmission has been reported [1].

The life cycle of *Leishmania* has two main morphological forms: flagellated promastigotes, which replicate and develop in the midgut of the sand fly vector, and rounded amastigotes, which live and multiply inside the macrophages of the vertebrate host. The establishment of the infection begins with the inoculation by the sand fly vector's bite of metacyclic promastigotes into the vertebrate host. From this wound site, the parasites encounter a variety of cell types including neutrophils, Langerhans and dendritic cells, keratinocytes, and tissue macrophages, all of which have been proposed to serve as the "first contact" host cell (reviewed in [2]). While *in vitro* and in some cases *in vivo* studies provide good support for these models, the complex nature of the sand fly bite makes it difficult to ascertain the quantitative importance of these to the final parasitic outcome. Ultimately, the metacyclic forms of the parasite are internalized and differentiate intracellularly to the amastigote form. In macrophages, amastigotes multiply inside the acidic vacuoles, and eventually are released after lysis, spreading the infection to uninfected cells [3]. Current knowledge about the steps leading to parasite escape is limited, for example, whether it is regulated by the parasite or occurs simply through overwhelming the capacity of the macrophage to harbor them.

Leishmania promastigotes are covered by a thick glycocalyx comprised of abundant glycoconjugates important for parasite survival and pathogenesis. These molecules include Lipophosphoglycan (LPG), proteophosphoglycan (PPG), gp63 metalloproteinase, and glycophosphatidylinositol lipids (GIPLs). One notable feature distinguishing the *Leishmania* surface from that of the host is that most parasite molecules are linked to the parasite surface through glycosylphosphatidylinositol (GPI) lipid anchors [4–8]. *Leishmania* also

secrete protein-linked phosphoglycans (PGs), such as the secreted proteophosphoglycan (sPPG) and secreted acid phosphatase (sAP) [9].

LPG is the most abundant glycoconjugate on the surface of *Leishmania* promastigotes. The GPI anchor which links LPG at surface of the parasite is constituted by a 1-*O*-alkyl-2-*lyso*-phosphatidyl(*myo*)inositol lipid anchor with a heptasaccharide glycan core, to which is joined a long PG polymer composed of 15–30 [6-Gal(β1,4)Man(α1)-PO_4-] repeating units, and terminated by a capping oligosaccharide (Figure 1). The PG repeating units are often modified by other sugars, which are typically species and stage specific. Procyclic and metacyclic promastigotes of all *Leishmania* species express high amounts of LPG on their surface, in contrast to amastigotes, whose LPG expression is highly downregulated [10]. In promastigotes, LPG plays an important role for parasite survival inside sand fly vector and for macrophage infection, as discussed below. In contrast, the survival of amastigotes inside host macrophages is improved by other PG-containing glycoconjugates, such as PPG, which are highly expressed on its surface. All of the LPG domains are shared with other parasite surface molecules, to varying extents and degrees of relatedness. The PG repeat, side chains, and caps can be found on PPG or sAP, and both the GPI glycan core and lipid anchor have similarities with those present in both GIPLs and GPI-anchored proteins [8, 11, 12]. As described below, the usual of mutants defective in specific steps of LPG biosynthesis have proven useful in resolving the role of LPG domains clearly from related ones borne by other molecules.

2. The Role(s) of LPG and PGs in the Sand Fly Vector

A number of obstacles present in the sand fly vector digestive tract are potentially able to impair the development of *Leishmania*, including digestive enzymes, the midgut peritrophic membrane barrier, avoidance of excretion along with the digested blood meal, and the anatomy and physiology of the anterior gut (Figure 2). These barriers have provided the evolutionary drive for expression of molecules by the parasite required for successful development in the sand fly vector. As in the mammalian stages emphasized in later sections, LPG and related PGs are key molecules important for survival inside the hostile environment of sand fly vector [9].

During the digestion of blood meal in the insect midgut, the intracellular amastigotes initiate their differentiation to the motile procyclic promastigotes. These forms of the parasite leave the macrophages and are exposed to the hostile environment of the midgut. The dense glycocalyx formed by LPG and PPG provides protection against the action of midgut hydrolytic enzymes and by inhibiting the release of midgut proteases [13]. Procyclic promastigotes are able to attach to midgut epithelial cells, which enable the parasite to be retained within the gut during excretion of the digested blood meal. Several findings have suggested that LPG plays an important role in attachment of promastigotes in midgut in some species or strains such as the *L. major* Friedlin line [14–16], which binds to the sand fly midgut lectin PpGalec [17]. However, in other species, LPG appears to play less of a role in attachment, as LPG-deficient mutants retain the ability to bind [18, 19]. The molecules mediating this attachment are unknown although a role for parasite lectins has been suggested [20, 21]. For those strains/species dependent upon LPG for binding, the parasite must then find a way to release from the midgut in order to be free for subsequent transmission. To do this, metacyclic parasites synthesize an LPG unable to interact with host lectins. For *L. major* strain Friedlin, the procyclic Gal-β 1–3 PGs of LPG are"capped" with D-arabinopyranose, resulting in an LPG unable to bind PpGalec [17, 22]. In contrast, in *L. donovani* which synthesizes an LPG lacking PG modifications, binding through the terminal capping sugar is "masked" through elongation of the LPG chain [23].

The promastigote stage of many *Leishmania* species elaborates a thick mucoid "plug" during infections, comprised primarily of PPGs along with other shed parasite molecules. At the time of transmission by biting, the plug contents are inoculated along with parasites and saliva into the host. Seminal studies by Bates and collaborators have suggested that the PG repeats borne on PPGs within the plug play key roles in exacerbating the subsequent infections in *L. mexicana*, thereby implicating PGs synthesized and secreted by *Leishmania* in the fly as important immunomodulators of the host response [24, 25]. Notably sand fly saliva can exacerbate *Leishmania* infections as well. It is worth pointing out that most experimental studies of *Leishmania* transmission are compromised to some extent by the use of needle inoculated parasites, lacking these key biological mediators as well as differing in the amount of local tissue damage.

3. The Role(s) of LPG and Related PGs in Mammalian Infectivity

As seen with the sand fly stages, LPG and related PGs have been implicated in a variety of key steps required for infectivity of mammalian hosts (Figure 2). Here, we summarize the current information regarding the role of LPG for subversion of mammalian protective responses by the parasite, and the recognition of parasite LPG by the mammalian innate immune cells.

4. The Role of LPG for Avoidance of Lysis by Complement

Before the internalization by host cells, metacyclic promastigotes must evade lysis by the mammalian complement system. Several studies using purified LPG or LPG-deficient parasites have shown that this molecule defends against complement-mediated lysis [26, 27]. *L. major* metacyclic promastigotes, the infective forms for mammals, are resistant to complement-mediated lysis while the procyclic forms, which reside inside the sand fly vector, are highly susceptible [28]. This difference is conferred by changes in the length of the metacyclic LPG PG polymer domain, which bears about

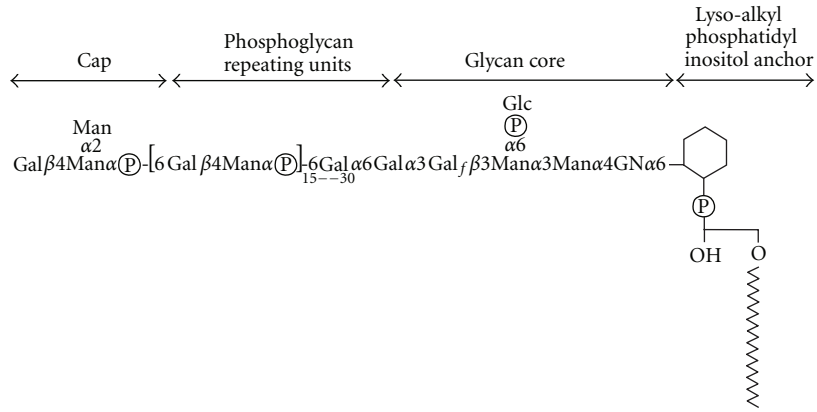

Figure 1: Structure of Lipophosphoglycan from *Leishmania donovani*. The four key domains (cap, phosphoglycan repeating units, glycan core and lipid anchor) are discussed further in the text. The number of phosphoglycan (PG) repeating units increases during metacyclogenesis, contributing to the role of LPG in complement resistance. In many *Leishmania* species, side chain modifications of the PG Gal residue are common, where they can play a role in sand fly transmission. The structure of the cap also differs amongst species. Gal, galactose; Man; Mannose; GN, glucosamine; Glc, glucose.

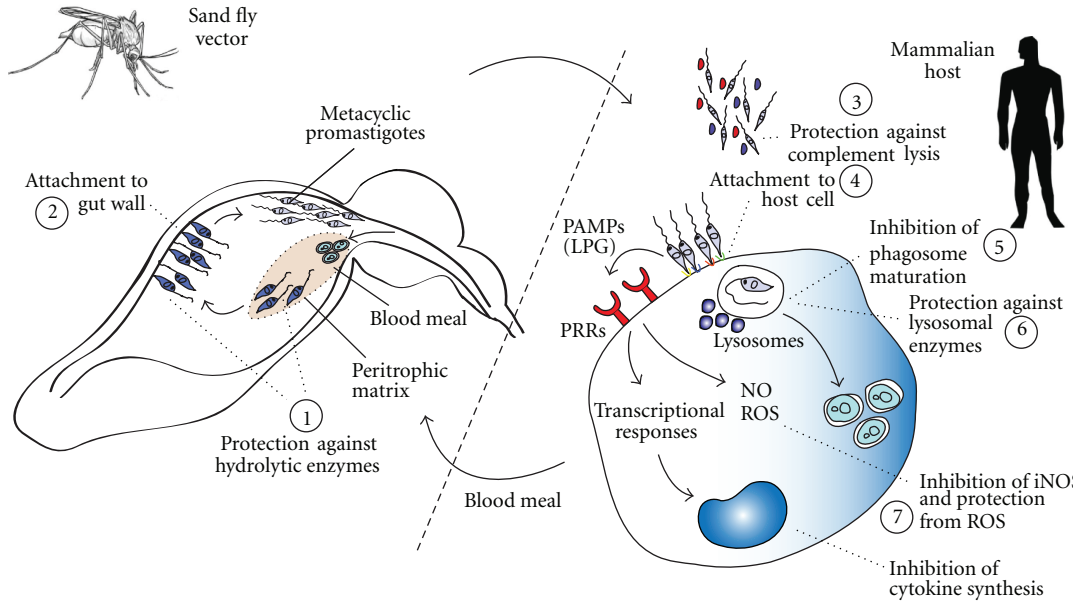

Figure 2: Role of LPG in *Leishmania* infectivity and virulence. Shown are putative and bona fide actions of *Leishmania* spp. LPG molecules in subversion of host and vector functions. These LPG functions include (1) physical protection to promastigotes against hydrolytic enzymes in the digestive tract of insect; (2) attachment of promastigotes to the gut wall; (3) In the mammalian host, promastigotes protection against lysis by complement proteins; (4) attachment of parasites to the macrophage membranes or alternative transiently infected cells, such as neutrophils, dendritic cells and perhaps others; (5) transient impairment of the phagosome maturation; (6) physical protection against degradation by lysosomal enzymes; (7) modulation of macrophages activation through impairing the synthesis of nitrogen species and cytokines related to the control of infection and protection from ROS.

twice as many repeating units as the procyclic promastigotes. This prevents the attachment of complement membrane attack complex (MAC) and pore formation on parasite surface [28]. However, earlier steps in the complement cascade may contribute in the entrance of *Leishmania* into macrophages through complement receptors. LPG, together with the protease gp63, is able to activate the complement system, leading to the generation of the C3b and C3bi opsonins. C3b and C3bi thus bind to *Leishmania* surface and mediate the parasite phagocytosis by complement receptor (CR) 1 and CR3 [29–35]. Phagocytosis of *Leishmania* via CR1 and CR3 receptors is considered as a means of "silent entry" into macrophages, because it does not prompt the oxidative burst and impairs the production of IL-12 [32, 36–38]. However, infections of CR3-deficient mice show very little attenuation of infection, suggesting that this step may be of lesser importance or redundant with other binding interactions in survival [39].

5. The Role of LPG in Parasite Invasion and Survival in Macrophages

After being inoculated into the mammalian host by the sand fly vector, the metacyclic promastigotes are internalized through interactions with a number of different receptors. While at various times interactions with one or another of these have been presumed or shown to be dominant in cellular or biochemical tests, genetic studies have typically led to conclusions that these interactions typically may instead be highly redundant in biological settings. In this scenario, the use of multiple receptors allows the promastigotes to be quickly internalized by macrophages (reviewed in [40]).

Importantly, the LPG plays an important role as a ligand during the attachment and invasion process of macrophages, either directly or indirectly through binding to other proteins.One example is the interaction of LPG with mannose-fucose receptor expressed by macrophages [41]. In addition, mannan-binding protein (MBP) is able to bind to mannose residues on LPG, enabling the formation of C3 convertase and generation of C3b, which helps promastigotes to attach to the macrophage as noted above [42]. C-reactive protein (CRP) binds to LPG of *L. donovani* metacyclic promastigotes triggering their phagocytosis by human macrophages via CRP receptor [43]. Commonly, the engagement of CRP receptor by its ligand leads to macrophage activation, resulting in proinflammatory cytokine production [44, 45]. However, phagocytosis of *L. donovani* by CRP receptor leads to an incomplete activation of macrophages, thus favoring parasite replication [46].

Following entry, promastigotes are contained in a phagosome known as parasitophorous vacuole (PV), which undergoes several fusion processes, giving rise to a phagolysosome-like organelle [47, 48]. During this process, LPG acts to delay PV fusion with lysosomes, promoting delay in PV acidification and acquisition of lysosomal enzymes [49]. Vacuoles harboring promastigotes of *L. donovani* and *L. major* genetically deficient for LPG fuse more extensively and rapidly with endosomes and lysosomes [27, 50]. While initially workers postulated that this delay protected promastigotes from acidic conditions and hydrolytic enzymes until they had differentiated to the more acidophilic amastigote stage, work with LPG-null *L. major* promastigotes provided little support for this model [27], as these parasites are able to survive under several conditions despite rapid fusion with host lysosomes. Instead, the delay in fusion reflects changes in membrane properties that result in delocalization of the host oxidative burst from its normal peri-PV location [51]. On top of this, LPG itself is able to interact and deflect oxidants directly [52]. Other roles of the delayed fusion may not only concern survival but immune recognition and antigen processing, which is dependent on host hydrolytic enzymes [50].

Whereas LPG seems to be important to protect *Leishmania* during differentiation from promastigote to amastigote forms, it does not play a significant role during the development of the amastigote form. Indeed, LPG expression on amastigotes of several species of *Leishmania* is highly downregulated (1000 fold or more) [10] suggesting that the protective role of LPG is transient and limited to the beginning of host cell infection. However, other PG-containing glycoconjugates and especially PPG are expressed at high levels in amastigotes, and act in PG-dependent manner to protect the amastigote [53, 54].

6. The Role of LPG for Inhibition of Macrophage Activation

Infected macrophages employ several microbicidal mechanisms to eliminate intracellular pathogens. When previously activated by interferon-gamma (IFN-γ) and tumor necrosis factor-α (TNF-α) or other microbial components, infected macrophages express high levels of the inducible nitric oxide synthase (NOS2), culminating with production of nitric oxide (NO), NO_2^-, and NO_3^- [55]. These nitrogen intermediates coordinate processes that lead to deprivation of important components, such as iron, which lead to restriction of intracellular parasites replication [56]. *L. major* is able to induce higher amounts of NOS2 in the cutaneous lesion and draining lymph nodes of the clinically resistant lineage C57BL/6 compared to the nonhealing BALB/c strain [57]. In addition, mice deficient for NOS2 are more susceptible to infection with *Leishmania*, compared to their littermate controls, as well as macrophages derived from these mice [58–60]. Thus, the production of NO is indispensable for the control of *L. major* infection and for maintaining life-long control of persisting *Leishmania* parasites [61–63].

In contrast, infection of unactivated macrophages typically leads to parasite survival and minimal levels of NOS2-dependent NO production, to the point that *L. major* was referred to as a "stealthy parasite" [64]. Thus, one of the challenges in experimental models is the need to distinguish infections where macrophages are naturally or experimentally activated from those situations where *Leishmania* exhibits successful parasitism and survival. Perusal of the literature suggests that many workers do not provide evidence about which fate meets *Leishmania* under their experimental infections, which may contribute occasionally to seemingly contradictory results.

Experimental studies have shown that similar to *Leishmania*, treatment of macrophages with *Leishmania* glycoconjugates can likewise regulate the activation of NOS2 and production of NO. LPG can synergize with IFN-γ for the induction of NO expression in murine macrophages *in vitro*. However, incubation of macrophages with LPG-derived PG before stimulation with LPG plus IFN-γ led to inhibition of NOS2 expression [65]. These studies provided evidence that the interaction between the macrophage and the parasite impairs the activation of the microbicidal mechanisms of macrophages after exposure to IFN-γ in a process that is replicated by PG treatments. Given these findings, it was surprising that despite the complete absence of LPG or all PGs in the $lpg1^-$ or $lpg2^-$ mutants (described further below), mutant parasites remained "stealthy" and able to down regulate host cell activation [27, 66]. A similar contradiction was seen in studies of the smaller GIPL, which are highly abundant in both parasites stages and had been shown

to inhibit NO synthesis by macrophage in a dose- and time-dependent manner, impairing its leishmanicidal activity [67]. However, mutants defective in the synthesis of the ether lipid anchor and thus lacking both LPG and GIPLs resembled the *lpg*1$^-$ and *lpg*2$^-$ mutants in remaining "stealthy" and inhibiting host cell activation [68]. This apparent paradox has not been resolved and has led to proposals that avoidance of host cell activation may be highly redundant amongst many parasite surface molecules, perhaps through their ability to interact with secondary ligands/mediators such as complement or other serum proteins. One attractive model is that macrophage deactivation is independent of surface molecules, instead depending on other processes such as secretion of parasite molecules through an exosomes-like or other pathways (reviewed in [69]).

In addition to NO, activated macrophages employ other antimicrobial molecules, such as ROS or antimicrobial peptides, to kill intracellular parasites. ROS such as superoxide, hydrogen peroxide, and hydroxyl radicals, are produced after activation of NADPH oxidase and interact with pathogen phospholipid membranes, inducing damage and dead of pathogens [70]. Some evidence highlights the importance of ROS in control of *Leishmania* growth [71, 72]. Upon infection by *L. donovani* promastigotes, peritoneal macrophages elicit a strong respiratory burst with release of superoxide anion, thus favoring the elimination of the intracellular amastigotes. When infected in the presence of catalase, an enzyme that catalyze the decomposition of hydrogen peroxide to water and oxygen, macrophages lost their ability to kill *L. donovani* [73]. These results show that ROS are central compounds that act to eliminate intracellular *Leishmania in vitro*.

Respiratory burst activity and NO production are regulated by phosphorylation events mediated by protein kinase C (PKC) [74]. Infection with *Leishmania* is able to inhibit PKC activity in macrophages and several findings suggesting that LPG is related to this activity, thereby favoring intracellular survival of the parasite through inhibition of both oxidative burst and NO production [10, 75–79]. Besides PKC, production of cytokines such as IL-12 was inhibited in bone marrow-derived macrophages after infection with *L. major* [80]. Furthermore, purified LPG plays similar inhibitory effect over IL-12 production, probably thought the activation of the mitogen-activated protein kinase (MAPK) Erk 1/2, which suppresses IL-12 gene transcription [81]. Besides IL-12, purified LPG also suppressed IL-1β gene expression in THP-1 monocytes induced by endotoxin, TNF-α or *Staphylococcus* stimulation [82].

7. Recognition of LPG by Mammalian Innate Immune Receptors

The initiation of immune response against invading pathogens starts upon the interaction of microbial molecules with receptors of innate immune cells. Glycoconjugates expressed by protozoans interact with macrophage receptors and are recognized as foreign by immune system. Purified GPI-anchored surface proteins of *Plasmodium falciparum*, *Trypanosoma brucei* and *L. mexicana*, initiate the rapid activation of macrophage protein tyrosine kinases (PTKs) [83–85]. GPI anchors expressed by protozoans, such as *Plasmodium* and *Trypanosoma*, can activate the secretion of cytokines, such as IL-12 and TNF-α, and NO synthesis by macrophages [83, 85–90].

The activation of Toll-like receptors (TLRs) by microbial ligands recruits the adaptor protein MyD88 (myeloid differentiation primary response gene 88) and triggers intracellular signaling events, culminating on the activation of the transcription factor NF-κB and its translocation to nucleus. NF-κB in turn induces innate immune mechanisms such as the production of reactive oxygen and nitrogen intermediates, chemokine/cytokine secretion, and cellular differentiation [91]. Several evidences have suggested that *Leishmania* expresses ligands able to stimulate the TLRs signaling pathways. RAW macrophages selectively upregulated the IL-1α mRNA expression in response to *L. major* infection, and this was not observed when macrophages were transfected with a dominant-negative of MyD88 or when peritoneal macrophages derived from MyD88-deficient mice were infected with *L. major* [92]. In addition, mice deficient for MyD88 infected with *L. major* showed an increase in lesion size compared to their littermate controls [93]. These results suggest that *L. major* may express ligands for TLR activation. Accordingly, *L. major* LPG activated NF-κB, the secretion of Th1-type cytokines, ROS, and NO by either human or murine macrophages in a mechanism dependent of TLR2 [93–95]. In addition, purified LPG upregulates TLR2 expression and stimulate IFN-γ and TNF-α secretion by human NK cells in a TLR2-dependent manner [96]. Thus, activation of TLR2 may contribute to host resistance against *Leishmania* and LPG is proposed to be a putative agonist for TLR activation.

In addition to macrophages and NK cells, LPG has been shown to exert stimulatory effects on dendritic cells (DCs). Purified *L. mexicana* LPG was able to induce the expression of CD86 and major histocompatibility complex class II (MHC-II) by DCs; furthermore, *L. major* LPG stimulated the expression of CD25, CD31, and vascular-endothelial cadherin by mouse Langerhans cells, albeit accompanied by inhibition of their migratory activity [97, 98]. Importantly, upregulation of stimulatory and costimulatory molecules in DCs occurs in response to activation of pattern recognition receptors; therefore, these studies corroborate the hypothesis that that *Leishmania* LPG triggers activation of these receptors.

Given the interaction of LPG with TLRs in the context of activated macrophages where this leads to a proinflammatory response and parasite control, an important but as yet unanswered question is how the LPG-TLR interaction fails to control parasite infection in unactivated macrophages. A variety of pathways are known which negatively regulate TLR signaling, and potentially one of these acts to mitigate TLR activation. A second question is whether LPG or related molecules are internalized into host cells, which would then place them in contact with variety internal sensors including the NOD-like receptors protein family in the cytosol. Early studies showed LPG trafficking into the interior of host cells [99] and recently several groups have provided evidence

suggesting that *Leishmania* molecules may gain access to the host cytosol through some routes, potentially including an exosome-like pathway [100, 101]. Further work is needed to confirm these provocative hypotheses and explore the role of LPG and related glycoconjugates in this process.

8. The Assessment of LPG Functions by Using LPG-Defective Mutants

As mentioned above, many studies have used purified LPG, fragments thereof, or related molecules, to investigate their role in *Leishmania* pathogenesis and host response. However, LPG preparations can include contaminating molecules including proteins, unless proper precautions are taken, and use of exogenous LPG may not properly mimic the physiological location and concentrations of LPG delivered by infecting parasites. Moreover, as noted above, many LPG domains are shared by other parasite molecules, raising the possibility that functions attributed to LPG *in vitro* may actually be fulfilled by LPG-related molecules *in vivo*.

In the last few years, the generation of LPG mutants of *Leishmania* has provided powerful tools to identify the function of these molecules [102–105]. Of note, the recent identification of genes related to LPG biosynthetic pathways allowed the generation of "clean" LPG mutant strains by specific gene targeting. *Leishmania* are typically diploid although recent studies suggest that many chromosomes may be aneuploid, at least transiently [106]. Thus, two or more successive rounds of gene replacement are required to generate full homozygous null mutants, as while feasible in some cases sexual crossing remains challenging [107]. Importantly, the phenotypes of the mutants chosen for biological studies were rescued by complementation of the specific LPG gene into the parasite [108–110]. This rules out the well-known problem of loss of virulence during transfection or culture of *Leishmania*, which occurs sporadically in all species. Thus far nearly 20 genes affecting various steps of LPG biosynthesis have been described through complementation of LPG mutants or through various reverse genetic strategies.

For the study of virulence, this repertoire of LPG genes has enabled researchers to concentrate on key mutants that cleanly affect LPG or related molecules. The first genetic assessment of the role of LPG in parasite virulence and host immunity followed the identification of the *LPG1* gene, which was recovered following complementation of the LPG-deficient R2D2 mutant of *L. donovani* [110]. This gene encodes a putative galactofuranosyl transferase involved in biosynthesis of the LPG glycan core, but not other galactofuranosyl-containing glycoconjugates whose synthesis depends on other LPG1-related transferases [111]. *lpg*1$^-$ mutants of *L. major* or *L. donovani* do not express LPG on their surface, while the expression of other glycoconjugates remains normal [112, 113], rendering these ideal for studies of the biological roles mediated exclusively by LPG. *lpg*1$^-$ mutants are highly susceptible to lysis by complement; sensitive to oxidative stress, and they fail to even transiently inhibit phagolysosomal fusion immediately after invasion [27]. Moreover, *L. major lpg*1$^-$ showed an impaired ability to survive inside macrophages [27, 113] and in mouse infections were highly attenuated, as represented by an extreme delay in lesion progression [27, 113].

Interestingly, the generality of the role of LPG or even PGs in parasite survival in all *Leishmania* has been questioned based on similar genetic studies in *L. mexicana*, where a proper *lpg*1$^-$ line shows no decrease in infectivity tests in macrophages or mice [112], although it is complement sensitive [114]. Despite these observations, the *lpg*1$^-$*L. mexicana* nonetheless showed some alterations in host response, with a poor ability to stimulate the expression of costimulatory molecules on mouse DCs, and it was found that *lpg*1$^-$*L. mexicana*-infected mice showed lower numbers of activated DCs in draining lymph nodes and were unable to control early parasite burden [97]. Thus, it appears that LPG plays a quantitatively or qualitatively different role in *L. mexicana* virulence, especially in directing the immune response. A similar contrast was found in studies of an *L. mexicana lpg*2$^-$, discussed below [115]. Amongst many potential explanations, the architecture of the PV has been proposed to be a factor, as it exists as a "spacious, multiparasite" compartment in *L. mexicana* infections versus a "tight, uniparasitic" compartment in *L. major* and *L. donovani* [114]. Thus, the roles of LPG appear to differ both quantitatively and qualitative amongst species.

While the LPG-deficient *lpg*1$^-$ parasites show severe attenuation in both *L. donovani* and *L. major,* studies in the latter species show that some parasites survive and go on to generate normal amastigotes, in keeping with the downregulation of LPG during development. Since other PG-containing glycoconjugates such as PPG are found throughout the life cycle, the role of the PG moieties generally was investigated by the use of a mutant globally affecting PGs. The *LPG2* gene was identified by complementation of the *L. donovani C3PO* mutant [109] and was shown in a series of seminal studies in Turco's laboratory to encode the Golgi GDP-mannose transporter [116–118], one of the founding members of what is now known to be a large family of nucleoside sugar transporters [119]. LPG2 was also the first multispecific nucleotide sugar transporters to be described, being able to carry both GDP-D-Arabinopyranose and GDP-Fucose in addition to GDP-Man [116]. As noted earlier, *L. major* utilizes D-Arabinopyranose as an LPG side chain "capping" sugar, but neither a role nor glycoconjugates bearing fucose has been described in *Leishmania*, although low levels of GDP-Fuc have been observed in promastigotes [120].

*lpg*2$^-$ mutant parasites lack all PGs, including LPG and PPG, but synthesize normal levels of GIPLs and gp63 [66]. *L. major* and *L. donovanI lpg*2$^-$ mutants failed to survive in the midgut of sand fly vector and were unable to establish infection in macrophages. In animal infections, *L. major* parasites showed "persistence without pathology," with parasites persisting at low levels for the life of infected animals—a situation reminiscent of the life-long infection following healing of *Leishmania* infections in experimental animals and humans [16, 66, 114]. This parallel was further extended by the demonstration that as in healed animals, *L. major lpg*2$^-$ induced long-term immunity against challenge with a virulent strain of *L. major* [121]. Observations that

lymphocytes isolated from *L. major lpg*2$^-$-infected mice produced less IL-4 and IL-10 after stimulation *in vitro*, compared to cells isolated from *L. major* WT-infected mice, provided evidences about the anti-inflammatory properties of PGs over immune cells [122]. Importantly, similar effects on cytokine expression were seen in the *lpg*5A$^-$/*lpg*5B$^-$ double mutant, which also lacks all PGs, but through inactivation of Golgi UDP-Gal transporter activity [122, 123]. However, the *lpg*5A$^-$/*lpg*5B$^-$ mutant shows a virulence defect comparable to that of the *lpg*1$^-$ rather than *lpg*2$^-$ mutant [123].This suggests that the "persistence without pathology" phenotype of the *lpg*2$^-$ mutant may arise from effects on gylcoconjugates other than PGs [123]. Thus, comparison amongst the well-characterized collection of LPG/PG mutants provides a "genetic sieve", allowing assignment of the roles of LPG and PGs separately and in immune interaction from their roles in general parasite infectivity. These studies using *lpg*2$^-$ mutant parasites provided evidence that PGs, in addition to LPG, play important roles in *Leishmania* virulence [122, 123].

9. Concluding Remarks

LPG is a key molecule mediating many important steps essential for *Leishmania* virulence, in the hostile environment of the sand fly vector midgut, or in the mammalian host. The identification of genes related to LPG synthesis allowed the generation of *Leishmania* strains defective in LPG. The uses of these mutants have provided valuable clues about the role of this glycoconjugate in the biology of *Leishmania*. We envisage that further studies using these and new mutants may elucidate important issues related to innate immune recognition and host cell activation by protozoan parasites. This information will greatly increase our understanding of both *Leishmania* pathogenesis and the recognition of protozoan parasites by the mammalian innate immune system.

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Clinical Study

Evidence for T Cell Help in the IgG Response against Tandemly Repetitive *Trypanosoma cruzi* B13 Protein in Chronic Chagas Disease Patients

Marcia Duranti,[1,2] Ludmila Camargo,[1,3,4] Gabriel Victora,[1,3,4,5] Barbara Ianni,[6] Paula Buck,[6] Charles Mady,[6] Jorge Kalil,[1,3,4] Bianca Zingales,[2] and Edecio Cunha-Neto[1,3,4]

[1] *Laboratory of Immunology, Heart Institute (InCor), School of Medicine, University of São Paulo, 05403-900 São Paulo, SP, Brazil*
[2] *Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, 05508-800 São Paulo, SP, Brazil*
[3] *Division of Clinical Immunology and Allergy, School of Medicine, University of São Paulo, 01246-903 São Paulo, SP, Brazil*
[4] *Institute for Investigation in Immunology (iii), INCT, 05403-900 São Paulo, SP, Brazil*
[5] *Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142, USA*
[6] *Myocardiopathies Unit, Heart Institute (InCor), School of Medicine, University of São Paulo, 05403-900 São Paulo, SP, Brazil*

Correspondence should be addressed to Edecio Cunha-Neto, edecunha@usp.br

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The tandemly repetitive *Trypanosoma cruzi* B13 protein is an immunodominant antigen among Chagas disease patients. Such repetitive domains may behave as T-independent antigens. However, T cells can recognize B13 epitopes in an HLA class II-restricted fashion and could potentially provide cognate T cell help and boost antibody titers. We assessed whether the presence of HLA class II molecules able to present B13 epitopes to T cells could affect anti-B13 IgG levels in a cognate fashion, in both major clinical forms of chronic Chagas disease. We found no difference between anti-B13 IgG antibody levels between patients carrying HLA class II molecules associated to T cell responses or other alleles. The predominant anti-B13 IgG subclass was IgG1, with negligible IgG2, suggesting a T-dependent, noncognate help for antibody production. In addition, the finding of increased anti-B13 IgG levels in sera from CCC patients indicates that clinical presentation is associated with increased anti-B13 antibody levels.

1. Introduction

Pathogenic protozoa like *Trypanosoma cruzi*, the causative agent of Chagas' disease, contain regions of tandemly repetitive amino acid sequences embedded in several immunodominant protein antigens [1]. The function of this type of protein domains is poorly understood. The repetitive elements are diverse in number, size, and distribution with several common features like their immunodominance, the bias in the component aminoacids, and an unusual evolutionary history [2]. Little is known about the molecular mechanisms that lead to the immunodominance of repeated sequences, except for their multivalency which can make them activate B cells directly and behave, in some cases, as T-independent antigens by crosslinking hapten-specific surface immunoglobulin [3]. It is known that the repetitiveness of a variety of agents causes MHC class II-independent, T cell independent activation of B cells, and it has been shown that tandemly repetitive carbohydrate antigens elicit IgG2 subclass antibody responses in humans [4–6]. B13 protein is an immunodominant recombinant antigen which encodes part of the tandemly repeated domain of the *T. cruzi* 140/116 kDa protein located on the surface of infective trypomastigotes from several strains. B13 protein from the Y strain of *T. cruzi* is formed by 19 tandemly repeated degenerate copies of a 12-amino acid motif P(L)P(S,A)P(L)FGQAAA(E)G(A)D(G)K, where residues in parentheses replace the preceding residue in different repeats [7]. B13 protein is recognized by IgG serum antibodies from 97% of *T. cruzi* infected individuals

in Latin America, bearing both forms of chronic Chagas' disease: chronic Chagas' disease cardiomyopathy (CCC) and the asymptomatic/indeterminate form (ASY) [8, 9]. Indeed, there is evidence supporting that the antibody response towards repetitive protozoan antigens is T cell-independent [10, 11].

However, it cannot be excluded that T cell help could boost antibody titers and promote IgG subclass switch. Cognate T cell help is provided when T cells and B cells of the same antigen specificity are in close contact, and wherein the antigen is presented directly to T cells in association with B cell surface HLA class II molecules. Cognate T cell help occurs only in individuals who carry HLA class II molecules that allow presentation and recognition of that antigen to T cells. Noncognate T cell help, on the other hand, occurs when T cells activated by one antigen presented by an antigen-presenting cell provide help for antibody production to a B cell with different antigen specificity [12, 13]. Repetitive sequences in protozoan parasite antigens have been shown to elicit T cell-dependent immune responses. It has been reported that peripheral T cells from *P. falciparum* malaria patients recognize T cell epitopes in EB200, a repetitive region of the *P. falciparum* antigen Pf332, the Pf155 repetitive domain of ring-infected erythrocyte surface antigen (RESA) and the repetitive domain of circumsporozoite CS protein [14, 15]. We have previously shown that PBMC proliferative and cytokine responses to B13 and its synthetic peptides are frequent among *T. cruzi*-infected subjects [8, 16]. We also found that T cell responses to B13 protein are restricted to patients carrying certain HLA class II alleles (HLA-DQA1*0501(DQ7), HLA-DR1, and HLA-DR2), which bind and present B13 peptides directly [8]. It is thus conceivable that B13-specific T cells could provide cognate help for the production of anti-B13 antibodies among *T. cruzi*-infected patients that carry such HLA class II molecules. To test this hypothesis, we will assess whether the presence of at least one of these HLA alleles increases the magnitude of IgG responses to the B13 antigen in Chagas patients stratified into ASY or CCC forms of disease. We will also assess whether the presence of B13-presenting HLA alleles would influence the IgG subclass profile, since distinct IgG subclass profiles have been associated to T-dependent or T-independent IgG responses [17, 18].

2. Methods

2.1. Study Population. Serum samples were taken from a total of 112 patients infected with *T. cruzi*, stratified into the two forms of Chagas' disease: chronic Chagas cardiomyopathy (CCC; $n = 72$) and the indeterminate/asymptomatic form (ASY; $n = 40$). The patients were recruited from the Heart Institute (InCor), University of Sao Paulo School of Medicine. Chronic Chagas cardiomyopathy (CCC) subjects fulfilled the following diagnostic criteria: positive *T. cruzi* serology, typical ECG abnormalities (left anterior hemiblock and/or right bundle branch hemiblock), with or without varying degrees of ventricular dysfunction, with all other etiologies of ventricular dysfunction/heart failure excluded. Indeterminate/asymptomatic subjects (ASY) showed positive *T. cruzi* serology but normal ECG and bidimensional echocardiography. The study protocol was approved by the Institutional Review Board of the University of São Paulo School of Medicine. All patients had given informed consent for the use of their blood samples for research.

2.2. HLA Class II Typing. DNA was extracted by either DTAB/CTAB or salting out methods [19]. DR typing was performed by low-resolution PCR-SSP as previously described [20–22]. DQA1, and DQB1 typing was performed by PCR-SSO using generic primers for exon-2 amplification [23].

2.3. Direct ELISA. 96-well polystyrene plates (Corning, USA) were coated with 30 ng/well of recombinant B13 protein in carbonate-bicarbonate buffer, pH 9.6, for 16 h at 4°C. Plates were washed with PBS and blocked with PBS-2% BSA for 30′ at 37°C. Serum samples at 1/50 dilution were incubated for 1 h at 37°C. After ten washing cycles with PBS-0.1% Tween 20 (PBS-T), wells were incubated with HRP-conjugated anti-human IgG for 30′ at 37°C. After ten additional washing cycles with PBS-T, samples were incubated with substrate solution (4 mg OPD in 10 mL citrate buffer, pH 5.0) for 30′ at 37°C. Absorbance at 490 nm wavelength was measured using an automated plate spectrophotometer.

2.4. IgG Subclass Measurement. Anti-B13 antibody subclass measurement was carried out by ELISA following essentially the same protocol described above except that a concentration curve was made by incubating different concentrations of IgG subclass (IgG1, 2, 3, and 4) standards. We also used HRP conjugated mouse anti-human IgG1, 2, 3, and 4 (Pharminrgen BD) diluted in PBS containing 1% calf serum PBS-T that was added to each well (Dilution of 1 : 1,000). After 1-hr incubation at 37°C, plates were incubated with substrate solution (4 mg OPD in 10 mL citrate buffer, pH 5.0) for 30′ at 37°C. Absorbance at 490 nm wavelength was measured using an automated plate spectrophotometer. The cutoff value was determined as the mean value of optical densities from control sera plus 3 standard deviations.

3. Statistical Analysis

Groups were compared by a nonparametrical test (Mann-Whitney Rank Sum Test) with GraphPad InStat software (version 5.0; GraphPad). Results were expressed as medians and interquartile ranges. P-values were considered significant if <0.05.

4. Results

4.1. Anti-B13 IgG Antibodies among Carriers and Noncarriers of B13-Binding HLA Class II Molecules. We divided the 112 *T. cruzi*-infected patients into two groups, 76 subjects bearing one or more of the HLA alleles required for presentation of B13 to T cells (HLA-DQ7, DR1, and DR2), termed HLA+, and 36 subjects lacking these alleles, HLA−.

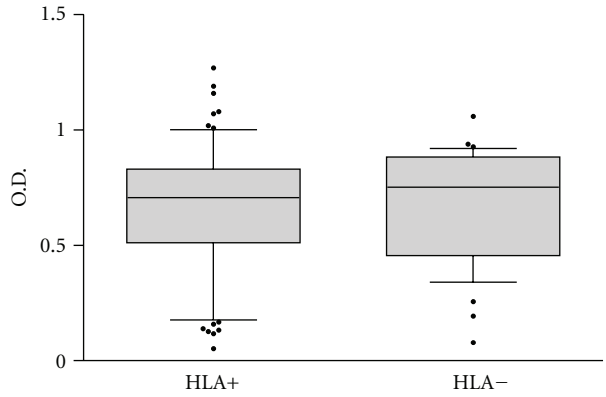

Figure 1: Direct ELISA for total IgG reactivity to recombinant B13 protein in the solid phase. 76 subjects bearing HLA alleles capable of presenting B13 peptides to T cells HLA-DQ7, DR1, and DR2 (HLA+), and 36 subjects bearing other HLA alleles (HLA−). The horizontal bar indicates median absorbance in each group. $P = 1.00$, *Mann-Whitney Rank Sum Test*.

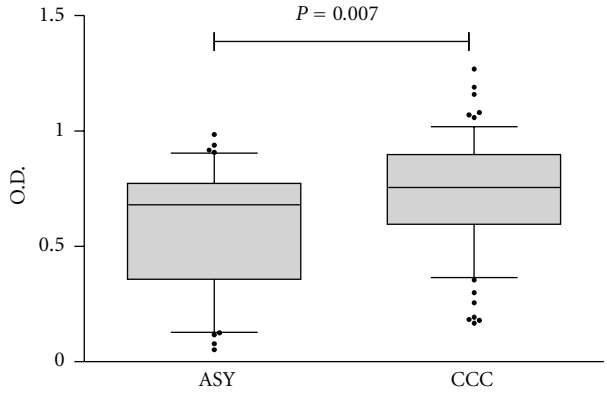

Figure 2: Direct ELISA for total IgG reactivity to recombinant B13 protein in the solid phase. Comparison between CCC (subjects with chronic Chagas Cardiomyopathy) and ASY (subjects with indeterminate/asymptomatic form of the disease). ASY and CCC subjects (median Abs. = 0.756 versus 0.681, resp.). The box plots whiskers show median values and 25–75% percentile; the 10% and 90% percentile; ou tlier points are shown above and below the figure.

Figure 1 shows that there was no significant difference in total IgG reactivity to B13 between HLA+ and HLA− patients. We then subdivided the group of subjects into different clinical presentations of Chagas disease, CCC ($n = 72$) and ASY ($n = 40$). Sera from CCC patients displayed a significantly higher total IgG anti-B13 reactivity than ASY patients, although there was some overlap between samples in both groups (Figure 2). When we further stratified the clinical groups in HLA+ and HLA− patients, we observed no significant difference in total IgG anti-B13 reactivity between samples from HLA+ to HLA− patients from the CCC and ASY groups (Figure 3).

4.2. Anti-B13 IgG Subclass Determination among Carriers and Noncarriers of B13-Binding HLA Class II Molecules. We evaluated whether the presence or absence of the relevant HLA alleles was associated with differences in distribution among the four IgG subclasses analyzing 87 subjects (HLA+; $n = 53$, and HLA−; $n = 34$). Figure 4 shows that there were no differences in the distribution of IgG subclasses between groups, with IgG subclass levels ranking from IgG1 > IgG3 > IgG2 > IgG4 in both HLA+ and HLA− subjects. We further stratified the subjects in our study separating them into the two clinical forms of Chagas disease, CCC ($n = 32$) and ASY ($n = 39$), inside the two HLA groups (HLA+ and HLA−), but no significant difference was observed (Figure 5).

5. Discussion

In this study, we further analyzed the magnitude of IgG responses and IgG subclasses to the *T. cruzi* tandemly repetitive B13 antigen in Chagas patients. We demonstrated that there is no difference in anti-B13 IgG antibody levels between patients carrying the B13 presenting HLA class II molecules, associated to T cell responses (HLA+) and those carrying other alleles (HLA−). Nevertheless, we found increased anti-B13 IgG levels in sera from CCC as compared to ASY patients, but no differences in anti-B13 IgG levels were observed between HLA+ and HLA− patients in the CCC and ASY groups. Regarding IgG subclasses, there was a predominance of IgG1 against B13 protein antigen, in both patient groups (HLA+ and HLA−); we also found no difference in levels of any of the IgG subtypes in the CCC and ASY groups, regardless of their HLA type.

The finding that anti-B13 IgG antibody levels of patients carrying the B13-presenting HLA class II molecules, associated to T cell responses, are similar to those carrying other alleles argues against a role for genetically restricted (thus, cognate) T cell help to anti-B13 antibody production. On the other hand, the IgG subclass distribution of anti-B13 antibodies—predominantly IgG1—argues against its being a strict T-independent (T1-2) antigen, since, even though it shows no genetic restriction, it fails to induce IgG2 antibodies as descript for tandemly repetitive carbohydrate antigens [4–6]. Antibodies against nonrepetitive (and presumably T-dependent) recombinant proteins [24] or *T. cruzi* epimastigote homogenate [25] in sera from chronic Chagasic patients were essentially of the IgG1 and IgG3 subclasses. This established the IgG1/IgG3 profile as the standard response against nonrepetitive *T. cruzi* proteins. Our finding that the predominant IgG subclass in the anti-B13 response is IgG1 supports the idea that the IgG antibodies may be generated under noncognate T cell help. This is further supported by the absence of genetic restriction of the anti-B13 IgG antibody responses. It is conceivable that B13-specific B cells may receive noncognate help from T cells specific for other *T. cruzi* proteins, cointernalized with B13, during *T. cruzi* infection. The same mechanism has been observed in systemic lupus erythematosus, where DNA-specific B cells may be helped by T cells specific for histones or other nuclear proteins [26]. However, since the nature of the B13 protein available for endocytosis by B cells during *T. cruzi* infection is unknown, this argument remains speculative.

The significant difference found in anti-B13 IgG antibody levels in CCC as compared to ASY is striking. This has

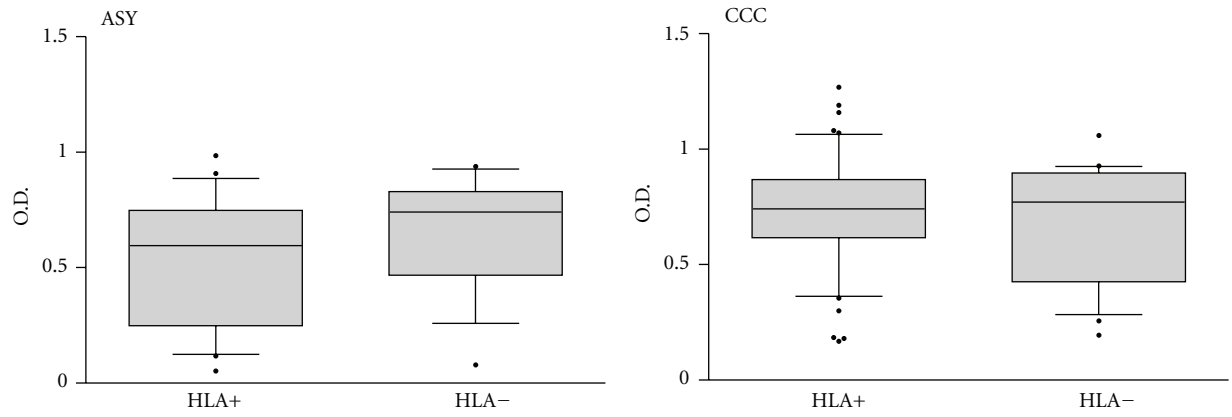

Figure 3: Direct ELISA for total IgG reactivity to recombinant B13 protein in the solid phase. The HLA+ group (subjects bearing HLA alleles capable of presenting B13 peptides to T cells,HLA-DQ7, DR1, and DR2); and the HLA− group (subjects bearing other HLA alleles). The horizontal bar indicates median absorbance in each group.

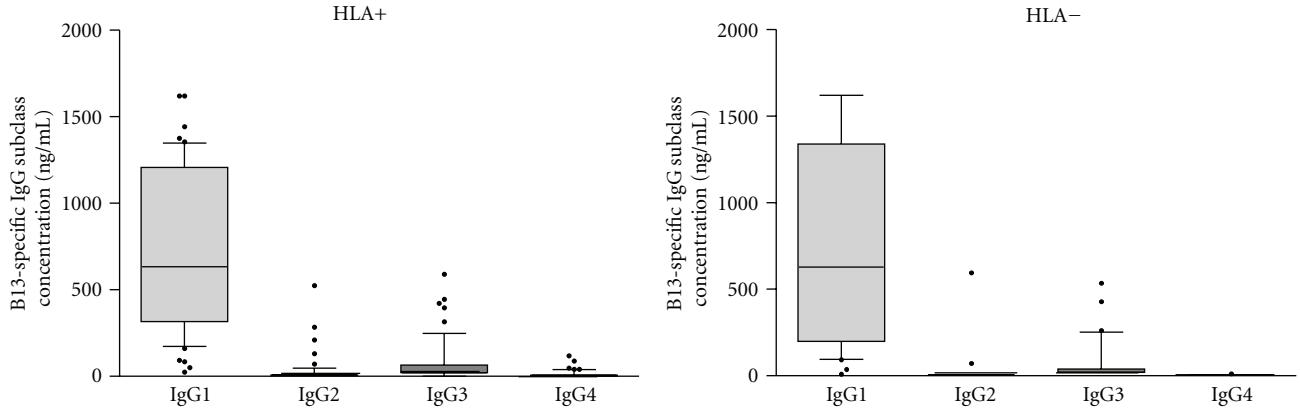

Figure 4: Direct ELISA for levels of the four subclasses of IgG reactive to recombinant B13 protein in the solid phase in HLA+ subjects ($n = 53$) and HLA− subjects ($n = 34$). The horizontal bar indicates median concentration of each IgG subclass in each group: IgG1; IgG2; IgG3; IgG4.

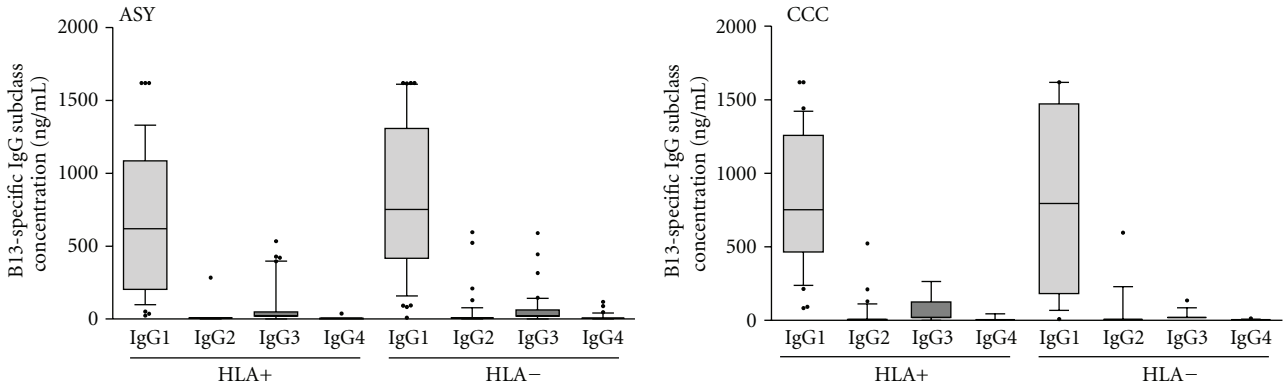

Figure 5: Direct ELISA for levels of the four subclasses of IgG reactive to recombinant B13 protein in the solid phase in ASY and CCC subjects; HLA+: subjects bearing HLA alleles capable of presenting B13 peptides to T cells (HLA-DQ7, DR1, and DR2); HLA−: subjects bearing other HLA alleles. The horizontal bar indicates median concentration of each subclass in each group: IgG1; 2: IgG2; 3: IgG3; 4: IgG4.

also been reported for a few other recombinant *T. cruzi* antigens, such as antigens JL5, JL9, and the C-terminal region of *T. cruzi* HSP70 [27]. B13 and JL5 have been described as cross-reactive with cardiac myosin [28–30] and cardiovascular beta-adrenergic and cholinergic receptors respectively [31]. It is possible that the higher levels displayed in sera from CCC patients of these two potentially pathogenic cross-reactive antibody systems are biologically relevant in the generation of pathological autoimmunity. Our results indicate that, even though B13 protein is a tandemly repetitive, TI antigen, the detectable IgG response to it apparently depends on noncognate T cell help. Further studies, including assessing direct B-cell activation by B13, may be necessary to definitively establish the nature of the serum IgG response to B13 found in chronic Chagas disease patients.

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Review Article

The Immune Response to *Trypanosoma cruzi*: Role of Toll-Like Receptors and Perspectives for Vaccine Development

Mauricio M. Rodrigues,[1] Ana Carolina Oliveira,[2] and Maria Bellio[3]

[1] *Centro de Terapia Celular e Molecular (CTCMol), Universidade Federal de São Paulo (UNIFESP), 04044-010 São Paulo, SP, Brazil*
[2] *Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro (UFRJ), 21941-902 Rio de Janeiro, RJ, Brazil*
[3] *Instituto de Microbiologia Paulo de Góes, Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro (UFRJ), CCS, Avenida Carlos Chagas Filho, 373 Bloco D, sala 35, Cidade Universitária, 21941-902 Rio de Janeiro, RJ, Brazil*

Correspondence should be addressed to Maria Bellio, mariabellioufrj@gmail.com

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In the past ten years, studies have shown the recognition of *Trypanosoma cruzi*-associated molecular patterns by members of the Toll-like receptor (TLR) family and demonstrated the crucial participation of different TLRs during the experimental infection with this parasite. In the present review, we will focus on the role of TLR-activated pathways in the modulation of both innate and acquired immune responses to *T. cruzi* infection, as well as discuss the state of the art of vaccine research and development against the causative agent of Chagas disease (or American trypanosomiasis).

1. Introduction

Trypanosoma cruzi is an intracellular trypanosomatid protozoan, which is transmitted to the human host by blood-feeding reduviid bugs, members of the insect subfamily Triatominae. Other modes of transmission include oral infection through contaminated food, congenital transmission, blood transfusions, organ transplants, and by accidental laboratory inoculation. This parasite, as well as its vector and the disease it causes, was first described by Chagas in 1909 [1]. Presently, the World Health Organization (WHO) estimates that approximately 10 million people are infected [2]. While Chagas disease is endemic to Central and South America, in the last years infected individuals have also been registered among immigrants in the United States, Europe, and Japan [3]. Although most of these cases were imported from the endemic regions, vector-transmitted autochthonous infections have also been documented in the United States. This fact and the lack of mandatory screening for all blood and tissue donors point to a possible altered epidemiology of Chagas disease in a near future.

The determinants of Chagas disease come from the burden and the lineage of the inoculated parasite, as well as the infection route and the immune competent status of the host. Two different phases of the disease follow the entrance of *T. cruzi* into the host (for a review see [4]). The acute phase lasts around two months and is asymptomatic in most infected individuals although some patients can present symptoms like prolonged fever, anorexia, nausea, vomiting, and diarrhea. During this phase, high numbers of parasites are frequently found in the host bloodstream and tissues, as well as high plasma levels of cytokines and intense activation of B and T lymphocytes. Also, lymphoadenopathy, splenomegaly, and intense inflammatory processes may be associated with parasite nests within tissues. A small percentage (5–10%) of infected individuals can develop a more severe condition, presenting myocarditis or meningoencephalitis, which they may die of. Most of the contaminated individuals remain asymptomatic (indeterminate form) often for years or even decades, but, then, around 30% of patients develop cardiac or gastrointestinal complications, characteristics of the chronic

phase of the Chagas disease. The pathological basis of chronic chagasic cardiomyopathy (CCC) has been a matter of intense debate for years. Immunopathology due to parasite persistence is considered a key element in the development of CCC although there is also evidence of a role for autoimmunity. During the chronic phase (indeterminate or not) few or no parasites are found in the circulation, but reactivation may occur by immunosuppression, particularly AIDS, and by pregnancy. The only effective and approved drugs in the treatment of the acute phase, or of the reactivation of the disease, are nitrofuran (nifurtimox, Lampit) and nitroimidazole (benznidazole, Rochagan), which are not fully satisfactory because of their limited efficacy in the chronic stage and of their important adverse side effects. Host control of *T. cruzi* has been shown to depend on both humoral and cell-mediated adaptive responses as well as on elements of the innate immune system [5]. To date, however, no human vaccine against infection with *T. cruzi* is currently available. Finally, the economic and social burdens due to early morbidity and mortality caused by Chagas disease are considerable, leading to high economic losses in Latin America. Understanding of the pathogenesis of Chagas disease will add to the development of new molecular targets for prophylactic vaccines and drug therapies, which are of extreme need for combating this emerging neglected disease.

2. Innate Immunity and TLRs

For a long time innate responses were believed to be nonspecific to the invading pathogen. In contrast, acquired immunity mediated by T and B lymphocytes was shown to display a fine specificity for the different pathogen-derived antigens through the employment of clonal receptors, which result from the genetic recombination of hundreds of different gene segments. The discovery in 1996 that the *Drosophila* transmembrane protein Toll specifically mediates the recognition and the response to fungal infection [6], followed by the cloning of several related receptors in other species, including human [7] and the discovery that one of these molecules (TLR4) is the receptor for lipopolysaccharide (LPS) [8], challenged the dogma that attributed nonspecificity to innate immunity. Owing to the new receptors' similarity to the Drosophila Toll, these molecules were called Toll-like receptors, or TLRs. So far, 10 and 12 different functional TLR-family members have been identified in man and mice, respectively, of which TLRs 1–9 are conserved in both species, TLR10 is selectively expressed in humans and TLR11, TLR12 and TLR13 are present in mice but not in humans (reviewed in [9]). Each TLR recognizes different chemical structures, which are highly conserved in microorganisms and collectively referred to as pathogen-associated molecular patterns (PAMPs). Among these are lipids, carbohydrates, nucleic acids, and various proteins derived from bacteria, viruses, fungi, protozoa, and helminth parasites. Moreover, TLR-signaling pathways may also be activated by self components released by tissue damage or inflammation, the so-called damage-associated molecular patterns (DAMPs), which alert the immune system to danger resulting either from sterile insult or from infection [10]. To learn the detailed mechanisms by which the innate immune system detects and responds to parasites is crucial to understanding how infection is controlled. However, only recently insights into how the TLR-signaling system responds to infection by protozoans, including *Trypanosoma cruzi, Trypanosoma brucei, Leishmania* spp., *Plasmodium* spp. *and Toxoplasma gondii* have emerged [11]. Different TLRs show a diverse expression pattern in a variety of cells and tissues, as well as different subcellular localization (either on the cell surface or within endosomal compartments). Although a certain degree of redundancy exists between signals induced by the various TLRs, recent studies have identified signaling pathways specific for individual TLRs, involving different adaptor molecules responsible for signal transduction. This leads to cytokine release profiles specific for particular PAMPs, and, thus, TLRs confer a certain degree of specificity to the innate-immune response. The formation of heterodimers among diverse TLRs (as TLR2/TLR6 or TLR2/TLR1) or the employment of accessory molecules (as CD14 or CD36), for the recognition of certain PAMPs but not others, creates a further degree of specificity [12]. Recognition of microbial components by TLRs triggers the initial innate immune response leading to inflammatory gene expression and, eventually, to the clearance of the infectious agent. Moreover, TLR-mediated recognition, by inducing the maturation of dendritic cells and, consequently, directing the T helper responses, represents a link between the innate- and acquired-immune systems [9]. Finally, as a result of studies searching for TLR agonists and antagonists, as well as for inhibitors of TLR-signaling pathways, drugs with these properties are currently being tested in a variety of therapeutic applications, and at least one TLR agonist (monophosphoryllipid A-MPL) has already been approved as adjuvant in vaccines [13].

Other germline-encoded innate receptor families were discovered in the last years and, together with TLRs, are collectively called pattern-recognition receptors (PRRs). These include membrane-bound C-type lectin receptors (CLRs), cytosolic proteins such as nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and RIG-I-like receptors (RLRs) (reviewed in [14]). Although TLRs play a central role in the initiation of immune responses against different pathogens, microbes display multiple PAMPs, which activate both TLRs and other PRRs, becoming evident that PRRs other than TLRs are also involved in the control of innate immunity. Moreover, while TLR ligand specificity, signaling pathways, and cellular trafficking have been broadly studied, less is known about the expected crosstalk between different PRR pathways, and the consequences that such an interaction would have for the induction of effective innate and acquired immune responses.

As reviewed here, after infection with *T. cruzi,* several inflammatory genes are activated through different TLR pathways. This leads to inflammatory response and induction of diverse effector mechanism of the adaptive immune response, which culminates with pathogen control, though the sterile cure is not achieved. On the other hand, very little is known about *T. cruzi* recognition by other PRRs. Recently, the first example of NLR-dependent response accounting for

host resistance against infection with a protozoan has been reported [15]. In this work, *Nod1*$^{-/-}$ mice were shown to be very susceptible to *T. cruzi*, succumbing to the infection and displaying higher parasitemia and parasite loads in the spleen and heart tissues, although NOD1 deficiency does not impair the production of different cytokines as IL-12, TNF-α, IFN-γ, or IL-10. As *T. cruzi* parasites lack peptidoglycan or any known agonist for NOD1, it would be interesting to determine whether NOD1 directly senses a *T. cruzi*-derived PAMP, or if the NOD1 pathway is indirectly activated during infection. Therefore, the detailed mechanism by which NOD1 confers resistance to infection with *T. cruzi* remains to be described and a possible cross-talk between NLR and TLR pathways during infection with *T. cruzi* waits for further investigation.

3. TLR Agonists Expressed by *T. cruzi*

In the past years, different groups have identified diverse *T. cruzi*-derived molecules that act as TLR agonists, inducing the production of nitric oxide (NO) and the secretion of inflammatory cytokines and chemokines by cells of the monocytic lineage. The first major class of *T. cruzi* molecules to be characterized as PAMPs was trypomastigote-derived glycosylphosphatidylinositol (tGPI) anchors of mucin-like glycoproteins, which are distributed at the cell-surface membrane of *T. cruzi* and were identified as potent activators of TLR2 from both mouse and human origin [16]. Proinflammatory activity of tGPI was shown to be dependent on its fine structure, mainly the unsaturated fatty acid at the sn-2 position of the alkylacylglycerolipid component. In contrast, another member of the GPI family purified from epimastigote forms, named glycoinositolphospholipid (eGIPL) and whose lipid moiety is instead composed by a *N*-lignoceroylsphinganine, was shown to induce NF-κB activation via TLR4 [17]. GIPLs are free anchors abundantly present at the surface membrane of all parasite stage forms, presenting different biological effects on different cell types [18, 19]. Importantly, the structure of GIPLs displayed by the infective metacyclic trypomastigote and by the epimastigote forms is very similar to each other, containing the same conserved Man4-GlcN glycan sequence and the myo-inositol-phosphate-lipid moiety predominantly (70%) formed by inositol-phosphoceramides, although its constitution may change depending on the *T. cruzi* strain [20]. For example, while GIPLs from Y, G, and Tulahuen strains contain ceramide, those from the CL strain are a mixture of dihydroceramide and alkylacylglycerol species [21]. Therefore, the variable lipid moiety composition of different GPI anchors determines whether their recognition is mediated by TLR2 (alkylacylglycerol) or TLR4 (dihydroceramide). Although tGPI (TLR2 agonist) and eGIPL from Y strain (TLR4 agonist) were not compared in the same assay for their relative capacity of inducing proinflammatory responses on cells expressing normal levels of TLR2 and TLR4 molecules, results obtained with human TLR2-transfected CHO cells, which also express endogenous levels of hamster TLR4, suggested a 100-fold superior activity of tGPI anchors [16]. An interesting point yet to be investigated is whether these different GPI anchors, which may be released by the parasite by shedding [22] and whose inflammatory activity depends on TLR2 or TLR4, present synergistic properties. Of note, genome-wise prediction analysis revealed that approximately 12% of *T. cruzi* genes possibly encode GPI-anchored proteins, a number much higher than in previously studied protozoa or mammalian species [23]. Moreover, the recent large-scale analysis of GPI-anchored molecules identified 78 GIPLs and 11 ptn-GPIs, of which 70 GIPLs and 8 ptn-GPIs were not previously described [23]. Among these, probably novel TLR2 and/or TLR4 agonists will be characterized. Other differences between *T. cruzi*-derived GIPL and tGPI anchors were determined concerning the participation of coreceptor molecules on their recognition and the triggered signaling pathway. For instance, while anti-CD14 antibodies blocked the production of TNF-α by human macrophages exposed to tGPI-mucin *in vitro* [24], neutrophil attraction to the peritoneal cavity triggered by the injection of eGIPL was maintained in CD14-deficient mice, indicating that eGIPL is recognized by TLR4 in a CD14-independent way (Bellio, M., unpublished results). Also, TNF-α and MIP-2 production in response to GIPL was shown to be significantly lower in CD1d-deficent mice (which lack NKT cells) when compared to WT mice [25]. Although the exact mechanisms for the observed response remain elusive, these results clearly implicate CD1d-restricted NKT cells in an early amplification step of cytokine and chemokine production during the innate response elicited by *T. cruzi* GIPL. Therefore, the *in vivo* effects of *T. cruzi* PAMPs deserve further investigation with regard to their mode of recognition by, and action on, different cell types.

Infective *T. cruzi* trypomastigotes invade host cells using at least two different strategies, either by an active process recruiting host-cell lysosomes to the area of parasite cell contact or by an alternative pathway, in which the parasite infects phagocytic cells through conventional phagocytosis/endocytosis mechanism [26–29]. While the general current view is that TLRs do not function directly as phagocytic receptors, studies have demonstrated that TLR signaling by means of MyD88 can enhance phagosome acidification and function, the so-called phagosome maturation, which is required for effective sterilization of its contents [30]. In accordance to that, we have demonstrated that the levels of *T. cruzi* internalization by macrophages is not affected in three different TLR4-deficient mouse strains (C3H/HeJ, C57BL/10ScN, and *Tlr4*$^{-/-}$), but TLR4 and parasite colocalize into acidic compartments, and, as soon as 4 h after infection, the percentage of TLR4-deficient macrophages infected with *T. cruzi* is significant higher when compared to WT cells, indicating the existence of an early trypanosomicidal mechanism triggered by TLR4, which was also shown to be dependent on the production of NO and reactive oxygen species (ROS) [31]. On the other hand, it was reported that during the invasion of *T. cruzi*, the activation of the Rab5-dependent phagocytic pathway is regulated by TLR2-dependent signals in macrophages [32]. Still, to our knowledge, there are no other studies on the participation of surface TLR pathways in the entrance of trypomastigotes into the host cells.

An additional TLR2 agonist with adjuvant properties, the *T. cruzi*-released protein related to thiol-disulfide

oxidoreductase family, called Tc52, was also described [33]. Surprisingly, however, despite the known *T. cruzi*-derived TLR2 agonists, no differences in parasitemia or mortality were noted following infection of mice genetically deficient in TLR2 [34]. Intriguingly, although TLR2 expression by macrophages stimulated *in vitro* with trypomastigote-derived GPI anchors appears to be essential for induction of IL-12, TNF-α and NO [16], when infected, the TLR2-deficient mice mount a robust proinflammatory cytokine and NO production by spleen cells, as well as higher serum levels of IFN-γ, when compared to WT mice [34]. This suggests an immunoregulatory role for TLR2 during the infection, maybe due to the action of TLR2 ligands on Tregs [35].

Interestingly, more recently, *T. cruzi*-derived nucleic acids have been also shown to act as PAMPs. Genomic DNA, which contains abundant oligodeoxynucleotide unmethylated CpG motifs, and total RNA purified from *T. cruzi* promote host cell activation via TLR9 and TLR7, respectively, stimulating cytokine response from macrophages and dendritic cells (DCs) [36–39]. Also, potential TLR7 ligands as guanosine- or uridine-rich single-strand RNA sequences were found by *in silico* analysis in the predicted parasite transcriptome [39]. Indeed, as discussed below, *Tlr9*$^{-/-}$ and *Tlr7*$^{-/-}$ mice were shown to be more susceptible than WT mice to infection with the parasite [37, 39].

4. Resistance to Infection Conferred by Different TLR Pathways

Directly testing the hypothesis that TLR triggering by the above-described PAMPs is crucial for host resistance against the infection is currently not possible, however, due to the absence of *T. cruzi* strains lacking the expression of any of the above-described TLR agonists. On the other hand, studying the course of infection in mice genetically deficient for different TLR-encoding genes, evaluating mortality, parasitemia, and several parameters of the innate and acquired immune responses have brought additional understanding of the impact of the lack of TLR-mediated recognition of *T. cruzi* for development of host susceptibility to the infection. In this context, the critical involvement of TLRs in the host resistance to *T. cruzi* was firstly highlighted in mice deficient for the MyD88 adaptor molecule, which is the main transducer of multiple TLR-signaling pathways [34]. In fact, *Myd88*$^{-/-}$ mice were shown to be highly susceptible to infection and to display lower production of proinflammatory cytokines, including IL-12p40 and IFN-γ, from innate immune cells [34]. In accordance, we first reported that C3H/HeJ mice, which express a nonfunctional natural mutant of TLR4, are highly susceptible to infection with *T. cruzi* [17], as evidenced by a higher parasitemia and earlier mortality. However, since classical genetic studies previously established that the resistance to *T. cruzi* is governed by multiple genetic factors, including H-2-linked genes [40, 41], the level of protection given by the TLR4 pathway during the infection of C3H/HeJ mice (whose C3H background is classified as "susceptible") could not be directly compared to the degree of susceptibility of infected *Myd88*$^{-/-}$ mice, which are of the resistant C57BL/6 genetic background. Therefore, we further investigated the impact of TLR4 deficiency in the *Tlr4*$^{-/-}$ (B6 background) mice [31]. We demonstrated that TLR4 signaling triggers an important early parasiticidal event against *T. cruzi*, which is dependent on the formation of NO and ROS and that splenocytes of *Tlr4*$^{-/-}$ infected mice display lower production of the proinflammatory cytokines IFN-γ and TNF-α, as well as of NO, when compared to WT B6 mice, what would explain the observed higher parasitemia levels in TLR4-deficient mice [31]. Together these results indicate that TLR4, as previously shown for TLR2 and TLR9, also contributes to resistance during the acute phase of infection in B6 mice. TLR4 deficiency by itself, however, does not lead to an earlier mortality in the B6 background [31].

An interesting study has demonstrated the involvement of TLR9 in the protection against *T. cruzi* infection [37]. TLR9 is one of the members of the TLR family located at the endolysosomal subcellular compartment and can recognize parasite-derived DNA sequences [38]. More importantly, since *Tlr2*$^{-/-}$*Tlr9*$^{-/-}$ double knockouts display higher parasitemia than the single *Tlr2*$^{-/-}$ or *Tlr9*$^{-/-}$ mice, this work was the first to demonstrate that TLR2 and TLR9 can cooperate, and/or that a degree of redundancy exist among different TLR family members, in the control of parasite replication. Nevertheless, although attaining parasitemia levels comparable to the observed in the *Myd88*$^{-/-}$ strain (which lack multiple TLR signaling), *Tlr2*$^{-/-}$*Tlr9*$^{-/-}$ double deficient mice did not show the acute mortality exhibited by *Myd88*$^{-/-}$ mice. This observation suggested that other TLR/IL-1R family members, in addition to TLR2 and TLR9, could be involved in the pathogenesis of *T. cruzi* infection. Furthermore, mice lacking both MyD88 and a second adaptor molecule which acts downstream TLR3 and TLR4, called TRIF, were shown to be even more susceptible than *Myd88*$^{-/-}$ mice. Contrary to *Myd88*$^{-/-}$, the *Myd88*$^{-/-}$*Trif*$^{-/-}$ double deficient mice were not able to control parasite levels in the bloodstream and die at an earlier time point after infection [42]. The TRIF-dependent pathway is indispensable for the induction of type 1 IFNs through TLR3 and TLR4, but the role of type 1 IFNs in the resistance to infection with *T. cruzi* is controversial [43, 44]. Curiously, although mice single deficient in TRIF or IFNAR1 (type 1 IFN receptor) were shown to be resistant to the infection with *T. cruzi*, *Myd88*$^{-/-}$*Ifnar1*$^{-/-}$ double deficient mice display the same highly susceptible phenotype as *Myd88*$^{-/-}$*Trif*$^{-/-}$ double deficient strain, pointing to a protective role for IFN-β and/or IFN-α that, however, only becomes apparent when the Myd88 pathway is absent [42]. Therefore, the high sensitivity demonstrated by the *Myd88*$^{-/-}$*Trif*$^{-/-}$ double deficient mice to infection is in accordance with a role for TLR4 and/or TLR3 in the response against *T. cruzi*, as these members of the TLR family are the only known to use TRIF as a transducer molecule.

A very recent work studying *Tlr3*$^{-/-}$ mice, however, has not supported any role for TLR3 in promoting control of *T. cruzi* parasitemia or host survival [39]. Yet, the possibility exists that a putative function of TLR3 would only become apparent in the concomitant absence of other TLR-family member with redundant function, by analogy to what was previously observed for TLR2, whose involvement in

protection against the parasite was only evident in the double *Tlr2*$^{-/-}$*Tlr9*$^{-/-}$ strain [37]. The article also provided, for the first time, evidences that TLR7 is a critical innate immune receptor involved in the recognition of *T.cruzi* RNA and in host resistance to a protozoan infection [39]. Caetano and collaborators analyzed the course of infection in different mouse strains lacking one or multiple endolysosomal TLRs. First, the authors followed the response to infection in a strain of mice called 3d, which has a loss-of-function point mutation in UNC93B1 (an endoplasmic reticulum (ER) resident protein that mediates the translocation of the nucleotide-sensing TLRs from the ER to the endolysosomes) and, consequently, is unresponsive to TLR3, TLR7 and TLR9 ligands (TLR8 is believed to be biologically inactive in mice). The phenotype of 3d mice was shown to be equivalent of the triple deficient *Tlr3*$^{-/-}$*Tlr7*$^{-/-}$*Tlr9*$^{-/-}$ strain and was intermediary between *Myd88*$^{-/-}$ (highly susceptible) and *Tlr9*$^{-/-}$ (moderately susceptible). This result suggested the contribution of TLR7, besides TLR9, for the resistance against *T. cruzi*, since, as mentioned, *Tlr3*$^{-/-}$ mice were not susceptible to the infection. In fact, *Tlr7*$^{-/-}$ mice were shown to display a degree of susceptibility comparable to *Tlr9*$^{-/-}$ mice [39].

Collectively, to date, the analysis of different mice strains lacking one or multiple TLR pathways demonstrated that TLR2, TLR4, TLR7, and TLR9 play a role in the resistance to infection with *T. cruzi,* with a degree of redundancy between them. The direct comparison between the levels of susceptibility displayed by the diverse TLR-deficient strains of mice is not always possible though, due to the fact that the above-cited studies employed different strains of the parasite, as Y [31, 34, 37], Tulahuén [42], or CL-Brener [39] strains, each of them presenting different virulence, tissue tropism, and time course of parasitemia, and which may also express PAMPs with different fine structures or levels of expression. Nevertheless, important issues have been revealed in those studies concerning the role of TLRs in innate and acquired immunity against *T. cruzi,* as discussed below.

5. TLRs in the Innate and Acquired Responses to *T. cruzi*

In the first 7 to 10 days following infection, before acquired immunity is fully activated, innate responses play a key role in containing parasitemia, through the action of microbicidal mediators (reactive nitrogen intermediates—RNI and ROS), whose production is enhanced by the action of proinflammatory cytokines (IL-12, TNF-α, and IFN-γ) released by macrophages, natural killer (NK), and $\gamma\delta$ T cells [45, 46]. Then, acquired immunity mediated by the T-helper 1 (Th1) cell response becomes crucial in parasitemia control and host survival. The release of IFN-γ by Th1 CD4$^+$ cells induces the activation of phagocytic cells for parasite killing. Th1 lymphocytes also provide help for the appropriate production of antibodies (cytophilic and complement-fixing immunoglobulin G2a) and for cytotoxic CD8$^+$T cells. The genetic absence, or the experimental blocking, of any of these adaptive responses (antibodies, CD4$^+$ or CD8$^+$ cells) results in uncontrolled parasite levels and decease [47–49]. Despite the control of parasite burden by different effector responses, however, its elimination is not achieved, leading to chronification of the infection. It is plausible that parasite persistence results from suppression of microbicidal immunity by anti-inflammatory responses mediated by IL-10 and transforming growth factor-β (TGF-β), as well as by infiltrating myeloid cells with suppressor activity, which succeed and counteract the potent inflammatory response that, otherwise, would lead to life-threatening injury to organs [50, 51].

It is a current paradigm that the activation of dendritic cells and other innate cells by TLR pathways is required for and play a role in the modulation of acquired responses although the precise function of each member of the TLR family in the responses against *T. cruzi* is still to be fully determined. All the strains of mice with single or multiple TLR deficiency tested to date, which display higher susceptibility to infection with *T. cruzi*, were found to display lower proinflammatory cytokine levels early during infection although the degree of susceptibility varies between the different TLR knockouts, as discussed above. Accordingly, serum levels of IFN-γ and IL-12 are low in *MyD88*$^{-/-}$ infected mice, as well as the *in vitro* production of IFN-γ, IL-12, TNF-α and NO by splenocytes obtained from these mice at day 10 postinfection [34]. Similar results were obtained with *Tlr4*$^{-/-}$, *Tlr9*$^{-/-}$, double *Tlr2*$^{-/-}$*Tlr9*$^{-/-}$, 3d, or *Tlr7*$^{-/-}$ mice [31, 37, 39]. These results confirmed others obtained *in vitro,* where lower levels of IL-12 (or NO) and higher number of trypomastigotes were released by splenocytes (or by *in vitro* infected macrophages) of MyD88-, double MyD88/TRIF-, TLR4-, 3d, TLR9-, or TLR7-deficient infected mice [31, 37, 39, 42]. Thus, with the apparent exception of TLR2, several TLRs contribute *in vivo* to the induction of proinflammatory cytokine secretion by infected host cells.

Beyond TLR's roles in modulation of innate immunity, the current paradigm strongly argues in favor of a critical role of these receptors in shaping the adaptive immune response [9]. This can be achieved mainly by their action on antigen-presenting cells (APCs), either by promoting cross-presentation for CD8 T-cell activation or by increasing the levels of costimulatory molecules and by stimulating the secretion of lineage-specific cytokines as IL-12, IL-6, IL-1β, IL-18, and IL-23 by APCs and, thus, promoting Th1 and Th17 differentiation. Although initially controversial, different groups demonstrated the expression of TLRs on activated T cells, as well as the effects of TLR agonists functioning as direct costimulatory signals during the initiation of the adaptive immune response or as an aid in the survival of memory T cells [35]. Therefore, one cannot rule out a direct role for *T. cruzi*-derived PAMPs on T-cell activation and survival during the infection, although to date, evidence favor the hypothesis that the major function of TLRs on T-cell activation during infection is an indirect one.

Presently, data on the detailed role of TLRs in the activation of acquired immunity during infection with *T. cruzi* are still scarce. Nonetheless, it was first demonstrated that CD4$^+$ T cells obtained from infected *Tlr9*$^{-/-}$, *Tlr2*$^{-/-}$*Tlr9*$^{-/-}$, or *MyD88*$^{-/-}$ mice strains produced lower IFN-γ when stimulated *in vitro* by infected syngeneic BMDC [37], while CD4$^+$ T cells from infected TLR2-deficient mice display levels of

IFN-γ comparable to WT, as expected due to their resistant phenotype [34]. Of note, the percentage of $CD4^+$ IFN-γ^+ T cells in the spleen of infected *MyD88*$^{-/-}$ mice at day 11 and 13 postinfection were shown to be significantly lower compared to WT mice, whereas the percentage of $CD4^+$ IFN-γ^+ T lymphocytes in the spleen of the *Tlr4*$^{-/-}$ strain resulted similar to that found in WT mice, in accordance with the relatively higher resistance of this strain, when compared to the other mentioned TLR-deficient mice [31]. Interestingly, the same picture of low $CD4^+$ T-cell activation was obtained when analyzing the IFN-γ production by $CD4^+$ T lymphocytes obtained from infected 3d or *Tlr3,7,9*$^{-/-}$ triple deficient mice, even when stimulated *in vitro* with antigen-pulsed WT DCs, suggesting the lower frequency of activated $CD4^+$ T cells in infected spleens of these susceptible strains [39]. In the particular case of *MyD88*$^{-/-}$ mice, the lower percentage of Th1 cells could also be due to nonresponsiveness to IL-18, since the receptor for this cytokine also relies on MyD88 for signaling, but the fact that mice deficient in IL-18 are not more susceptible to experimental infection with *T. cruzi* [52] argues against this hypothesis. Therefore, in all the TLR-deficient strains tested, susceptibility to infection correlates with lower levels of serum IL-12 and decreased frequency of activated Th1 cells in the spleen.

A nonexpected result was found, in contrast, when the percentage of $CD8^+$ IFN-γ^+ T cells (measured either by cytometry or by ELISPOT), and the CD8-dependent *in vivo* cytotoxic activity was measured in *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, *Tlr9*$^{-/-}$, and in *MyD88*$^{-/-}$ infected mice, as both parameters were preserved to WT levels in all the above-cited deficient strains [31]. More recently, the maintenance of the frequency of $CD8^+$ T cells specific for an immunodominant peptide to WT levels has been also demonstrated in *MyD88*$^{-/-}$, *Tlr9*$^{-/-}$, and 3d mice [39] although *in vitro* the levels of IFN-γ secretion were lower in cell cultures of *MyD88*$^{-/-}$ and 3d, but not of *Tlr9*$^{-/-}$ mice. A first possible interpretation of these results is that none of the tested TLR pathways is essential for the generation of cytotoxic $CD8^+$ T cells during *T. cruzi* infection. Of note, the TLR3- and TLR4-triggered TRIF pathway is preserved in *Myd88*$^{-/-}$ mice, hence, their activation would lead to type I IFN secretion and consequent DC maturation, resulting in the normal $CD8^+$ T-cell response observed in these mice. Also in accordance with this hypothesis, extensively discussed by us in a previous work [31], doubly MyD88/TRIF-deficient (as MyD88/IFNAR1 DKO) mice are more sensitive to infection and do not control parasitemia [42]. Alternatively, other signaling molecules and innate recognition systems can be involved in the generation of $CD8^+$ T-cell responses. For example, it was described that NFATc1 activation and IFN-γ production in a TLR-independent pathway may lead to DC maturation during *T. cruzi* infection [53]. Also, DC maturation may be induced by bradykinin B2 receptors (B2Rs) after the release of pro-inflammatory bradykinin peptide by the parasite proteases during infection [54]. Thirdly, a recent work, cited above, has demonstrated the activation of NOD receptors by *T. cruzi* infection [15] though it is still not clear whether these two latter pathways would function independently of TLRs for licensing $CD8^+$ T-cell effector functions.

Therefore, the lower levels of $CD4^+$ effectors observed in infected MyD88- or TLR-deficient mice seem sufficient for their help to $CD8^+$ T cells but might not be enough for inducing the necessary B-cell-mediated response, or for $CD8^+$ T lymphocyte mobilization to parasite-infected tissue other than spleen, as heart or liver, for example. Although a recent report that described a role for IL-17A in host protection against acute infection [55] and a role for Th17 cells in regulating parasite-induced myocarditis has been shown during *T. cruzi* infection in mice [56], nothing is known at the present time about the possible modulation of this T helper subset, and its consequences to infection with *T. cruzi*, in the absence of TLR signaling. Undoubtedly, more work is necessary for a full understanding of the effects of *T. cruzi*-induced TLR signaling in the control of adaptive immunity against the parasite.

In summary, the present data support the idea that a degree of redundancy exists among different TLR family members, meaning that each of the TLR pathways may not be individually essential for the resistance to infection. *T. cruzi* displays various ligands for different TLRs (see Figure 1) and only the concomitant absence of signaling through multiple TLR receptors, but not their individual deficiency, results in a high degree of susceptibility to the infection.

No discussion about the role of TLRs in the infection by *T. cruzi* could be complete without some speculation concerning the possibility that the immunological response elicited through TLR pathways might have a role in the progression of the disease toward its chronic phase, CCC. Both *T. cruzi*- and heart tissue-specific responses have been put in evidence and may be important for the pathology of CCC although a consensus does not exist about the relative contribution of each of these responses for CCC [57]. Whatever the answer to this question might be, TLR signaling could be implied in the process, since beside their role in the triggering of the adaptive response to pathogens, as above discussed, several studies have also reported the contribution of TLR-family members in the induction of autoimmunity [58]. However, studies on the chronic stage of infection with *T. cruzi* are difficult in mice of C57BL/6 genetic background (as all the available TLR knockout strains), due to the scarcity of good experimental models capable of inducing in these mice the pathophysiologic traits observed in the human condition. Notwithstanding, a study of 169 patients with chronic chagasic cardiomyopathy and 76 *T. cruzi*-infected asymptomatic individuals revealed that *T. cruzi*-infected patients who are heterozygous for the MAL/TIRAP S180L variant (which leads to a decrease in signal transduction upon ligation of TLR2 or TLR4 to their respective ligand) may have a lower risk of developing CCC [59]. Interestingly, it was also demonstrated that TLR2 functions as the main upstream regulator of hypertrophy triggered in isolated murine cardiomyocytes by *T. cruzi* [60]. Therefore, the study of the involvement of TLR signaling in experimental models of the chronic phase of the Chagas' disease could be of considerable value in elucidating the pathophysiology of CCC, which remains one of the major causes of heart failure among younger individuals in Latin America today. Moreover, determining precisely how TLR-TRIF-MyD88 activation could trigger and

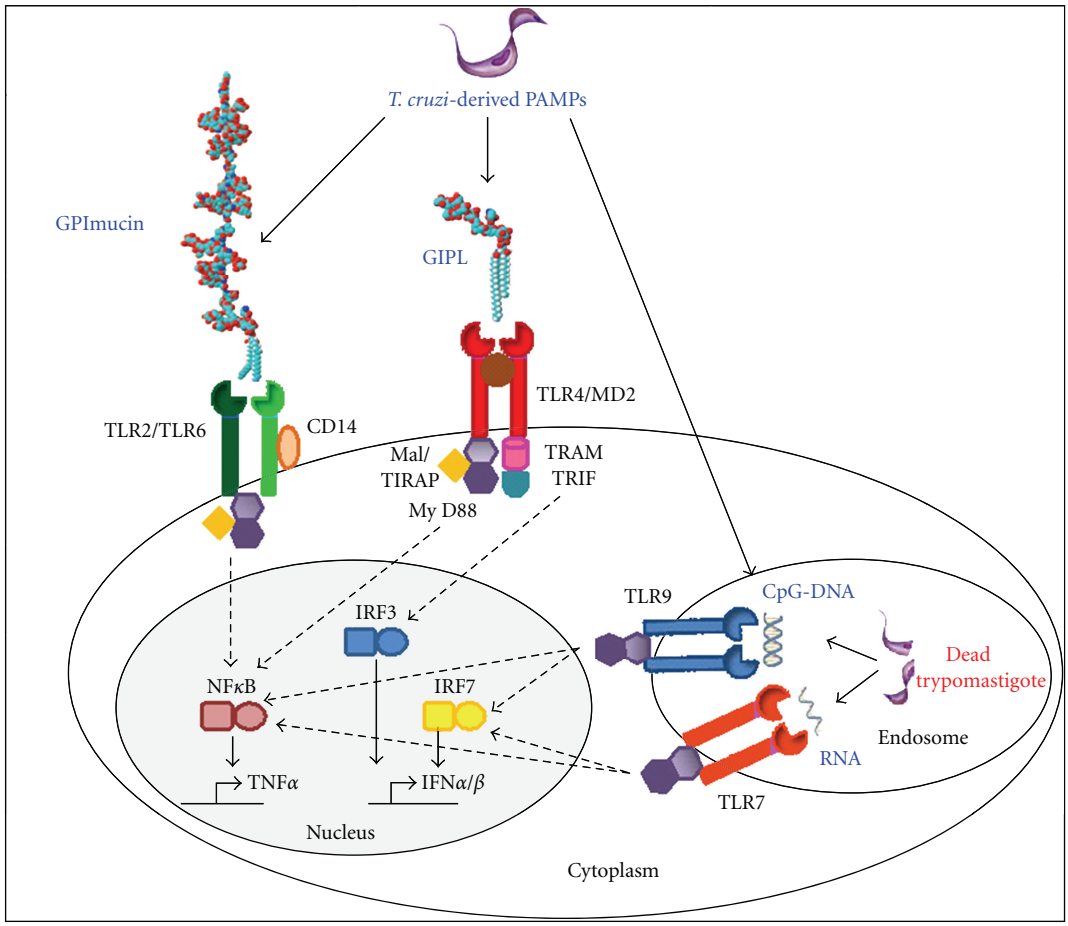

Figure 1: *T. cruzi*-derived PAMPs are recognized by different TLRs. The recognition of different *T. cruzi* molecules, like parasite surface glycoconjugates and nucleic acids, occurs through distinct Toll-like receptors, which are localized at the cellular plasma or endoplasmic membranes, respectively, and are differentially expressed by various innate immune cell types. GPI anchors of mucin-like glycoproteins activate TLR2/TLR6 heterodimer, GIPL is an agonist for TLR4, genomic DNA activates endosomal TLR9, and TLR7 is involved in parasite RNA recognition. TLRs induce NF-κB and/or IRFs activation via their interaction with different TIR domain-containing adaptor molecules. Of these, MyD88 and Mal/TIRAP are required for TLR2 and TLR4 activation of NF-κB. In a MyD88-independent way, TRIF and TRAM signal downstream TLR4, activating IRF3. TLR7 and TLR9 activate NF-κB and IRF7 via MyD88. NF-κB activation leads to proinflammatory cytokines production, such as TNF-α and IL-12, whereas IRFs are required for type I IFN gene transcription.

modulate the immune response against *T. cruzi* will be of critical relevance for vaccine development against this important human parasite.

6. Vaccination against *Trypanosoma cruzi* Infectiona

The strong specific immune response developed in most hosts following *T. cruzi* infection does not eliminate the parasite, and parasite persistence is considered to be the main factor contributing to the late symptoms of Chagas disease. Therefore, eliminating the parasite at the early stage (acute phase) prevents parasite survival and may be an interesting route to avoid chronic phase immunopathology. Prophylactic vaccination would help to reduce or completely eliminate the parasite burden and thus represents a desirable method to restrict the development of chronic symptoms of the disease. Until recently, vaccination was not considered a cost-effective measure for containment of the disease transmission because other methods of prevention would be simpler and cheaper. Nevertheless, recent detailed analyses have proved that indeed vaccination can be cost effective in a variety of scenarios including the regions where the prevalence is as low as 1% by using a vaccine which the efficacy was only 25% [61]. Based on that, future research programs should consider these calculations to support this type of biotechnological alternative.

Because $CD4^+$ and $CD8^+$ T cells are critical mediators of the acquired immune response, over the past 20 years, we and others have tested the hypothesis that non-antibody-mediated cellular immune responses to the antigens expressed in the mammalian forms of the parasite could indeed be used for the purpose of vaccination. Using a mouse model of the disease, we confirmed this hypothesis by inducing protective immunity against *T. cruzi* infection specifically mediated by $CD4^+$ Th1 and $CD8^+$ Tc1 cells specific for antigens

expressed by trypomastigotes and amastigotes of *T. cruzi* (reviewed by [62–64]).

T. cruzi antigens recognized by immune sera from immune or infected humans or animals served as the basis for researchers to conduct studies using recombinant proteins. These recombinant proteins included members of the large *trans*-sialidase (TS) surface protein family expressed mainly in the infective trypomastigote and amastigote forms of the parasite. The second group of genes belonged to the family of cysteine-proteases (cruzipain) expressed in all of the different forms of the parasite. Other antigens formed a heterogeneous group including molecules such as the flagellar calcium-binding protein, paraflagellar rod protein-2, LYT-1 antigen, ribosomal protein L7a-like protein, and KMP11, among others (reviewed by [62, 63]).

To induce *T. cruzi*-specific T lymphocytes and protective immunity against an experimental infection, several delivery antigens were used successfully such recombinant proteins mixed in the presence of distinct adjuvants, plasmid DNA, recombinant viruses, and bacteria. Very recently, genetically attenuated parasites have been also successfully generated for the purpose of the development of an oral veterinary vaccine [65]. Protective immune response in the mouse model was measured by the reduction in acute phase parasitemia, tissue parasitism, and mortality. In most cases, immunity elicited by these antigens was associated with type I immune response, generated by IFN-γ-producing $CD4^+$ and/or $CD8^+$ T cells. Some of the mechanisms mediating protective immunity were investigated. Following intranasal immunization with (TS) in the presence of the TLR9 activator CpG ODN, the absence of $CD4^+$ or $CD8^+$ T cells renders the vaccinated animals completely susceptible to infection. Because these animals were genetically deficient, these cells can be required for the induction or the effector phase of the immune response, or both. Similarly, CD8-deficient mice failed to generate protection after immunization with native Par-2 protein emulsified in CFA or recombinant adenovirus expressing TS or ASP-2 genes [66, 67]. Upon plasmid immunization, the depletion of either $CD4^+$ or $CD8^+$ T cells completely reversed protective immunity, thus demonstrating a nonoverlapping role for these two subpopulations [68, 69]. Following vaccination with recombinant protein of ASP-2 in alum and CpG ODN, only depletion of $CD8^+$, but not $CD4^+$, T cells reversed protective immunity [70]. Finally, vaccination with a single *T. cruzi* epitope, recognized by $CD8^+$ T cells [71], elicited a protective immune response using a heterologous prime-boost strategy with recombinant adenovirus and vaccinia virus. These experimental systems found type 1 $CD4^+$ and $CD8^+$ T cells to be necessary, confirming the general paradigm that type 1 $CD4^+$ and $CD8^+$ T cells do play a key role in protective immunity. In agreement with this hypothesis, recent observations have pointed to IFN-γ as a critical mediator of the protective immune response [72]. Also relevant is the fact that protective T cells can be long lived and stable and display a phenotype of effector memory T cells [73, 74]. Another recently added information that might be of general importance for vaccine development has been the fact that the target of these protective $CD8^+$ T cells is not only the immune-dominant epitopes, but they can also be subdominant/cryptic T-cell epitopes [75, 76].

The question as to whether other cell types are also critical for the adaptive immunity induced by these recombinant vaccines is currently being investigated. Still, noteworthy is the fact that infection itself elicits strong type 1 immune response, and it is not capable of clearing the parasite completely. This apparent contradiction suggests that there may be qualitative differences between immune responses elicited by infection or vaccination that are not revealed by the analyses of the cytokine pattern. In fact, ongoing experiments strongly argue that there are qualitative differences that account for the protective properties of the T cells expanded after infection in genetically vaccinated mice (Vasconcelos, unpublished results).

In spite of clear evidence that immunization with *T. cruzi* antigens can provide protective immunity as measured by a reduction in acute phase parasitemia, tissue parasitism, and mortality, it is not clear whether immunization will lead to either remission or a cure of the chronic phase symptoms of the disease. To determine the role of immunization in reducing chronic phase disease symptoms, a number of experiments using different animal models must be performed. In many of the models described above, tissue inflammation and parasitism in the late chronic phase were significantly reduced following prophylactic vaccination [69, 77, 78]. Therefore, it is possible that prophylactic vaccinations indeed halt the development of the chronic phase immunopathologies. Nevertheless, the most compelling evidence of a vaccine's ability to reduce the immunopathology was obtained by therapeutic immunization with *T. cruzi* genes encoding the TSA and Tc24 genes [79]. Whether these results are reproducible using different combinations of mouse and parasite strains remains to be seen.

In conclusion, in spite of the pessimism of certain researchers, there are a number of experimental evidences that support the fact that a vaccine against Chagas disease can be obtained for veterinary use. This type of vaccine could have a definitive impact on disease transmission. Whether this knowledge can be translated into a vaccine for a human use will still require considerable body of experimental and clinical studies [80].

7. TLRs and the Development of New Adjuvants

Understanding how pathogens initiate and direct immune responses can provide useful perspectives for vaccine development. In fact, in the last twenty years, the increasing knowledge of the cellular and molecular mechanisms by which innate immunity signaling triggers particular responses from APCs has allowed the design of new defined adjuvants. For example, monophosphoryl lipid A (MPL) is a detoxified lipid A derivative of lipopolysaccharide from *Salmonella enterica*, which acts as a TLR4 partial agonist. It preferentially induces the TRAM/TRIF-signaling pathway and, consequently, has lower toxicity when compared to LPS but retains its adjuvant properties [81]. MPL adsorbed to aluminium salts has been used as adjuvant in prophylactic vaccines against different infectious agents, including an antihuman papillomavirus (HPV) vaccine approved in Australia, Europe, and the USA for the prevention of cervical

cancer [82]. Therefore, research focused on the identification and characterization of PAMPs from *T. cruzi*, as well as from other pathogens, may provide us with new TLR agonists, which combined to known adjuvant molecules will allow the creation of a new generation of vaccines, which will be able, for example, to direct the immune response toward a dominant Th1 profile (required for protection against intracellular pathogens) and will be endowed with long-lasting immunological memory. TLR agonists may also be employed not only in prophylaxis but also in therapeutic approaches. This fascinating subject is however beyond the scope of the present review and has recently been discussed in detail by other authors [13, 83].

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Review Article

New Insights on the Inflammatory Role of *Lutzomyia longipalpis* Saliva in Leishmaniasis

Deboraci Brito Prates,[1,2] Théo Araújo-Santos,[2,3] Cláudia Brodskyn,[2,3,4] Manoel Barral-Netto,[2,3,4] Aldina Barral,[2,3,4] and Valéria Matos Borges[2,3,4]

[1] *Departamento de Biomorfologia, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Avenida Reitor Miguel Calmon S/N, 40110-100 Salvador, BA, Brazil*
[2] *Centro de Pesquisa Gonçalo Moniz (CPqGM), Fundação Oswaldo Cruz (FIOCRUZ), Rua Waldemar Falcão 121, 40296-710 Salvador, BA, Brazil*
[3] *Faculdade de Medicina da Bahia, Universidade Federal da Bahia, Avenida Reitor Miguel Calmon S/N, 40110-100 Salvador, BA, Brazil*
[4] *Instituto Nacional de Ciência e Tecnologia de Investigação em Imunologia (iii-INCT), Avenida Dr.Enéas de Carvalho Aguiar 44, 05403-900, São Paulo, SP, Brazil*

Correspondence should be addressed to Valéria Matos Borges, vborges@bahia.fiocruz.br

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When an haematophagous sand fly vector insect bites a vertebrate host, it introduces its mouthparts into the skin and lacerates blood vessels, forming a hemorrhagic pool which constitutes an intricate environment of cell interactions. In this scenario, the initial performance of host, parasite, and vector "authors" will heavily influence the course of *Leishmania* infection. Recent advances in vector-parasite-host interaction have elucidated "co-authors" and "new roles" not yet described. We review here the stimulatory role of *Lutzomyia longipalpis* saliva leading to inflammation and try to connect them in an early context of *Leishmania* infection.

1. Introduction

Leishmaniasis remains a serious problem in public health, endemic in 88 countries on four continents, but most of the cases occur in underdeveloped or developing countries [1]. Visceral Leishmaniasis (VL) is a progressive infection with fatal outcome in the absence of treatment. Approximately 90% of the VL cases registered in the Americas occur in Brazil and are concentrated in the Northeast region. In the New World, *Lutzomyia longipalpis* is the principal vector of *Leishmania infantum chagasi*, the agent of American Visceral Leishmaniasis [2].

The causes related to development of distinct clinical manifestations in leishmaniasis are multifactorial and reflect the complexity at the vector-pathogen-host interface [3]. Protozoan parasites of the genus *Leishmania* are the causative agents of the disease and are transmitted to the mammalian hosts by the bite of female phlebotomine sand flies during blood repast. For blood meal obtainment, sand flies introduce their mouthparts into the skin, tearing tissues, lacerating capillaries, and creating haemorrhagic pools upon which they feed [4]. The presence of sand fly saliva in the blood pool, the environment where the parasite encounters host cells, influences the development and functions of several leukocytes. In recent years, the importance of the interaction between components of sand fly saliva and host immune mechanisms in regulating infectivity and disease progression has become clearer and suggests their consequences to disease outcome in leishmaniasis [5].

The aspects involved in immune response resulting in resistance or susceptibility widely depend on the first attempt of host's innate response to contain infection that may influence on the predominance of a pattern of future host's immune adaptive response against *Leishmania*. Many

studies have been performed to understand the mechanisms leading to protection or exacerbation of the disease however; relatively few studies have investigated the role of the sand-fly-derived salivary compounds in the innate immunity. In this paper we integrate the influence of sand fly bite with current ideas regarding the role of early steps of host inflammatory response against *Leishmania*.

2. Sand Fly Saliva: A Rich Field of Study

Sand fly vectors display a rich source of salivary biological active components to acquire blood from vertebrate hosts, a task not easy due the haemostatic, inflammatory and immune responses resultant from the bite [6]. Thus, it is not unexpected that many scientists have progressively investigated several aspects of sand fly saliva, concerning its composition and the range of mammalian response to it.

Among the New World species of sand fly which are vectors of *Leishmania*, *L. longipalpis* and its salivary gland content are the best studied. One of the first components related to *L. longipalpis* salivary gland was maxadilan [7], the most potent vasodilator peptide known and one of the two phlebotomine salivary proteins more extensively studied. Maxadilan is recognized by causing typical erythema during the feeding of *L. longipalpis* [8]. Further, it was described that maxadilan is able to modulate the inflammatory response by inhibiting cytokines such as TNF-α, by inducing IL-6 production, and by stimulating hematopoiesis [9–11]. Charlab et al. (1999) reported nine full clones and two partial cDNA clones from salivary gland from *L. longipalpis* [12]. In that work, they reported for the first time a hyaluronidase activity from sand fly saliva, an activity not yet described on phlebotomine sand flies, helping the diffusion of other pharmacological substances through the skin matrix [13]. It was also described an apyrase activity on *L. longipalpis* saliva which hydrolyses ATP and ADP to AMP, functioning as a potent antiplatelet factor [12, 14]. Interestingly, a 5′-nucleotidase activity is also present in *L. longipalpis* saliva exert vasodilator and antiplatelet aggregation role by converting AMP to adenosine [12]. One of the most abundant protein found in the *L. longipalpis* saliva is the *Yellow*-related protein [12, 13, 15, 16]. Our group has demonstrated that this family of proteins are the most recognized in sera from children living in an endemic area of visceral Leishmaniasis in Brazil [17] and by normal volunteers exposed to laboratory-reared *L. longipalpis* bites [18]. Recently, Xu et al. (2011) described the structure and function of a *yellow* pro-tein LJM 11 [19]. In this report, the authors described that yellow proteins from *L. longipalpis* saliva act as binder of proinflammatory biogenic amines such as serotonin, histamine, and catecholamines [19]. One member of the D7 family of proteins (commonly found in dipterans saliva) is present in *L. longipalpis* [12]. The exact function of this protein in sand fly saliva is still unknown. However, its role on mosquito's saliva suggests that it could act as anticoagulant or binding biogenic amines avoiding host inflammatory events [12, 15].

Herein, we present some of the most studied proteins related to *L. longipalpis* saliva. (See [6, 15, 16, 20] for more details about this topic). Although many of them have been associated with blood-feeding, their biological functions remain undefined. Nevertheless, by modulating the host haemostatic and inflammatory response, this yet unreported sand fly salivary content remains as a research challenge, acting on host immunity to *Leishmania* during transmission and establishment of infection.

3. Immune Response to *Lutzomyia longipalpis* Saliva against *Leishmania*

There are several studies contributing to a better understanding of *L. longipalpis* saliva effects on host immunity to *Leishmania* infection. A brief exposition of these major contributions in the last 10 years is shown in Figure 1.

In mice, salivary products seem to exacerbate the infection with *Leishmania* and may, in fact, be mandatory for establishment of the parasite in vertebrate hosts. It has been shown that components of *L. longipalpis* or *Phlebotomus papatasi* salivary gland lysates mixed with *Leishmania major* resulted in substantially larger lesions compared to controls [21, 22]. Our group have shown that repeated exposure of BALB/c mice to *L. longipalpis* bites leads to local inflammatory cell infiltration comprised of neutrophils, macrophages and eosinophils [23]. Total IgG and IgG1 antibodies react predominantly with three major protein bands (45, 44, and 16 kD) from insect saliva by Western blot [23]. The injection of immune serum previously incubated with salivary gland homogenate induced an early infiltration with neutrophils and macrophages, suggesting the participation of immune complexes in triggering inflammation [23].

We have shown that in endemic areas natural exposures to noninfected sand fly bites can influence the epidemiology of the disease [17, 24]. We observed that people who presented antibodies against saliva of *L. longipalpis* also showed DTH anti-*Leishmania,* suggesting that the immune response against saliva of the vector could contribute to the induction of a protective immune response against the parasite. Recently, in a prospective study this data was reinforced by Aquino et al. (2010) evaluating 1,080 children from 2 endemic areas for VL [25]. There was a simultaneous appearance of antibodies anti-saliva and an anti-*Leishmania* DTH, or a cellular response against the parasite [25], sup-porting the idea that eliciting immunity against saliva could benefit the induction of a protective response against the parasite. The anti-sand fly antibodies can serve as epidemiological marker of vector exposure in endemic areas. In fact, we demonstrated that two salivary proteins, called LJM 17 and LJM 11, were specifically recognized by humans exposed to *L. longipalpis*, but not *Lutzomyia intermedia* [26]. We also evaluated the specificity of anti-*L. longipalpis* in a panel of 1,077 serum samples and verified that LJM 17 and LJM 11 together in an ELISA assay identified the effectiveness of these proteins for the prediction of positivity against salivary gland sonicate (SGS) [27]. In experimental model using C57BL/6 mice, immunization with LJM 11 triggered DTH response and decrease the diseased burden after *L. major* infection [19].

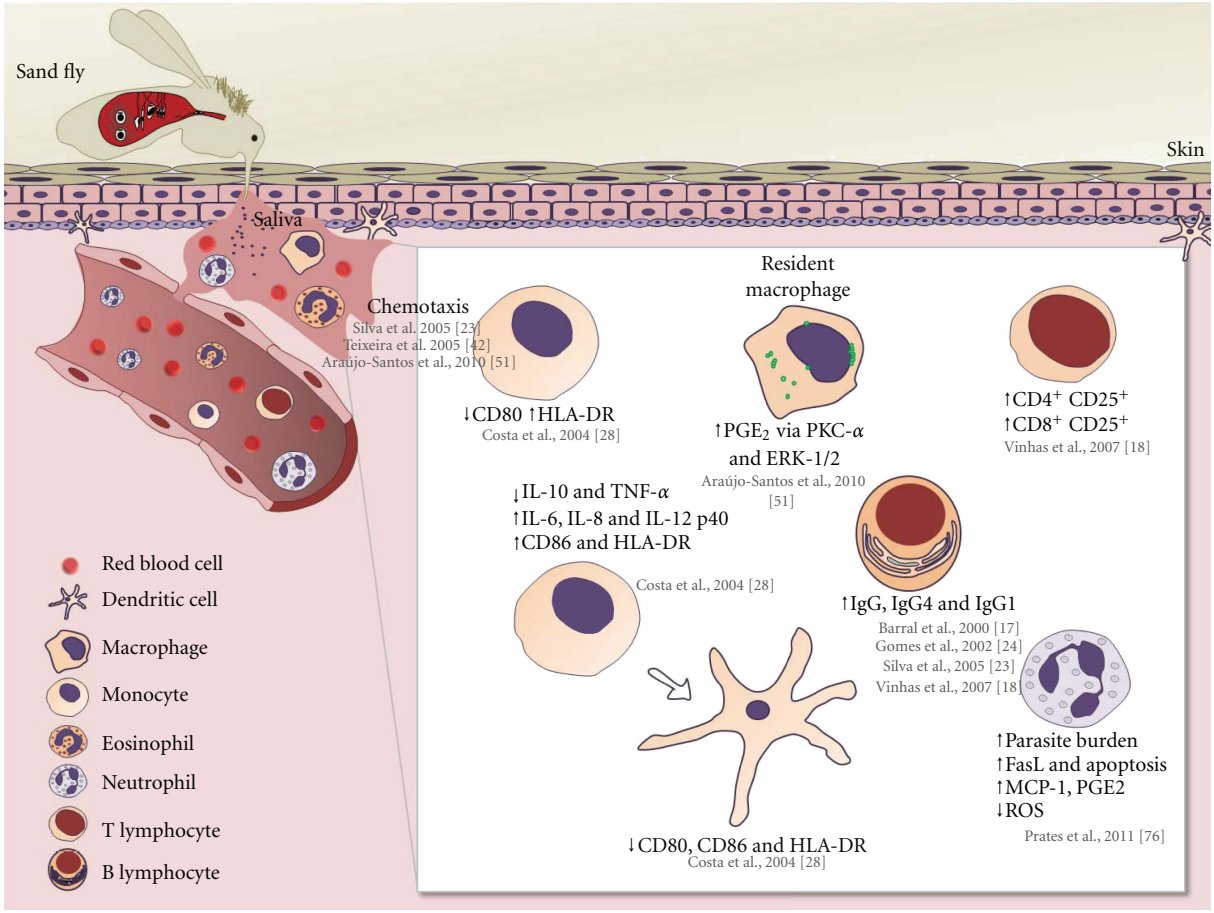

Figure 1: Roles of *Lutzomyia longipalpis* saliva in host immune response cell. After *L. longipalpis* saliva injection a set of events can be triggered in the host immune response. Herein, we summarized the roles of saliva on major cell populations involved in the host immune response against *Leishmania* infection.

We also characterized the immunological patterns following sand fly saliva exposure, using healthy volunteers exposed to laboratory-reared *L. longipalpis* [18]. We noticed high levels of IgG1, IgG4, and IgE antibodies anti-saliva. Furthermore, following *in vitro* stimulation with salivary gland sonicate, there was an increased frequency of CD4(+)CD25(+) and CD8(+)CD25(+) T cells as well as IFN-γ and IL-10 synthesis. Strikingly, 1 year after the first exposure, PBMC from the volunteers displayed recall IFN-γ responses that correlated with a significant reduction in infection rates using a macrophage-lymphocyte autologous culture. Together, these data suggest that human immunization against sand fly saliva is feasible and recall responses are obtained even 1 year after exposure, opening perspectives for vaccination in man [18].

Sand fly saliva also seems to exert a direct effect on human antigen presenting cells. *L. longipalpis* SGS inhibited IL-10 and TNF-α production but induced IL-6, IL-8, and IL-12p40 production by LPS-stimulated monocytes and dendritic cells [28]. Besides cytokine production, sand fly saliva also interfered with the expression of costimulatory molecules in macrophages (reduced CD80 and increased HLA-DR expression) and in monocytes (increased CD80 and HLA-DR expression). During dendritic cell differentiation induced by CD40L, a slight reduction in CD80, CD86, HLA-DR, and CD1a expression were also observed [28].

Whereas enhancement of *Leishmania* transmission by saliva is probably due to immunomodulatory components of sand fly saliva, an explanation of the anti-*Leishmania* effect resulting from host immunization against salivary antigen is not straightforward. Immunity in this system could derive from neutralization of salivary immunomodulators such as the peptide maxadilan from *L. longipalpis* (as reviewed in [22]). Alternatively, immunity could derive from a DTH reaction at the site of the bite generated by a cellular response to salivary antigens injected by the fly [29, 30]. This particular reaction could turn the lesion and its surroundings into an inhospitable site for the establishment of *Leishmania* infection in the new host, or it could modify the environment priming the initial events of the host immune reaction to *Leishmania*.

The disease exacerbative properties of saliva, often resulting from the bioactive property of one or more of its molecules, should not be confounded with antigenic molecules in saliva that induce an adaptive immune response in the host. This acquired immunity can be either protective

or exacerbative depending on the nature and dominance of the salivary components of a vector species. Exposure to uninfected bites of the sand fly *P. papatasi* induces a strong delayed-type hypersensitivity response and IFN-γ production at the bite site that confers protection in mice challenged by *L. major*-infected flies [29]. By contrast, acquired immunity to *L. intermedia* saliva results in disease exacerbation not protection [31]. Moreover, *P. papatasi* saliva, despite its overall protective property, contains molecules that alone induce a protective (PpSP15) or exacerbative (PpSP44) im-mune response in the host [32, 33]. It is likely that *L. intermedia* saliva also contains molecules with similar profiles despite the overall exacerbative effect of total saliva.

Recently, we developed a model for visceral Leishmaniasis (VL) in hamsters, using an intradermal inoculation in the ears of 100,000 *L. chagasi* parasites together with *L. longipalpis* saliva to mimic natural transmission by sand flies [34]. Hamsters developed classical signs of VL rapidly, culminating in a fatal outcome 5-6 months postinfection. Immunization with 16 DNA plasmids coding for salivary proteins of *L. longipalpis* resulted in the identification of LJM19, a novel 11-kDa protein that protected hamsters against the fatal outcome of VL. LJM19-immunized hamsters maintained a low parasite load that correlated with an overall high IFN-γ/TGF-β ratio and inducible NOS expression in the spleen and liver up to 5 months post-infection. Importantly, a delayed-type hypersensitivity response with high expression of IFN-γ was also noted in the skin of LJM19-immunized hamsters 48 h after exposure to uninfected sand fly bites. Induction of IFN-γ at the site of bite could partly explain the protection observed in the viscera of LJM19-immunized hamsters through direct parasite killing and/or priming of anti-*Leishmania* immunity. Recently, Tavares et al. [35] showed that LJM19 was also able to protect hamsters against an infection composed by *Leishmania braziliensis* plus saliva of *L. intermedia*, the vector responsible for the transmission of this parasite in Brazil [35]. The immunization also induced a higher ratio of IFN-γ/TGF-β production in the cells from lymph nodes draining the infection site. Collin et al., (2009) immunized dogs using intradermal injections of DNA codifying salivary proteins of *L. longipalpis* (LJM17 and LJL 143), followed by injection of recombinant *Canarypox virus* containing the same genes [36]. They also observed a potential protective response against *Leishmania*, showing high concentrations of IFN-γ in PBMC stimulated with recombinant salivary proteins. Importantly, the bite of uninfected sand flies resulted in a strong DTH characterized by high amount of IFN-γ and low levels of TGF-β [36]. Together, these results point out the possibility to immunize against leishmaniasis using defined proteins of vector's saliva against *Leishmania*.

4. Early Steps of Host-Vector-*Leishmania* Interplay: Cell Recruitment Induced by Saliva

It is well established that the first steps in leishmaniasis are critical in determining the development of the disease. In order to understand this critical moment, several reports have investigated the early recruitment of cells induced by both *L. longipalpis* saliva alone or coinoculated with *L. chagasi*. Sand fly saliva is able to induce an inflammatory process in the host by recruiting different cells into the bite site. In fact, it was verified that *L. longipalpis* salivary gland lysate markedly modifies the inflammatory response to infection with *L. braziliensis* in BALB/c mice [37]. The saliva-associated lesions progressed to extensive accumulations of heavily parasitized epithelioid macrophages, with persistent neutrophilia and eosinophilia [37]. Eosinophilia has also been described in dogs intradermally inoculated with *L. longipalpis* saliva associated with *L. chagasi* promastigotes [38]. Interestingly, this inflammatory response was not observed in animals that received saliva or parasites alone [38]. The significance of this in the context of Leishmaniasis remains to be investigated. However, this phenomena is not exclusive to *L. longipalpis* saliva once eosinophils were described in the inflammatory course at the site of immunization of mice with the salivary recombinant 15-kDa protein from *P. papatasi*, the sand fly species vector of *Leishmania major* [32]. It is well established the abundant presence of eosinophils in both inflammatory site and allergic response. Activated eosinophils release lipid mediators as PAF, prostaglandins, leukotrienes, and lipoxins, as well as cytokines IL-10 and IL-8 that, in conjunct, trigger vasodilatation and leukocyte chemotaxis (reviewed in [39]). In the context of sand fly bite, this eosinophilic reaction could favor vector feeding but creates an unfriendly environment for *Leishmania* parasites.

Host cell infiltration induced by sand fly bite is the most physiologic approach to reinforce the inflammatory role of vector saliva. This event has been explored using *P. papatasi*, in which saliva-induced DTH response observed was associated to a possible fly adaptation to manipulate host immunity for the vector's own advantage [30]. Concerning *L. longipalpis* saliva, our group investigated the initial vertebrate reactions against sand fly saliva. We demonstrated that repeated exposures of BALB/c mice to *L. longipalpis* bites lead to an intense and diffuse inflammatory infiltrate characterized by neutrophils, eosinophils, and macrophages [23]. This response was observed by histological analysis of the ear dermis from exposed mice as early as 2 hours and was sustained up to 48 hours after challenge with the *L. longipalpis* salivary sonicate [23]. Moreover, the injection of immune serum previously incubated with salivary gland homogenate induced an early infiltration with neutrophils and macrophages, suggesting the participation of immune complexes in triggering inflammation [23]. An elegant and remarkable visual advance obtained by two-photon intravital imaging has recently demonstrated that the neutrophils represent the first cell population which is recruited to *Phlebotomus duboscqi* bite site [40]. Although the participation of vector salivary components had not been directly attributed to this inflammatory event by the authors, we could not discharge this possibility considering diverse data showing that saliva from different sand flies species exert chemotaxis. As neutrophils were observed on *L. longipalpis* bite site [23] the implications of its saliva on this cells will be further discussed in this paper.

In addition to *in vivo* models, cell chemotaxis induced by saliva has also been observed *in vitro*. This is of particular interest, indicating that *L. longipalpis* salivary components can act directly as inflammatory mediator. Using transwell system, Zer et al. (2001) showed the direct chemotatic effect of saliva on BALB/c peritoneal macrophages. In the same work, it was demonstrated that *L. longipalpis* saliva is able to both increase the percentage of macrophages that became infected with *Leishmania* in BALB/c and C3H/HeN mice and exacerbate the parasite load in these cells [41]. The authors discuss the possibility that, during natural transmission, saliva could reduce the promastigote exposure to the immune system by attracting host cells to the bite site and by accelerating the uptake of these parasites.

Exploring a straightforward and consistent model—the mouse air pouch—to investigate the inflammatory response induced by *L. longipalpis*, our group has described that *L. longipalpis* salivary gland sonicate was able to induce not only macrophages, but also neutrophil and eosinophil recruitment after 12 h in BALB/c [42]. The increased macrophage recruitment was linked to production of chemokine CCL2/MCP-1 and expression of its receptor CCR2 in the air pouch lining tissue. It was observed that *L. longipalpis* also synergizes with *L. chagasi* to recruit more inflammatory cells to the site of inoculation [42]. This is noteworthy because it increases the availability of "safe targets," the macrophages, for parasite evasion of the effector immune responses [43]. Interestingly, the recruitment profile observed in BALB/c was not observed in C57BL/6 mice, indicating that the same salivary components can induce diverse inflammatory effects depending on the host background [42]. However, because of limited number of cells that can be recovered on the air pouch model, some questions concerning early inflammatory events could not be investigated. Alternatively, the peritoneal cavity has been employed to this kind of study allowing the collection of high number of immigrating cells [44, 45]. In this regard, leukocyte recruitment into peritoneal cavity induced by *L. longipalpis* saliva has been evaluated in both BALB/c and C57BL/6 mouse strains [45]. In this work, significant neutrophil recruitment was observed six hours after administration of saliva, *L. major*, or saliva plus *L. major*. However, in BALB/c mice, all stimuli were able to induce more neutrophil migration than in C57BL/6 mice. Seven days later, it was observed that all stimuli were able to induce higher numbers of eosinophils and mononuclear cells in BALB/c when compared with C57BL/6 mice [45]. This study focused on the effect of saliva from *L. longipalpis* on adaptive immunity, evaluating CD4+ T lymphocyte migration and production of IL-10 and IFN-γ cytokines [45].

4.1. Inflammatory Events Triggered by L. longipalpis Saliva. Neutrophils rapidly accumulate at the inflammatory site (as reviewed in [46]) and have been described on the sand fly bite site [23, 40]. Focusing on inflammatory events triggered by *L. longipalpis* saliva using the peritoneal model, we could observe a distinct kinetic of neutrophil recruitment to the peritoneal cavity of BALB/c and C57BL/6 mice (Figure 2). A late neutrophil influx was observed in BALB/c mice (Figure 2(a)), whereas in C57BL/6 mice neutrophils were already evident in the first hours after *L. longipalpis* saliva inoculation compared to mice injected with endotoxin-free saline (Figure 2(b)).

The link between neutrophil recruitment induced by *L. longipalpis* saliva and other events which initiate and switch off the inflammatory response is an attractive field to be explored. Inflammation resolution is regulated by the release of mediators that contribute to an orchestrated sequence of events [47]. For simplicity, they result in predominance of neutrophils in the inflamed area which are later replaced by monocytes that differentiate into macrophages. During the resolution, inflammatory cells undergo apoptosis and are phagocytosed. Clearance of apoptotic cells by macrophages drives a response characterized by release of anti-inflammatory mediators [48]. Such safe removal of apoptotic cells has been implicated in exacerbation of *Leishmania* infection [49, 50]. The influence of *L. longipalpis* saliva in the time course of inflammation could be observed in cytospin preparations of the peritoneal cells from C57BL/6 mice. Neutrophils in contact with or phagocytosed by macrophages were observed at six hours (Figures 2(c) and 2(d)) and leukocyte phagocytosis by macrophages was an early event as well (Figure 2(e)). Moreover, apoptotic neutrophils were evident in C57BL/6 mice in the presence of saliva (Figure 2(f)). Therefore, components of sand fly saliva are able to both recruit and induce proapoptotic effects on neutrophils. These findings, in the scenario of anti-inflammatory clearance of apoptotic cells, add to the notion of beneficial effects of vector saliva on *Leishmania* transmission. Further work on mediators and mechanisms involved in this process is necessary.

5. Host Macrophage Response to *L. longipalpis* Saliva

Sand fly saliva displays an important role in the macrophage response by triggering the recruitment [42, 51] and suppressing the killing of parasites within macrophages [41, 52]. In this regard, *P. papatasi* saliva inhibits the NO production in macrophages treated with IFN-γ [52] and *L. longipalpis* saliva hampers *Leishmania* antigen presentation to T lymphocytes by macrophages [53] as well as upregulates the IL-10 production related with NO suppression in macrophages infected with *L. amazonensis* [54]. Moreover, pure adenosine from *P. papatasi* saliva decreases NO production in murine macrophages [55] and maxadilan peptide present in *L. longipalpis* saliva upregulates IL-6, IL-10, and TGF-β cytokine responses of LPS-activated macrophages and downregulates IL-12, TNF-α, and NO associated with *L. major* killing [56]. Despite this, few research reports cover the cellular pathways involved in sand fly saliva modulation of macrophage response. Previous study showed that maxadilan acts on PAC-1 receptor in LPS-activated macrophages and inhibits TNF-α production whereas it increases IL-6 and PGE_2 [11], and the authors suggest the participation of cAMP activation by maxadilan in this process.

Although the literature abounds with reports on the effects of sand fly saliva in the immune response and infection,

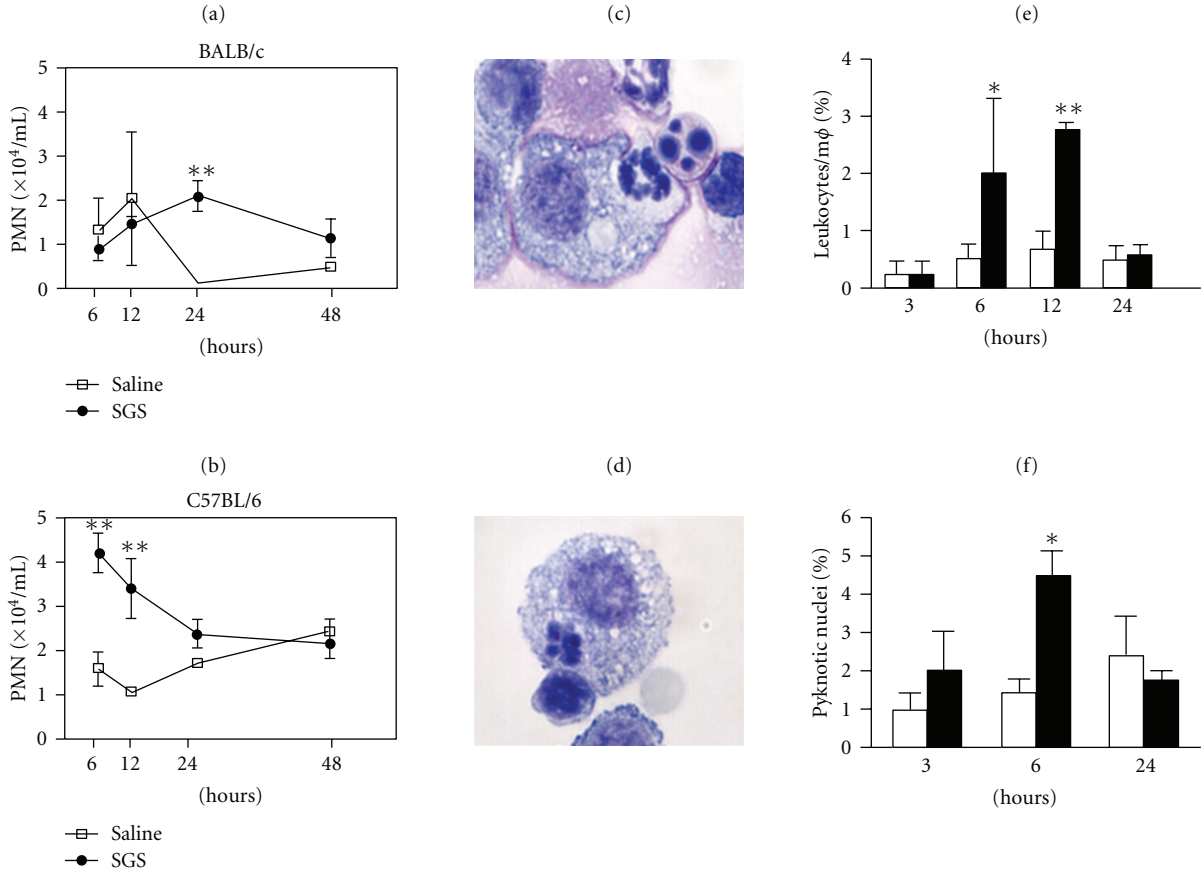

Figure 2: Neutrophil influx, apoptosis, and phagocytosis into BALB/c and C57BL/6 peritoneal cavity in response to *L. longipalpis* saliva. Mice were injected with endotoxin-free saline or *L. longipalpis* salivary gland sonicate (SGS) (0.5 pair/animal). After stimulation, peritoneal cavities were washed and differential cell counts were performed on Diff-Quik stained cytospin preparations. (a-b) Kinetics of neutrophil recruitment in BALB/c (a) and C57BL/6 (b) mice. (c-d) Representative events of C57BL/6 neutrophil phagocytosis by macrophages on Diff-Quik stained cytospin (magnification 1000x). (e-f) Phagocytosis of C57BL/6 leukocytes by macrophages (e) and neutrophil apoptosis (f) after stimulation with SGS (•) or saline (□). Data shown are from a single experiment representative of three independent experiments. Values represent means ± SEM of five mice per group. $^{*}P < 0.05$ and $^{**}P < 0.01$.

the effect of whole sand fly saliva on macrophages is poorly understood. Recently, we showed that *L. longipalpis* saliva activates lipid body (LB) formation in resident macrophages committed with PGE_2 production by COX-2 enzyme (Figure 3) [51]. Lipid bodies are intracellular sites related with eicosanoid production, and their formation can be triggered by activation via different intracellular pathways (as reviewed in [57]). In this context, *L. longipalpis* saliva activated ERK-1/2 and PKC phosphorylation and the inhibition of both pathways resulted in blockade of saliva-induced PGE_2 production by macrophages [51]. PGE_2 modulates the macrophage response during *Leishmania* infection in macrophages [58, 59] and is related with parasite dissemination after infection; however, the role of saliva in the PGE_2 released by macrophages during *Leishmania* infection remains to be addressed. Further studies will be necessary to clarify the importance of eicosanoids stimulated by sand fly saliva in macrophage clearance of parasites and consequently in parasite transmission after sand fly bite.

6. Neutrophils and *L. longipalpis* Saliva: A Neglected Interaction on Scenery of *Leishmania* Infection

Looking to the neutrophils as a significant host-defense cell player in both innate and adaptive response of immune system, it is surprising that few works have attempted to investigate the consequences of vector's saliva and neutrophils interaction in the pathogenesis of leishmaniasis. The reasons to encourage this special attention rise from several lines of evidence showing that neutrophils participate in *Leishmania* immunopathogenesis, by uptaking promastigote forms, producing cytokines and inflammatory mediators or interacting with macrophages enhancing infection (as reviewed in [60, 61]).

Neutrophils are considered as an initial target of *Leishmania* infection [40, 62], and they are implicated in the immunopathogenesis of murine leishmaniasis [50, 63, 64]. Moreover, significant numbers of neutrophils are present at

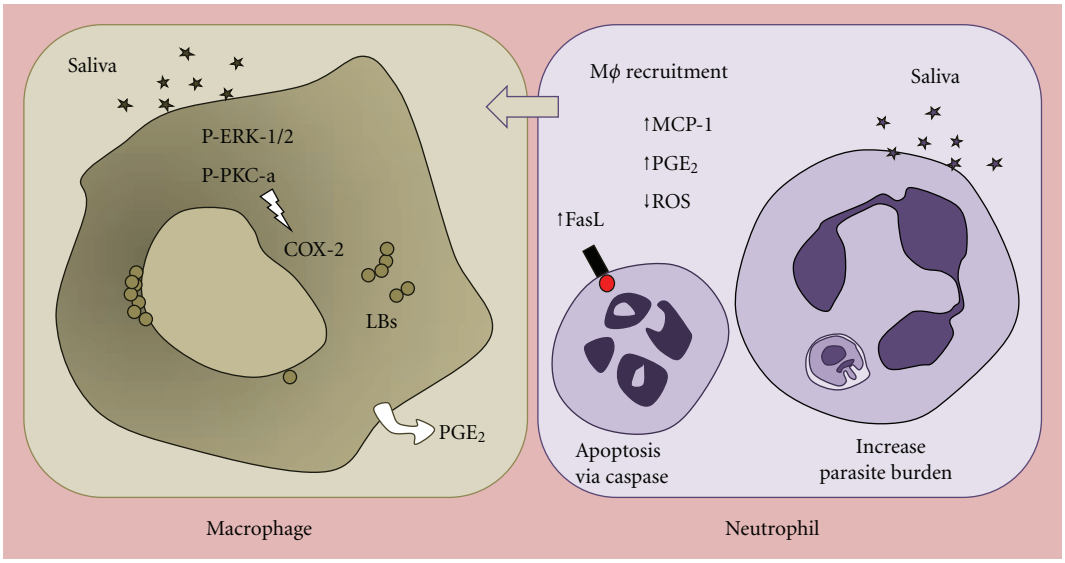

Figure 3: Effects of *Lutzomyia longipalpis* saliva on macrophage activation and neutrophil apoptosis. Macrophages and neutrophils are the first host cells to contact *Leishmania* after sand fly bite. Saliva triggers macrophages activation by lipid bodies formation committed with the PGE_2 production via COX-2 after phosphorilation of kinases. On the other hand, saliva induces neutrophil apoptosis by caspase and FasL activation. In addition, neutrophils activated by saliva become susceptible to *Leishmania chagasi* and release MCP-1, which is associated with macrophage recruitment. This scenario promoted by *L. longipalpis* saliva can contribute to *Leishmania* transmission in the early times of infection.

the inoculation site, lesions, and draining lymph nodes from *Leishmania*-infected mice [31, 63, 65–67]. In addition, *Leishmania* parasites undergo a silent entry into macrophages inside phagocytosed neutrophils, thus reinforcing the role of neutrophils on establishment of *Leishmania* infection [68]. *Leishmania donovani* inhibition of traffic into lysosome-derived compartments in short-lived neutrophils was suggested as a key process for the subsequent establishment of long-term parasitism [69]. On the other hand, neutrophils have also been implicated in parasite control. Phagocytosis of *L. major* by human neutrophils led to parasite killing [70]. Human neutrophils were capable to kill *L. donovani* by oxidative mechanisms [71], and, more recently, it was described the involvement of NET's (Neutrophil Extracellular Traps) on *L. amazonensis* destruction [72].

One elegant approach that reinforced the essential role for neutrophils in leishmaniasis revealed the presence of *Leishmania*-infected neutrophil on the sand fly bite site [40]. However, in that work, although the sustained neutrophil recruitment had been evident only in response to the sand fly bite, the authors did not attribute the neutrophil influx to vector salivary components. Surprisingly, besides neutrophil recruitment, there are no previous reports on further effects of sand fly saliva on neutrophil inflammatory response. Interestingly, studies performed with tick saliva disclose that the inhibition of neutrophil functions favors the initial survival of spirochetes [73–75].

Our group has recently shown the first evidence of direct effect of *L. longipalpis* salivary components on C57BL/6 mice neutrophils [76]. In summary, we described that saliva from *L. longipalpis* triggers apoptosis of inflammatory neutrophils obtained from C57BL/6 peritoneal cavity (Figure 3). The proapoptotic effect of saliva was due to caspase activation and FasL expression on neutrophil surface. Although salivary glands from blood feeding vectors have a variety of components [76], it seems that the proapoptosis compound in *L. longipalpis* saliva is a protein. However, further work is required to elucidate which protein or proteins act in this process. Additional helpful information from this study is that preincubation of *L. longipalpis* saliva with anti-saliva antibodies abrogated neutrophil apoptosis. This allows us to propose that proapoptotic component from *L. longipalpis* saliva could be target for the host's antibodies.

Moreover, neutrophil apoptosis induced by *L. longipalpis* saliva was also increased in the presence of *L. chagasi* [76]. This is particularly interesting by reinforcing the synergistic effect of both vector component and parasite on host inflammatory response, as have been observed in cell chemotaxis [42]. Interestingly, saliva from *L. longipalpis* enhanced *L. chagasi* viability inside neutrophils. This effect was attributed to modulation of neutrophil inflammatory response [76], as treatment of neutrophils with a pan caspase inhibitor (z-VAD) and a COX-2 inhibitor (NS-398) abrogated the increased parasite burden observed. Finally, we also described a novel inflammatory function of *L. longipalpis* saliva on neutrophils, stimulating MCP-1 production, able to attract macrophages *in vitro*. Even though chemotatic activity from *L. longipalpis* saliva has been previously reported, this is the first demonstration that saliva modifies directly the neutrophil inflammatory function, inducing the release of chemotatic factors by these cells.

7. Future Directions

In this paper, we explored the new inflammatory events induced by *L. longipalpis* in the recruitment and cellular function of leukocytes, as well as the repercussion to *L. chagasi* infection. The understanding of protective mechanisms regarding the initial steps of host's response to salivary molecules that can correlate with resistance or susceptibility to *Leishmania* has been poorly explored. Further investigation should address factors that determine the success of *Leishmania* infection. Identifying new escape mechanisms used by *Leishmania* associated to the pharmacological complexity of the sand fly saliva remains a challenge. In this scenario, phylogenetic implications between vector and *Leishmania* species can result in distinct action under host cells. The insights from the inflammatory scenery approached here, as lipid body induction in macrophages and apoptotic death of neutrophils, need to be investigated during the interaction between saliva from other sand fly and *Leishmania* species. Another important point is that these inflammatory effects were detected in salivary gland extract of sand fly vector. However, recombinants proteins from *L. longipalpis* saliva that presented known immunogenic role should be tested as inducers of these inflammatory events during infection by *Leishmania* sp. The studies discussed here suggest that saliva components can act on virulence factors from parasite surface in the first steps involved the recognition, resistance to oxidative mechanisms, and modulation of inflammatory mediators' produced by host cells. However, this finding seems to be part of a "large puzzle," since they are viewed in isolation, by methodological limitations. Recent emerging imaging technologies have opened the possibility to monitor the process of *Leishmania*-host cell interaction in real time from the first moment upon sand fly bite, allowing understanding of molecular and cellular mechanisms in *Leishmania* experimental infection. These advances will enable future integrated studies that may increase understanding of immunopathogenic mechanisms induced by saliva in this intricate and fascinating interaction.

Conflict of Interests

The authors have no financial or other conflicts to declare.

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Review Article

Macrophage Migration Inhibitory Factor in Protozoan Infections

Marcelo T. Bozza, Yuri C. Martins, Letícia A. M. Carneiro, and Claudia N. Paiva

Laboratório de Inflamação e Imunidade, Departamento de Imunologia, Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, 21941-902, Rio de Janeiro, RJ, Brazil

Correspondence should be addressed to Marcelo T. Bozza, mbozza@micro.ufrj.br

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Macrophage migration inhibitory factor (MIF) is a cytokine that plays a central role in immune and inflammatory responses. In the present paper, we discussed the participation of MIF in the immune response to protozoan parasite infections. As a general trend, MIF participates in the control of parasite burden at the expense of promoting tissue damage due to increased inflammation.

1. Introduction

The immune/inflammatory response triggered during infection has an essential role in eliminating the infectious agent and in promoting tissue repair [1]. The very existence of multicellular organisms in an environment replete of infectious agents is made possible by an effective immune system, indicating that the ability to control infections has been throughout evolution an important selective pressure to mold the immune system. However, it is not unusual that the tissue damage observed during infectious processes is caused by the immune/inflammatory response itself. Innate immune receptors recognize conserved microbial molecules from all classes of microorganisms [1, 2]. The activation of these receptors elicits selective intracellular signaling cascades that result in the production of cytokines, chemokines, lipid mediators, and reactive oxygen/nitrogen species. Both the intensity and the quality of the inflammatory responses are determined by the detection of combinations of microbial molecules and molecules from host origin such as cytokines, ATP, and ROS [3, 4]. This activation of the immune system is considered essential for pathogen killing but, on the other hand, is also critically involved in tissue damage and sepsis [1–4]. Thus, the pathology of infectious diseases can result either from a direct effect of the infectious agents or from the immune/inflammatory response, both of which can cause metabolic changes, cellular malfunctioning, and cell death. In fact, the pathology of most infectious diseases is the intricate result of these two forces.

Macrophage migration inhibitory factor (MIF) activity was described in the sixties and it is considered one of the first cytokines to be described [5, 6]. The MIF gene was cloned in 1989 using a functional assay based on its ability to inhibit the random migration of macrophages [7]. A major breakthrough in the characterization of MIF was achieved by a remarkable study that identified proteins secreted by the pituitary gland upon stimulation by LPS [8]. Among these proteins was MIF, and the authors went on to show that blockade of MIF protected mice from LPS-induced lethality, indicating its prominent proinflammatory role in endotoxemia. These studies led to renewed scientific interest on the biology of MIF and opened research avenues in several fields. In the 20 years of research following the cloning of MIF a complex scenario of its biology has emerged and it is now clear that MIF is an important inflammatory mediator that participates in both innate and adaptive immune responses [9].

Preformed MIF protein is found in many cell types and is released in response to different stimuli, such as infections and cytokine stimulation [9]. Physiological increases in glucocorticoid concentrations induce immune cells to secrete MIF, and, once released, MIF can counterregulate the anti-inflammatory effects of steroids on cytokine production [10, 11]. The pro-inflammatory activities of MIF include the induction/production of inflammatory mediators such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and nitric oxide (NO) by macrophages, the production of arachidonic acid and eicosanoids through the induction of phospholipase

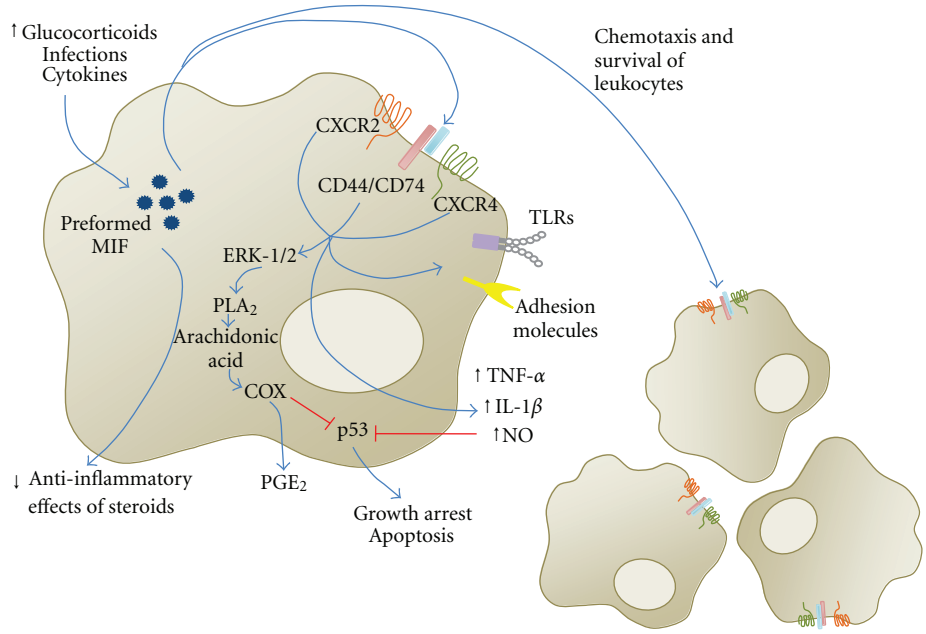

Figure 1: The effects of MIF on macrophage activation. Release of preformed MIF induced by different types of stimuli, such as infections, cytokines, and variations on glucocorticoid levels, has paracrine and exocrine effects: triggering of the CD44/CD74 receptor complex and the CXCR2 and CXCR4 chemokine receptors results in the production of tumor-necrosis-factor-α (TNF-α), interleukin-1 (IL-1), and nitric oxide (NO,) as well as of arachidonic acid and eicosanoids through the induction of phospholipase A_2 and cyclooxygenase, and in increased expression of TLRs and adhesion molecules in macrophages. The exocrine effects of MIF include induction of chemotaxis and promoting the survival of leukocytes.

A_2 and cyclooxygenase via a protein kinase A and ERK-dependent pathway, the increased expression of TLRs and adhesion molecules, antagonistic effects on glucocorticoids activity, and its role as a chemoattractant and in promoting the survival of leukocytes (Figure 1) [12–19]. These effects of MIF are, at least in part, mediated by activation of the CD74-CD44 receptor complex, as well as of the CXCR2 and CXCR4 chemokine receptors (Figure 1) [18–21]. MIF also increases macrophage survival through inhibition of p53 activity, thus reducing activation-induced apoptosis [22]. Interestingly, the inhibitory effect of MIF on p53 is dependent on COX-2 and autocrine production of PGE_2 by macrophages [17]. This increased survival of macrophages promoted by MIF might affect the immune response to intracellular parasites.

Studies using antibody neutralization, antagonists, or gene deletion demonstrated that MIF plays a critical role in the pathogenesis of several inflammatory disorders, such as sepsis, glomerulonephritis, arthritis, colitis, encephalomyelitis, atherosclerosis, and asthma [8, 9, 14, 18, 23–27]. Indeed, MIF has been shown to influence the pathogenesis of infectious diseases, participating in the protective immune response or playing a critical role in its immunopathogenesis [8, 9, 14, 19, 28–35]. Similarly, polymorphisms of the human MIF gene have been associated with increased susceptibility to or severity of a number of inflammatory diseases [36]. In the present paper we discuss the role of MIF in the host-parasite interaction upon infection caused by protozoan parasites (Table 1).

2. The Role of Host MIF in the Pathogenesis of Malaria

Malaria is caused by protozoan parasites from the genus *Plasmodium*. Presently, it is accepted that five species can cause disease in humans: *Plasmodium malariae*, *Plasmodium vivax*, *Plasmodium falciparum*, *Plasmodium ovale*, and *Plasmodium knowlesi* [48]. Together, these species are responsible for around 225 million cases of malaria and nearly one million deaths per year [49]. Although all five species can cause severe diseases [50], *P. falciparum* infections are the most prevalent in the world and are the most likely to complicate, which makes this species responsible for over 90% of the deaths [49]. Severe malarial anemia (SMA) and cerebral malaria (CM) are the most common and life-threatening complications caused by *P. falciparum* infections [51].

3. Host MIF Is Detrimental in Experimental Malaria

Experimental murine models of malaria infection have provided an invaluable resource for studying the role of inflammatory and immune responses in the pathology of malaria [52]. Infections caused by the four rodent parasite species (*P. chabaudi*, *P. yoelii*, *P. berghei*, and *P. vinckei*) vary in virulence and pathology depending on the strains of *Plasmodium* and laboratory mouse used [52]. For example, BALB/c mice develop a lethal infection with rapidly increasing parasitemia and anemia that peak approximately on day

Table 1: Role of MIF in the control of parasite burden and in the pathogenesis of protozoan infections.

Intracellular pathogen	Experimental system of MIF manipulation	Effects of MIF on parasite burden	Control of parasite burden	Pathogenesis	Ref.
Leishmania major	Murine macrophages, anti-MIF, rMIF	↓	MIF increases macrophage activation through enhancement of TNF and NO production	—	[37]
	Mif^{-}/$^{-}$ mice (C57BL/6)		MIF decreases lesion sizes and mediates leishmanicidal effects of IFN-γ on macrophages, reduces their NO and ROS production, but does not alter Th1 polarization	MIF decreases lesion sizes, a finding associated with decreased parasite burden	[38]
Toxoplasma gondii	*Mif*$^{-}$/$^{-}$ mice (BALB/c and C57BL/6 systemic infection; virulent RH and avirulent ME49)	↓	MIF stimulates production of IL-1β, IL-12, TNF, NO, and IFN-γ	MIF prevents tissue pathology, including liver lesions, a finding associated with parasite burden control and reduction of mortality	[39]
	Mif^{-}/$^{-}$ mice (C57BL/6; peroral infection ME49)		MIF controls parasite burden in ileum, while it increases TNF, IL-12, IFNγ, IL-23, and TGF-β and reduces IL-22 expression	MIF increases morbidity and mortality, increases MMP9 in ileum, contributes to its damage, and is involved in a sepsis-like response with liver impact	[40]
	Mif$^{-/-}$ (BALB/c peroral infection; ME49)		MIF improves maturation of DC and controls parasite burden in brain and livers	MIF prevents mortality	[41]
Trypanosoma cruzi	*Mif*$^{-/-}$ mice (BALB/c)	↓	MIF stimulates production of IL-1β, IL-12, and IL-18, Th1 polarization and specific IgG2a production	MIF prevents classical heart and striated muscle lesions, a finding associated with parasite burden control and prevention of mortality	[42]
Plasmodium chabaudi AS	*Mif*$^{-/-}$ mice (BALB/c); recombinant MIF	=	—	MIF inhibited erythropoiesis *in vitro* alone and synergizing with TNF and IFNγ *Mif*$^{-/-}$ had less severe anemia and increased survival	[43]

Table 1: Continued.

Intracellular pathogen	Experimental system of MIF manipulation	Effects of MIF on parasite burden	Control of parasite burden	Pathogenesis	Ref.
Plasmodium chabaudi adami	*Mif*$^{-/-}$ mice (BALB/c); Ab-neutralized MIF	↑	MIF promotes Th2 polarization (in its absence, cells react better to IL-12/anti-IL-4 with Th1 polarization)	—	[44]
Plasmodium falciparum	Human volunteers submitted to infection; correlation	=	—	MIF is associated with a number of circulating lymphocytes	[45]
	Infected children in endemic zone; correlation	↓	—	MIF concentrations in plasma and MIF produced by leukocytes *in vitro* inversely correlated with severity of malarial anemia	[46]
	Infected children in endemic zone; association between polymorphism of MIF promoter and pathology	=	—	MIF peripheral levels are associated with promoter polymorphisms and with susceptibility to severe malarial anemia	[47]

8 of infection when inoculated with *P. chabaudi chabaudi* AS [53]. For this reason, this parasite-mouse combination is considered an experimental model of SMA. On the other hand, the same strain of mouse develops a nonlethal self-resolving infection with peak parasitemia also on day 8 of infection followed by cell-mediated parasite killing and total parasite clearance on day 15 when inoculated with *P. chabaudi adami* DK [43]. This second model is considered suitable to study the interactions between macrophages and T cells involved in parasite elimination. Alternatively, C57Bl/6 mice develop a neurologic syndrome similar to human CM and characterized by ataxia, convulsions, and coma upon infection with *P. berghei* ANKA or *P. yoelii* 17XL [54–56]. Interestingly, *P. yoelii* 17XL, but not *P. berghei* ANKA, also induces CM when inoculated in BALB/c mice [52].

However, none of the rodent *Plasmodium* strains are natural pathogens of laboratory mouse strains and the course of infection and complications observed in some mouse-parasite combinations including SMA and CM differ from the human spectrum of disease [52]. For instance, peak anemia in the *P. c. chabaudi* AS-BALB/c model correlates with a peak parasitemia of around 20%, which makes the destruction of infected erythrocytes a major contributor to the physiopathogenesis of anemia in this model [52, 57]. Although it also occurs in acute hyperparasitemic infections, the development of SMA in humans occurs mainly in chronic infections with low parasitemias (<5%) and appears to be more related to other mechanisms such as the destruction of uninfected erythrocytes and the suppression of the erythropoietic response [57]. The majority of mouse models of CM are characterized by the adhesion of leukocytes, instead of infected erythrocytes in the brain microvasculature as occurs in human CM [58].

Studies using mouse models of malaria indicate that MIF plays a detrimental role during the infection [43, 44, 53]. *Mif*$^{-}$/$^{-}$ mice in the BALB/c background or animals treated with anti-MIF neutralizing monoclonal antibodies are more resistant to *Plasmodium chabaudi adami* infection than wild-type controls presenting a significant reduction in peak and cumulative parasitemia [44]. Accordingly, the infection of BALB/c mice with *P. chabaudi chabaudi* AS, a mouse model of SMA, revealed that elevated concentrations of MIF in the plasma are associated with severity of anemia and suppression of erythropoiesis [43, 53]. In addition, *Mif*$^{-}$/$^{-}$ mice infected with *P. c. chabaudi* develop a parasitemia curve similar to that of wild-type controls but present less severe anemia, less inhibition of erythroid colony formation, and a higher survival [43].

It is not clear why blockade of MIF reduces parasitemia during *P. c. adami* but not *P. c. chabaudi* infection. As the development of immunity and/or anemia in mouse

and human malaria result from a complex process that involves multiple factors [57, 59], these findings indicate that MIF could act modulating different mechanisms during *Plasmodium* infection. For example, MIF attenuates the development of Th1 responses following *P. c. adami* infection in BALB/c mice by decreasing T $CD4^+$ IFN-γ production and enhancing IL-4 [44]. Nevertheless, experimental evidence has suggested no role for IFN-γ and TNF as inhibitors of erythropoiesis in mice [60] and serum concentrations of these cytokines are the same during the critical period of anemia in $Mif^{-/-}$ and wild-type mice infected by *P. c. chabaudi* [43], indicating that the role of MIF in this mouse model of SMA is independent of the contribution of TNF or IFN-γ.

Production of MIF in mouse SMA seems to be triggered by hemozoin, which is an insoluble heme polymer produced by parasite catabolism of host hemoglobin [43, 53]. Hemozoin contributes to the suppression of erythropoiesis in several ways, including the induction of MIF [61–63]. Mouse macrophages secret MIF in response to *P. c. chabaudi*-infected erythrocytes and synthetic hemozoin in a dose response manner [43, 53]. MIF inhibits erythroid colony formation and differentiation in mouse and human bone marrow cell cultures containing erythropoietin by modulating MAP kinase activation [10, 43]. Taken together these data indicate that MIF plays a role in the physiopathology of mouse SMA by decreasing red blood cell production during the infection.

Nevertheless, the role of MIF in semi-immune mouse models of SMA (multiple cycles of *P. berghei* ANKA infection followed by antimalarial treatment in C57Bl/6 mice) that present low levels of parasitemia during anemic episodes and are believed to be more closely related to the human pathology [64, 65] was not investigated yet. It is also still unclear what is the relationship, if any, between MIF production during *Plasmodium* infection in mice and other mechanisms known to be involved in mouse SMA such as lysis of infected erythrocytes due to schizogony and destruction of noninfected erythrocytes [66], by phagocytosis [64], and auto-antibodies [65].

4. Host MIF Seems to Be Protective in Human Malaria

Studies conducted in Africa have reported lower concentrations of MIF in *P. falciparum*-infected children when compared to asymptomatic ones [46, 67, 68]. These studies have shown an inverse correlation between MIF concentrations and parasite burden [68] and suggested a protective role for MIF during noncomplicated malaria [67] and human SMA [46]. Furthermore, the data above is corroborated by an experimental work with healthy European volunteers showing that MIF concentrations are decreased in response to early *P. falciparum* infection but are increased in response to antimalarial treatment [45]. Several other reports in children [43, 69] and adults [70, 71] infected with *P. falciparum* showed conflicting results adding to the controversy on the role of MIF on malaria pathogenesis. The reasons behind these discrepancies are not obvious but one should consider that factors such as the previous degree of immunity of the studied population, which can change the pattern of response to the infection [59], and the presence of *Plasmodium*-derived MIF homologues [71] were not accessed and, consequently, not taken into account when analyzing the different studies.

Furthermore, an inverse correlation between MIF plasma concentrations and hemozoin accumulation was showed in children affected by severe anemia indicating that, different from mouse models, hemozoin may decrease MIF production in human malaria [46]. The explanation for this paradox may lie in a feedback loop involving long-lasting hemozoin activation of macrophages in these children. In fact, PBMC from malaria-naïve patients can react to hemozoin by either increasing or decreasing MIF production [46], depending on whether they have a generally well-preserved MIF production. Additionally, MIF polymorphisms also give rise to variable magnitudes of response to hemozoin [43] and could also help to explain the variability found in these studies regarding MIF production in response to hemozoin. Accordingly, there is an association between certain MIF haplotypes of the −173G/C and −794CATT5-8 polymorphisms and susceptibility to SMA [43, 47].

Few studies in humans indicate that MIF is involved in the pathogenesis of cerebral malaria [72–74]. Necropsy studies show a decrease in endothelial cell expression of MIF in brain vessels of cerebral malaria patients when compared to endothelial cells from axillary and chest vessels [72, 73]. A clinical study in India showed that high concentrations of MIF in the plasma are associated with death in cerebral malaria patients [74]. Finally, women with placental malaria infection presented significantly higher levels of MIF in the placental intervillous blood when compared to uninfected pregnant women also indicating a role for this cytokine in malaria infection during pregnancy [69, 75].

5. The Role of Plasmodium MIF in the Pathogenesis of Malaria

MIF homologues have been identified in all species of *Plasmodium* examined to date—*P. falciparum* [76–78], *P. vivax* [71], *P. berghei* [54, 79], and *P. yoelii* [55, 56]. The data from the studies cited above indicate that *Plasmodium* MIF (pMIF) is structurally similar to mammalian MIF with around 30% amino acid sequence identity and possesses some, but probably not all, activities normally attributed to the latter. pMIF expression increases with blood-stage parasite maturation: minimal in ring stage and peaking in the trophozoite and schizont stages [54, 55, 76]. There is no evidence showing that pMIF is actively or passively secreted to the blood stream by the parasite, but it is released extracellularly upon schizont rupture, when it becomes available to interact with the host immune cells [54, 55]. Indeed, pMIF has been detected in culture supernatant and plasma of *Plasmodium*-infected mice and humans [55, 56, 67, 71]. However, due to a low conservation of the aminoacid residues in the molecular region known to be involved

in the catalytic sites, it seems that the tautomerase and oxidoreductase activities are highly depressed in pMIF when compared to mammalian MIF. Alternatively, pMIF might have a different substrate specificity and the physiological substrate has yet to be identified [54–56].

In terms of functional studies, *in vitro* assays and animal models have shown that pMIF shares some biological properties with mammalian MIF. Indeed, both pMIF and mammalian MIF reduce AP-1 expression, interact with human CD74, induce macrophage chemotaxis, and inhibit erythropoiesis and macrophage apoptosis [54–56, 79, 80]. On the other hand, pMIF does not stimulate the release of IL-8, TNF, or IL-12 from mice and human monocytes or enhance the response of these cells to LPS [55, 78], a key function of mammalian MIF. Finally, a study showed that pMIF and mouse MIF act synergistically to activate the MAPK-ERK1/2 signaling pathway at very low concentrations but act antagonistically at higher concentrations [56], indicating that pMIF and mammalian MIF can interact in a complex way.

The role of pMIF during malaria infection is also not completely understood. Although counterintuitive, studies in mouse models indicate that pMIF attenuates *Plasmodium* virulence by modulating host immune responses [54–56]. C57Bl/6 and BALB/c mice showed a reduction in disease severity when infected with transgenic strains of *P. yoelii* 17X and *P yoelii* 17XL that constitutively overexpress *P. yoelii* MIF (PyMIF) [55] or when treated with recombinant PyMIF [56]. This was phenotypically manifested by a decrease in peak and cumulative parasitemia in mice infected with the nonlethal strain *P. yoelii* 17X and prolonged course of infection with a reduction in overall mortality rate in animals infected with the lethal strain *P yoelii* 17XL [55, 56]. On the other hand, the development of cerebral complications in C57BL/6 mice and hyperparasitemia and severe anemia in BALB/c mice did not differ upon infection with *P. berghei* wild-type or *P. berghei* MIF knockout parasites [54]. Once again, studies in humans failed to recapitulate observations from mouse models as pMIF amounts in uncomplicated malaria patients are positively correlated with parasitemia, disease severity, and plasma concentrations of TNF, IL-10, and MCP-1 [71]. Thus, future studies are required to define the role of host and *Plasmodium* MIF in the pathogenesis of malaria.

6. Critical Role of MIF in *Toxoplasma gondii* Infection

Toxoplasma gondii is an intracellular parasite of the phylum Apicomplexa that is highly adapted to infect different cell types and tissues. *T. gondii* enters its host via the gastrointestinal tract and the innate immune response in the intestine is triggered by the recognition of parasite molecules by enterocytes, macrophages, and dendritic cells (DCs) [81]. The establishment of an antigen-specific Th1 response is essential to protective immunity but also potentially detrimental as excessive intestinal inflammation and tissue necrosis can lead to bacterial translocation and death [81]. The proinflammatory cytokines IL-12, TNF, IFN-γ, and IL-1β promote resistance against *T. gondii* in part due to the generation of NO by macrophages, an important mechanism responsible for parasite elimination.

A model of systemic infection with *T. gondii* through the intraperitoneal route demonstrated an increased susceptibility of $Mif^{-}/^{-}$ mice when compared to wild-type mice [39]. $Mif^{-}/^{-}$ mice presented higher parasite burden in brains and peritoneal macrophages and reduced plasma concentrations of IL-12, TNF, IFN-γ, IL-1β, and nitrite during infection [39]. These findings were expected considering that MIF is an enhancer of IL-12 and TNF production by macrophages. A recent study using a model of oral *T. gondii* infection in the BALB/c background also demonstrated an increased lethality and tissue parasitism with reduced IL-12 production and DC activation on $Mif^{-}/^{-}$ mice compared to wild-type mice [41]. DCs obtained from spleens and mesenteric lymph nodes from $Mif^{-}/^{-}$ mice orally infected with *T. gondii* had impaired maturation, with decreased expression of CD80, CD86, CD40, and MHC class II [41]. Thus, the protective role of MIF in *T. gondii* infection is apparently related to the production of proinflammatory cytokines, the activation of DC, and the better control of parasite burden.

BALB/c mice are naturally resistant to oral infection with *T. gondii,* while those of C57BL/6 are highly susceptible displaying intestinal inflammation especially in the ileum [82–84]. This increased lethality of C57BL/6 is related to the extensive intestinal inflammation, tissue necrosis, and a sepsis-like syndrome. Using the peroral route of infection in C57BL/6 mice, it was shown that $Mif^{-}/^{-}$ mice have reduced intestinal and systemic inflammation and survive longer compared to wild-type mice, despite an increase in intestinal parasite burden [40]. Lack of MIF caused a reduction of TNF, IL-12, IFN-γ, and IL-23 and an increased expression of IL-22 in ileal mucosa. Signs of systemic inflammation including the increased concentrations of inflammatory cytokines in the plasma and liver damage were less pronounced in $Mif^{-}/^{-}$ mice compared to wild-type mice [40]. Although MIF has been regarded as essential in host protection during *T. gondii* infection, these findings demonstrated a pathogenic role of MIF in natural *T. gondii* infection in susceptible hosts. This dichotomy seems to depend on the route of infection and the genetic background of the host. Thus, MIF is necessary to control parasite burden in resistant and susceptible hosts, but it increases intestinal tissue damage causing death in susceptible hosts while it is essential for survival in resistant hosts.

A major consequence of human *T. gondii*-infection is the severe congenital malformations when the primary infection occurs in the first trimester of pregnancy. A series of studies demonstrated a putative role of MIF on placental biology upon infection with *T. gondii*. Infection or stimulation of chorionic explants with molecules of *T. gondii*, IFN-γ, and IL-12 evoked the secretion of MIF [85, 86]. MIF and its receptor, CD74, are present in the syncytiotrophoblast layer and mesenchyme [86]. MIF induces ICAM-1 expression increasing the interaction of villous explants with monocytes [85]. These results suggest that MIF, by influencing the recruitment of *T. gondii* infected monocytes, could facilitate

the dissemination of the infection into the deep placental tissues or increase the tissue damage due to inflammation. The same group recently demonstrated, however, that MIF is important for control of placental *T. gondii* infection in first trimester of pregnancy [86].

7. MIF Is Protective in *Leishmania* Infection

Leishmaniasis, caused by the protozoan parasites from the genus *Leishmania*, comprises a large spectrum of clinical manifestations including benign ulcer, destructive mucocutaneous lesions, disseminated cutaneous lesions, and systemic visceral forms [87]. In the mammalian host, *Leishmania sp*. is an obligatory intracellular parasite infecting mainly macrophages. Parasite killing requires macrophage activation with ensuing NO and ROS production [87]. Infection with *Leishmania major* causes skin lesions, which in general parallel the parasite load. A highly polarized Th1 response is effective against *L. major*, activating macrophages to produce NO and resulting in resolving skin lesions. Addition of MIF to macrophage cell cultures results in increased *L. major* elimination [37]. Though the MIF concentration required to reduce *L. major* burden in macrophages is high (1 μg/mL, 100 times that of other cytokines with leishmanicidal effects, such as IFN-γ), this concentration is within the range reached in inflammatory conditions. The MIF-induced leishmanicidal effect requires the production of TNF and NO by infected macrophages, and can be reversed by the addition of IL-10, TGF-β or IL-13, indicating that it depends on an M1 activation status [37]. The expression of MIF increases during *L. major* footpad inoculation in popliteal lymph node, but the kinetics of its expression compared to that of MIF secretion by T cells upon antigenpresentation suggests that lymph node MIF comes from another cellular source [37]. Consistent with the observed role of MIF as an enhancer of macrophage leishmanicidal function, oral administration of *Salmonella typhimurium* transfected with MIF reduces the size of skin lesions [88], while $Mif^{-}/^{-}$ mice are highly susceptible to *L. major*, developing severe skin lesions late after infection [38]. MIF does not affect Th polarization in *L. major* infection, as indicated by the similar IFN-γ and IL-4 production among T cells from $Mif^{-}/^{-}$ and wild-type mice. However, IFN-γ-activated macrophages from $Mif^{-}/^{-}$ mice infected *in vitro* with *L. major* have slightly decreased parasite clearance [38], indicating that either they are somewhat insensitive to IFN-γ or MIF production is partially required as an intermediary step to IFN-γ-induced leishmanicidal activity. The contribution of MIF produced by $CD4^{+}$ lymphocytes to protective immunity against cutaneous leishmaniasis was demonstrated using a model of vaccination with the *L. pifanoi* antigen P-4 [89]. BALB-c mice immunized with P-4 expressed around 10-fold higher amounts of MIF, TNF, and IFN-γ mRNAs than the adjuvant controls. Moreover, blockage of MIF with anti-MIF antibody significantly reduced the leishmanicidal ability of macrophages cultured with $CD4^{+}$ lymphocytes obtained from P-4-immunized mice.

Patients with visceral leishmaniasis due to infection with *L. donovani* presented $CD4^{+}$ lymphocytes expressing low amounts of CD2, IFN-γ, and MIF [90]. Antileishmanial treatment caused immunological recovery with increased expression of CD2 and production of MIF. On the other hand, a recent study demonstrated that patients with visceral leishmaniasis caused by *L. chagasi* have increased plasma concentrations of MIF [91]. The MIF concentrations were higher in patients with the active form compared to patients in remission. Interestingly, the authors identify an increase of LPS in the plasma of patients with active disease and the LPS concentrations positively correlated with MIF.

8. Identification of Leishmanial MIF and Its Role in Infection

The complete genome sequencing of *L. major* revealed two genes with significant sequence similarities to human MIF (22% identity) [92]. Cloning and expression of one of these leishmanial orthologues of MIF allowed detailed functional and structural characterizations [93, 94]. The X-ray crystal structure of Lm1740MIF/LmjMIF1 demonstrated an overall global topology similar to that of human MIF, but the catalytic site has substantial differences that correlate with the low tautomerase activity of Lm1740MIF/LmjMIF1 and the lack of inhibitory effect of ISO-1, a MIF antagonist that binds to the catalytic site [93, 94]. Similar to the other MIF structures, the *L. major* orthologue proteins adopt trimeric ring architecture. Lm1740MIF binds to CD74, the MIF receptor, indicating a putative role of *L. major* MIF affecting host immunity [93]. In fact, LM1740 induces a signaling cascade on monocytes dependent on CD74 and similar to the one triggered by mammalian MIF. This includes the ability of *L. major* MIF orthologues to induce ERK1/2 phosphorylation, to cause the reduction of Ser^{15}-p53 in the cytoplasm, and to protect macrophages from NO-induced apoptosis [93]. Since the macrophage is the main cell type hosting *Leishmania*, the ability of *L. major* MIF to increase the survival of macrophages might represent an important selective advantage that guarantees more efficient amastigote replication.

9. MIF Is Protective in *Trypanosoma cruzi* Infection

Trypanosoma cruzi is an intracellular protozoan that can infect many cell types, including macrophages. The effective response to *T. cruzi* comprises innate activation of macrophages to induce NO production and, ultimately, the establishment of antigen-specific Th1 CD4 and CTL CD8 responses [95]. Mice genetically deficient in *Mif* also are more susceptible to *Trypanosoma cruzi* infection [42]. This increase in susceptibility is accompanied by decreased plasma concentrations of IL-12 and IFN-γ along with acute infection and also decreased IL-12 and IFN-γ production by splenocytes stimulated with *T. cruzi* antigens early in the acute phase, indicating that in contrast to the trypanosomatid, *L. major*, MIF participates in Th1 polarization in *T. cruzi* infection. This deficient Th1 polarization is reflected by decreased titers of anti-*T. cruzi* IgG2a (but not IgG1). Also,

Mif^{-}/$^{-}$ mice have decreased plasma concentrations of TNF, IL-1β, and IL-18, suggesting that decreased production of proinflammatory cytokines underlies their susceptibility to *T. cruzi* infection. The deficient Th1 polarization, specific IgG and pro-inflammatory cytokine secretion are all highly compatible with susceptibility to *T. cruzi* infection, but there is currently no functional data to support this hypothesis. In fact, IFN-γ-activated macrophages have a prominent role in *T. cruzi* clearance through NO production, a function that can be enhanced by TNF production. As MIF controls TNF production by macrophages in a number of cases and, along with TNF, enhances production of NO by macrophages and the elimination of trypanosomatid *L. major* [37], it seems likely that MIF enhances macrophage trypanocidal activity. Interestingly, increased expression of MIF was observed in myocardium and skeletal muscles from acutely *T. cruzi* infected BALB/c mice and positively correlated with parasite burden and myopathic alterations [96].

A prior intracellular infection can sensitize the organism to septic shock by priming monocytes to overreact in the presence of very low amounts of TLR ligands, as happens in influenza [97], VSV [98], LCMV infection [99], among others. *T. cruzi*-infected mice are highly susceptible to systemic inflammation, which can be caused by infection itself in mice lineages that develop severe inflammatory response or by administration of TNF, anti-CD3 [100], SEB [98], or LPS [101]. The lethal synergism between *T. cruzi* infection and LPS inoculation likely results from redundant lethal pathways induced by TNF and MIF: although both *Mif*$^{-}$/$^{-}$ and *Tnfr1* $^{-/-}$ infected mice succumb to LPS administration, treatment with anti-MIF rescues *Tnfr1*-deficient mice from lethal shock [102]. However, at present there are no studies demonstrating a contribution of MIF to human mortality in Chagas disease.

Almost no information is available on MIF biology in Chagasic patients. The only study that addressed this issue demonstrated that the MIF-173G/C polymorphism confers susceptibility to Chagas disease in two cohorts from Colombia and Peru [103]. Future studies are essential to characterize the participation of MIF in the physiopathology and immunity to *T. cruzi* infection.

10. Future Directions

In this paper we described the involvement of MIF in several models of protozoan infections, considering common themes and certain peculiarities specific to each parasite. In general, MIF seems to participate in the control of parasite burden but, in many cases, with the cost of promoting tissue damage due to increased inflammation. The essential role of MIF in the pathogenesis of infectious diseases and, consequently, the concept that it might be used as therapeutic target still require extensive clinical studies. Thus, for the years to come, several aspects of the biology of MIF and its participation in the response to infectious diseases, including parasitic diseases, need to be addressed opening up new highways of research and, possibly, novel therapeutic strategies.

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Review Article

Subversion of Immunity by *Leishmania amazonensis* Parasites: Possible Role of Phosphatidylserine as a Main Regulator

Joao Luiz Mendes Wanderley,[1] Jaqueline França Costa,[2] Valéria Matos Borges,[2,3,4] and Marcello Barcinski[5]

[1] *Universidade Federal do Rio de Janeiro, Pólo Universitário Macaé, Brazil*
[2] *Centro de Pesquisa Gonçalo Moniz, Fundação Oswaldo Cruz, Salvador, BA, Brazil*
[3] *Faculdade de Medicina, Universidade Federal da Bahia, Salvador, BA, Brazil*
[4] *Instituto Nacional de Ciência e Tecnologia de Investigação em Imunologia, Salvador, BA, Brazil*
[5] *Laboratório de Biologia Celular, Instituto Oswaldo Cruz, Rio de Janeiro, RJ, Brazil*

Correspondence should be addressed to Joao Luiz Mendes Wanderley, lmwjoao@yahoo.com.br

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Leishmania amazonensis parasites cause progressive disease in most inbred mouse strains and are associated with the development of diffuse cutaneous leishmaniasis in humans. The poor activation of an effective cellular response is correlated with the ability of these parasites to infect mononuclear phagocytic cells without triggering their activation or actively suppressing innate responses of these cells. Here we discuss the possible role of phosphatidylserine exposure by these parasites as a main regulator of the mechanism underlying subversion of the immune system at different steps during the infection.

1. *Leishmania* Parasites

Leishmania parasites are heteroxenous kinetoplastid protozoan organisms, which undergo complete differentiation upon a cycle of proliferation/differentiation in the midgut of phlebotomine sand flies followed by the transmission of infective metacyclic promastigotes [1, 2] to mammalian hosts during the insect blood meal. Once infecting mammalian hosts, these organisms, from free-living protozoa, become obligate intracellular parasites, residing and proliferating inside phagolysosomes of mononuclear phagocytic cells as amastigote forms.

In humans, *Leishmania* parasites can cause a broad spectrum of clinical manifestations from mild, self-resolving skin diseases to potentially fatal, disseminated visceral diseases. The outcome of the infection is dependent on multiple, interdependent factors, such as vector species, parasite species and strain, genetic background, and immunological status of the host. There are two main groups of parasites, stratified upon the clinical outcome of the infection: the ones capable of causing tegumentar and the ones capable of causing visceral diseases. In both cases, disease is initiated by the bite of an infected sand fly, followed by the generation of a skin lesion, mainly caused by the inflammatory response induced on that site. In some cases the disease is confined to the skin or mucosal tissues, and is termed cutaneous (CL) or mucocutaneous (MCL) leishmaniasis, respectively. In addition, diffuse cutaneous leishmaniasis (DCL) occurs when the parasite disseminates causing the appearance of multiple skin lesions, in distal sites relative to the transmission site [3]. In a similar way, in visceral leishmaniasis, there is parasite dissemination through blood and lymphatic vessels from the initial lesion site. However, these parasites establish in organs that comprise important populations of mononuclear phagocytes, such as bone marrow, spleen, and liver [4]. Among the clinical manifestations observed in humans with the tegumentary disease, diffuse and mucocutaneous leishmaniasis are the most severe forms. In both cases, most patients were found in the South and Central America, associated with *L. amazonensis* infection for DCL and *L. braziliensis* infection for MCL.

1.1. Diffuse Cutaneous Leishmaniasis. Diffuse cutaneous leishmaniasis (DCL) is a rare clinical manifestation and is characterized by the appearance of several nonulcerated nodular skin lesions, uncontrolled parasite proliferation, an inefficient cellular immune response against parasite antigens, and resistance to most therapeutic strategies [5, 6]. The lesions are characterized by a dense dermal infiltrate of vacuolated macrophages heavily parasitized. The intense parasitism in the DCL lesions reflects the functional state of macrophages, which are considered permissive. The deficient macrophage activation in DCL hinders the elimination of *Leishmania* resulting in a disorganized inflammatory process, unable to control the infection. The determinants of DCL are multifactorial and may be associated with both immunologic and genetic events of the patient and the pathogenic factors related to the parasite and vector. The participation of factors associated with the parasite has been shown by some authors although it is a point that still remains to be further explored. In this context, the exhibition of markers of apoptosis by the parasite could be a contributing factor during host-parasite interactions as a possible immunosuppressive mechanism of DCL [7].

2. Immune Response

2.1. Classical L. major Infection. Experimental infection models with *Leishmania* parasites have been extensively used as a tool to study immune responses, especially regarding T-cell differentiation [8, 9]. This is due to the fact that inbred mice strains demonstrate specific patterns of susceptibility and resistance to the disease [9, 10] which correlate with the immune response built by these animals. The classical experimental model that generated this knowledge was infection with *L. major* parasites. C57BL/6 mice infected with this parasite develop a Th1 $CD4^+$ T-cell response, which is highly effective to activate leishmanicidal and inflammatory mechanisms in macrophages, leading to intracellular parasite destruction. In this case, a skin lesion is formed, which regresses, becoming undetected around 6–8 postinfection [9]. Nevertheless, latent parasites remain in the infected tissue, providing antigens to maintain a protective immune response that prevent reinfections [11]. On the other hand, BALB/c mice infected with the same parasite species and strain developed a Th2 $CD4^+$ T-cell response, which is not efficient to promote macrophage classical activation, leading to progressive disease. At the cellular level, this difference is mainly due to the activation of a population of cells that express a highly restricted T-cell receptor, Vβ4 Vα8, which recognizes the LACK (*Leishmania* homologue of receptors for activated kinase) antigen and rapidly produces IL-4, necessary to deviate the immune response towards Th2 [12]. Currently, it is clear that the proposed model of susceptibility and resistance to *Leishmania* infection is quite reproducible when working with some specific strains of *L. major* though, for other strains and/or species, the picture is relatively more complex. Indeed, effective macrophage activation is the key to control the infection; however, the phenotype displayed by T cells in different situations is not as polarized as observed in the classical model. Actually, there are several papers that suggest that most correlations between $CD4^+$ T-cell response and disease development are not straightforward. BALB/c IL-4 receptor knock-out (KO) mice remained susceptible to *L. major* infection when infected with LV39 strain, which seems to be due to an increased production of IL-10 by T cells [13]. C57BL/6 mice infected with a *L. major* strain, isolated from a patient with nonhealing lesions, still developed a Th1 response but displayed a progressive disease [14]. In addition, when infected with the IR173 strain of *L. major*, $CD4^+$ T cells from both BALB/c and the resistant mice strain B10.D2 produce IL-4 very rapidly [15]. Other factors such as infection route, number of parasites inoculated, and type of infection (needle versus sand fly inoculation) are crucial to determine the type of response elicited (reviewed in [9]). The complexity of the interactions that determines the clinical and immunological outcome of the disease is much less known, and apparently much more multifactorial in other infection systems, such as the ones that involve *L. amazonensis* infection.

2.2. L. amazonensis Infection: Beyond the Paradigm. Experimental infection with *L. amazonensis* parasites leads to progressive disease and uncontrolled lesion development in all inbred mouse strains, including those ones that are highly resistant to *L. major* infection. However, there is a gradient of disease severity, ranging from BALB/c mice, which develop a very fast lesion, that ulcerate, generating extensive areas of necrotic tissue, to C3H.HeN mice that still develop nonhealing lesions, however, displaying slow progression rates [16, 17]. Nonetheless, the phenotype displayed by different mouse strains does not correlate with dichotomic Th1/Th2 responses. Actually, in the analyzed mouse strains such as BALB/c, C57BL/6, and C3H.HeN, it was possible to observe $CD4^+$ T cells capable of producing different types of cytokines such as Th2 cytokines (IL-4, IL-5, and IL-13), Th1 cytokines (IFNγ, and TNFα), and regulatory cytokines (TGFβ and IL-10), which characterizes an unpolarized cellular response [18–20]. Targeted deletion of the *Il4* or *Il10* gene [21, 22] causes minimal effects on lesion development and parasite tissue loads as well as treatment of infected mice with IFNγ [23] or IL-12 [22]. Interestingly, *L. amazonensis* promastigotes and, especially amastigotes, are able to get through the innate immune response almost unnoticeable. As mentioned before, the main host cell for *Leishmania* proliferation in the mammalian host is the macrophage, which is, together with dendritic cells (DCs), the main antigen presenting cells of the innate immune response. When compared to *L. braziliensis* parasites, for example, *L. amazonensis* parasites are much less capable of triggering the expression of CD40 and CD80 [24], both costimulatory molecules for T-cell activation, and the production of IL-12p40 [24]. Actually, amastigote infection is able to downregulate the expression of MHC class II molecules [25], which, during macrophage infection, is depending on sequestering these molecules inside the parasitophorous vacuole for degradation [26, 27]. During the first week of infection in C57BL/6 mice, chemokines such as CCL5, CCL3, CCL2, CCL4, and CCL11 as well as their receptors, are not upregulated when compared to

L. major infection, both at the lesion site and draining lymph node [19]. Additionally, amastigote infection downregulates several intracellular pathways that lead to DC activation such as STAT 1, STAT 3 and Erk 1/2 phosphorylation and the expression of the interferon-responsive elements IRF8 and 1, suggesting a global inhibition of inflammatory responses of these cells [25]. The most well-characterized ligand for amastigote recognition and internalization in macrophages is the opsonizing antibodies produced throughout infection. Triggering of Fc receptors on the host cells lead to IL-10 production and has a pathogenic role [28]. These events are necessary to evade the early immune response culminating with the ineffective T-cell response observed in most cases. In parallel to *L. major* infection in BALB/c mice, where the oligoclonal Vβ4 Vα8 CD4$^+$ T-cell population is necessary to the development of susceptibility in the host and is considered as pathogenic T cells, in *L. amazonensis* infection, T cells, in a general way, seem to be highly pathogenic. First of all, there is no clonal or oligoclonal T-cell population involved, since there is no predominance of a single or a group of Vβ chains expressed on T cells that respond to *L. amazonensis* infection [29]. However, the disruption of CD4$^+$ T cell effector functions, as observed in recombinase-activating gene KO mice (RAG KO), MHC class II transactivator KO mice (CIITA KO) and nude mice leads to transient resistance to *L. amazonensis* infection, measured by lesion development [30]. In addition, the adoptive transfer of regulatory T cells also restrains pathogenic effector T cells, diminishing lesion size and parasite tissue loads [31]. The mechanisms underlying pathogenic role of T cells for the disease need to be determined.

3. Phosphatidylserine Exposure

3.1. Homeostasis and Efferocytosis. Phosphatidylserine (PS) is a structural phospholipid present in virtually all membranes and cell types. In normal cells these molecules face the cytoplasmic leaflet of the plasma membrane, whereas during apoptotic cell death these molecules translocate to the outer surface. Once outside the cell, PS becomes one of the ligands recognized by surrounding phagocytes to clear dying cells [32]. However, PS in this model is not just one eat-me signal [33]. PS is the most characterized tickling [34] ligand of apoptotic cells, which means that PS provides the signals for the phagocyte to activate immunosuppressive and anti-inflammatory mechanisms. PS recognition is mandatory to prevent the establishment of a response to the self-antigens engulfed by these cells during apoptotic cell clearance and to avoid triggering inflammatory responses, especially during the embryogenesis, when massive amounts of apoptotic cells are generated and therefore, cleared [35–37], but also in adults to prevent inflammatory immunopathologies [32]. The intracellular events, receptors, and soluble factors involved in this mechanism are still being deciphered and are not the focus of this discussion. However, the effects of PS recognition in macrophages and DCs have a direct impact in immune responses. Apoptotic cells actively induce the production of the anti-inflammatory cytokines TGFβ, PGE2, and PAF [35] and actively inhibit the production of TNFα and IL-1β, even upon LPS challenge [35, 38]. Recognition of apoptotic cells also decreases the expression of several activation markers and costimulatory molecules by both human and murine DCs [39, 40] and regulates the expression of cytokines involved with T-cell differentiation at the transcriptional level [41, 42]. At the single cell level, DCs that ingested an apoptotic cell and bacteria at the same time are able to discern between them and only present bacterial antigens. This is possible because the generation of peptide-MHC class II complexes is controlled by toll-like receptors (TLRs) in a strictly phagosome autonomous manner. Since apoptotic cells do not trigger TLR activation, the generation of stable complexes is inhibited or abrogated [43]. All these effects are fundamental to maintain homeostasis and comprehend the last step of the efferocytosis [44] or apoptotic cell clearance. However, it seems that intracellular parasites elegantly make use of these mechanisms to establish in the host [45–47]. Furthermore, some parasites mimic the features of apoptotic cells to avoid host immune response, as discussed in the next section.

3.2. Conserved Immune-Evasion Mechanism? One of the most common PCD phenotypes is phosphatidylserine (PS) exposure, which can be observed upon chemotherapy, starvation, and heat shock conditions in several unicellular organisms [48–51] or is actively displayed in normal conditions [52]. Our group observed that lesion-derived amastigotes of *L. amazonensis* actively expose high levels of PS, and by blocking this molecule there is a drastic decrease in the ability of these parasites to infect and establish in the macrophages [52]. These parasites are viable and capable of differentiating into promastigote forms *in vitro* (unpublished data) and inside the sand fly vector [53] and to infect macrophages and mice [52, 54] and did not display other markers of PCD. Therefore we denominated this mechanism as apoptotic mimicry. PS exposure on amastigotes of *L. amazonensis* occurs in virtually 100% of the parasites; however, the amount of PS molecules depends on the infected host. Parasites obtained from BALB/c mice expose higher amounts of PS than the ones obtained from C57BL/6 mice [54]. This observation demonstrates that the amount of PS at the surface of the amastigotes has a positive correlation with the severity of the disease and suggests that the host is able to modulate this phenotype of the parasite. Following our description several other groups demonstrated the role of PS exposure and recognition in different infection models. Blood and cell-derived trypomastigotes of *Trypanosoma cruzi* are able to expose PS, in contrast with epimastigotes, which are not. In addition, infection with PS-exposing trypomastigote forms induces Smad nuclear translocation and inducible nitric oxide synthase inhibition (iNOS), suggesting an autocrine modulation of the host cell dependent on TGFβ [55]. It is interesting to note that, among all *T. cruzi* parasite stages, only the ones that are infective for mammalian cells evolve the ability to expose PS, suggesting the presence of an evolutionary link between PS exposure and the ability to infect host cells. Similarly, *Toxoplasma gondii* peritoneal tachyzoites expose PS at their surface, and the recognition of this molecule seems to be necessary to downmodulate

iNOS expression and activity upon macrophage infection [56]. More recently, several papers have demonstrated the role of exposed PS molecules for the infection by enveloped viral particles. For human immunodeficiency Virus-1 (HIV-1), PS at the viral envelope is a cofactor for monocyte infection [57]; in vaccinia virus infection, PS recognition modulates the activity of proteins involved in cytoskeleton reorganization such as p21-activated kinase (PAK) and the small Rho GTPase Rac, leading to increased macropinocytic activity and uptake of viral particles [58]. In addition, PS exposure by tumor cell, microvesicles shed by transformed cells, or endothelial cells in the intratumor environment seems to be involved in different events in tumor development, maintenance, and metastasis [59, 60]. This knowledge stimulated some researchers to evaluate the efficacy of anti-PS antibodies to treat viral and tumoral diseases. Actually the results so far are promising. In murine models of Lassa fever (Pichinde virus) or murine cytomegaloviruses the treatment efficacy was very high, reaching complete cure (total absence of detectable viral loads) in combination with available antiviral drugs [61]. For experimental tumoral disease, lung cancer, pancreatic tumors, and glioblastomas were efficiently treated, decreasing tumor growth and metastasis in some cases or potentiating the effect of chemo- and radio-therapies [62–64].

3.3. Leishmania amazonensis Infection: New Insights. Our group has been committed to study the role of PS exposure on the surface of different isolates of *L. amazonensis*. We worked with the hypothesis that *L. amazonensis* isolates from DCL patients would have higher PS exposure compared with localized cutaneous leishmaniasis (LCL), and this would contribute to macrophage deactivation, favoring parasite replication. For this, we compared PS exposure in *L. amazonensis* isolates from DCL clinical cases in the active phase of the disease, reported in Maranhão state in Brazil, to those isolated from LCL patients of clinical cases from Bahia. The results indicate that the isolates obtained from DCL patients indeed displayed more PS than isolates from LCL patients at early times postinfection. In addition, isolates from DCL patients were more infective than the ones obtained from LCL patients (França-Costa et al., unpublished results). On the other hand, independent of parasite strain analyzed, the parameters of infectivity correlated positively with the exposure of PS in the parasites. These data suggest that in human infections the pattern observed in mice when comparing BALB/c versus C57BL/6 mice is maintained. However, it is necessary to investigate the mechanisms by which the recognition of PS on the surface of the isolates of *L. amazonensis* deactivate the macrophage response. Particularly, it would be necessary to evaluate whether freshly isolated parasites display this phenotype to validate our analysis made on amastigotes derived from macrophages infected *in vitro* with cultured promastigote parasites isolated from human lesions. We believe that understanding the dynamics of PS expression, along with identification of the mechanisms involved in the immunosuppression of DCL patients, can result in therapeutic targets for intervention in the immunopathogenesis of this chronic and severe form of leishmaniasis.

In a similar way we are interested in the immunomodulatory mechanisms underlying PS exposure in different inbred mice strains. For that we are currently evaluating these mechanisms during BALB/c infection, which induces high levels of PS exposure on intracellular amastigotes. We observed that PS exposure is intrinsic to the intracellular parasite and cannot be observed in axenically cultured amastigotes but upregulates very fast after internalization. However, these levels are dramatically increased when infected macrophages are in the presence of previously primed T cells or their soluble products. We confirmed these results by infecting BALB/c nude mice where we observed that the amastigotes obtained from these mice display minimal levels of PS, which are completely restored if we adoptively transfer primed $CD4^+$ T cells to nude mice (Wanderley et al. unpublished results). Interestingly, these data indicate that one possible role for the previously reported pathogenic T cells [31] would be to induce PS exposure on intracellular amastigotes and, therefore, contributing to the generation of highly infective parasites. The T-cell-dependent PS exposure on amastigotes seems to be dependent on the induction of iNOS expression on host macrophages, and parasite survival is dependent on the concomitant induction of arginase 1 expression (Wanderley et al. unpublished results). We propose that high levels of PS exposure are induced by parasite stress delivered by iNOS activity. In this case, it is still unknown whether PS exposure on amastigotes is indeed a phenotype triggered by PCD or a specific process involving modulation of PS translocation. Under PS-inducting conditions, macrophages express high levels of arginase 1 (Wanderley et al. unpublished results), that is the enzyme necessary to produce ornithine, the precursor of polyamines. In this situation, polyamines could protect the parasite from the iNOS-dependent stress, stimulating parasite growth [66, 67] and increasing DNA stabilization [68, 69]. We understand that the unique characteristics of the T-cell response to *L. amazonensis* infection contribute to the generation of a perfect environment to stimulate and maintain increased levels of PS on the surface of intracellular parasites. Probably the balance observed in infected BALB/c mice, when disrupted, leads to the differences observed among different mouse strains. In Figure 1 we summarized our hypothesis regarding the T-cell-dependent modulation of PS exposure on intracellular amastigotes of *L. amazonensis*.

4. Final Remarks

The observation of PS exposure as a strategy to evade the immune system and persist in the mammalian host, made initially in the experimental model of *L. amazonensis* infection, was a breakthrough since it stimulated different groups around the world to look for the possibilities for basic and applied research on the field. Our group is still studying the immunological, cellular, and molecular mechanisms underlying control of PS exposure in parasites and the effects of its recognition by parasitized cells and organisms. We believe that this could be a major strategy in different systems

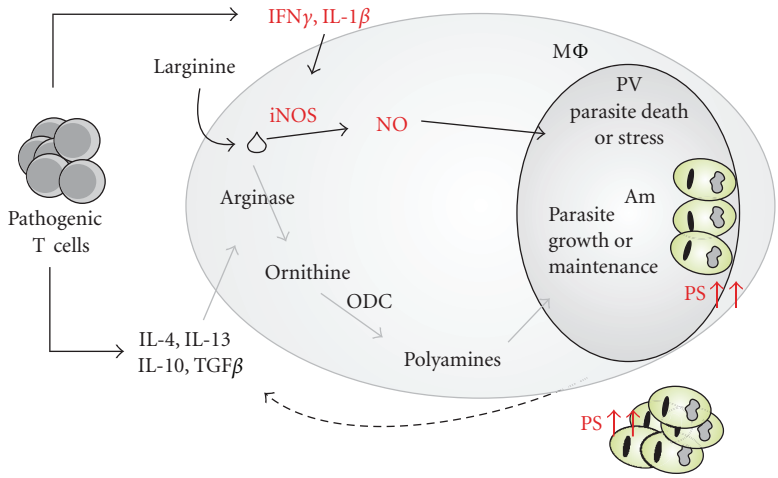

Figure 1: PS exposure on intracellular amastigotes of *L. amazonensis:* hypothesis for T-cell-dependent modulation. T cells primed by leishmanial antigens display a pathogenic phenotype, characterized by the production of unpolarized cytokines [18, 31]. These cytokines are able to activate both iNOS- and arginase 1-dependent intracellular macrophage pathways (Wanderley, JL et al. unpublished). In this environment, amastigotes receive stress from iNOS-derived nitric oxide (NO) which triggers high levels of surface PS on the parasite (Wanderley, JL et al. unpublished). Simultaneously, arginase 1 is also induced, and the outcome of this activation is an increase in polyamine intracellular levels [65]. Polyamines are indispensable for parasite survival and proliferation, maintaining them even in the presence of NO (Wanderley, JL et al. unpublished, [63]). Upon macrophage disruption, highly infective PSHIGH amastigotes are released, being capable of infecting new host cells and of spreading the anti-inflammatory signals derived from PS recognition (dashed arrow). PV: parasitophorous vacuole, ODC: ornithine decarboxylase, iNOS: inducible nitric oxide synthase, MΦ: macrophage, Am: amastigote.

where avoidance from immune surveillance is necessary to establish a disease.

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Review Article

Thymus Atrophy and Double-Positive Escape Are Common Features in Infectious Diseases

Juliana de Meis,[1] Désio Aurélio Farias-de-Oliveira,[1] Pedro H. Nunes Panzenhagen,[1] Naiara Maran,[2] Déa Maria Serra Villa-Verde,[1] Alexandre Morrot,[1,2] and Wilson Savino[1]

[1] *Laboratory on Thymus Research, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Avenue Brasil 4365, Manguinhos, 21045-900 Rio de Janeiro, RJ, Brazil*
[2] *Department of Immunology, Microbiology Institute, Federal University of Rio de Janeiro, 21941-590 Rio de Janeiro, RJ, Brazil*

Correspondence should be addressed to Wilson Savino, w_savino@hotmail.com

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The thymus is a primary lymphoid organ in which bone marrow-derived T-cell precursors undergo differentiation, leading to migration of positively selected thymocytes to the T-cell-dependent areas of secondary lymphoid organs. This organ can undergo atrophy, caused by several endogenous and exogenous factors such as ageing, hormone fluctuations, and infectious agents. This paper will focus on emerging data on the thymic atrophy caused by infectious agents. We present data on the dynamics of thymus lymphocytes during acute *Trypanosoma cruzi* infection, showing that the resulting thymus atrophy comprises the abnormal release of thymic-derived T cells and may have an impact on host immune response.

1. Introduction

The thymus is a primary lymphoid organ in which bone marrow-derived T-cell precursors undergo differentiation, leading to migration of positively selected thymocytes to the T-cell-dependent areas of secondary lymphoid organs [2]. Interactions between thymocytes and specialized thymic microenvironmental cells (thymic epithelial cells, macrophages, dendritic cells, and fibroblasts) support and drive T-cell differentiation from bone marrow-derived precursors, by means of a series of interactions including receptor/coreceptor interactions, cytokines, chemokines, and hormones [3–7], as illustrated in Figure 1.

Thymopoiesis starts at the time that a T-cell precursor enters the thymus and interacts with local microenvironmental cells, which ultimately lead to their proliferation and further differentiation to the T-cell lineage. Various types of interactions take place, including those mediated by the class I and class II major histocompatibility complexes (MHC) expressed by microenvironmental cells, extracellular matrix proteins (ECM) such as laminin, fibronectin, and collagen, chemokines (as CCL25, CXCL12, CCL21), lectins such as galectin-3, various typical cytokines (IL-1, IL-2, IL-3, IL-6, IL-7, IL-8, IFN-gamma, and others), sphingosin-1-phosphate (S1P1), and hormones (thymulin, thymopoietin, thymosin-a1) [2, 5, 8–13]. T-cell differentiation depends on T-cell receptor (TCR) gene rearrangement and membrane interaction with MHC molecules.

The mechanisms by which progenitors home to the thymus have been suggested to be similar to those used by leukocytes to enter lymph nodes (selectins, chemokines receptors, and integrins) [1, 14, 15]. As soon as these thymic settling progenitors (TSP) enter the thymus close to the corticomedullary junction, they generate early T-cell progenitors (ETP) or double-negative DN1 thymocytes, known to be CD117/c-KIT$^+$, CD44$^+$ CD25$^-$ [16]. ETP or DN1 thymocytes evolve to DN2 and DN3 thymocytes that migrate to the subcapsular zone of the thymic lobules, where they rearrange the genes encoding the TCR beta chain, express pre-TCR receptor, and proliferate.

At the DN3 stage, the CXCL12/CXCR4 interaction contributes thymocyte proliferation and differentiation towards the DN4 and subsequently CD4$^+$CD8$^+$ (DP) stage [1, 17]. Double-negative thymocytes, TCR$^-$CD4$^-$CD8$^-$, represent 5% of total thymocytes. Maturation progresses with the definite acquisition of TCR, CD4, and CD8 expression

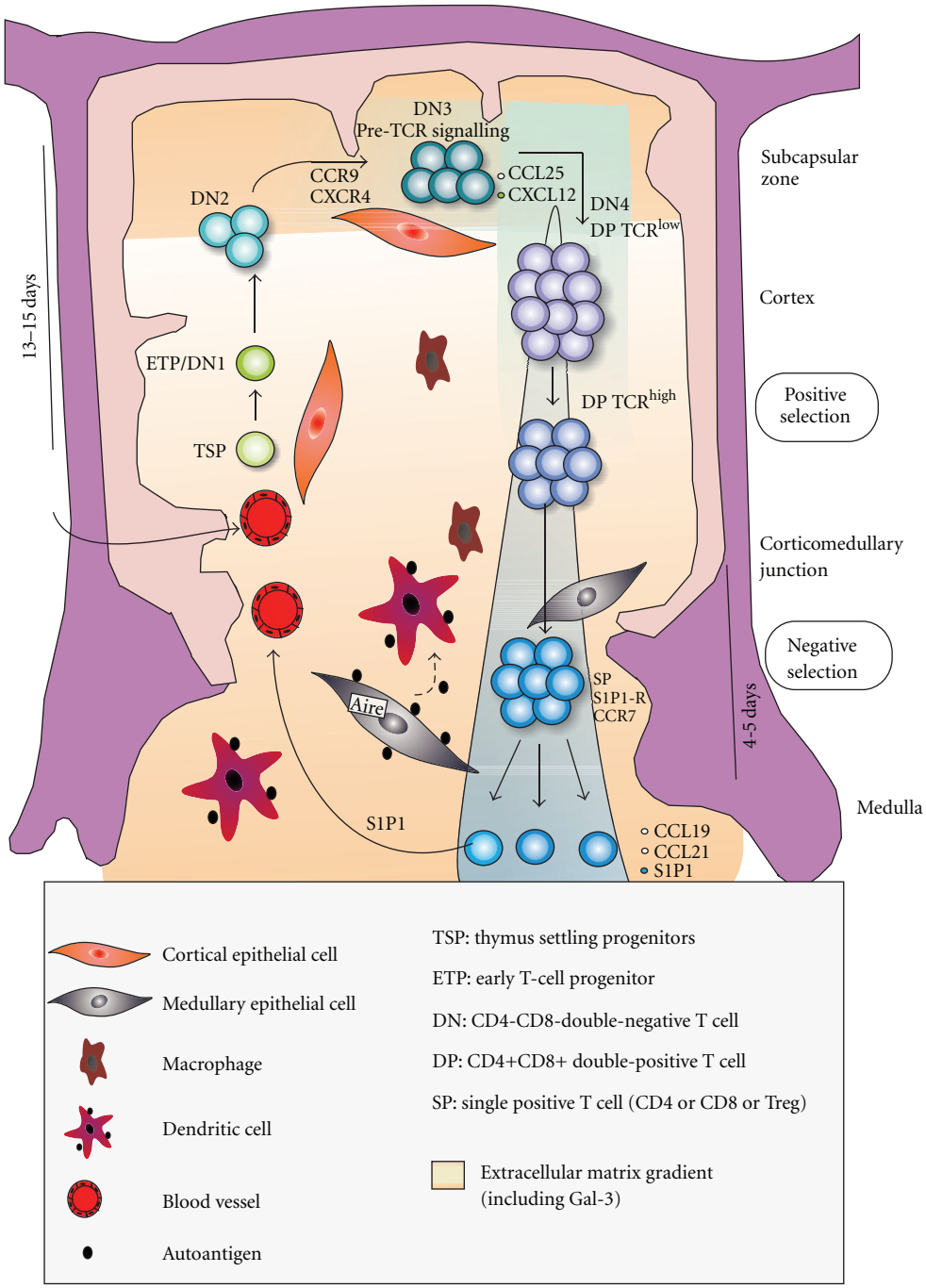

Figure 1: *Intrathymic differentiation of T cells.* Lymphocyte differentiation initiates when T-cell precursors enter the thymus through postcapillary venules located at corticomedullary junction. After entering the organ, cells interact with the thymic microenvironment (thymic epithelial cells, macrophages, dendritic cells, and fibroblasts), which ultimately lead to their proliferation and TCR rearrangement. Interactions between thymocytes and specialized thymic microenvironmental cells support and direct T cell differentiation by means of a series of interactions including receptor/coreceptor interactions (MHC-TCR, Integrin/ECM Proteins), cytokines (IL-1, IL-2, IL-3, IL-6, IL-7, IL-8, IFN-gamma), chemokines (as CCL25, CXCL12, CCL21), and hormones, with corresponding receptors. At the subcapsular zone, these thymocytes undergo TCR beta chain rearrangement and selection. Double-positive thymocytes migrate through the cortex and initiate TCR testing (positive selection). Positively selected thymocytes, located at the medulla, are screened for self-reactivity through negative selection. Residence in the medulla is followed by emigration, which is regulated by sphingosine-1-phosphate and its receptor (S1P1). Adapted from [1].

generating DP; double cells, which constitute 75–80% of the whole thymocyte population. Thymocytes that do not undergo a productive TCR gene rearrangement die by apoptosis, whereas those expressing productive TCRs interact with peptides presented by molecules of the major histocompatibility complex (MHC), expressed on microenvironmental cells. The result of this interaction determines the fate of thymocytes [2, 9, 18]. The positively selected thymocytes will escape from apoptosis and become mature $CD4^+$ or $CD8^+$ single-positive (SP) T cells (Figure 1). This is a highly rigorous process, and only a small proportion of the double-positive population survives [19]. Positive selection also results in lineage commitment so that the lymphocytes can be committed to either the CD4 or CD8 single-positive phenotype, depending on the class of MHC molecule with which the TCR interacts.

Intrathymic negative selection is essential to establish self-tolerance in the T-cell repertoire, deleting high-avidity TCR signaling thymocytes reacting to self-peptides presented by microenvironmental cells [2, 11, 18, 20].

Interestingly, along with $CD4^+$ T-cell differentiation, two distinct groups of cells, with opposite roles, have been reported: the classical $CD4^+$ T helper cells (cells that are able to trigger and/or enhance an immune response in the periphery) and regulatory $CD4^+CD25^+FOXP3^+$ T cells, which are able to impair a given immune response [9, 21].

The data summarized above clearly demonstrate that the thymus is vital for the homeostatic maintenance of peripheral immune system, maturing both effector and regulatory T cells (Figure 1).

It has been well documented that the thymus undergoes an age-related atrophy [22]. Under normal circumstances, the decline in thymic cellularity in healthy subjects promotes minimal consequence. Nevertheless, over time, reduced efficacy of the immune system with age increases the rise of opportunistic infections, autoimmunity, and cancer [22–24].

In this paper, we present emerging data regarding accelerated thymus atrophy caused by infected agents and possible impact of this thymic atrophy to the host immune response. Moreover, we show that thymic-derived T cells are involved in the dynamics of lymphocyte populations in secondary lymphoid organs during acute *Trypanosoma cruzi* infection.

2. Parasite Infection Promotes Thymic Atrophy with $CD4^+CD8^+$ Thymocyte Depletion

As mentioned above, the thymus senses several exogenous agents, responding with atrophy, promoted by viruses (HIV, rabies virus), parasites (*Trypanosoma cruzi*, *Plasmodium berghei*, *Schistosoma mansoni,* and *Trichinella spiralis*), and fungi (*Paracoccidioides brasiliensis* and *Histoplasma capsulatum*) [9, 22, 25–40]. The mechanisms involved in the thymic atrophy in infectious disease are not completely elucidated and may vary. Nevertheless, common histological features occur, including decrease of cortical thymocytes and loss of clear-cut distinction in the corticomedullary region [9, 38, 41–47]. At least in some cases, such atrophy may be transient: biphasic reactions of the thymic cortex, characterized by initial atrophy and further restoration, were reported in experimental infections by *Histoplasma capsulatum* and *Toxoplasma gondii* [48, 49].

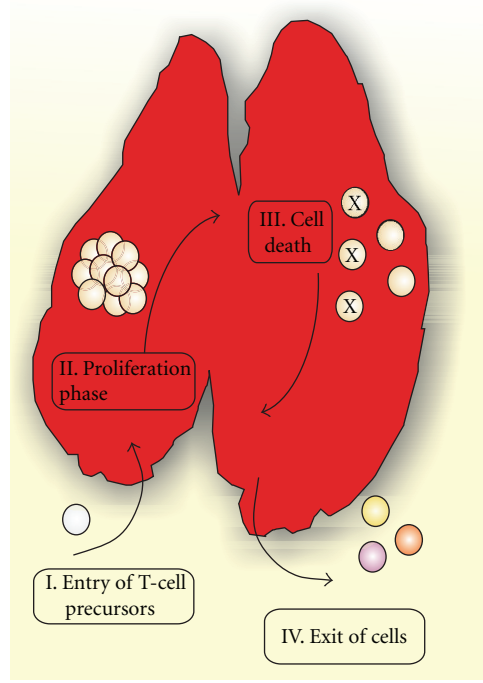

Figure 2: Possible mechanisms involved in thymic atrophy. I. Decreased number of precursor cells migrating into the thymus, II. Lower capacity in thymocyte proliferation during T-cell differentiation, III. Increased thymocyte death, and/or IV. Exit of immature T cells to peripheral tissues.

Thymic atrophy in infectious disease may reflect distinct nonmutually excluding events: decreased number of precursor cell entry into the thymus, lower capacity in thymocyte proliferation, increased thymocyte death, and/or increased exit of thymocytes to peripheral lymphoid tissues (Figure 2).

Although the migratory capacity of T-cell precursors to colonize the thymus in infectious disease remains unknown, data from the literature suggest that parasite-induced thymus atrophy comprises changes in involvement of proliferation, death, and exit of thymocytes.

3. Impaired Thymocyte Proliferation in *T. cruzi*-Infected Mice

It has been shown that mitogenic responses of thymocytes from *T. cruzi* acutely infected mice are reduced due to decrease in interleukin (IL)-2 production, which in turn is associated with high levels of IL-10 and interferon-γ [50]. It has also been suggested that changes in thymocyte subset proportions induced by *T. spiralis* infection are reflected in a reduced capacity of thymocytes to respond to the T-cell mitogen concanavalin A [45]. In contrast, thymocytes from *S. mansoni*-infected mice apparently exhibit similar concanavalin A-induced proliferative response, as compared to controls [38]. Conjointly, these data suggest that some (but not all) parasites induce decrease in the ability of thymocytes

Box 1. *Immunoneuroendocrine interactions involving cytokines and Hypothalamus-Pituitary-Axis in infectious diseases.* Infectious agents lead to activation of innate and adaptive immune response. Proinflammatory cytokines (IL-1, IL-6, and TNF-α) are key mediators of immune response and stimulate the Hypothalamus-Pituitary-Adrenal (HPA) axis. This HPA activation leads to increasing corticotrophin-releasing hormone (CRH) by the hypothalamus and further production of Adrenocorticotropic hormone (ACTH) by the pituitary gland. ACTH stimulation promotes adrenal production of steroids as glucocorticoids (GCs), dehydroepiandrosterone (DHEA), and its sulphate ester (DHE). GCs trigger apoptotic signals to T- and B-cell precursors as well as immature T cells [51–55]. In murine Chagas disease, there is an imbalance of the HPA axis, with increase in GCs levels, in the absence of rise in CRH and ACTH [56, 57].

Box 1

to proliferate, which in turn account for the resulting thymic atrophy.

4. Thymocyte Apoptosis Is a Common Feature in Acute Parasite Infections

In the vast majority of infectious diseases coursing with thymic atrophy, the major biological event associated with thymocyte loss is cell death by apoptosis, as seem, for example, in experimental models of *Trypanosoma cruzi* and *Plasmodium berghei* infection [9]. Although $CD4^+CD8^+$ thymocytes are the main target population in infection, other subsets as DN and SP cells also depleted in infected thymus [30, 32, 42, 63, 64].

Glucocorticoid hormones are strong candidates to promote thymic atrophy and thymocyte death in parasitic infections. Serum glucocorticoid levels are upregulated in acute infections and promote DP thymocyte apoptosis through caspase-8 and caspase-9 activation [9, 56, 57, 65, 66] (Box 1). Such rise in serum glucocorticoids has been reported in experimental parasitic diseases such as malaria, American tripanosomiases or Chagas disease, African trypanosomiases or sleeping sickness, toxoplasmosis, leishmaniasis, and schistosomiasis [51, 56, 67–72]. In experimental acute *T. cruzi* infection, thymic atrophy and thymocyte depletion have been associated with both TNF and glucocorticoid serum levels [44, 65, 73].

Nevertheless, at least in *T. cruzi* infection, various and different biological mechanisms seem to be involved. *T. cruzi*-derived transsialidase, as well as host-derived galectin-3, extracellular ATP, and androgens have been pointed out as candidate molecules to enhance thymocyte death [44, 64, 69, 74–77]. Conversely, typical cytotoxic molecules such as Fas and perforin are not involved in thymus atrophy in *T. cruzi* infection [78].

5. Acute Infection Can Promote Abnormal Escape of Immature Thymocytes to the Periphery

T-lymphocyte migration is controlled by several molecular ligand/receptor interactions, including those involving ECM proteins, chemokines, and lectins [12, 13, 79–82].

In the thymus of mice acutely infected by *T. cruzi* or *P. berghei* alterations in expression of ECM proteins, chemokines, and/or galectin-3 have been described [5, 63, 64, 79, 83], which is in keeping with the abnormal appearance of thymus-derived immature DP lymphocytes in peripheral lymphoid organs and blood from infected hosts. These findings suggest that the premature scape of immature cells from the organ also contributes to the establishment of the thymic atrophy [38, 42, 84, 85]. Accordingly, it has been shown that thymocytes from *T. cruzi* acutely infected mice exhibited increased migratory responses to fibronectin and that abnormally high numbers of DP T cells migrate from the thymus to peripheral lymphoid organs. [42, 64, 83–86] (Box 2). Studies performed in experimental *P. berghei* infection have also demonstrated increased expression of ECM proteins, CXCL12 chemokine production, and enhanced migratory response of thymocytes from infected mice, when compared to controls [87].

6. Thymic Changes May Impact on the Immune Response of Infected Animals

Acute *T. cruzi* infection in mice leads to strong activation of innate and adaptive immune responses. Splenomegaly and expansion in subcutaneous lymph nodes (SCLN) were reported, mediated by persistent T- and B-cell polyclonal activation [63, 88–91]. Conversely, atrophy in thymus and mesenteric lymph nodes (MLN) has been observed along with infection [9, 43, 92]. We have previously demonstrated that MLN atrophy in *T. cruzi* infection mice was associated with massive lymphocyte apoptosis, mediated by TNF, Fas, and caspase-9 [63, 88, 92]. The role of thymus-derived T cells in secondary lymphoid organ dynamics remains unclear. In order to analyze the role of the thymus upon regional immune response in secondary lymphoid organs from acute *T. cruzi* infected mice, thymectomized male BALB/c mice or sham-operated counterparts were infected with 100 blood-derived trypomastigotes from Tulahuén strain of *T. cruzi*. In the peak of parasitemia (18–21 d.p.i), mice were killed, and subcutaneous, mesenteric lymph nodes as well as spleen were analyzed. As demonstrated in Figure 3, thymectomy in noninfected mice does not alter lymphocyte counts in the spleen, SCLN, and MLN. However, absence

Box 2. Thymic atrophy and negative selection in experimental acute Chagas disease.
It is largely established that interactions between TEC and thymocytes control the development of the thymic microenvironment and T-cell development. Furthermore, many tissue-specific self-proteins are known to be synthesized by medullary thymic epithelial cells (mTEC) that express Aire. For this reason, Aire-expressing mTEC have a central role in the deletion of self-reactive thymocytes during the process of negative selection [58–61]. In T. cruzi infection we showed that the expression of Aire and highly selective tissue restricted antigens was readily detectable in whole thymus by real-time PCR analysis from infected mice, suggesting an expression of peripheral antigens which would be sufficient to modulate the tolerance induction by the negative selection process [62].
During the acute phase of infection, as the thymic atrophy becomes evident, there is an increase in numbers of apoptotic intrathymic DP cells, compared to their respective normal counterparts. Although this phenomenon may be a consequence of the changes observed in the organ, our data show that along the DP depletion there is sustained expression of Bim, a proapoptotic factor essential for thymocyte negative selection. Further analysis, by using an OTII TCR transgenic system, revealed that the administration of the cognate OVA peptide in the acutely infected mice undergoing thymic atrophy can induce TCR-stimulation-induced apoptosis of semimature thymocytes. These data point out that negative selection operates normally during infection-promoted thymic atrophy, since the DP cells can be negatively selected in the infected thymus by antigen-induced depletion [62].

Box 2

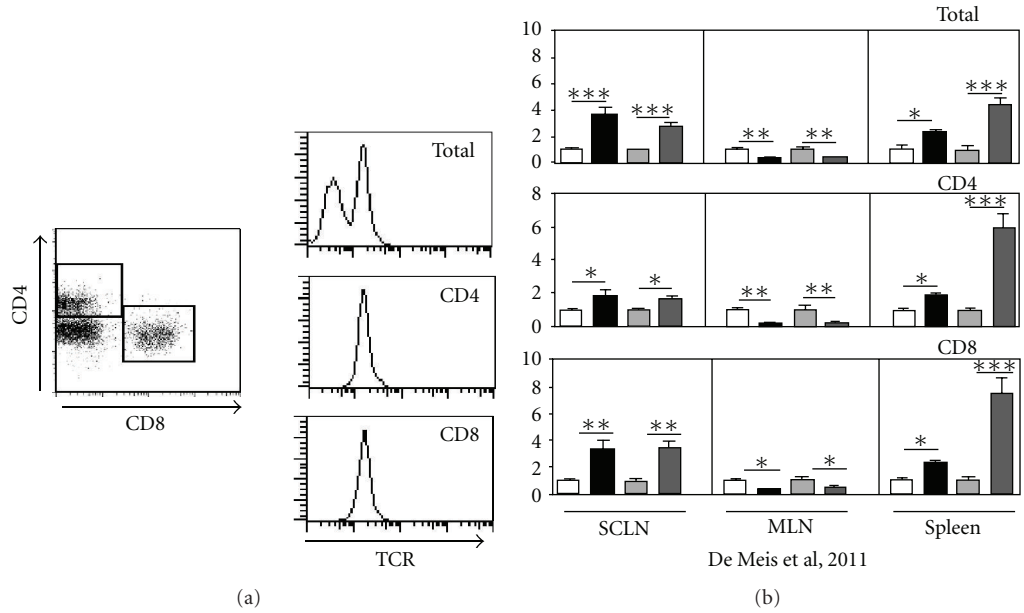

Figure 3: Thymectomy modulates splenic cell numbers during acute *Trypanosoma cruzi* infection. Mice were thymectomized and, six days later, were infected intraperitoneally by the Tulahuén strain of *T. cruzi*. Animals were killed at 19 days postinfection, and subcutaneous (SCLN), mesenteric (MLN), lymph nodes and spleen cell numbers were evaluated. (a) Representative data demonstrating TCR expression in CD4 and CD8 T cells in SCLN, MLN, and spleen, analyzed by flow cytometry. (b) Data show fold change of 6–8 animals/group where (white rectangle) represents sham-operated control, (black rectangle) sham-operated infected, (light grey rectangle) thymectomized control, and (dark grey rectangle) thymectomized infected mice. Results were representative of three different experiments and were expressed as mean ± standard deviation, ns: not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, after comparison by One Way ANOVA.

of thymic-derived T cells during acute infection increased the number of splenocytes (Figure 3). In this respect, it has been demonstrated that thymus-derived $\gamma\delta$TCR^{+} T cells removed from the spleen exhibit suppressor activity for T lymphocytes [93]. Moreover, as showed in thymectomized *T. cruzi* chronically infected animals, thymic removal may act by downregulating immunoregulatory mechanisms, leading to an exacerbation of autoimmune reactions believed to be involved in the generation of myocardial damage [94].

Interestingly, no changes were observed in SCLN cell expansion and MLN atrophy between infected sham and thymectomized mice, suggesting that suppressor T cells migrate

preferentially to the spleen (Figure 3). All together, these data indicates that thymic-derived T cells can exert immunoregulatory in the spleen during acute *T. cruzi* infection.

7. Conclusion

Several pathogens, including *T. cruzi*, cause thymic atrophy. Although the precise mechanisms underlying this phenomenon are not completely elucidated, most likely it is linked to a particular pathogen-host relationship. Recently, we addressed whether the changes of the thymic microenvironment promoted by an infectious pathogen would also lead to an altered intrathymic negative selection of the T-cell repertoire. By using a *T. cruzi* acute infection model, we have seen that, despite the alterations observed in the cortex and medullary compartments undergoing a severe atrophy during the acute phase, the changes promoted by the infection in the thymic architecture do not affect the negative selection.

Although the intrathymic checkpoints necessary to avoid the maturation of T cells expressing potentially autoreactive "*forbidden*" T-cell receptors are present in the acute phase of murine Chagas disease, circulating $CD4^+CD8^+$ T cells have been reported in humans as well as in animals such as mice, chicken, swine, and monkeys [9, 62, 85]. The existence of this unconventional and rare lymphocyte population in the periphery was explained as a premature release of DP cells from the thymus into the periphery, where their maturation into functionally competent single-positive cells continues.

Most importantly, there is considerable evidence of an increased frequency of peripheral $CD4^+CD8^+$ T cells not only during acute *T. cruzi* infection but also in viral infections. For example, in human immunodeficiency virus or Epstein-Barr virus infections, the percentage of DP cells can increase to 20% of all circulating lymphocytes [95–97]. This fluctuation is also present in the secondary lymph nodes as we demonstrated in the experimental model of Chagas disease, in which DP-cell subset increases up to 16 times in subcutaneous lymph nodes [83, 85]. During the course of infection, these peripheral DP cells acquire an activated phenotype similar to what is described for activated and memory single-positive T cells with high IFN-γ production, $CD44^+CD69^+$ expression, and cytotoxic activity [62].

Furthermore, similar to previous studies showing high cytotoxic activity and effector memory phenotype of extrathymic DP cells in *cynomolgus* monkeys and in a chimpanzee experimental infection with hepatitis C virus [95], our results indicate that the DP cells purified from peripheral lymphoid tissues of chagasic animals show cytotoxic activity as compared to naïve single-positive $CD4^+$ or $CD8^+$ T cells.

Most likely, the presence of peripheral, mature, and activated DP lymphocytes challenges the perception of the T-cell populations involved in adaptive immune responses during the infection. The presence of peripheral activated DP cells with potentially autoreactive TCR may contribute to the immunopathological events possible related to several pathogen infections. In the Chagas disease model, we have demonstrated that increased percentages of peripheral blood subset of DP cells exhibiting an activated HLA-DR^+ phenotype are associated with severe cardiac forms of human chronic Chagas disease [62]. The role of these HLA-DR^+ DP T cells in myocardial damage and host pathologies is unknown. However, correlations between the changes in the numbers of DP T-cell subsets and the extent of inflammatory lesions may represent a clinical marker of disease progression in parasitic infections and may help the design of novel therapeutic approaches for controlling infectious diseases.

Abbreviations

T. cruzi:	*Trypanosoma cruzi*
DP T cells:	$CD4^+CD8^+$ double-positive T cells
AIRE:	Autoimmune regulator gene
TRAs:	Tissue-restricted antigens
TCR:	T cell receptor
TEC:	Thymic epithelial cells.

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Review Article

B-Cell Response during Protozoan Parasite Infections

María C. Amezcua Vesely, Daniela A. Bermejo, Carolina L. Montes, Eva V. Acosta-Rodríguez, and Adriana Gruppi

Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI-CONICET), Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, Ciudad Universitaria, 5000 Córdoba, Argentina

Correspondence should be addressed to Adriana Gruppi, agruppi@fcq.unc.edu.ar

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In this review, we discuss how protozoan parasites alter immature and mature B cell compartment. B1 and marginal zone (MZ) B cells, considered innate like B cells, are activated during protozoan parasite infections, and they generate short lived plasma cells providing a prompt antibody source. In addition, protozoan infections induce massive B cell response with polyclonal activation that leads to hypergammaglobulnemia with serum antibodies specific for the parasites and self and/or non related antigens. To protect themselves, the parasites have evolved unique ways to evade B cell immune responses inducing apoptosis of MZ and conventional mature B cells. As a consequence of the parasite induced-apoptosis, the early IgM response and an already establish humoral immunity are affected during the protozoan parasite infection. Moreover, some trypanosomatides trigger bone marrow immature B cell apoptosis, influencing the generation of new mature B cells. Simultaneously with their ability to release antibodies, B cells produce cytokines/quemokines that influence the characteristic of cellular immune response and consequently the progression of parasite infections.

1. B Cells Can Play Protective and Pathogenic Roles in Protozoan Infections

Host resistance in protozoan infections is dependent on both innate and acquired cell-mediated immune responses. In addition, several studies have implicated B cells and antibodies (Abs) in host survival and protozoan parasite clearance [1–3]. B cells can function as Ab-producing cells but they can also modulate immune responses through critical Ab-independent mechanisms that include secretion of cytokines and chemokines as well as antigen presentation [4–6]. Furthermore, B cells can directly modulate dendritic cells and T-cell subsets, and, consequently, they can influence adaptive immunity and the progression of the infection [7]. Accordingly, in protozoan infections B cells may play a protective and a pathological role. In malaria and trypanosome infections, Abs appear to play a famajor role in immunity. In *Trypanosoma cruzi* and *T. brucei gambiense* infections, Ab-dependent cytotoxic reactions against the parasite have been reported [8]. Several studies demonstrated that Abs are responsible for the survival of susceptible animals in the initial phase of *T. cruzi* infection and for the maintenance of low levels of parasitemia in the chronic phase [9, 10]. Although Abs were shown to be responsible for clearing the African trypanosomes from the blood of infected animals, recent evidence suggests that the survival time of infected mice does not necessarily correlate with the ability of the animal to produce trypanosome-specific antibody. In general, the parasite-specific immune response mounted during protozoan infections is insufficient to completely eradicate the pathogen, allowing chronic infection.

B cells do not only play protective roles in protozoan infections. In fact, they are required for the development of Th2 cell response and, consequently, for the susceptibility to infection with *Leishmania major* [11]. BALB/c uMT mice infected with *L. major* LV39 mount a Th1 response and present restricted lesion development and contained parasite replication. Adoptive transfer of B cells from BALB/c mice in

B-cell-deficient BALB/c uMT mice before infection restores susceptibility to *L. major* LV39 and Th2 cell development in resistant mice.

2. B-Cell Development

Given the role for B cells in conditioning the progression of protozoan infections, it is important to understand the kinetics and regulation of the whole B-cell cycle from the development to the differentiation into mature and memory B cells and plasma cells. The humoral immune response has been shown to be two branched providing an innate-like response (involving B1 and marginal zone (MZ) B cells) and an adaptive immune response (involving conventional B2 cells). In the adult, B cells are generated in the bone marrow (BM) and migrate to the periphery at the transitional B-cell stage, when they are still short lived and functionally immature [12, 13]. Conventional B2-cell development occurs via a series of BM stromal cell-facilitated processes that begin within the hematopoietic stem cell pool and proceed in hierarchical steps of lineage commitment [14]. B lymphopoiesis yields several developmental stages of pre-pro-B, pro-B, pre-B, and, eventually, immature B cells, which show a high expression of the IgM form of the antigen receptor and low or no expression of the IgD maturation marker. To complete their development, immature B cells migrate through the periphery; however, only 10% reach the spleen as transitional B cells of the T1 type [15]. In the spleen, transitional B cells develop into conventional and MZ B cells [16]. B1 cells are efficiently generated in fetal life and during the first few weeks after birth. The fetal liver is an efficient source of B1 cells [17]; however, it is not the only one as a recent study identified a B1-cell precursor in adult BM [18]. Interestingly, protozoan parasite infections can affect the different compartments of B-cell development (summarized in Figure 1), influencing the generation of new mature B cells or their survival and, consequently, the cellular immune response.

3. Protozoan Parasite Infections Affect BM B-Cell Development

BM is the main hematopoietic organ of an adult organism and is able to provide cells of immune systems rapidly in cases of infection. Immature B-cell reduction in BM during an infection would limit the Ab source cells and favour parasite replication and chronicity, so the identification of the mechanisms ruling B-cell depletion represents an important challenge in biomedical research.

We have reported that *T. cruzi* infection induces a marked loss of immature B cells in the BM and also compromises recently emigrated B cells in the periphery [19]. The depletion of immature BM B cells was associated with an increased rate of apoptosis, and we established that *T. cruzi* trypomastigotes failed to directly induce immature B-cell apoptosis. We proved that this cell death process occurs in a Fas/FasL-independent fashion but depends on the presence of $CD11b^+$ myeloid cells that secrete a product of the cyclooxygenase pathway that depletes immature B cells [19].

In addition, BM is compromised in other protozoan parasite infections. In fact, infections with *Neospora caninum* [20] and *T. brucei* [21] also cause a general decrease in BM cells. Recently, the *T. brucei* infection upshot on B lymphopoiesis has been examined using a C57BL/6 mouse *T. brucei* AnTat 1.1E infection model [22]. Using this model, Bockstal et al. [23] observed that the number of hematopoietic stem cells was minimally affected, but BM B lymphopoiesis was severely affected in *T. brucei*-infected mice, starting with the common lymphoid progenitor fraction. The pre-pro-B-cell population showed a 50% reduction by day 20 after infection, while the subsequent B-cell maturation stage, that is, the pro-B, pre-B, and immature B-cell populations reached more than 95% depletion by day 10 after infection and failed to recover throughout the further course of infection. In *T. brucei* infection, mice do not present increased apoptosis of BM B-cell precursors nor alteration in the expression of B-cell-development-specific transcription factors like Icaros, PU.1, EBF and E2A and the IL-7. However, *T. brucei*-infected mice show a reduction in BM CXCL12 levels [23], indicating that during early *T. brucei* infections, B-cell precursors prematurely migrate out of the BM as a result of the initiation of inflammation. Similarly, CXCL12 decreased production by BM cells was determined in *Plasmodium chabaudi* infection [24]. Furthermore, the significant reduction in CXCL12 expression in the BM of 10 days *P. chaubaudi*-infected mice correlates with a reduction in B-cell precursor cells. At days 20 and 30 of infection, a significant recovery in CXCL12 expression in BM is detected, coinciding with a slow recovery of B lymphopoiesis.

4. B1 and MZ B-Cell Response in Protozoan Parasite Infections

Among the mature B cells, MZ and B1 B cells appear to be evolutionarily selected and maintained to facilitate prompt Ab responses. Due to this, they provide a bridge between the innate and the adaptive arms of the antipathogen immune response. B1 cells, distinguished from B2 cells by their phenotype ($B220^{low}$ $CD5^{+/-}$ $CD11b^+$) and anatomic location and functional properties, are the dominant population of B cells in the pleural and peritoneal cavities, but represent only a small fraction of splenic B cells [25]. B1 cells produce most of the natural serum IgM and much of the gut IgA and express a BCR repertoire that is enriched for highly polyspecific receptors with low affinities to a broad range of antigens [26]. Despite the fact that B1 cells are very efficient in the control of bacterial and viral infections, they apparently do not play a role in the control of protozoan parasite replication. Indeed, BALB/c Xid mice carrying an X-linked mutation (that prevents B1 cell development) infected with *T. cruzi* display poor B-cell responses to the infection, accompanied by low levels of specific and nonspecific immunoglobulins in the serum [27]. Surprisingly, Xid mice infected with *T. cruzi* were able to control parasitemia and did not show the wasting syndrome observed in wild-type mice. In addition, they developed almost no pathology early in the chronic phase. The resistance of these mice to experimental Chagas disease was associated with the absence of

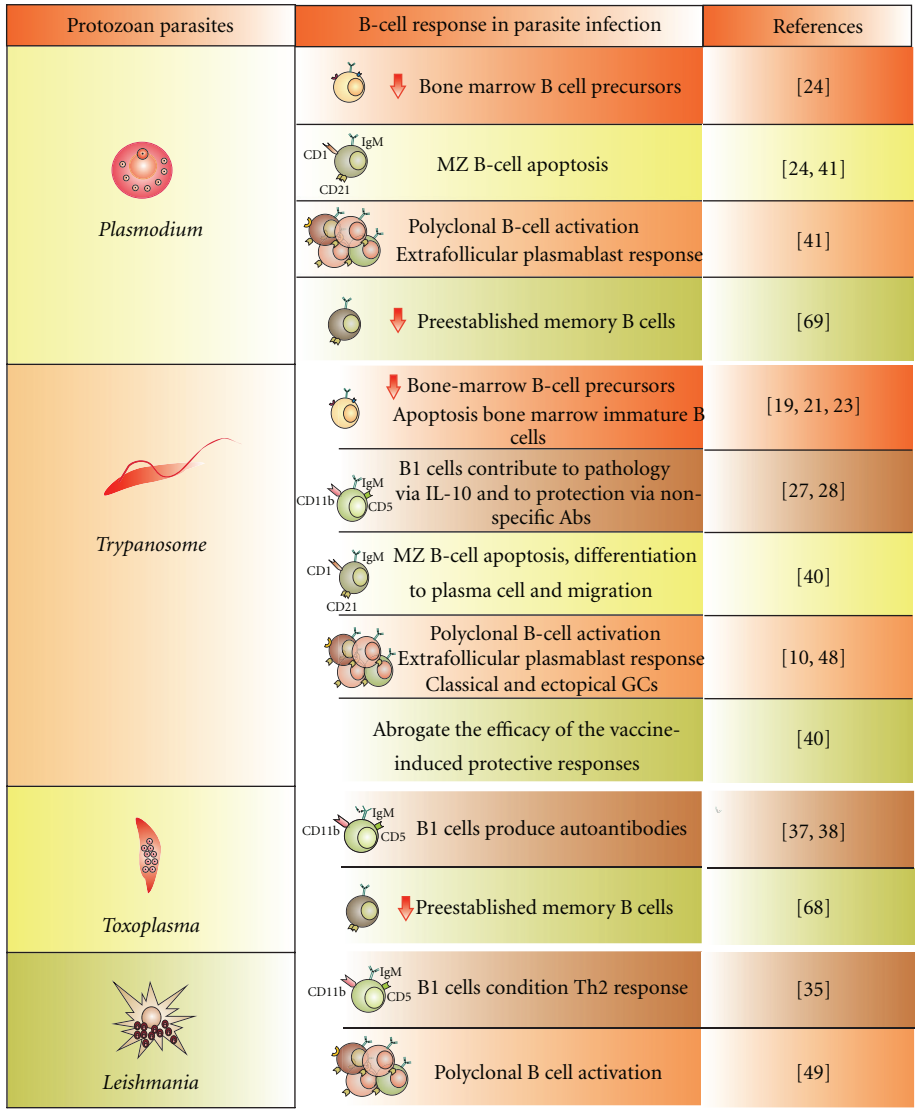

Figure 1: Protozoan parasites affect the different B-cell compartments. MZ: marginal zone B cells, GCs: germinal centers.

IL-10-secreting B1 cells and high levels of IFN-gamma [28]. These results suggested that B1 cells play a pathological rather than protective role in Chagas' disease. Additionally, in *T. cruzi* infection, we observed a disappearance of peritoneal B1 cells, due to an enhanced differentiation into a particular type of plasma cells, the "Mott-like cells" [29]. Nevertheless, the specific role of these cells in the experimental Chagas disease has not been elucidated yet; their association with autoimmune manifestations in CD22-deficient mice [30] and lupus [31, 32] suggests that these cells may be involved in the autoimmune responses observed in *T. cruzi* infection. We and others have reported that the peritoneal B-cell response observed in *T. cruzi* infection is almost not specific for the invading pathogen [29, 33]. However, these "sticky" antibodies could unspecifically bind to parasites providing protection.

B1 cells are not only implicated on Ab secretion; in fact, they may modulate T-cell response. In this sense, O'Garra et al. [34] have reported that B1 cells secrete large amounts of IL-10 and, consequently, can contribute to the susceptibility of BALB/c mice to *L. major* infection by skewing the T-helper cell network towards a Th2 phenotype. In this way, it has been observed that *L. major* infection of B-cell-defective BALB/c Xid mice induces a less severe disease compared to wild-type control mice [35]. Another report indicates that the behavior of *L. major*-infected Xid mice can be explained more in relation to the high endogenous IFN-gamma production than to the lack of B1 cells. Indeed, B1-cell-depleted irradiated mice showed similar or even worse disease progression compared to control BALB/c mice [36].

B1 cells would also be implicated in the pathogenesis of toxoplasmosis through the production of Abs against the heat shock protein 70 of *T. gondii* that also recognize mice HSP70 [37]. These Abs seem to have a pathogenic role in toxoplasmosis as their injection in *T. gondii*-infected mice clearly increases the number of parasites in mice brain [38]. Moreover, IL-10 produced by B1 cells could, in turn, favor *T. gondii* survival. Then, fine tune regulation of the exacerbated

Th1 response by IL-10 is important during *T. gondii* infection.

MZ B cells are also considered innate-like cells that can be induced to differentiate into short-lived plasma cells in the absence of BCR ligation. Splenic MZ B cells can be distinguished from the other splenic B cells by $CD24^{high}$, IgM^{high}, IgD^{high}, $CD23^{-}$ expression, as well as by their higher expression of CD21. It is known that these B cells mediate humoral immune responses against blood-borne type 2 T-independent antigens [39] but their role in parasite infection has been scarcely studied. Induction of a T-independent anti-trypanosome IgM response has been shown to be a crucial factor in *T. brucei* parasite elimination [1]. Even when increased splenic cellularity occurs after *T. brucei* infection, a significant reduction of splenic IgM+ MZ B-cell numbers takes place right after the first week of infection [40]. The infection-associated disappearance of the MZ B cells from the spleen could be explained by two independent mechanisms, namely, cell differentiation and/or cell death. Supporting the first possibility is the observation that the rapid disappearance of MZ B cells coincided with the temporary accumulation of IgM+ plasma cells. The analysis of MZ B cells that remain in the spleen in the days following the clearance of the first peak of parasitemia revealed that these cells upregulated Annexin V expression. In addition, these cells exhibit caspase 3 gene expression as well as the conversion of procaspase 3 into the cleaved 12 kD and 17 kD caspase 3 activation products suggesting the induction of trypanosomiasis-associated apoptosis in the splenic MZ B-cell population [40]. As in African trypanosomes, *P. chabaudi chabaudi* infection also caused a severe depletion of MZ B cells in the spleen [41] and this loss is mainly the result of the highly increased rate of apoptosis [24]. As MZ B cells serve as an important source for T-cell independently generated IgM+ plasma cells during early stages of infection, apoptosis induction of MZ B cells can be used by parasites as strategy to avoid early IgM protective response and, consequently, prolong their survival.

5. Protozoan Infections Induce Massive B-Cell Response with Polyclonal Activation of Splenic B Cells

Whereas the BM of some protozoan-infected mice suffers from a strong B-lineage-cell depletion, the spleens show a marked cellular hyperplasia as a consequence of an intense B-cell response. A detailed analysis of splenic B-cell response was performed in experimental Chagas disease and malaria [10, 41]. An extrafollicular Ab response, in mice infected with *T. cruzi,* is evident a few days after infection and reached a peak after 18 days of infection. This extended kinetics of the extrafollicular response could be characteristic of infections caused by blood circulating protozoan parasites since in *P. chabaudi chabaudi* infection extrafollicular plasmablasts are visible from day 4, and by day 10 they are unconventionally sited in the periarteriolar region of the white pulp. In this region, in both *T. cruzi* and *Plasmodium* infection, extrafollicular plasmablasts form clusters occupying part of the area normally filled by T cells. The kinetics of the appearance of GCs during *T. cruzi* and *Plasmodium* infection are similar to those observed after immunization with classical haptenated proteins, where GCs are visible within the 8 days of immunization [42]. In addition, we detected functional (Ab producing) GCs in atypical sites. The GCs in the spleens of *T. cruzi*-infected mice persisted for at least 32 days resembling the kinetics of the response seen in *P. chabaudi* [41] and *L. amazonensis* [43] infections. We observed that even though *T. cruzi* infection induces early, persistent, and massive extrafollicular and follicular plasmablast responses together with classical and ectopic GCs, infected mice have a delayed parasite-specific Ab response. A key finding of our study [10] is that, while an important amount of Abs is rapidly secreted during infection, antigen specific antibodies were not detected until the third week of infection.

The consequence of the massive extrafollicular and follicular B-cell response is the polyclonal B-cell activation that leads to hypergammaglobulinemia with serum Abs specific for the parasite and self- and/or nonrelated Ags [44–46]. In leishmaniasis, hypergammaglobulinemia was described in both *L. major*-susceptible and -resistant mouse strains. *T. congolense* infection also results in a strong production of non-parasite-specific Abs characterized by the predominance of IgG2a- and IgG2b isotypes [47]. All the mouse strains infected with *T. congolense* present a marked increase in splenic B cells resulting in a nonspecific polyclonal activation of lymphocytes that affects primarily B cells. In strains of *T. congolense* mice which survived longest, that is, C57B1/6J and AKR/A, the increase in splenic B cells is less marked.

Different roles are proposed for polyclonal B-cell activation, which can be crucial for early host defense by contributing with Abs specific for a spectrum of conserved structures present in the pathogens. Additionally, polyclonal activation can be a mechanism triggered by microorganisms to escape the host-specific immune response by diluting pathogen-specific Abs while increasing irrelevant antibodies. Accordingly, recently it has been reported that C57Bl/6 mice, resistant to *T. cruzi* infection, had improved parasite-specific humoral responses that were associated with decreased polyclonal B-cell activation. In the context of parasite infection, Bryan et al. [48] study shows that Th2 cytokine responses were associated with amplified polyclonal B-cell activation and diminished specific humoral immunity. This report demonstrate, that polyclonal B-cell activation during acute experimental Chagas disease is not a generalized response and suggests that the nature of humoral immunity during *T. cruzi* infection contributes to host susceptibility. In leishmaniasis visceral, at early times after infection, there is a marked B-cell expansion in the draining lymph nodes of the site of the infection, which persists throughout infection. As early as day 7 after infection, polyclonal antibodies (TNP, OVA, chromatin) were observed in infected mice and the levels appeared comparable to the specific antileishmania response. Although B-cell-deficient JhD BALB/c mice are relatively resistant to infection, neither B-cell-derived IL-10 nor B-cell antigen presentation appears to be primarily responsible for the elevated parasitemia. Interestingly, passive transfer and

reconstitution of JhD BALB/c with secretory immunoglobulins (IgM or IgG; specific or nonspecific immune complexes) results in increased susceptibility to *L. infantum* infection [49].

Another potential deleterious role for polyclonal activation is that it could potentially turn on anti-self-responses and lead to autoimmune manifestations during chronic infections. IgG autoantibodies to brain antigens are increased in *P. falciparum*-infected patients and correlate with disease severity in African children [50]. Autoreactive Abs against endocardium and nerves can be detected in mice and humans infected with *T. cruzi* [51, 52] and are thought to be responsible for much of the Chagas' disease pathological damage. Recently, we reported that BAFF-BAFF-R signaling in *T. cruzi* infection partially controls polyclonal B-cell response but not parasite-specific class-switched primary effectors B cells. BAFF (TNF superfamily B lymphocyte stimulator), a crucial factor for the survival of peripheral B cells [53–55] associated to the development of autoimmune disorders [56], is produced early and persists throughout the infection with *T. cruzi*. By BAFF blockade we observed that this cytokine mediates the mature B-cell response and the production of non-parasite-specific IgM and IgG and influences the development of antinuclear IgG [57].

In addition, polyclonal B-cell activation can be responsible for maintenance of memory B-cell responses because of the continuous, unrestricted stimulation of memory B cells whose Ab production may be sustained in the absence of the antigens binding-specific BCR [58].

6. B Cells Influence the Characteristic of Cellular Immune Response Because They Act as APC and Cytokine/Chemokine Producers

Besides being the precursors of the Ab-secreting cells, B cells are committed to do other immune functions such as Ag presentation to T cells or cytokine/chemokine production. It has been widely studied that $CD8^+$ CTL are important for protective *T. cruzi* immunity [59, 60] but generally they are not induced by soluble protein vaccines. However, a mechanism known as cross-priming has been described whereby certain professional APC can induce $CD8^+$ T-cell responses after the uptake of exogenous Ag [61]. Hoft et al. [62] have demonstrated that the APC functions of B cells may be important for the induction of optimal vaccine-induced responses in mice immunized with a mix of CpG and *T. cruzi* transsialidase, an enzyme involved in parasite infectivity. They also demonstrate that mice deficient in B cells (uMT mice) fails to induce protective immunity when they were immunized with CpG and *T. cruzi* transsialidase. This failure of uMT mice to be protected was associated with the absence of *T. cruzi* transsialidase-specific $CD8^+$ T cell response, suggesting that B cells may be important for the cross-priming of $CD8^+$ CTL. In addition, it has been reported that *T. cruzi*-infected B-cell-deficient mice have reduced numbers of $CD8^+$ splenic T cells and impaired generation of central or effector splenic memory T cells [63]. *T. gondii*-infected C57BL/6 mice develop a robust and uncontrolled Th1 response, and it has been reported that *T. gondii*-primed B cells, but not naive B cells, were able to increase IFN gamma production by splenic T cells *in vivo*. The mechanisms involved may be linked to the presence of membrane-bound TNF on B-cell surface [64].

The fine tune regulation of migratory cells during infection is an important event in which chemokines and their receptors play a leading role. Different reports have demonstrated that the chemokine receptor CCR5 plays a role in systemic protection and cardiac inflammation during *T. cruzi* infection [65, 66]. $CCR5^+$ cells migrate to both mucosal and systemic sites in response to the chemokines CCL3 (MIP-1a), CCL4 (MIP-1b), and CCL5 (RANTES). In line with these reports, Sullivan et al. [67] showed that neutralization of CCL5 in $CCR5^{-/-}$ *T. cruzi*-immune mice results in decreased levels of *T. cruzi*-specific B cell responses and decreased mucosal protection in these mice. They also showed that CCL5 produced by B cells acts in an autocrine manner to increase B cell proliferation and total IgM secretion.

7. Established Memory B Cell Response Is Affected by Protozoan Parasite Infections

A hallmark of adaptive immunity is the ability to generate humoral immunological memory by which memory B cells could respond more rapidly and robustly to re-exposure to a new infection. Interestingly, it has been reported that *T. brucei* infection is capable of abrogating the efficacy of the vaccine-induced protective responses against non-related pathogens such as *B. pertussis* [40]. In the same line, Strickland and Sayles [68] showed that *T. gondii* infected mice which were immunized with SRBC had a depression not only in the primary, but also in the secondary humoral immune response, since they showed less IgM and IgG splenic Ab-secreting cells than non-infected control mice. All the data discussed in the present review indicate that protozoan parasites not only affect the development of the cells involved in Ab production [69] but also affect an already established humoral response against other pathogens. Then, the identification of mechanisms able to improve B cell response and, consequently, parasite control will also be beneficial to avoid the deterioration of a memory response to other pathogens.

Conflict of Interests

The authors have declared that no competing interests exist.

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Review Article

Lysophosphatidylcholine: A Novel Modulator of *Trypanosoma cruzi* Transmission

Mário A. C. Silva-Neto,[1,2] Alan B. Carneiro,[1,2] Livia Silva-Cardoso,[1,2] and Georgia C. Atella[1,2]

[1] *Instituto de Bioquímica Médica at Universidade Federal do Rio de Janeiro (UFRJ), 21940-590 Rio de Janeiro, RJ, Brazil*
[2] *Instituto Nacional de Ciência e Tecnologia em Entomologia Molecular (INCT), 21940-902 Rio de janeiro, RJ, Brazil*

Correspondence should be addressed to Mário A. C. Silva-Neto, maneto@bioqmed.ufrj.br

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Lysophosphatidylcholine is a bioactive lipid that regulates a large number of cellular processes and is especially present during the deposition and infiltration of inflammatory cells and deposition of atheromatous plaque. Such molecule is also present in saliva and feces of the hematophagous organism *Rhodnius prolixus*, a triatominae bug vector of Chagas disease. We have recently demonstrated that LPC is a modulator of *Trypanosoma cruzi* transmission. It acts as a powerful chemoattractant for inflammatory cells at the site of the insect bite, which will provide a concentrated population of cells available for parasite infection. Also, LPC increases macrophage intracellular calcium concentrations that ultimately enhance parasite invasion. Finally, LPC inhibits NO production by macrophages stimulated by live *T. cruzi*, and thus interferes with the immune system of the vertebrate host. In the present paper, we discuss the main signaling mechanisms that are likely used by such molecule and their eventual use as targets to block parasite transmission and the pathogenesis of Chagas disease.

1. Immune Response to *Trypanosoma cruzi* Infection in the Vertebrate Host

T. cruzi infects the vertebrate host through bite wounds produced in skin by a feeding bug or through the interaction of the parasite with conjunctival mucosa. Such interaction sometimes produces visible signs called Romaña's sign or chagoma inoculation. The histology of this initial site of infection is defined by an elevated number of mononuclear cells [1]. This first sign of infection suggests that *T. cruzi* can stimulate skin cells to produce mediators that trigger a local inflammatory response. Despite controversies about the mechanism of the pathogenesis of Chagas disease [2–5], until recently, some authors believed that the disease was limited to an acute phase, followed by a chronic phase that was considered an autoimmune disease, where the parasites would be physically linked to sites of inflammation in the heart and esophagus [6–8]. However, nowadays, the disease is considered multifactorial, with multiple and continuous interactions between pathogen and host [9]. After the incubation period of 2 to 3 weeks, infection with *T. cruzi* is manifested by the presence of a large number of parasites in the blood and tissues. Acute infection is accompanied by an excessive activation of the immune system that includes the production of high levels of cytokines, intense activation of T and B cells, lymphadenopathy, splenomegaly, and intense inflammation associated with tissue infection niches. The acute phase induces the development of an effective acquired immunity leading to the control of parasitemia. The chronic phase is considered lifelong and is associated with only a few parasites in the host. The beginning of chronic infection with *T. cruzi* is asymptomatic in most patients. However, with the advance of the disease, clinical manifestations become variable, ranging from no symptoms to the involvement of cardiovascular and/or gastrointestinal symptoms [10, 11]. Before the acquired immunity is established, the innate immune system appears to be essential for at least two important aspects of Chagas disease: control of replication of the parasite in the host tissue and progress of the inflammatory reaction. The latter, in turn, has been considered to be

the main cause of tissue damage and dysfunction of certain organs in the host [11]. Some studies in experimental models of infection of *T. cruzi* suggest that the potent immune response to Th-1 CD4 and CD8 cells, with the production of specific inflammatory cytokines, such as interferon gamma (IFN-γ), tumour necrosis factor (TNF-α), and interleukin 12 (IL-12), as well as the production of reactive nitrogen species such as nitric oxide (NO), plays an important role in the control of parasitemia during the initial stage of the disease [4, 10–13]. Moreover, cells of innate immunity, such as natural killer (NK) cells, dendritic cells, and macrophages, are also key elements in the initial control of parasite replication [10–13].

In recent years, research on Chagas disease has focused on the investigation of the role of pathogen-associated molecular patterns (PAMPs) of protozoa, which are the targets of innate immune receptors. Also, the problem of identifying relevant receptors in innate immunity-parasite interactions during the evolution of the disease in the host has been addressed by several laboratories. This strategy ultimately aims at the development of therapeutic interventions through the use of PAMPs derived from parasites. Glycosyl-phosphatidyl-inositol (GPI) is the name given to the first glycoconjugate in *T. brucei* that was identified with the function of anchoring proteins on the cell surface [14–17]. PAMPs widely studied in *T. cruzi* are, in fact, GPI anchors. All evolutive forms of this parasite express on their surface GPI-anchored glycoproteins [14–17]. Some studies have identified GPI anchors isolated from trypomastigote-derived mucin-like glycoproteins (GPI-mucins) of *T. cruzi* as the molecules primarily responsible for stimulating the host immune system [18, 19]. Thus, *T. cruzi* GPI-mucins are able to activate macrophages and stimulate the production of proinflammatory cytokines, chemokines, and NO [20–22]. Innate immune response to *T. cruzi* has been studied extensively and is based on the activation of signaling pathways triggered by Toll-like receptors (TLRs). TLRs are proteins that recognize conserved motifs associated with several different pathogens; they trigger intracellular signaling cascades that ultimately lead to a complex host immune response [11, 12]. There are 10 TLRs described in humans and 12 in mice [11, 12]. Generally, the stimulus induced by GPI molecules occurs during the early phase of infection, where macrophages respond to trypomastigotes in a TLR-dependent mechanism and ultimately induce the production of IL-12 and TNF-α and trigger the responses of CD4 and CD8 cells through the production of IFN-γ [23]. Thus, macrophages activated by TNF-α and IFN-γ seem to have an important role in controlling parasite growth. Free GPI anchors or glycoinositolphospholipids (GIPLs) are also able to stimulate the host immune system. GIPLs are similar to GPIs but contain instead ceramide in their lipid moiety [18, 19].

TLRs 2, 4, and 9 are the major TLRs involved in innate immune response to *T. cruzi* [18, 24–29]. TLR2 has been identified as the main receptor responsible for macrophage activation by GPI mucins [18, 24–29]. According to Ropert and Gazzinelli [27], the receptor heterodimer composed of TLR2 and TRL6 is activated by GPI mucin and the CD14 coreceptor. Oliveira et al. [25] observed that GIPL from *T. cruzi* confers an inflammatory response via TLR4, promoting the recruitment of neutrophils into the peritoneal cavity of mice. Later, Medeiros et al. [26] demonstrated that this effect was partially dependent on the production of IL-1β. The genomic DNA of *T. cruzi* also plays an important role in proinflammatory response of the vertebrate host during infection, since TLR 9 is activated by CpG motifs from nonmethylated DNA [28, 29]. Besides the innate immune response mediated by TLRs, *T. cruzi* can also stimulate TLR-independent pathways that lead to the production of IFN-β and IFN-γ. In this case, this occurs due to a surge in intracellular calcium concentration which ultimately leads to the activation of calcineurin and calmodulin [30–32].

2. Lysophosphatidylcholine and Modulation of NO Production and Host Immunity

Lysophospholipids such as lysophosphatidylcholine (LPC), sphyngosylphosphoryilcholine (SPC), lysophosphatidic acid (LPA), and sphingosine-1-phosphate (S1P) regulate a large number of cellular processes. LPC is a derivative of phosphatidylcholine (PC) that arises by the loss of a fatty acid through the action of a phospholipase A_2 (PLA_2) or by transferring it to cholesterol by the action of a cholesterol-acetyltransferase [33]. LPC is involved in several physiological events and is already known as a central molecule in several pathological states, but it is especially present during the deposition and infiltration of inflammatory cells and deposition of atheromatous plaque [34–36]. Research directed towards LPC has increased greatly since the finding that these molecules are involved in atherosclerosis [37]. The idea that various phospholipases secreted by circulating leukocytes participate in this pathology was soon proposed. Thus, the current model suggests that diabetes and hypercholesterolemia contribute to generate a large number of LDL particles in plasma that can undergo oxidation of unsaturated fatty acids, generating an oxidized particle (oxLDL). Since on average 50% of LDL fatty acids are arachidonic acid and linoleic acid, the chances of such an oxidative event are huge. The oxLDL is a potential cause of the increased expression of inflammatory markers such as TNF-α, MCP-1, and MCSF that will attract differentiating monocytes to the lesion site. In this sense, LPC is one of the most powerful chemotactic signals for macrophages and is also generated by cells in the apoptotic process as mentioned above. OxLDL particles are recognized by various secretory PLA_2 in the plasma, including type IIA, V, and X. Our group showed for the first time the presence of phospholipids and lysophospholipids in saliva and feces of the hematophagous organism *Rhodnius prolixus,* a triatominae bug vector of Chagas disease [38]. The major lipids present in *R. prolixus* saliva are PC and LPC [38]. Salivary LPC is an additional antihemostatic molecule that is part of the pharmacological arsenal injected into the bite site to allow the insect to feed. It inhibits platelet aggregation and increases the production of NO in endothelial cells. Thus, LPC was initially described as a molecule with antiplatelet and vasodilatory activities, and

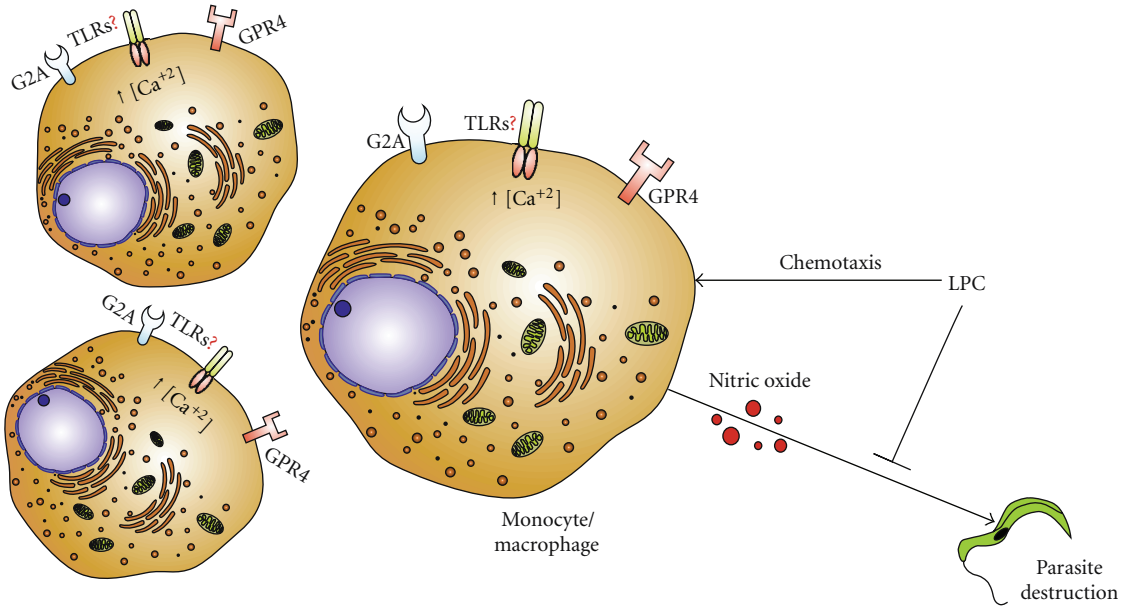

Figure 1: LPC-induced signaling on monocytes and macrophages. LPC is a signaling molecule that may act through different receptors on cell surface such as G2A and GPC4. Despite its description as a proinflammatory molecule, LPC-mediated signaling through TLRs is not demonstrated yet. LPC induces cell chemotaxis which ultimately increases the number of cells in the wound site. Also, LPC-treated cells undergo a decrease on NO synthesis when stimulated by parasite or LPS. Finally, a transient increase on intracellular calcium is also reported in such cells. These combined effects enhance the number of cells prone to *T. cruzi* invasion.

a few years later, its effect as an immunomodulator of *T.cruzi* infection was demonstrated [38, 39].

The role of LPC as a modulator of *T. cruzi* transmission occurs by three main mechanisms summarized on Figure 1 and mentioned as follows.

(1) LPC is a vector-derived molecule. It acts as a powerful chemoattractant for inflammatory cells at the site of the insect bite. This event will provide a concentrated population of cells available for *T. cruzi* infection [38, 39].

(2) LPC increases macrophage intracellular calcium concentrations that ultimately enhance parasite invasion.

(3) LPC inhibits NO production by macrophages stimulated by either live *T. cruzi*, LPS, or LPS in the presence of IFN-γ, and thus interferes with the immune system of the vertebrate host [39].

The above findings demonstrate that LPC is now a signaling molecule with effects beyond that of counteracting host blood hemostasis, since it acts as modulator of NO biology and parasite transmission [40–43]. Macrophages are intimately related to the establishment of acute infection with *T. cruzi*, since the success of the infection depends on the initial invasion of these cells [44–46]. This leads to the assumption that salivary LPC may facilitate the parasite infection, favoring not only insect feeding, but also preparing the environment for the arrival of the parasite, minutes or hours after the initial bug bite. Recent results obtained by our group demonstrated that injection of salivary LPC into host skin followed by parasite inoculation in the same site minutes later ultimately increases blood parasitemia from 3- to 6-fold in animals infected with *T. cruzi*. LPC's effect on parasitemia is mainly achieved by the activation of macrophage chemotaxis and immunosuppression of NO production induced by the parasite. We also showed an increase in the rate of association of the parasite with macrophages induced either by 500-fold diluted saliva or by LPC. This was the first demonstration of a potentiating factor of transmission of Chagas disease and the first implication of a lysophospholipid in an infectious disease [39].

The activation of receptors that recognize the parasite by the presence of specific structures on its surface stimulates host cells to produce TNF-α, IL-12, and NO, as mentioned above. Depending on the MyD88 adapter protein, TLRs 2, 4, and 9 have been implicated in the network used by the immune system of the mammalian host to control infection by *T. cruzi* [14–18]. Campos et al. [18, 23] were the first to demonstrate the involvement of TLR2 in the interaction between the parasite and host macrophages. The expression of TLR2 is essential for the induction of IL-12, TNF-α, and NO, and this receptor is activated by parasite-derived molecules such as GPI anchors, which have been isolated from the surface of trypomastigotes of *T. cruzi* [14–18]. The production of NO but not IL-12 by *T. cruzi*-exposed macrophages is not affected by bug saliva [39]. Curiously, in bone-marrow-derived macrophages obtained from TLR2-deficient mice, the production of IL-12 is largely suppressed by LPC. These data indicate that in some cell types, the production of this cytokine may be affected by this lysophospholipid through a TLR2-independent mechanism.

Moreover, GIPLs from *T. cruzi* are TLR4 agonists with proinflammatory effects [25, 26]. We showed that NO production, induced by the parasite or by lipopolysaccharide (LPS), another ligand of TLR4, either in murine peritoneal

macrophages or bone marrow-derived macrophages, is blocked in both cases by LPC even in the presence of IFN-γ *in vitro* [39]. The ability of LPC to reverse the induction of NO production in all cases, almost independently of the ligand type, suggests that this lysophospholipid must act by a unique pathway. In this regard, the receptors involved in cell signaling induced by LPC, in general, exhibit a certain promiscuity with respect to the ligand and vice versa. In the case of LPC, different receptors have been proposed for this molecule, including G2A, a G protein-coupled receptor, and GPR4, another important candidate [45, 47–49]. Despite the controversy generated in the literature due to the low reproducibility of the studies using radioactive LPC and its interaction with candidate receptors, the ability of G2A to bind fatty acids and protons is noteworthy [33]. Thus, G2A remains in the literature as the best-known receptor involved in the adaptation of the signal induced by LPC [47–49]. Moreover, the redistribution of G2A receptor itself and the exposure of TLR4 are influenced by LPC metabolism [33, 50]. In this case, the content of intracellular LPC is finely controlled by the activity of a lysophosphatidylcholine acyltransferase (LPCAT), an enzyme that uses LPC as a substrate and generates phospholipids as the product of its action. The treatment of monocytes with LPS activates this enzyme and increases the transport of TLR4 to membrane rafts in these cells [50]. Since the LPCAT inhibitor used, 5-hydroxyethyl 5′3′ thiophenol pyridine (HETP), increases the lysophospholipids/phospholipids ratios, it reverses the effect of LPS [50]. Thus, it seems appropriate to propose that in the presence of *T. cruzi*, one should conduct a map of the distribution of both receptors, G2A and TLR4, in the presence and in the absence of LPC using both proteomic and immunological methods [33, 50].

During programmed cell death, LPC is generated by a calcium-independent PLA_2 activated by caspase-3. Thus, LPC acts as a chemotactic *find-me* signal that attracts the phagocyte to the apoptotic cells [49, 51, 52] and as an *eat-me* signal involving recruitment of complement proteins for recognition by phagocytes [49, 51, 52]. Such LPC-induced chemotaxis is very interesting, because in Chagas disease, the uptake of apoptotic cells by macrophages infected with *T. cruzi* stimulates parasite growth [53]. In addition, it has been shown that *T. cruzi* infective stages are able to generate lipid messengers, including LPC, that modulate host cell signaling [54]. Regarding adaptor molecules mobilized in response to LPC, it is known that in most cell types, there is the involvement of isoforms of protein kinase C [55]. Probably it is the type of isoform activated in each cell that directs the intensity and type of response triggered by LPC in that specific cell type. When combined with different types of TLRs and adapters, LPC-mediated signaling must produce a specific and still poorly understood repertoire of immunosuppression.

3. Vector Phospholipases and Eventual Target to Block *T. cruzi* Transmission

PLA_2 is an enzyme family present in various organisms such as viruses, bacteria, plants, and animals. According to studies done on mammals, the action of PLA_2 is important for the remodeling of cell membranes, lipid digestion, cell signaling, and immune defense of the host as well as production of various lipid mediators [56–61]. In insects, the phospholipases that have been studied are related to the venom injected into their prey, the physiology of digestion, immunity, and reproduction [61]. Among the published studies on phospholipase activity in arthropods are those reporting the presence of such enzymes in the salivary glands of *Manduca sexta* [62] and in the saliva and salivary glands of *Amblyoma americanum* [63, 64]. These studies have found a correlation between PLA_2 activity and digestion. In addition, Zhu et al. [64] suggested another role for this activity, linking it to the production of prostaglandins, promotion of vasodilation and the suppression of inflammation and immunity. The production of prostacyclin may also lead to the inhibition of platelet aggregation and the induction of vasodilation. Furthermore, platelet-activating factor (PAF) acetyl hydrolase, a member of the GVII family of PLA_2 enzymes, is a serine-dependent hydrolase that does not require Ca^{2+} for activity. This enzyme cleaves the acetyl group from the *sn*-2 position of the phospholipid, and in the case of PAF, there is the hydrolysis of the *sn*-2 ester bond, releasing acetate and biologically inactive lyso-PAF [65, 66]. Cat flea (*Ctenocephalides felis*) salivary gland homogenate has a PAF-acetylhydrolase activity, and the estimated amount of activity in a single pair of salivary glands (~5 pmol/min) is of the right order of magnitude to induce a localized anti-inflammatory/allergic effect [67]. Extracellular PAF is proinflammatory and acts via very high affinity G-coupled protein receptors, causing activation of platelets, neutrophils, and monocytes [66]. The hypothesis is that PAF-acetylhydrolase activities from saliva can downregulate inflammatory and immune reactions mediated by PAF released from host cells. This may happen as a reaction to injected cat flea saliva and may be interrupted by host grooming or scratching the locale of the bite. Besides these reports, phospholipases also have been identified in transcriptomes of saliva or salivary glands of some hematophagous arthropods such as the soft ticks *Ornithodoros coriaceus* [67, 68] and *O. parkeri* [69], of hard ticks such as *Ixodes pacificus* [70], and in insects such as *Anopheles funestus* [71], *Phlebotomus arabicus* [72], and *Glossina morsitans* [73].

Zeidner et al. [74] have suggested that aside from facilitating some tick digestive processes, it is possible that secretion of PLA_2 into the feed site creates some protective barrier against bacteria that can be carried into the wound. They demonstrated that the borreliacidal activity found in *A. americanum* saliva is most probably due to the enzymatic effects of PLA_2 and that it would directly and rapidly kill *Borrelia burgdorgeri* through the digestion of membrane lipids, composed by a majority of PC and phosphatidylglycerol. The authors hypothesize that high level of PLA_2 enzymatic activity present in saliva is related to *A. americanum*'s refractoriness to *B. burgdorgeri*. Other studies have demonstrated the importance of PLA_2 in the infection processas elicited by pathogens such as *Toxoplasma gondii*, *Cryptosporium parvum*, *Entamoeba histolytica*, *Leishmania amazonensis*, and *T. cruzi* [75–77]. Moreover, Connelly and Kierszenbaum [44]

showed that the presence of PLA_2 significantly increased the association between *T. cruzi* and macrophages, and they suggest that this effect relies on alterations of the parasite membrane, since it was induced by pretreatment of parasite membranes with PLA_2 but not macrophages. But nowadays, as cited above, the presence of PLA_2 in salivary secretions of *T. cruzi* vectors implies LPC generation and its further involvement in the inflammatory process that occurs during infection.

PLA_2 enzymes from snake venom induce a wide spectrum of pharmacological effects, including anticoagulant proprieties that can be mediated by hydrolysis of phospholipid or by a nonenzymatic mechanism, such as when PLA_2 from *Naja nigricollis* venom binds factor Xa in the coagulation cascade through the specific anticoagulant site on its surface [78]. Our group is investigating a further role for the LPC present in the saliva of vectors, which we believe is related to muscular paralysis. Rigoni et al. [79] have shown that lysophospholipids, in particular LPC, can block the exocytosis of neurotransmitters, thus paralyzing the muscle. In this context, using the predator insect *Belostoma anurum* as model, we showed that the salivary LPC also has this property. Our hypothesis is that *B. anurum* uses lysophospholipids as a way to paralyze the prey while it feeds, since it makes an extraoral digestion [80]. We obtained similar results with LPC from *R. prolixus*, with less pronounced blockage of exocytosis. LPC action may be more local in order to avoid disturbing the host. Thus, the above data show that the presence of LPC generated by PLA_2s in salivary secretions of predators and blood-sucking arthopods is widespread in the animal kingdom, and this molecule may be a surviving trace of ancient feeding habits.

Another aspect that should be emphasized is that PLA_2s also generate free fatty acids that can be converted to eicosanoids. Eicosanoids are polyunsaturated fatty acids of 20 carbons that act as local mediators of short half-life; they are derived from arachidonic acid (20 : 4 n-6) or other polyunsaturated 20-carbon precursors (20 : 3 n-6 and 20 : 5 n-3). Arachidonic acid is esterified in phospholipids of plasma membranes, these being released by the action of PLA_2. The biological action of arachidonic acid products requires its oxygenation, which can take place in three different ways: (a) via the cyclooxygenases that generate prostaglandins and thromboxanes, (b) via the lipooxygenases that generate leukotrienes and lipoxins, and (c) via the cytochrome P-450, which generates epoxides [81]. Physiological processes that usually involve autacoids, hormones, and growth factors may stimulate the release of arachidonic acid, as already widely described in mammals, as in mediating immune and inflammatory response of late vertebrates [82]. Recently, the involvement of thromboxane A_2 (TXA_2) in the process of vertebrate host infection by *T. cruzi* [83] was demonstrated. The same group showed that the eicosanoid TXA_2 is prevalent in all life stages of the parasite. Thus, in infected mice, the parasite itself may account for 90% of the total TXA_2 in plasma. In this regard, it is noteworthy that the production of TXA_2 from arachidonic acid occurs by the cyclooxygenase pathway. Accordingly, results from our laboratory indicate that half of the fatty acids ingested along with blood are unsaturated and about half of them are arachidonic acid. So, if a pool of TXA_2 is a prerequisite for the process of infection of host cells by any pathogen, that pool could be generated during the final stages of blood digestion in the vector at the expense of fatty acids released there. A triacylglycerol-lipase activity was identified in the gut lumen of blood-fed insects and is probably involved in the digestion of lipids from the blood meal. These lipase activities and also the metabolism and fate of lipids that are generated during digestion of ingested blood were studied and characterized [84]. However, neither the dynamic generation of free fatty acids in insects infected with *T. cruzi* nor their processing to TXA_2 in the final stage of the digestive process has ever been assessed in any vector. Thus, an attractive model for the future might involve the silencing of a PLA_2 gene in the saliva of Chagas disease vectors to obtain LPC-depleted or LPC-free saliva. The saliva of these insects would be expected to lower the rate of infection of the vertebrate host.

4. The Role of Host Plasma LPC in *T. cruzi* Infection

The original studies that implicated LPC in the pathogenesis of atherosclerosis tended to highlight the presence of this phospholipid in atheromatous lesions [37]. However, the origin and dynamics of the formation of this molecule remained unknown for many years. Wilensky et al. [85] identified the main enzyme responsible for the generation of LPC, Lp-PLA_2, also known as PAF-acetyl hydrolase or phospholipase VIIA, which is secreted by leukocytes and associated with plasma lipoproteins, especially LDL. This enzyme recognizes and cleaves oxLDL and oxidized phospholipids, generating LPC and free fatty acid oxidation (oxNEFAS, or oxidized nonesterified fatty acids). The LPC, as previously mentioned, is a potent proinflammatory molecule capable of leukocyte recruitment and activation with induction of apoptosis. Demonstration of Lp-PLA_2 in the necrotic core of atheromatous lesions and fibrous cap of vulnerable plaques supports our current views of the importance of this enzyme in atherogenesis. An important therapeutic option is the selective inhibition of Lp-PLA_2. Wilensky et al. [85] showed that the administration of darapladib (GlaxoSmithKline) in experimental models selectively reduces the activity of this enzyme, attenuates the formation of LPC, and reduces the formation of atherosclerotic plaques with negative regulation of proinflammatory genes in macrophages and T lymphocytes. Chagas disease treatment aims to slow the progression of myocardial impairment caused by invasion of the patient's heart by the parasite. Some of the drugs used to treat Chagas disease cause changes in the patients plasma lipid profile, leading to high concentrations of LDL. During treatment, these patients are likely to present favorable conditions for LDL oxidation and generation of LPC, which will certainly trigger the proinflammatory phenotype, thereby maintaining levels of reinfection of myocardial cells. In this sense, it would be important to evaluate the generation of LPC in Chagas disease patients treated with various categories of drugs in order to verify the formation of this lipid mediator. Likewise, chronic treatment with the drug

in healthy experimental animals should be carried out to identify any effects on the levels of LPC and subsequent susceptibility to infection by *T. cruzi*. In conclusion, darapladib may constitute a novel tool with dual use: to optimize the current therapeutic treatment of chronic chagasic patients and to experimentally modify the plasma levels of LPC in mice to determine whether the reduction of such levels decreases the susceptibly to infection by *T. cruzi*.

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Research Article

The Role of Vitamin D and Vitamin D Receptor in Immunity to *Leishmania major* Infection

James P. Whitcomb,[1] Mary DeAgostino,[1] Mark Ballentine,[1] Jun Fu,[1] Martin Tenniswood,[2] JoEllen Welsh,[2] Margherita Cantorna,[3] and Mary Ann McDowell[1]

[1] *Eck Institute for Global Health, Department of Biological Sciences, University of Notre Dame, Notre Dame, IN 46556, USA*
[2] *Cancer Research Center, University at Albany, 1 Discovery Drive, Rensselaer, NY 12144, USA*
[3] *Department of Veterinary and Biomedical Sciences, Pennsylvania State University, University Park, PA 16802, USA*

Correspondence should be addressed to Mary Ann McDowell, mcdowell.11@nd.edu

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Vitamin D signaling modulates a variety of immune responses. Here, we assessed the role of vitamin D in immunity to experimental leishmaniasis infection in vitamin D receptor-deficient mice (VDRKO). We observed that VDRKO mice on a genetically resistant background have decreased *Leishmania major*-induced lesion development compared to wild-type (WT) mice; additionally, parasite loads in infected dermis were significantly lower at the height of infection. Enzymatic depletion of the active form of vitamin D mimics the ablation of VDR resulting in an increased resistance to *L. major*. Conversely, VDRKO or vitamin D-deficient mice on the susceptible Th2-biased background had no change in susceptibility. These studies indicate vitamin D deficiency, either through the ablation of VDR or elimination of its ligand, 1,25D3, leads to an increase resistance to *L. major* infection but only in a host that is predisposed for Th-1 immune responses.

1. Introduction

The initial metabolism of vitamin D_3 occurs in the skin where 7-dehydrocholesterol is converted to previtamin D_3 after exposure to UVB radiation. After isomerization, vitamin D_3 is metabolized by hepatic 25-hydroxylase (CYP2R1) to form 25-hydroxyvitamin D_3 (25OHD3), which is the major circulating form of vitamin D_3. $25OHD_3$ is metabolized by 1α-hydroxylase (CYP27B1) that is present in the kidney and many other target tissues including the skin. The active form of vitamin D_3, 1α,25 dihydroxyvitamin D3 (1,25D3) translocates to the nucleus of target cells, where it binds to the vitamin D receptor (VDR), a member of the nuclear receptor supergene family. The VDR heterodimerizes with the retinoid X receptor and this complex recognizes vitamin D response elements in the promoters of many genes to modulate their transcription.

1,25D3 is well characterized for its function in maintaining appropriate serum calcium concentrations as well as its critical requirement for proper bone formation. In addition to these roles, 1,25D3 is important in the regulation of immune responses. Vitamin D has important roles in leukocyte differentiation, dendritic cell maturation, and modulation of the T-helper cell dichotomy [1–5]. Symptoms of Th-1-mediated autoimmune diseases can be reduced or eliminated by treatment with 1,25D3 [6–8]. In addition to its role in autoimmune disease, recent studies have explored a role for vitamin D-mediated signaling in infectious disease resistance [9]. For example, *Mycobacterium tuberculosis* infection induces both VDR and 1-α hydroxylase, which are necessary for production of cathelicidin as part of an antimicrobial peptide response against the *M. tuberculosis* infection in vitro [10]. Here, we investigated the role of VDR in immunity to murine experimental cutaneous leishmaniasis (CL), the prototypical *in vivo* model of the Th-1/Th-2 dichotomy. Parasites from the genus *Leishmania* are responsible for a spectrum of diseases ranging from self-healing cutaneous disease to the potentially fatal visceral disease. Immunity to *L. major,* a parasite responsible for cutaneous leishmaniasis, is highly dependent on a strong

IFNγ-mediated T-helper 1 (Th-1) response. This Th-1 response induces production of nitric oxide (NO) which is critical in elimination of parasites.

We report that VDRKO mice exhibit decreased lesion development as well as decreased parasite loads at the height of *L. major* infection. Additionally, we demonstrate that resistant mice that are rendered vitamin D-deficient by ablation of the 1-α hydroxylase enzyme in CYP27B1 KO mice mimic VDRKO mice with regards to decreased lesion development, suggesting that the effect is dependent on the presence of the ligand. Surprisingly, deficiency of vitamin D signaling in genetically susceptible mice has no effect on *L. major* infection.

2. Materials and Methods

2.1. Mice. VDRKO [11], CYP27B1KO [12], and WT mice, on both BALB/c and C57BL/6 backgrounds, were maintained at Friemann Life Sciences Center at the University of Notre Dame (Notre Dame, Ind, USA). To generate the BALB/c VDRKO strain, female C57BL/6 VDRKO mice were bred with WT male BALB/c mice. The female offspring from this mating were genotyped by PCR [11] and the mice identified as heterozygous for the VDRKO allele were backcrossed to WT male BALB/c mice. This process was repeated 7 times resulting in mice with a greater than 99.2% BALB/c background. Male and female heterozygous mice were bred to each other to generate offspring that were homozygous for either the VDRKO allele or for the WT allele. These homozygous mice were then used to establish a breeding colony of both VDRKO and WT mice on a BALB/c background. VDRKO and WT mice were maintained from weaning on a high-calcium, high-lactose rescue diet (TD96348; Teklad, Madison, Wis, USA) to prevent hypocalcemia associated with VDR deficiency [13]. All animal protocols were approved and reviewed by the University of Notre Dame Institutional Animal Care and Use Committee (IACUC). All mice used in the experiments described below were females between 1 and 4 months of age. WT female mice were age-matched for all experiments.

2.2. Parasites and Infection. *L. major* NIH Friedlin V1 strain (MHOM/IL/80/FN) was used in this study. The parasites were cultivated and infective stage, metacyclic promastigotes were generated as previously described [14]. For infections, 1×10^5 metacyclic promastigotes in 20 μL of PBS were injected intradermally into the outside surface of the ear. Lesion diameter and thickness and ulcer diameter was measured weekly with a digital vernier caliper. A lesion "score" was calculated by adding the values obtained for lesion diameter, ulcer diameter, and lesion thickness. Infected ear tissue was homogenized to determine relative parasite load by performing a limiting dilution assay as previously described [15].

2.3. Histology. Segments of ear tissue were embedded in Tissue-Tek OCT freezing media (Fisher Scientific, Pittsburgh, PA), sectioned and stained with Mayer's hematoxylin and eosin to distinguish cytoplasm and nuclei, respectively. Images were obtained using a Nikon E400 light microscope (Nikon, Melville, NY, USA).

2.4. IgG Subclass Determination. Collected sera was used in a soluble *Leishmania* antigen (SLA) specific ELISA. IgG subclass specific secondary antibodies (Southern Biotech, Birmingham, Ala, USA) were used to determine IgG subclass levels. Absorbances were recorded using a SpectraMax M2 plate reader (Molecular Devices, Sunnyvale, Calif, USA). Sera from mice 12 weeks after post-infection were pooled and run as a normalizing sample on each plate. For each isotype, the absorbance for each normalizing sample was used to generate a mean absorbance (ABS). Plate to plate variation was eliminated by using the equation (mean ABS/plate ABS) $\times$ (plate ABS) = normalized ABS.

2.5. Quantitative RT-PCR Analysis. Total RNA was isolated from pulverized ears using an RNeasy Mini kit (Qiagen, Valencia, Calif, USA) according to the manufacturer's instructions and contaminating DNA was removed via DNase I treatment (Invitrogen, Carlsbad, Calif, USA). Reverse transcription was performed using 1 μg of DNA-free total RNA, 250 ng random primers (Invitrogen), and SuperScript III kit (Invitrogen). Real-time PCR was performed on an ABI 7900HT sequence detection analyzer (Applied Biosystems, Foster City, Calif, USA) using the 2x SYBR Green Kit (Applied Biosystems). The primers (300 nM; IDT, Coralville, Iolua, USA) used were HPRT, IFNγ, IL-4, IL-12p40 [16], Arg1 [17], iNOS [18], IL-10: 5′-CAC AAA GCA GCC TTG CAG AA-3′/ 5′-CTG GCC CCT GCT GAT CCT-3′, TNFα: 5′-GAA ACA CAA GAT GCT GGG ACA GT-3′/ 5′-CAT TCG AGG CTC CAG TGA ATT C-3′. For FoxP3, gene expression was determined using a premade gene expression assay (Applied Biosystems) according to manufacturer's instructions. Relative copy number was determined using the comparative CT method [19].

2.6. Restimulation Assay. Lymph nodes from infected mice were disrupted using a syringe plunger and a cell strainer (BD Biosciences, San Jose, Calif, USA) and cells from 4 mice per condition were pooled and stimulated with 50 μg of SLA. Cell supernatant was harvested at 120 hr after treatment and was analyzed on a Luminex 200 instrument using a multiplex biomarker immunoassay (Millipore, Billerica, Mass, USA). Two biological replicates were assessed, the first in quadruplicate on two separate Luminex plates and the second in duplicate on one plate.

2.7. Flow Cytometry. FcReceptors were blocked with 10% normal mouse serum and stained using 1% BSA in PBS. Cell surface labeling was performed using the following anti-mouse antibodies: α-CD45, α-CD11b, α-CD11c, α-LY-6C/G, α-CD4, α-CD8, α-TCR (BD Biosciences), α-F4/80 (Invitrogen), and α-FoxP3 (eBioscience, San Diego, Calif, USA). Appropriate isotype controls were used as negative controls. Flow cytometry was performed using an FC-500 flow

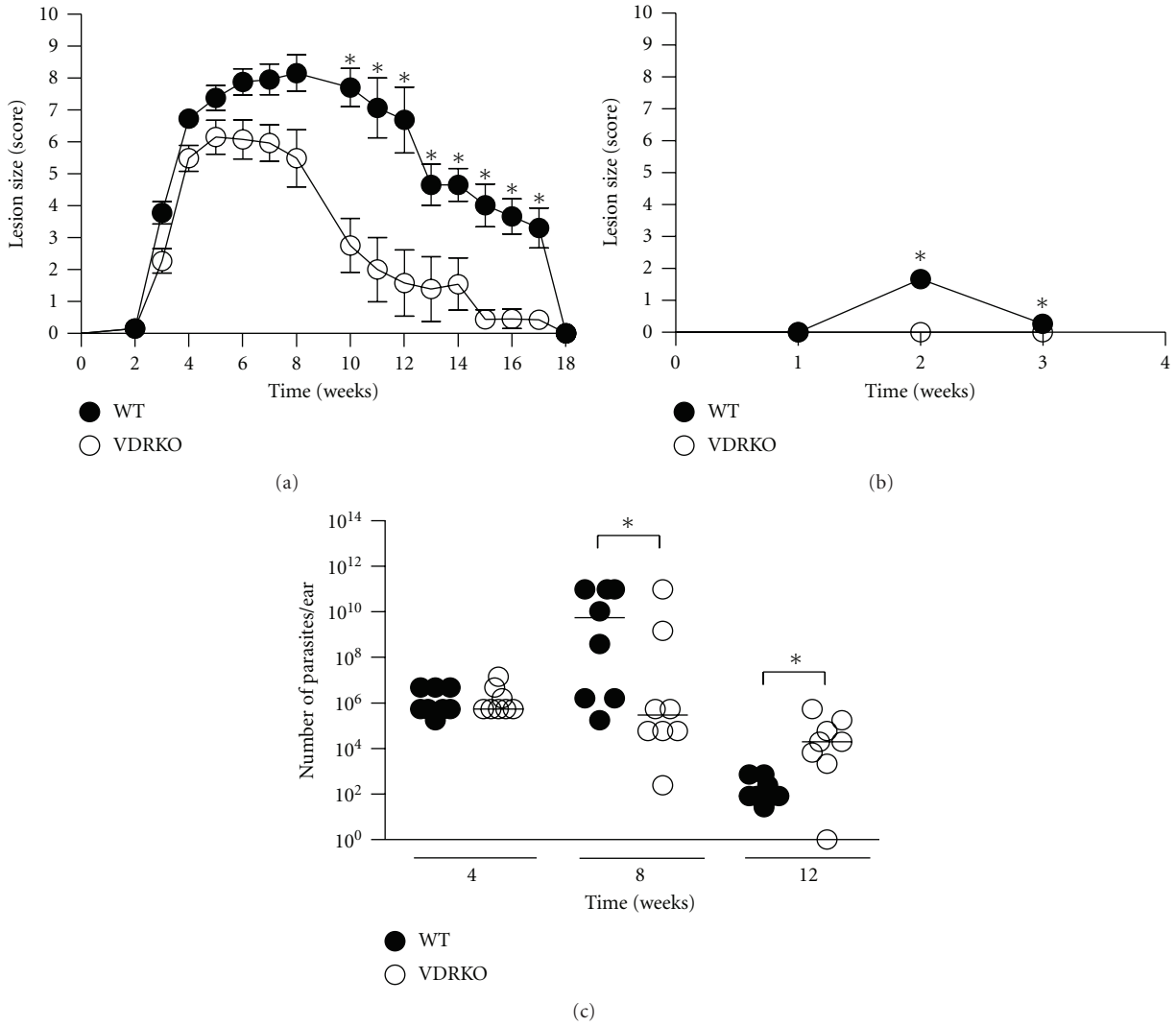

Figure 1: VDRKO mice develop smaller lesions when infected with *L. major*. C57BL/6 WT (closed circles) and VDRKO (open circles) mice were infected in the ear with 10^5 *L. major* parasites. (a) Cumulative measurement of lesion size (score) was measured over the course of infection. One representative of 5 independent experiments is presented; $^*P \leq 0.05$. (b) Mice were reinfected upon complete resolution of the primary infection (wk 18) and lesions were measured until completely healed. Mean lesion score ± SE is presented; $n = (11–21$ ears). One representative of 2 independent experiments is presented. (c) Parasite burden of infected ears from VDRKO and WT mice was determined at 4, 8, and 12 weeks after infection by performing a limiting dilution assay. Each point indicates the parasite load in a single ear. One representative of 2 independent experiments is presented. $^*P \leq 0.05$.

cytometer (Beckman Coulter, Fullerton, CA) and analyzed with MXP analysis software (Beckman Coulter).

2.8. Diet Studies. For vitamin D deficiency experiments, mice were fed TD.04179, a vitamin D-deficient diet (Harlan Teklad) from birth prior to mating with male mice from the same background. Once weaned, the offspring from these mice were fed solely using the vitamin D-deficient diet throughout the study. Mice were infected in the ears and lesions were measured as previously described. Vitamin D analysis of serum was performed using both a 25(OH)D3 ELISA and a 1,25D3 ELISA (IDS, Fountain Hills, AZ, USA) following the manufacturer's instructions.

2.9. Generation of Bone Marrow Derived Macrophages and Bone Marrow-Derived Dendritic Cells (DC). Bone marrow was flushed from femurs with RPMI-C. Red blood cells were lysed using ice-cold sterile ACK lysis buffer (0.15 M Nh_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA). Macrophages were generated as previously described [14]. For the generation of DC, progenitor cells were counted and resuspended at 2×10^5/mL in RPMI-C containing 20 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF) (Peprotech, Rocky Hill, NJ, USA). The cells were supplemented with fresh RPMI-C containing 20 ng/mL GM-CSF on days 3, 6, 8, and 10. On day 12, the cells were transferred to 24 well plates for the duration of the experiment.

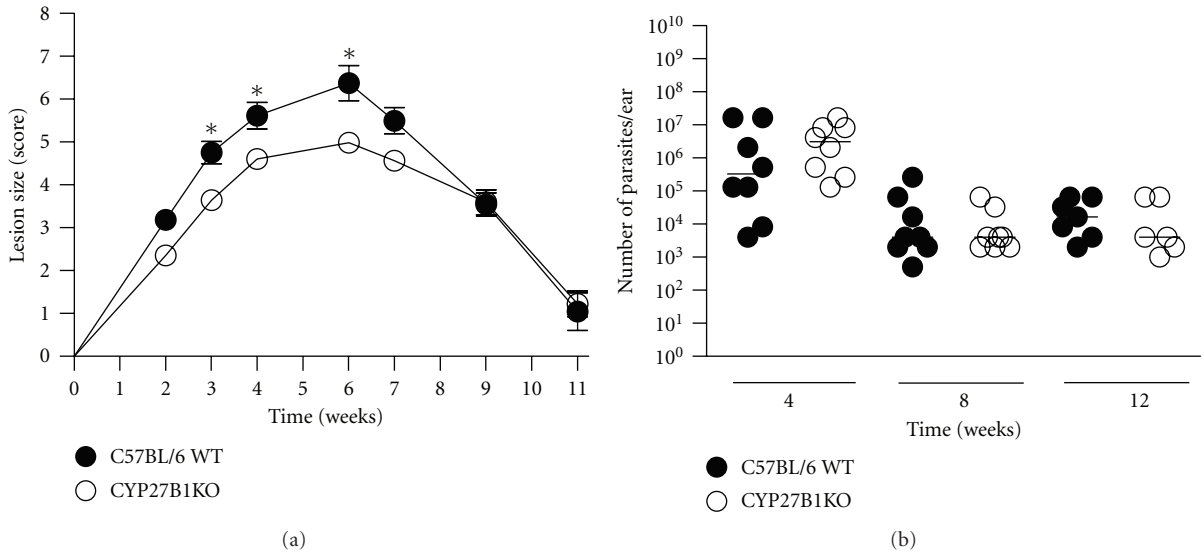

Figure 2: Lesion size is reduced in CYP27B1KO mice. C57BL/6 WT (closed circles) and CYP27B1KO (open circles) mice were infected with 10^5 *L. major* parasites and the resulting lesions were measured at the indicated time points. Mean ± SE is presented; $n = (8–17)$. (b) Infected ears were harvested at 4, 8, and 12 weeks after infection, and parasite load was determined by limiting dilution assay. Each point indicates parasite burden in one ear. One representative of two independent experiments is presented. $^*P \leq 0.05$.

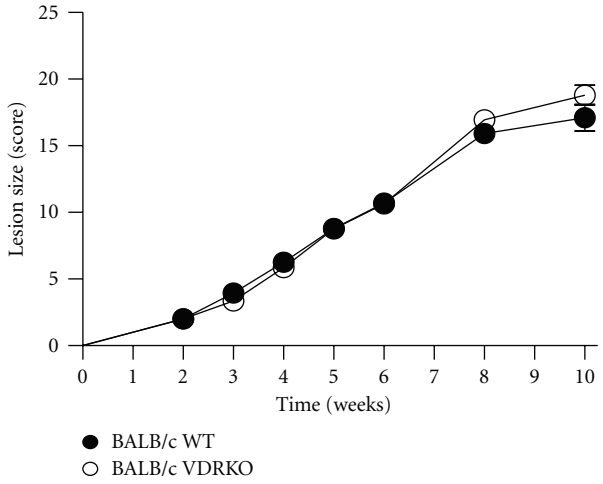

Figure 3: Deficiency of vitamin D signaling does not modulate resistance in a susceptible mouse strain. VDRKO mice on a susceptible BALB/c background (open circles) or WT BALB/c (closed circles) were infected with 10^5 *L. major* parasites and the resulting lesions were measured at the indicated time points. Mean ± SE is presented; $n = 14$. One representative of two independent experiments is presented.

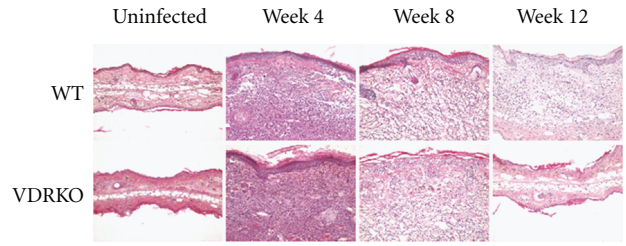

Figure 4: Inflammation is decreased in the infected ears of VDRKO mice. C57BL/6 WT and VDRKO were infected in the ear with 10^5 *L. major* parasites. Histological cross sections were H&E stained at the indicated time points and inflammation of the dermis was assessed visually by microscopy. One representative of 2 independent experiments is presented.

2.10. Phagocytosis Assays. Macrophages or DC were treated with IFN-γ (500 U/mL) and/or 1,25D3 (Biomol, Plymouth Meeting, Pa, USA) (40 nM) for 24 hr or left untreated. Cells were infected at a ratio of 5 : 1 with V1 strain *L. major* metacyclic promastigotes. Parasites were opsonized with 5% normal mouse serum for 30 minutes, washed, then coincubated with the macrophages or dendritic cells for 1, 2, 4, and 24 hr. Coverslips were stained with Diff-Quick (Fisher Scientific).

2.11. IL-12 ELISA/Nitric Oxide (NO) Assays. Levels of IL-12 were analyzed using anti-IL-12p40 ELISA (Pierce, Rockford, Ill, USA). NO production was assessed by Griess reaction according to the manufacturer's instructions (Promega, Madison, Wis, USA).

2.12. Statistical Analyses. For statistical analysis of lesion size, analysis of variance (ANOVA) between WT and KO strains was utilized with a subsequent Bonferonni's post-test to determine at what time points the strains were different. Paired *t*-tests were performed to determine differences between WT and KO populations for parasite burdens, IgG levels, cytokine regulation, and all *in vitro* analyses. All statistical analyses were performed using GraphPad Prism 4.0 software. In all cases, a P value ≤ 0.05 was considered statistically significant.

3. Results

3.1. VDRKO Mice on a C57BL/6 Background Exhibit Increased Resistance to L. major Infection. To investigate the role of VDR in immunity to *L. major* infection, C57BL/6 VDRKO and WT mice were infected in the ear dermis with 10^5 *L. major* metacyclic promastigotes. Both groups of mice developed lesions that eventually healed; however, VDRKO mice developed lesions that were significantly smaller and healed 3 weeks faster than their WT counterparts (Figure 1(a)). Upon reinfection, VDRKO mice did not develop lesions at the site of secondary infection whereas the WT mice developed small lesions that resolved within 3 weeks (Figure 1(b)). These results indicate that VDRKO mice have an increased resistance to *L. major* infection compared to WT mice as demonstrated by decreased lesion development. Additionally, VDRKO mice have an intact and possibly heightened memory response to secondary infection.

Because VDRKO mice had decreased lesion development, we compared the parasite load at the site of infection between VDRKO and WT mice using a limiting dilution assay. At 4 weeks after infection, both groups of mice had similar levels of parasites at the site of infection and at week 8, the VDRKO mice had significantly fewer parasites compared to the WT mice (Figure 1(c)). However, by 12 weeks after infection, VDRKO mice harbored significantly elevated parasite loads, suggesting that wound healing and parasite killing may be occurring via different mechanisms [20].

3.2. 1-α Hydroxylase-Deficient Mice Exhibit Heightened Resistance to L. major Infection. CYP27B1KO mice, which lack the 1-α hydroxylase enzyme required to produce 1,25D3, displayed prototypical lesion development that is observed in resistant (C57BL/6) strains of mice; that is, lesions develop after a few weeks and eventually resolve. Similar to VDRKO mice, CYP27B1KO mice developed significantly smaller lesions compared to their WT counterparts throughout the first 6 weeks of infection (Figure 2(a)). CYP27B1KO mice resolved their infections at a similar rate as WT mice and both groups were completely healed by week 11 after infection. Parasite quantification of infected ears from these groups of mice demonstrated that that CYP27B1KO and WT mice possess similar parasite burdens throughout the infection (Figure 2(b)).

3.3. VDR Ablation Does Not Affect L. major Infection in BALB/c Mice. C57BL/6 mice have the propensity to completely heal after *Leishmania* infection, whereas BALB/c mice are unable to heal or resolve *L. major* induced lesions. Unlike C57BL/6 VDRKO mice, VDRKO mice on a susceptible BALB/c background did not develop smaller lesions compared to their WT counterparts (Figure 3).

3.4. VDRKO Mice Exhibit Decreased Inflammation at the Site of Infection. To investigate the causes of reduced lesions and parasite burden in C57BL/6 VDRKO mice, we performed further analyses on lesions and immune responses to *L. major* infection. VDRKO mice exhibit alopecia, develop dermal cysts, and do not recycle epidermal layers properly [21, 22]. To assess if these skin differences were involved in the differential lesion development, we performed a histological study to explore lesion architecture. Uninfected C57BL/6 VDRKO and WT mice do not have any differences in their skin architecture and displayed no characteristics indicative of inflammation (Figure 4). At 4 weeks after infection, both VDRKO and WT mice exhibit similar levels of inflammation at the site of infection. Although numbers of infiltrating cells were similar in the two mouse strains through 8 weeks of infection (data not shown), VDRKO mice appeared to display decreased inflammation by 8 weeks after infection (Figure 4). Inflammation continued to decrease in both groups of mice, and by 12 weeks after infection lesions from VDRKO mice exhibited little signs of inflammation. Inflammation was still observed in the WT mice at 12 weeks after infection although greatly reduced.

There were no significant differences between WT and VDRKO mice in terms of macrophage (CD11b+/F480+), neutrophil (Ly-6C/G+/F480-), T cell (CD4+ or CD8+), or dendritic cell (CD11c+) cell infiltration (data not shown).

WT mice produced more IFNγ and IL-10 mRNA at the infection site 2 weeks after *L. major* infection (Figure 5). In addition, IL-4 and IL-12 mRNA was elevated in WT mice compared to VDRKO mice. Inducible nitric oxide synthase (iNOS) was upregulated during *L. major* infection, however no differences between WT and VDRKO were detected. As previously reported [17], WT mice express more arginase mRNA than VDRKO mice (Figure 5).

3.5. Systemic Immune Responses in VDR KO and WT Mice. Flow cytometry analysis of the draining lymph nodes indicated that T-helper cells (CD4+), cytotoxic T-cells (CD8+), and FoxP3+ Treg (CD4+/CD25+) cells are elevated in VDRKO mice at most time points relative to WT animals (Suppl. Figure 1 which is available online at doi:10.1155/2012/134645). The number of each of these cell types increased as the lesions progressed and then decreased during healing. No obvious differences in other cell types such as macrophages (CD11b+/F480+), dendritic cells (CD11c+), or neutrophils (LY-6C/G+/F480-) were observed in the draining lymph nodes (data not shown).

VDRKO lymph node cells produced significantly lower amounts of inflammatory cytokines IFNγ, TNF-α, GM-CSF, and MIP-1α at the height of infection than the cells from WT mice (Suppl. Figure 2). Conversely, more MCP-1 was produced by lymph node cells from VDRKO mice compared to the cells from WT mice. Early after infection (2 weeks), cells from both mouse strains up regulated IL-4, IL-5, and IL-13, production that decreased by 12 wks post-infection (Suppl. Figure 2). Furthermore, VDRKO and WT lymph nodes generated equivalent amounts of IL-1β, IL-6, IL-10, Rantes, and KC at all time points.

Total IgG serum levels increased in both groups of mice as lesion development progressed and remained elevated even after the mice had healed (Figure 6). Significantly elevated levels of IgG2a/c were detected in VDRKO mice beginning at week 4 after infection and remained significantly elevated

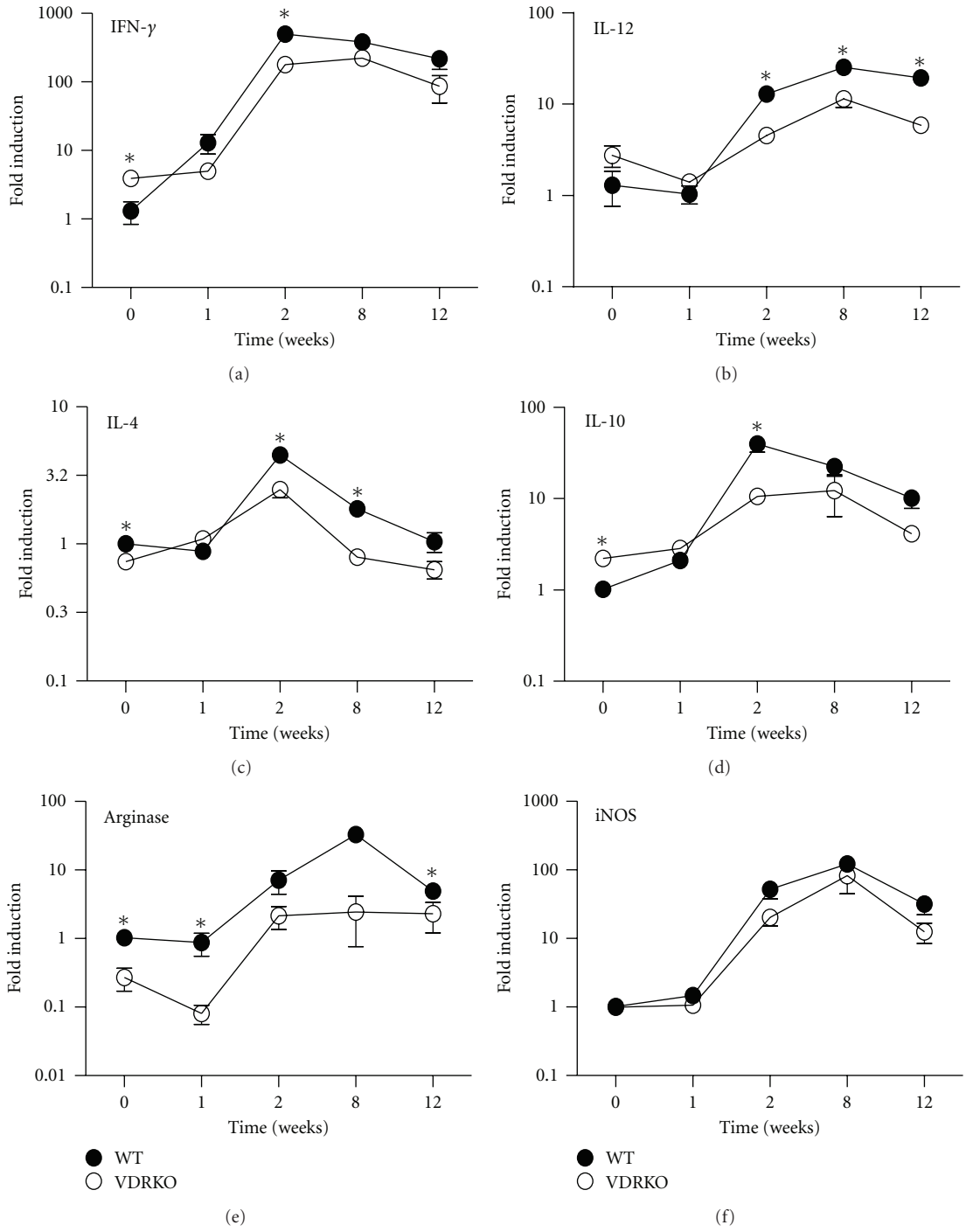

Figure 5: Quantitative RT-PCR analysis of cytokine production at the site of infection. C57BL/6 WT (closed circles) and VDRKO (open circles) mice were infected with 10^5 *L. major* parasites and their ears were harvested at the indicated time points. Quantitative RT-PCR was performed to determine the fold increase over uninfected WT ears for each cytokine. One representative of two independent experiments is presented. $^*P \leq 0.05$. $n = 4$ mice/time point.

throughout infection (Figure 6). No differences in IgG1 titers were observed between the VDRKO and WT mice at any time point (data not shown).

3.6. NO and IL-12 Production Are Increased in DC from VDRKO C57BL/6 Mice. NO production is a potent mechanism used by antigen presenting cells to eliminate *L. major* parasites. DC from both groups of mice were either left untreated or preincubated with combinations of IFN-γ and/or 1,25D3 prior to *L. major* infection and 48 hours after infection, cell culture supernatant was analyzed for levels of NO production. DC from VDRKO generate significantly more NO upon stimulation with IFNγ or IFNγ and 1,25D3 (Figure 7(a)). Preincubation of IFNγ or a combination

of IFNγ and 1,25D3 resulted in significantly more NO production as compared to untreated cells in both cell types. We did not observe an inhibition of NO production by the DC upon treatment with 1,25D3 in either the VDRKO or WT derived cells. In addition, there is no effect of either the VDR or 1,25D3 on NO production in macrophages (Suppl. Figure 3(a)).

Regardless of the IFNγ and/or 1,25D3 treatment, IL-12p40 production by VDRKO DC is significantly increased compared to WT DC, suggesting that VDR may contribute to regulation of IL-12p40 production by DC (Figure 7(b)).

4. Discussion

Leishmania are obligate intracellular parasites that are eliminated by a strong Th-1 host response. Vitamin D treatment reduces inappropriate Th-1 responses thus decreasing or eliminating symptoms of autoimmune diseases [6–8, 23]. As vitamin D exerts these effects through the VDR, we hypothesized that ablation of the VDR would tilt the Th-1/Th-2 balance towards a Th-1 bias and lead to enhanced resistance to *L. major* infection. Using a mouse model of cutaneous leishmaniasis, in which the parasites were injected into the ear dermis, we observed that VDRKO mice developed significantly smaller lesions and have fewer parasites at the site of infection during the height of infection than their WT counterparts. These results are similar to those observed by previous studies using a foot pad model of cutaneous leishmaniasis [17]. As VDRKO mice healed earlier than WT mice, we anticipated that VDRKO mice would exhibit decreased parasite loads compared to WT mice, because VDRKO mice resolve their lesions more quickly than WT mice. However, at 12 weeks after infection, VDRKO mice had elevated levels of parasites at the infection site even though they have decreased lesion size. This disparity may indicate that different processes are contributing to wound healing and parasite killing. The ability of VDRKO mice to produce increased dermal depositions of collagen may contribute to the increased healing phenotype we observed in VDRKO mice, as research indicates that orderly collagen fiber deposition in the skin is one factor that may contribute to increased healing in *L. major* infected mice [20].

Successful clearance of a *L. major* infection depends on initiation of a robust Th-1 response that leads to parasite killing via production of NO. VDRKO mice produce significantly lower levels of inflammatory cytokines locally at the infection site and following restimulation in vitro suggesting that VDRKO mice generate a decreased Th-1 response to *L. major* infection. We expected to detect upregulated transcription of iNOS in VDRKO mice as this enzyme leads to production of the NO necessary for killing of *L. major*; however, iNOS is not upregulated at any point post *L. major* infection in VDRKO compared to WT mice. Rather, arginase transcript levels are higher in WT compared to VDRKO mice, supporting the suggestion by others that upregulation of arginase antagonizes the metabolism of NO by competing with iNOS for a common substrate, L-arginine [17]. The lack of such competition would allow VDRKO mice to generate more NO than WT mice, leading to increased parasite killing. This hypothesis is further supported by studies demonstrating that inhibition of arginase by N-hydroxy-L-arginine, a precursor of NO, results in increased macrophage killing of *L. major* parasites *in vitro*, while induction of arginase contributes to the growth of *L. major* by providing the parasites with the polyamines required for replication [24, 25].

Toll like receptor activation initiates upregulation of VDR and CYP27B1 leading to an antimicrobial peptide response against *Mycobacteria* [10, 26], which contributes to killing of *M. tuberculosis* in human macrophages [27]. This pathway is unlikely to play a role in resistance to *L. major* as antimicrobial peptides have little effect in killing of *Leishmania* [28, 29]. VDR expression, treatment with IFNγ and/or vitamin D does not affect the ability of macrophage and DC to phagocytose *L. major* (data not shown and [17]). Furthermore, 1,25D3 treatment alone had no effect on IFNγ-induced NO production in infected macrophage and DC in vitro. This contrasts with other published data suggesting that NO production by *L. major* infected macrophages is inhibited by 1,25D3 [17]. These authors suggest that enhanced production of arginase competes with iNOS for a common substrate, ultimately resulting in decreased production of NO in macrophage. Stimulation of VDRKO macrophage with IFNγ and LPS resulted in significantly less NO than similarly treated WT macrophage (data not shown) suggesting that VDR may play a role in modulation of NO production in macrophage. Conversely, IFNγ-induced NO production by infected VDRKO DC was significantly higher than WT DC implying that VDR ablation increases rather than decreases NO production in DC. Our data suggests that vitamin D and the VDR differentially regulate NO production in macrophage versus DC.

In addition to overproducing NO, VDRKO DC overproduce IL-12p40 compared to WT. Other studies demonstrate that 1,25D3 inhibits IFNγ signaling in both T cells, macrophages and DC [17, 30–33]. Indirect inhibition of DC produced NO then could result via VDR inhibition of IFNγ activated STAT1 signaling. The ablation of the VDR results in elimination of this inhibition resulting in upregulation of NO and IL-12p40 production. As a similar increase of IL-12p40 and NO production was not observed in VDRKO macrophage this implies that macrophage differ from DC in their IFNγ induced production of IL-12p40.

VDRKO mice generate significantly more antigen specific IgG2a/c antibodies, that serve as an indicator of Th-1 biased immune responses [34, 35] than WT mice. The elevated IgG2a/c production in VDRKO mice is not surprising as treatment with 1,25D3 inhibits IgG2a production in mice [36], in cattle [37], and in pigs [38]. These data suggest that elimination of inhibitory effects of 1,25D3 skews antibody response towards production of IgG2a/c. The higher levels of IgG2a/c possibly explain the increased resistance to secondary infection with *L. major* observed in the VDRKO mice (Figure 1(b)). Indeed, elevated antigen specific and nonspecific induction of IgG2a/c has been observed in older VDRKO mice infected with *Listeria monocytogenes* [39]. However, the exact relationship between IgG2a/c production and vitamin D signaling remains to be elucidated.

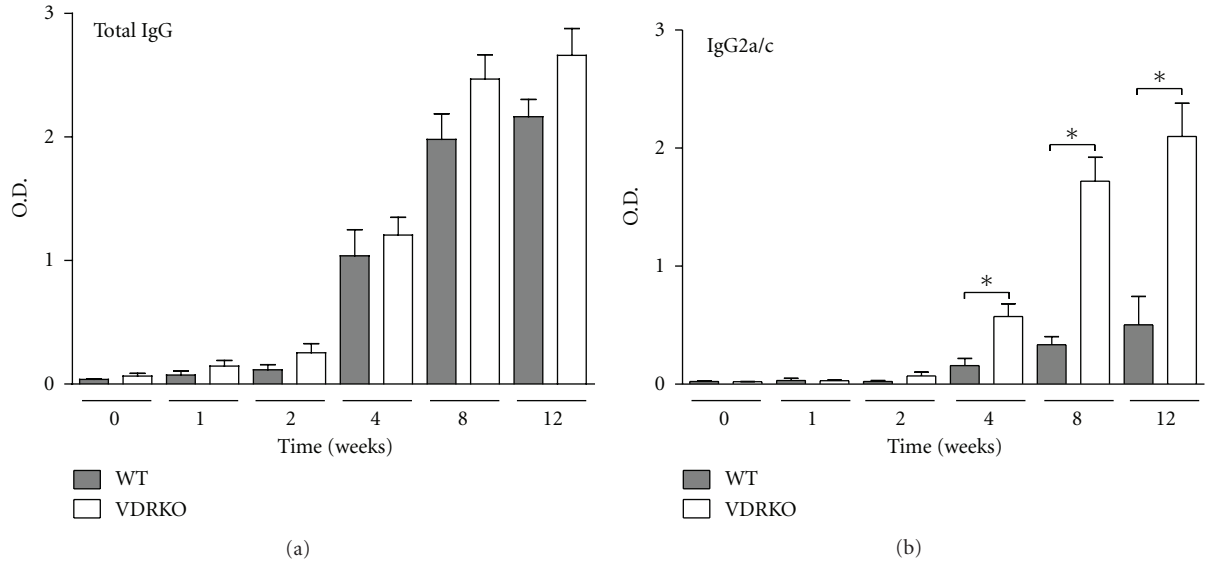

Figure 6: VDRKO mice exhibit elevated IgG2a/c production in response to *L. major* infection. C57BL/6 WT (closed bars) and VDRKO (open bars) mice were infected with 10^5 *L. major* parasites and serum samples were collected at the indicated time points. Isotype specific ELISA was performed to determine levels of total IgG and IgG2a/c in the serum. Mean ± SE is presented; $n = (8–10)$. $^*P \leq 0.05$. One representative of two independent experiments is presented.

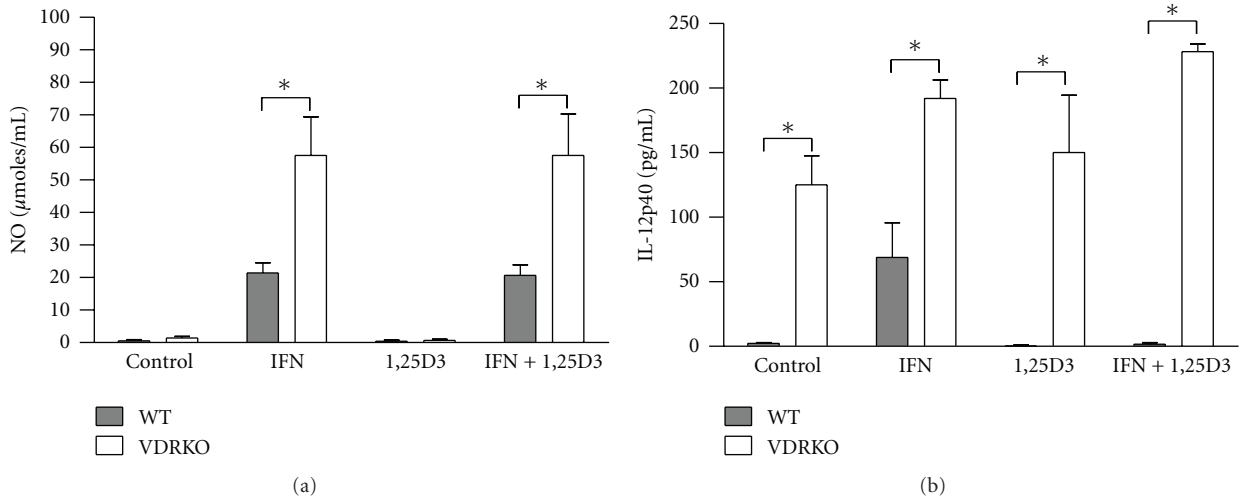

Figure 7: NO and IL-12p40 production by host cells. C57BL/6 WT (closed bars) and VDRKO (open bars) mice. DC were treated with IFNγ and/or 1,25D3 for 24 hr prior to being infected at a ratio of 5 : 1 with *L. major*. The cells were infected for 4 hr and washed to remove extracellular parasites. Supernatants were harvested 48 hr after infection and analyzed for production of NO (a) and IL-12p40 (b). Mean ± standard error is presented; $(n = 4)$. $^*P \leq 0.05$.

We demonstrate that neither genetic removal of the VDR or depletion of vitamin D from the diet (data not shown) of mice on the BALB/c background does not alter susceptibility to *L. major* infection. Susceptibility in WT BALB/c mice is attributed to their inability to mount a Th-1 response against infection. Our results indicate that the effect of the VDR may depend on the nature of the host immune response. Additional mechanisms, such as wound healing and genetic background, have also been shown to be important in resistance to *L. major* infection [20, 40]. Our data clearly show that the absence of vitamin D signaling cannot overcome the susceptibility traits of BALB/c mice.

Contrary to the results observed in VDRKO or CYP27B1KO mice, dietary deficiency of vitamin D in resistant C57BL/6 mice did not reduce the severity of *L. major* lesions (data not shown). A similar disparity has been observed in studies investigating the role of VDR deletion versus dietary vitamin D deficiency in the development of diabetes [23, 41] and MS [8, 42]. Our data may indicate that some of the effects of VDR on the course of *L. major*

infection are ligand independent, as has been shown in the case of alopecia [43], or that sufficient 1,25D3 persists in mice maintained on the deficient diet to activate the receptor.

In summary, we have demonstrated that VDRKO mice on the C57BL/6 background develop smaller lesions than WT mice upon infection with *L. major*, but this phenotype is not observed on the BALB/c genetic background. Enzymatic depletion of 1,25D3 also enhances resistance to *L. major* infection in C57BL/6 mice. The data further suggest that increased IL-12p40 and NO production by VDRKO dendritic cells may contribute to increased resistance to *L. major*. These studies indicate that ablation of VDR or elimination of its ligand, 1,25D3, is able to increase resistance to *L. major* infection, but only in a host that is predisposed for Th-1 immune responses. Our data confirm an important role for vitamin D for regulating immune responses that depend on Th-1 cells.

Acknowledgments

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Research Article

Modeling the Effects of Relapse in the Transmission Dynamics of Malaria Parasites

Ricardo Águas,[1] Marcelo U. Ferreira,[2] and M. Gabriela M. Gomes[1]

[1] *Instituto Gulbenkian de Ciência, 2781-901 Oeiras, Portugal*
[2] *Department of Parasitology, Institute for Biomedical Sciences, University of São Paulo, 05508-000 São Paulo, SP, Brazil*

Correspondence should be addressed to Ricardo Águas, rjaaguas@gmail.com

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Often regarded as "benign," *Plasmodium vivax* infections lay in the shadows of the much more virulent *P. falciparum* infections. However, about 1.98 billion people are at risk of both parasites worldwide, stressing the need to understand the epidemiology of *Plasmodium vivax*, particularly under the scope of decreasing *P. falciparum* prevalence and ecological interactions between both species. Two epidemiological observations put the dynamics of both species into perspective: (1) ACT campaigns have had a greater impact on *P. falciparum* prevalence. (2) Complete clinical immunity is attained at younger ages for *P. vivax*, under similar infection rates. We systematically compared two mathematical models of transmission for both Plasmodium species. Simulations suggest that an ACT therapy combined with a hypnozoite killing drug would eliminate both species. However, *P. vivax* elimination is predicted to be unstable. Differences in age profiles of clinical malaria can be explained solely by *P. vivax*'s ability to relapse, which accelerates the acquisition of clinical immunity and serves as an immunity boosting mechanism. *P. vivax* transmission can subsist in areas of low mosquito abundance and is robust to drug administration initiatives due to relapse, making it an inconvenient and cumbersome, yet less lethal alternative to *P. falciparum*.

1. Introduction

Plasmodium falciparum has traditionally been the main focus of malaria control programs worldwide, mainly because this parasite is the major cause of severe morbidity and mortality in tropical Africa. However, at a time when global eradication is advocated as the ultimate goal of malaria control strategies worldwide, *P. vivax* needs to be given much more attention from researchers, policy makers, and funding agencies. *P. vivax* is a major public health challenge in Central and South America, the Middle East, Central, South, and Southeast Asia, Oceania, and East Africa, where 2.85 billion people are currently at risk of infection [1] and as many as 250 million infections may be due to this species each year [2]. The emergence of drug-resistant strains and severe (sometimes fatal) disease challenges the traditional view of vivax malaria as a benign infection [3].

Although cytoadhesion of *P. vivax*-infected erythrocytes to endothelial cells has been recently demonstrated and might contribute to the pathogenesis of severe vivax malaria [4], this phenomenon is likely to be much less common than with *P. falciparum* infections [5]. Compared to *P. falciparum*, *P. vivax* has a slightly longer incubation period (12 days to several months) and a similarly phased erythrocytic cycle (42–48 hours) that yields fewer merozoites per schizont [6]. However, the distinct ability of *P. vivax* to stay dormant in host's liver cells and cause relapses weeks or months after the primary infection is the most striking difference between *P. vivax* and *P. falciparum*. Although the molecular mechanism of relapse remains undisclosed, progression of the parasite through its life cycle is fairly well described [5, 6]. A proportion of sporozoites remain dormant in the liver, as hypnozoites, for prolonged periods of time before developing and causing recurrent infection.

The ability to relapse is thought to render *P. vivax* resilient to eradication efforts. In fact, in areas where both species are present, ACT (artemisinin-based combination therapy) campaigns have had a greater impact on *P. falciparum* than on *P. vivax* prevalence [7, 8]. We demonstrate

how this is evident when contemplating elimination scenarios, with *P. vivax* elimination being extremely difficult to achieve by mass drug administration.

Immunity to human malaria is largely species specific. Epidemiological studies have accumulated evidence that clinical (antidisease) and antiparasite immunity is attained at younger ages for *P. vivax*, when compared to *P. falciparum*, under similar infection rates [9–11]. This suggests that immunity is acquired through different mechanisms [12]. We put forward an alternative hypothesis arguing that differences between the observed age profiles lie in the characteristic life cycles of both parasites. Explicitly, we propose that the ability of *P. vivax* to relapse can accelerate the piecemeal acquisition of clinical immunity.

2. Methods

We developed a model representing the transmission dynamics of *P. vivax* by adding new elements to the foundation laid by previous work in *P. falciparum* [13]. We have thus a model structure for *P. vivax* transmission, represented by Figure 1(a), which contains the topology representing *P. falciparum* as a submodel (Figure 1(b)). The falciparum dynamics are retrieved by equating p_1 to 1 in the *P. vivax* model. Natural history of *P. vivax* infection is generally similar to that of *P. falciparum*, with a few but crucial idiosyncrasies.

Susceptible individuals (S) are subject to a certain rate of infection (here represented by the force of infection Λ), which depends on local environmental and socioeconomic factors. In *P. vivax*, after a mosquito infectious bite, an indeterminate proportion of the inoculated sporozoites remains dormant in the liver, whilst the remaining develops into erythrocyte invading merozoites. The model describing the transmission dynamics of *P. vivax* must then include a latent class, representing those individuals who, after recovering from infection, keep a remnant of dormant liver forms, called hypnozoites (subject to reactivation at rate ω) rather than clearing all parasites while acquiring clinical immunity. Reactivation is still a rather cryptic process, and most relapses seem to result from activation of heterologous hypnozoites [14], which suggests that genotype-specific immunity somehow modulates the occurrence of relapses, much to the resemblance of how the clinical outcome of a given infection is determined [15]. Here, parameter p_1 accounts for episodes not followed by a relapse, either because no hypnozoites were formed or because the remaining hypnozoites do not reactivate during their lifespan. For illustration purposes, throughout the paper we keep $p_1 = 0.25$ for *P. vivax* (and $p_1 = 1$ for *P. falciparum*).

It is generally accepted that the severity of malaria episodes decreases as the host accumulates exposures to the parasite. We implement this aspect of malaria immunity by discretizing the malaria clinical spectrum into two compartments: I_1 represents the severe end of the spectrum, and is labeled "clinical malaria," while I_2 represents the less severe part and is labeled "asymptomatic malaria". Naturally, there is a degree of arbitrariness in this compartmentalization,

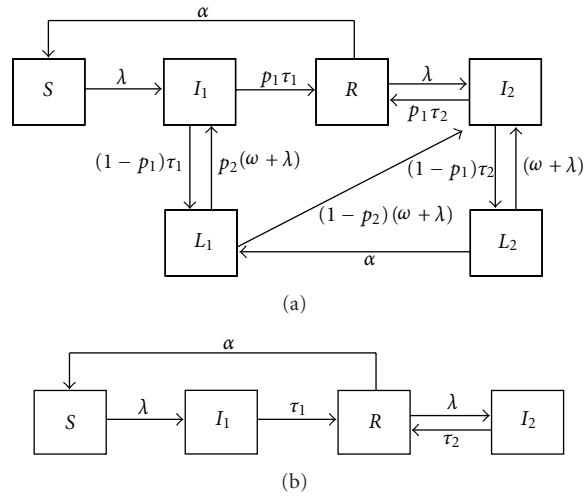

Figure 1: Plasmodium transmission dynamics. (a) Illustration of the natural history of infection with the *P. vivax* parasite. The variables represent a classification of the population at any given age and time into six states: completely susceptible (S); clinical malaria resulting from an infection in a completely susceptible individual (I_1); recovered with clinical immunity without any hypnozoites (R); mild or asymptomatic infection resulting from exposure of recovered individuals (I_2); recovered with a certain degree of clinical immunity, carrying hypnozoites (L_1); recovered with clinical immunity, carrying hypnozoites (L_2). Description and values for the parameters can be found in Table 1. (b) *P. falciparum* transmission dynamics. This is a subset of the previous system which is retrieved by making $p_1 = 1$.

and the results should be interpreted in this context. We consider that immunologically naïve individuals will display clinical symptoms when infected (I_1). Although *P. vivax* infections are generally not as severe as those caused by *P. falciparum*, they are far from benign. Community studies have revealed that the proportion of *P. vivax* infections presenting with fever is similar to the one registered for *P. falciparum* [16]. Relapse originates either new clinical episodes or asymptomatic infections in accordance with empirical data [17]. The probability of clinical outcome upon relapse in individuals that kept hypnozoites (L_1) from a previous clinical infection is determined by parameter p_2. Latent individuals carrying hypnozoites are subject to reinfection at rate Λ, with the resultant infection phenotype being determined by parameter p_2 as well.

Individuals who have just recovered from a clinical malaria episode are said to have acquired temporary clinical immunity (R). This means that they do not display clinical symptoms upon reinfection and that, unless they are challenged again within a given time frame, they will lose that clinical protection. The same applies to those who have just recovered from an asymptomatic infection. In fact these infections are crucial in boosting acquired clinical immunity, and the interplay between the rate of infection in clinically immune individuals and the rate of clinical immunity loss is fulcrum in determining the number of expected clinical malaria cases during one's lifespan [18]. As such, those

Table 1: Model parameters.

Parameter	Description	Value
μ	Birth and death rate	0.02 years^{-1}
β	Transmission coefficient	varying
λ	Force of infection	varying
τ_1	Recovery rate from clinical infection	14.12 years^{-1}
τ_2	Recovery rate from asymptomatic infections	2.23 years^{-1}
ω	Relapse rate	12 years^{-1}
α	Rate of loss of clinical immunity	1.07 years^{-1}
p_1	Proportion of vivax infections which recover with hypnozoites	varying
p_2	Proportion of relapses which are clinical	varying

recovering from an asymptomatic infection are also subject to loss of immunity at rate α. These include individuals that either clear all parasite forms and return to R (a proportion p_1) or keep a remnant of hypnozoites and go to the L_2 class. We consider that, in the latter case, subsequent infections and relapse will give rise to asymptomatic malaria.

The described dynamics can be written as the following system of differential equations:

$$\begin{aligned}
\frac{\partial S}{\partial t}+\frac{\partial S}{\partial a} &= \alpha R-(\lambda(a)+\mu)S,\\
\frac{\partial I_1}{\partial t}+\frac{\partial I_1}{\partial a} &= \lambda(a)S+p_2(\omega+\lambda(a))L_1-(\tau_1+\mu)I_1,\\
\frac{\partial R}{\partial t}+\frac{\partial R}{\partial a} &= p_1\tau_1 I_1+p_1\tau_2 I_2-(\alpha+\lambda(a)+\mu)R,\\
\frac{\partial I_2}{\partial t}+\frac{\partial I_2}{\partial a} &= \lambda(a)R+(\omega+\lambda(a))L_2\\
&\quad +(1-p_2)(\omega+\lambda(a))L_2-(\tau_2+\mu)I_2,\\
\frac{\partial L_1}{\partial t}+\frac{\partial L_1}{\partial a} &= (1-p_1)\tau_1 I_1+\alpha L_2-(\omega+\lambda(a)+\mu)L_1,\\
\frac{\partial L_2}{\partial t}+\frac{\partial L_2}{\partial a} &= (1-p_1)\tau_2 I_2-(\omega+\lambda(a)+\alpha+\mu)L_2,
\end{aligned} \tag{1}$$

with boundary conditions at age $a = 0$: $S(t,0) = \mu$ and $I_i(t,0) = L_i(t,0) = R(t,0) = 0$ for $i = 1, 2$.

The *P. falciparum* transmission dynamics are retrieved by making $p_1 = 1$:

$$\begin{aligned}
\frac{\partial S}{\partial t}+\frac{\partial S}{\partial a} &= \alpha R-(\lambda(a)+\mu)S,\\
\frac{\partial I_1}{\partial t}+\frac{\partial I_1}{\partial a} &= \lambda(a)S-(\tau_1+\mu)I_1,\\
\frac{\partial R}{\partial t}+\frac{\partial R}{\partial a} &= \tau_1 I_1+\tau_2 I_2-(\lambda(a)+\alpha+\mu)R,\\
\frac{\partial I_2}{\partial t}+\frac{\partial I_2}{\partial a} &= \lambda(a)R-(\tau_2+\mu)I_2.
\end{aligned} \tag{2}$$

The force of infection was constructed as an age-dependent parameter [13]

$$\lambda(a) = \lambda_0\left(1-ce^{-ka}\right). \tag{3}$$

The function is strictly increasing with age, with a minimum $\lambda_0(1-r)$ (at age zero) converging asymptotically to λ_0 as age increases. Parameter k determines how steeply the force of infection increases with age, and r controls the magnitude of that increase. A summary measure of transmission is obtained by integrating the force of infection over age as

$$\Lambda = \int \lambda(a)P(a)da, \tag{4}$$

where $P(a) = \mu e^{-\mu a}$ is the total population distributed over age and μ is the birth and death rate. Adopting standard assumptions, Λ is proportional to the frequency of infectious individuals, the proportionality constant being the transmission coefficient,

$$\beta = \frac{\Lambda}{I_1+I_2}. \tag{5}$$

This standard assumption allows us to analyze how the equilibrium behavior of the system depends on β, which is a critical transmission parameter, representing the sylvatic portion of the classical Macdonald formulation of the basic reproduction number for vector-borne diseases [19]. The basic reproduction number for the *P. vivax* model presented here assumes the form

$$R_0 = \frac{\beta(\varepsilon p_2 N_1+\tau_1\varepsilon p_1 N_2+\tau_2\varepsilon p_1 N_3+\mu(\varepsilon+\mu)N_4+\tau_1\varepsilon N_5)}{\varepsilon^2\mu D_1+\varepsilon\mu\tau_2 D_2+\tau_1\alpha D_3+\tau_1\varepsilon^2 p_1 D_4+\mu^2 D_5+\mu^3 D_6}, \tag{6}$$

where

$$\begin{aligned}
N_1 &= \tau_1(1-p_1)(-\alpha-\mu)+\alpha\tau_2,\\
N_2 &= \varepsilon p_2-\alpha-\mu,\\
N_3 &= \mu+\alpha+\varepsilon-\alpha p_2,\\
N_4 &= (\varepsilon+\mu)+\alpha+\tau_2,\\
N_5 &= \alpha+\mu-\varepsilon(p_1+p_2)+\varepsilon,\\
D_1 &= \tau_1+\tau_2 p_1-\tau_1 p_2,\\
D_2 &= \alpha(p_2+p_1-p_1p_2)+\tau_1(1+p_1p_2+p_1-p_2),\\
D_3 &= \mu(\varepsilon+\tau_2-p_2\varepsilon+p_2\varepsilon p_1)+\varepsilon\tau_2 p_1,
\end{aligned}$$

$$D_4 = \tau_2 - p_2\tau_2 + p_2\mu + p_2\tau_2 p_1,$$
$$D_5 = (\tau_1 + \varepsilon)(\alpha + \tau_2) + \tau_2(\alpha + \varepsilon p_1) + \varepsilon\tau_1(2 - p_2 - p_2 p_1 + \varepsilon^2),$$
$$D_6 = \mu + \tau_2 + \alpha + \tau_1 + 2\varepsilon. \quad (7)$$

Epidemiological changes are often attributed to thresholds in transmission, and these are detected through longitudinal trends and comparative studies across multiple communities. We consider the transmission coefficient, β (or the basic reproduction number, R_0) as a control parameter and describe the significance of these indices of transmission on selected epidemiological variables. To do so, we calculated the endemic equilibria for systems (1) and (2), without age dependence. Age profiles were obtained by following a cohort under the pressure of an age-dependent force of infection defined by (3), for 20 years. The common variables between systems (1) and (2) have the same boundary conditions. We used the escalator boxcar train (EBT) technique to simulate the dynamics in our age-structured population [20].

To assert the benefits of specific interventions we first simulate our age-structured model in equilibrium conditions to obtain the age profile of clinical disease prevalence without intervention. We use that age profile as the initial condition for the simulation in which the drug administration trial is in vigor. The simulated antimalarial treatment consists of treating every infected individual, regardless of symptoms. In such a setup, asymptomatic malaria is treated as effectively as clinical malaria, and thus the recovery rate from an asymptomatic infection, τ_2, takes the same value as the recovery rate from a clinical infection, τ_1.

3. Results

The expected prevalence of malaria cases of each Plasmodium species is highly dependent on local environmental and socioeconomic factors that can be summarized into some transmission index. In Figures 2(a) and 2(c), infectious proportions are plotted in terms of a transmission coefficient, β, which encapsulates information on contact rates and infectivity. Comparing the model outputs for the *P. falciparum* (red) and *P. vivax* (blue) systems, we verify that, if a significant proportion of individuals recovers from infection with dormant forms of the parasite which reactivate later on, it is easier for the parasite to be transmitted in a sustainable manner (Figure 2(a)). Latency then generates a mechanism by which the parasite population can be maintained in scenarios where vectors are not very abundant or where human-mosquito contacts are sparse. Strikingly, for any value of transmission coefficient that sustains both species, the proportion of individuals with a clinical episode due to *P. vivax* is lower than that due to *P. falciparum* (Figure 2(a)), while the overall parasite prevalence (measured as proportion of individuals in the population which carry parasites in the blood stream and are thus potentially infectious) is expected to be higher for *P. vivax* (Figure 2(c)). Figures 2(b) and 2(d) display how equilibrium solutions depend on the basic reproduction number, R_0, as a proxy for transmission.

Figure 3 simulates the introduction of malaria control measures in an area supporting *P. vivax* transmission versus in an area supporting *P. falciparum* transmission, with the same parasite prevalence. The intervention consists of applying an MDA (mass drug administration) campaign using ACT aimed at reducing the infectious period of asymptomatic infections, making all infections, regardless of clinical outcome, last the same. Figure 3 portrays that by treating asymptomatic infections equally for both species one can reduce falciparum prevalence (red lines) to a much greater extent than vivax prevalence (blue lines) within the same time frame (dot-dashed lines). This intervention alone leads to sustained elimination of *P. falciparum* if implemented for 274 days (time for the system to enter the basin on attraction of the disease-free equilibrium). In the case of *P. vivax*, elimination is only possible through a stochastic event, and even then the risk of reemergence will be high as the disease-free equilibrium is unstable for this system. If a drug that can eliminate the dormant forms of the parasite (Primaquine) is included in the MDA strategy, then the *P. vivax* dynamics approach those for *P. falciparum*. However, once the intervention is halted the risk for reemergence would still be much higher for *P. vivax* due to instability of the disease-free equilibrium.

In Figure 4, we simulate the age profiles for *P. vivax* and *P. falciparum* for an equal risk of infection (λ_0). The predicted age profiles for *P. vivax* (blue) display a higher value for clinical malaria cases at the peak, when compared with *P. falciparum* (red), and reveal a decrease in the average age at infection. This means that the risk of a *P. vivax* episode relative to the risk of having a *P. falciparum* episode is greater in very young children and lower throughout childhood. This is consistent in the two transmission settings chosen for this illustration as well as for the entire transmission spectrum.

4. Discussion

The true impact of *P. vivax* transmission on human populations stands in the shadow of the overwhelming mortality and morbidity burden exerted by *P. falciparum* worldwide. *P. vivax* importance has been increasingly recognized over the years, and new estimates of the global malaria burden revealed that there are slightly more people at risk of having a *P. vivax* infection than a *P. falciparum* infection [1]. However, probably more interesting is to consider the importance and impact of *P. vivax* under the scope of its ecological interactions with *P. falciparum*, especially considering that coinfection with these species might somehow modulate the clinical outcome of infection [21–23], and that there might be cross-specific immunity [24–27]. Understanding the transmission dynamics of *P. vivax* is crucial to understand the current epidemiological scenario and the potential long-term impact of control interventions.

We have previously developed a mathematical model to represent the dynamics of *P. falciparum* transmission

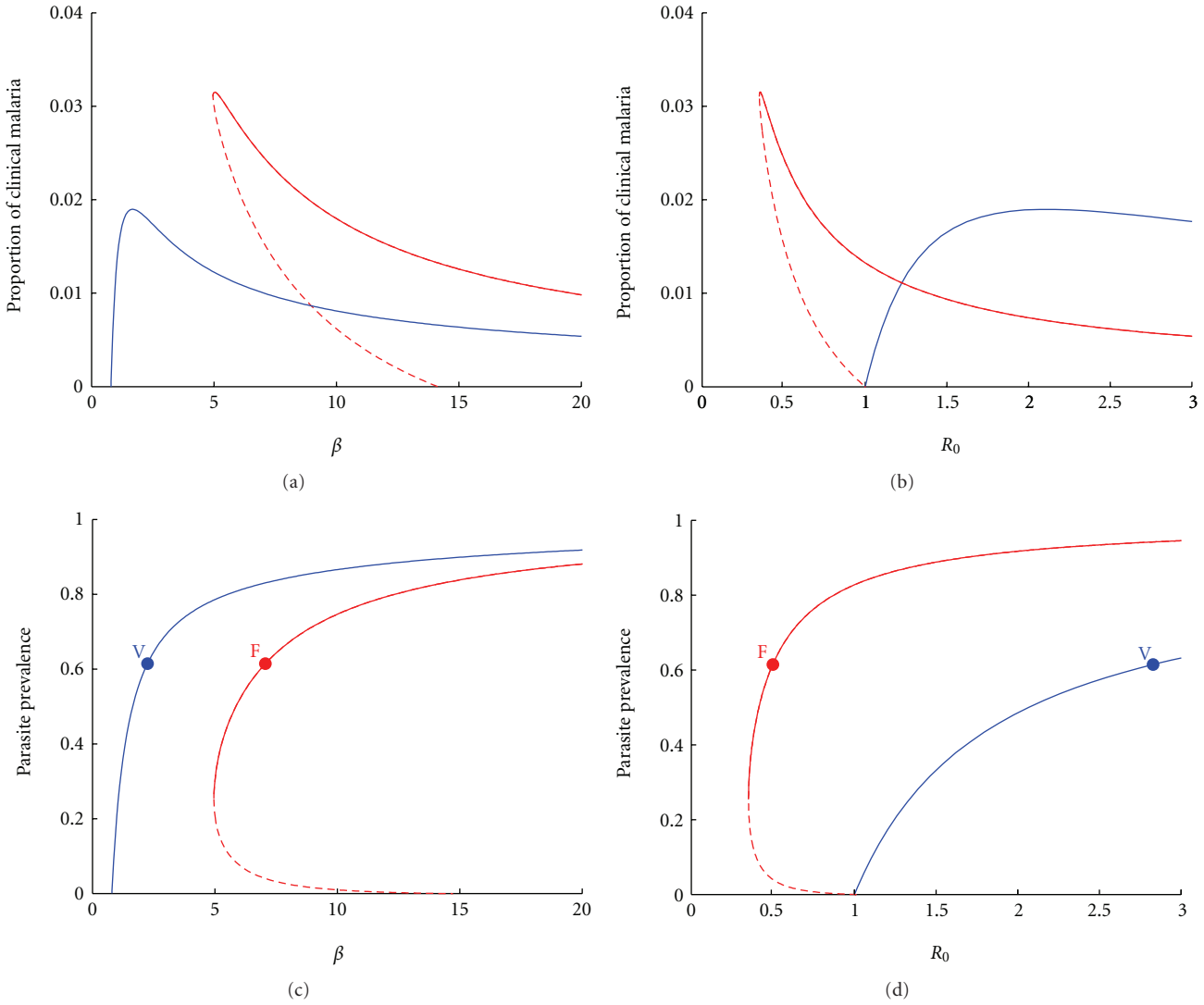

Figure 2: Expected clinical malaria episodes and parasite prevalence at equilibrium, for both *P. vivax* and *P. falciparum*. (a) Bifurcation diagram, showing the influence of β on the equilibrium levels of clinical malaria for *P. vivax* (blue) and *P. falciparum* (red). (c) Influence of β on the equilibrium levels of parasite prevalence ($I_1 + I_2$) for *P. vivax* (blue) and *P. falciparum* (red). (b) and (d) are similar to (a) and (c), respectively, but use R_0 as control parameter. For *P. vivax*, we used $p_1 = p_2 = 0.25$. Dashed lines represent unstable equilibrium solutions, whilst full lines refer to stable endemic equilibria.

in human populations [13]. The model was calibrated on hospitalization data from 8 endemic regions in sub-Saharan Africa [28], estimating a fundamental difference between the duration of clinical and asymptomatic infections. This result led to the identification of a deterministic elimination threshold for *P. falciparum* malaria in areas of low to moderate transmission. We adopted the same generic model for *P. vivax* while adding latency classes to represent those individuals who recover from infection with a remnant of dormant parasites, called hypnozoites. These parasites can reactivate at any given time causing a relapse.

Model outputs were generated and compared with and without latent classes, to mimic vivax and falciparum, respectively, while all other features were unchanged. The expected levels of clinical episodes of both *P. vivax* and *P. falciparum* for the same levels of the transmission coefficient, β, indicate that *P. vivax* transmission can be sustained for much lower values of this parameter, when compared with *P. falciparum* (Figure 2(a)). This becomes intuitive in light of *P. vivax*'s ability to relapse, which can transform a single infectious bite into more than one malaria episode leading to higher parasite prevalence in the *P. vivax* system (Figure 2(c)). These relationships are inverted, however, when parasite prevalence is represented against the basic reproduction number, R_0, (Figures 2(b) and 2(d)) attesting the importance of standardizing transmission indices. More importantly, we have uncovered a qualitative change due to the latency classes. The deterministic elimination threshold described for the *P. falciparum* system is no longer present under the conditions simulated for *P. vivax*. The parameter

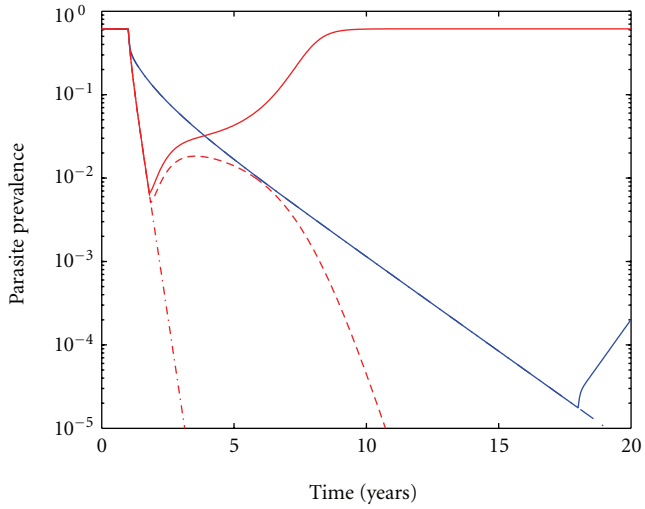

Figure 3: Mass drug administration (MDA) strategy applied in regions where *P. falciparum* (red) and *P. vivax* (blue) are equally prevalent. The initial parasite prevalence is equal for both *P. vivax* and *P. falciparum* as highlighted Figures 2(c) and 2(d) by V and F, respectively. Asymptomatic infections are treated at a constant rate so they, on average, last as long as a clinical case. The red solid and dashed lines display interventions with durations just below and above the deterministic elimination threshold, respectively, for *P. falciparum*. For *P. vivax*, the model indicates no deterministic elimination threshold. The blue solid line represents continuing the intervention for 18 years and then halting, under $p_1 = p_2 = 0.25$. The blue and red dot-dashed lines represent uninterrupted 20-year MDA campaigns.

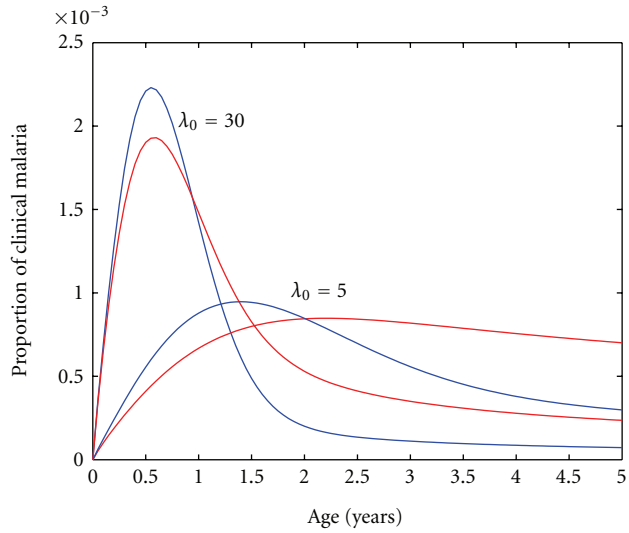

Figure 4: Age profiles for two transmission settings. Clinical *P. vivax* malaria age profiles (blue lines) compared with *P. falciparum* profiles (red lines) for equal risks of infection.

regime sustaining the bistability phenomenon that gives rise to the elimination threshold is contracted when latency comes into play.

A major advance in the search for effective malaria drug treatment, following the demise of most known drugs in the battle against resistant parasites, came in the form of artemisinin, a very potent and effective drug against chloroquine and sulphadoxine-pyrimethamine-resistant infections, which can clear parasites and resolve fever faster than any other licensed antimalarial [29]. However, artemisinin derivatives have a very short half-life, translating into substantial treatment failures when used as monotherapy [30], which motivated the combination of artemisinin with longer-lasting partner drugs in the so-called Artemisinin Combination Therapies (ACTs), assuring that there is substantial antimalarial pressure to deal with the residual parasite biomass that may persist when the artemisinin derivatives have fallen below therapeutic levels [31]. Curiously, empirical studies have revealed that the deployment of ACT as a control strategy affects *P. falciparum* transmission much more than it does *P. vivax* transmission [8, 9]. Furthermore, unexpected resurgences of *P. vivax* malaria in areas where elimination attempts were thought to have been successful question to what extent *P. vivax* control is sustainable [32, 33]. Figure 3 supports these results in emphatic fashion. Our simulations suggest that, by treating all infections (equally for both species) with an ACT therapy, one can reduce falciparum prevalence to a much greater extent than vivax prevalence (in the same time scale). This is intrinsically associated with ACT's inability to kill the dormant forms of *P. vivax*, which escape drug action, subsist, and can cause a relapse later on, thus sustaining the parasite pool. In such scenario one should invoke the use of drugs targeting hypnozoites (Primaquine is the only approved and available drug at the moment) as a means of counteracting the parasite's ability to relapse. If everyone was to receive a dose of Primaquine to kill the hypnozoite parasite forms, the dynamics would be similar to that of *P. falciparum*. A remaining difference, however, is that *P. vivax* elimination is predicted to be unstable, meaning that any perturbation in the system (introduction of infectious individuals from neighboring populations for instance) would drive it back to the endemic equilibrium.

Another epidemiological observation that deserves careful consideration is the differential speed at which complete clinical immunity is attained when comparing *P. vivax* to *P. falciparum* for scenarios of equal risk of infection [9–11]. While others have interpreted this phenomenon as an evidence of there being different mechanisms by which immunity is acquired [12], our results suggest that the difference in age profiles of clinical malaria can be accounted for by the intrinsic transmission dynamics inherent to the natural history of infection of each species. In Figure 4, we can clearly see that the model topologies we used to describe the *P. vivax* and *P. falciparum* transmission dynamics can account for differences in the age profiles of clinical malaria, specifically for low levels of p_1 and p_2. We then propose that differences in age profiles of clinical malaria can be explained solely by *P. vivax*'s ability to relapse, which converts a single infectious mosquito bite into one or more malaria infections, thus accelerating the acquisition of clinical immunity. Relapse also serves as an immunity

boosting mechanism that prevents onsets of malaria episodes in older ages.

On a final note, we recall that all parameters other than those governing hypnozoite formation and relapse were unchanged between the two scenarios. *P. vivax* and *P. falciparum* transmission dynamics might, however, be modulated by a number of biological characteristics such as gametocyte production and antigenic variation. Although our model was not designed to explore these processes, some analogies can be made as detailed in the Supplementary Material available online at doi:10.1155/2012/921715. Two aspects are especially urging. First, it should be acknowledged that *P. vivax* produces gametocytes earlier than *P. falciparum* during a human infection and these gametocytes are shorter lived in the bloodstream [34]. As a consequence, vivax patients are expected to have transmitted more by the time malaria infection is confirmed and treatment is provided. Second, the acquisition of immunity to *P. vivax* relies on expectations regarding strain specificity of natural immunity and antigenic similarity between primary infections and relapses that deserve further attention [35, 36].

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Review Article

Comparison of Protective Immune Responses to Apicomplexan Parasites

Sonja Frölich,[1] Rolf Entzeroth,[2] and Michael Wallach[1]

[1] *The ithree Institute, University of Technology Sydney, P.O. Box 123, Broadway, Sydney, NSW 2007, Australia*
[2] *Institute of Zoology, Technical University Dresden, Mommsenstraße 13, 01062 Dresden, Germany*

Correspondence should be addressed to Michael Wallach, michael.wallach@uts.edu.au

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Members of the phylum Apicomplexa, which includes the species *Plasmodium, Eimeria*, *Toxoplasma,* and *Babesia* amongst others, are the most successful intracellular pathogens known to humankind. The widespread acquisition of antimicrobial resistance to most drugs used to date has sparked a great deal of research and commercial interest in the development of vaccines as alternative control strategies. A few antigens from the asexual and sexual stages of apicomplexan development have been identified and their genes characterised; however, the fine cellular and molecular details of the effector mechanisms crucial for parasite inhibition and stimulation of protective immunity are still not entirely understood. This paper provides an overview of what is currently known about the protective immune response against the various types of apicomplexan parasites and focuses mainly on the similarities of these pathogens and their host interaction. Finally, the evolutionary relationships of these parasites and their hosts, as well as the modulation of immune functions that are critical in determining the outcome of the infection by these pathogenic organisms, are discussed.

1. Introduction

Parasitic protozoans of the phylum Apicomplexa are the most prevalent and successful pathogens known to humankind. Today, half of the world's population is at risk of malaria caused by four *Plasmodium* species [1], and more than 50 billion livestock reared for food production suffer from debilitating intestinal diseases caused by many species of *Eimeria, Theileria,* and *Babesia*, amongst others [2]. *Eimeria* is the cause of coccidiosis in chickens, a parasite that infects the intestinal mucosa of the infected bird leading to severe weight loss and even death of the host. Cryptosporidiosis is caused by *Cryptosporidium* species and, like *Eimeria,* is transmitted by accidental ingestion of highly resistant and environmentally stable oocysts that contaminate the food and water. The disease is marked by self-limiting diarrhoea in immunocompetent individuals, but in immunocompromised patients, the disease can be fatal. *Babesia* is related to the malaria parasite in that it infects the reticulocytes of the infected cow and causes severe pathology and can cause death as well. *Toxoplasma* is the cause of toxoplasmosis in humans, a disease characterized by mild flu-like symptoms in healthy hosts. However, immunocompromised individuals, such as HIV/AIDS patients and organ transplant recipients, often suffer from ocular toxoplasmosis or even encephalitis. *Theileria*, an important cattle parasite transmitted by ticks, is characterized by anaemia and high mortality rate especially in pregnant cows.

Plasmodium infects red blood cells and is the cause of malaria in humans as well as in several other vertebrate and bird species. Nearly one million human deaths are attributed to malaria each year, meaning that every 30 seconds a child dies of this disease in Africa. This high toll in human and animal life and wellbeing has been further exacerbated by the inappropriate use of antimicrobial compounds over the years. Thus, widespread resistance to most (if not all) drugs used to date makes control of these parasites extremely difficult [3, 4]. The novel artemisinin-based therapies are considered to be the new hope for malaria control and have proved to be successful in interrupting the maturation

of the infectious stages (oocysts) in the related parasite, *Eimeria*, thereby reducing or blocking the transmission and spread of the parasite [5–7]. However, there are fears that overuse of these novel compounds will also facilitate the selection of even more potent strains.

Over the past three decades, a number of putative protective antigens from several members of the phylum has attracted a great deal of research and commercial interest in the hope to develop vaccines and alleviate the burden on public health and world economy imposed by this class of parasites [8–17]. The first antiprotozoan subunit vaccine developed to date, CoxAbic, which contains antigens isolated from the sexual stages of development of *Eimeria* is a proof of principle for transmission-blocking immunity and an example of a strategy that has been proved successful in helping to tackle one of these important apicomplexan diseases [18–21].

Despite the enormous efforts to characterise the apicomplexan immunostimulatory antigens and genes encoding them, the fine cellular and molecular details of the effector mechanisms crucial for parasite inhibition and stimulation of protective immunity are still not fully understood. It is hoped that unravelling the proteomes and genome sequences of these protozoan pathogens will facilitate our understanding of the mechanisms involved in the infectious process and lead to the design of new effective control strategies [22].

Early studies concerning the developmental biology and immunology of the Apicomplexans have provided valuable insights into the immune mechanisms responsible for the inhibition of parasite growth and development and in the establishment of host resistance to infection [23–27]. Efforts by research laboratories across the globe have demonstrated that, in order to control the infection, both the innate and adaptive arms of the immune system are crucial for resistance and cross-protection [28]. This paper provides an overview of Apicomplexan biology and focuses on the protective immune response against the various types of apicomplexan parasites, from *Eimeria* to *Plasmodium* including *Toxoplasma, Cryptosporidium*, *Theileria,* and *Babesia*. It also addresses the evolutionary relationship of these parasites and their hosts and the modulation of the host immune response that are critical in determining the outcome of an infection.

2. Apicomplexan Life Cycle and Parasite-Host Relationship

The apicomplexan life cycle includes both asexual multiplication (schizogony, merogony) and sexual reproduction (gametogony) [25, 29, 30]. While some members of the phylum require an intermediate host and a variety of cell types to complete their developmental life cycle (i.e., *Plasmodium, Babesia, Theileria, Toxoplasma*), others lead a monoxenous life style with the asexual and sexual stages of development restricted to specific tissues of a single host (i.e., *Eimeria* and *Cryptosporidium* species). Thus, the possibility of culturing asexual stages in vitro [31], as well as the feasibility of isolating relatively large numbers of sexual forms (gametocytes), has granted *Eimeria* species a status of an attractive and a relatively simple model for investigating parasite-host interactions, as well as applying transmission-blocking immunity [32].

Apicomplexan parasites affect all classes of vertebrates, including fish, amphibians, reptiles, birds and mammals. The apparently long coevolutionary history of the apicomplexans means that targeting the metabolic reactions and pathways of the parasite also harms the host, making the identification of therapeutic targets extremely difficult. Recent advancements in molecular biology have shed new light into the coevolutionary history of the apicomplexa and their hosts. This is based on the finding that the branching of the evolutionary tree of at least some of these parasites coincided with the evolution of the vertebrate host [33]. In addition, it was found that several enzymes involved in a variety of metabolic pathways are highly conserved between the parasite and its host. Furthermore, comparative studies of whole genome nucleotide sequences in several members of the phylum have revealed that surface proteins, unlike the house-keeping proteins and enzymes, have evolved rapidly over the past 500 million years due to functional and immune selective pressure [33]. This is especially evident in the molecules comprising the apical complex, the invasion machinery mediating physical recognition, cytoadherence, and penetration of the host, which are parasite specific.

The successful invasion of the host cell by the apicomplexan parasite is dependent upon the sequential secretion of proteins and other molecules from the rhoptries, micronemes, and dense granules and results in the formation of the parasitophorous vacuole (PV) (i.e., *Toxoplasma, Cryptosporidium, Plasmodium,* and *Eimeria spp*.). This in turn provides access to intracellular nutrients and protection from the host's immune system [34]. Although the PV shields the parasite from the host defences, at the same time it restricts access to nutrients in the host's cytoplasm. Thus, the Apicomplexa has adopted different tactics to circumvent this problem, including biochemical modification of the PV making it permeable to essential nutrients. In contrast, some parasites, such as *Theileria spp*., do not form the PV and proliferate freely in the cytoplasm with direct access to host nutrients [35].

Lateral gene transfer, best known for its role in antibiotic resistance in bacteria, has been proposed as a mechanism by which these opportunistic organisms acquire new genes that confer parasite fitness [36–38]. For example, the apical complex, actin-myosin-powered motor, evolved as a result of the nuclear transfer of genes acquired during the secondary endosymbiotic event. It is believed that origin of the apicoplast can be attributed to endosymbiotic partnership in which the plastid-containing eukaryote was engulfed by a second eukaryotic cell. In addition, sexual replication appears to be another contributing factor to the diverse functions of the apicomplexan surface proteins and the adaptations to genera-specific niches [39–41]. Therefore, it is not surprising that *Plasmodium* and *Babesia* species share evolutionarily conserved mechanisms of erythrocyte invasion [23]. Moreover, transmembrane proteins (thrombospondin-related anonymous proteins—TRAP) bridging the apical complex to the host cell in *Plasmodium, Eimeria,*

and *Toxoplasma* also share a high degree of homology [34, 42–44], suggesting that Apicomplexa use the same molecular machinery to invade a wide variety of cells.

A great deal of research has also been carried out to study the role of surface antigens in parasite growth, development, and survival. Passive transfer of polyclonal and monoclonal antibodies raised to asexual stage surface antigens of *T. gondii* are capable of conferring resistance against lethal challenge with this parasite [45, 46]. Similarly, *Eimeria* and *Plasmodium* antisporozoite and antimerozoite antibodies that recognize surface antigens, namely, glycosylphosphatidylinositol- (GPI-) anchored antigens in *E. tenella* (EtSAG1) and *P. falciparum* (MSP1), respectively, are able to induce a strong inhibitory response and provide protection against infection [47, 48]. In addition, the antigens found on the surface of sporozoites have been implicated in the recognition and invasion of the hepatocytes in malaria and, therefore, represent promising targets for vaccine developers [10, 42, 49, 50] (a detailed description of apicomplexan invasion and egress has been reviewed recently by Westwood and colleagues with a special emphasis on elements of the apical complex and their potential role as vaccine targets [51]). Thus, both asexual-stage surface proteins and the molecules associated with the apical complex have been proposed as potential candidates for vaccine development.

Another conserved feature of the phylum is the transmissible, environmentally durable oocyst/cyst, the zygote stage of the Coccidia (i.e., *Toxoplasma, Eimeria, Cryptosporidium,* and *Neospora* species) [29, 52]. It is intriguing that asexual reproduction in *Eimeria* is tightly regulated, and although signals initiating the start of sexual reproduction have not yet been identified, the 2nd, 3rd, and 4th generation merozoites (depending upon which *Eimeria* species infects the host chicken) differentiate into male (micro-) and female (macro-) gametes in a synchronised manner. The trademark of apicomplexan gametogenesis is the synthesis of numerous lipid bodies and polysaccharide granules, believed to be acquired from the host cell, serving as an energy source for the developing zygote. Moreover, increased DNA synthesis and RNA transcription to produce multinucleated microgametocytes and heightened protein synthesis in the macrogametocytes to produce wall forming bodies (WFBs) are characteristics of sexual stage development at the molecular level [53–56]. *Eimeria, Cryptosporidium,* and *Toxoplasma spp*. gametogenesis is completed by the formation of environmentally resilient oocysts in the mucosae lining the gastrointestinal tract of chickens, humans, and cats, respectively. The oocysts are then excreted with the faeces where they mature (sporulate) becoming infectious. *Cryptosporidium* is slightly different from *Eimeria* and *Toxoplasma* in that the *Cryptosporidium* oocysts can also release sporozoites in the gut which are capable of infecting new epithelial cells (i.e., autoreinfection). In all three cases, transmission is primarily by the accidental ingestion of sporulated oocysts.

Unlike the intestinal parasites described above, gametogenesis of *Plasmodium* and *Babesia spp*. is completed in the gut of their arthropod hosts. During development, after fertilization of macrogametes by the microgametes and invasion of the gut by the ookinete, the zygotes encase themselves in a protective single-layered cyst wall to form an oocyst. Flagellated sporozoites then exit the oocyst and migrate from the midgut to the salivary gland of their arthropod host. From there, they are injected into the blood and subcutaneous tissue of the next vertebrate host the arthropod bites.

3. Naturally Acquired Immunity to Apicomplexan Parasites Is Exclusively Dependent upon Cell-Mediated Immunity

Studies to elucidate the mechanism(s) of the protective immune response to apicomplexan parasites have been limited mainly due to the lack of being able to carry out such studies in the definitive host such as cattle or human beings. Thus, much of our understanding of the protective immune response to apicomplexan infections has been derived from murine models which can be genetically modified [26, 57]. Generally speaking, parasite replication in the host eventually leads to host cell lysis and parasite egress. It is now widely accepted that cells of the innate immune system and the molecules they produce and/or secrete are important controlling factors of parasite infectivity and in limiting the extent of parasitemia [58–61]. It is not clear exactly how these molecules, particularly in the case of parasites infecting host erythrocytes (i.e., *Plasmodium* and *Babesia spp*.), interfere with parasite development. What does appear to be the case is that this inhibition is accomplished by the production of gamma interferon (IFN-γ), by natural killer cells (NK), and tumour necrosis factor alpha (TNF-α), nitric oxide (NO) and reactive oxygen species (ROS) by macrophages [62–64]. Despite the fact that the mechanisms by which IFN-γ mediates protection are not completely understood, studies using IFN-γ and iNOS knock-out mice infected with *T. gondii* indicated that activation of p47 guanosine triphosphatases (GTPases) leads to degradation of the PV in infected cells [65].

While the immune response in healthy hosts is often but not always able to control parasite replication and limit the disease, immunocompromised individuals fail to stop parasite growth, and clinical disease develops in nearly all cases. This is especially evident in toxoplasmosis where immunocompetent hosts control parasite replication causing tachyzoites (the rapidly replicating asexual stages) to migrate to muscle and brain tissues where they differentiate into bradyzoite cysts (the slow-replicating form) and persist throughout the host's life. Although the tissue cysts can become reactivated periodically, most healthy hosts never develop clinical disease. In contrast, immunocompromised patients, such as those suffering from AIDS, remain chronically infected, whereby reactivation of the tissue cysts can lead to toxoplasmic encephalitis with severe pathological consequences.

The ability to clear acute and chronic infections with the Apicomplexa seems to correlate with host CD4+ T-cell levels [62, 66, 67]. Thus, studies involving athymic animals have shown that T cells play a crucial role in the infectious process [62, 68].

The population of T cells coexpressing $\alpha\beta$ markers appears to be important for host defence against apicomplexan infections [69, 70]. Thus, it was demonstrated that TCRα-deficient mice developed severe disease when compared to controls [70]. Furthermore, mice lacking major histocompatibility complex class II expression (i.e., CD4+ T-cell deficient) appeared more susceptible to apicomplexan infections [62, 67].

CD8+ T cells also appear to play a role in parasite growth and dissemination because they provide sporozoite transport from their initial infection site, as is the case for *Eimeria* and *Toxoplasma* infections [69, 71]. During the course of primary infection with *Eimeria*, CD8+ T cells appear to play a role in parasite growth and dissemination because they provide sporozoite transport from their initial infection site to the crypt cells. On the other hand, reports have also shown that increased numbers of CD8+ T cells in the crypt epithelium act as cytotoxic killer cells facilitating the clearance of the parasite-infected cells [72, 73]. Furthermore, studies in infected chickens have shown that the contribution of both CD4+ and CD8+ T-cell populations differs according to the infective species used [70]. Nevertheless, during avian coccidiosis and babesiosis, CD4+ T-cell subsets were found to be elevated in animals following challenge infections [67, 70, 72]. An intriguing question arises from all of these observations: why are some species or strains of parasites extremely "immunogenic" and induce protective immunity, while others seem to be invisible to the immune system?

Studies on naturally acquired immunity to malaria have shown that adequate protective immunity to *P. falciparum*, the etiological agent of the most severe malaria in humans, usually required repeated infections. Thus, protection against this particular strain appeared to be acquired more slowly than against the less pathogenic *P. vivax* or *P. malariae* [28]. Moreover, numerous studies have shown that immunity appeared to be species specific and did not confer protection against challenge with heterologous species. However, it was reported that heavy exposure to parasites induces the development of antigenic memory [74]. Although the molecular and cellular mechanisms driving the onset of protective host immunity against malaria or any other pathogenic protozoan are not entirely understood, it is believed that susceptibility to infection is driven by extrinsic factors such as antigenic variation and also by intrinsically inappropriate immune responses [75]. This is particularly evident in *Theileria* infections of immunocompromised cattle, which often results in the death of the host animal. It is theorised that the ability of *Theileria* to interfere with the host's apoptotic pathways is the crucial factor contributing to mortality [35, 76]. Once inside the host cell, *Theileria* resides free in the cytoplasm and induces uncontrolled proliferation of the infected cell. Some have even compared *Theileria* infections with cancer development and metastasis. Thus, it appears that during *Theileria* infection the immune system fails to control this proliferation in time, in turn resulting in potent, nonspecific lysis of both infected and noninfected cells.

Studies using immunocompetent animals have shown that, in addition to innate host resistance, IFN-γ plays a key role in the development of adaptive immunity and clearing of Apicomplexa infections. For example, *Cryptosporidium*-infected mice, with faulty IFN-γ gene expression, suffered from severe mucosal destruction, and as a result they also secreted more oocysts [60, 77]. Furthermore, mopping-up the secreted IFN-γ by antibodies in immunocompromised animals seemed to worsen *C. parvum* infection. In addition, it is now widely accepted that IL-12, known to increase host IFN-γ production, can reduce the severity and even prevent infection by apicomplexan parasites [63]. Although believed to be mediated by an IFN-γ-dependent mechanism, the pathways or downstream molecules crucial in this process have not been well defined to date.

4. Masters of Disguise

Although viruses, which entirely depend on the host machinery for replication and assembly of new viral particles, are the experts in host cell manipulation, the Apicomplexa are considered to be the masters of disguise. This is because they have evolved to evade the host immune system to aid in their own survival. Antigenic variation has been proposed as a key factor in this process. Unlike allelic polymorphism, which results in different phenotypes or so-called parasite strains [78], antigenic variation is the tightly regulated expression of different genes of a clonal population of parasites over the natural course of infection [75]. Antigenic variation amongst malarial and *Babesia* parasites is a prime example of sophistication apicomplexans employed to avoid antibody-mediated inhibition [79–81]. *P. falciparum* achieves this by secreting a single type of a variant molecule (parasite-derived erythrocyte membrane protein 1-PfEMP1) on the surface of the infected erythrocyte at any one time. The PfEMP1 surface proteins are encoded by a family of genes, called *var* genes, and each individual parasite expresses only a single *var* gene, keeping all other members of *var* gene family in a transcriptionally silent state [82–84]. This strategy in turn induces adhesion of the parasite-infected erythrocytes to the blood vessels to avoid reaching the spleen, whose main function is to rid the body of damaged and/or infected blood cells. Similarly, sequestration of *Babesia*-infected erythrocytes in the microvasculature enables the *Babesia* to persist within the host maximizing its chances of transmission [81]. This cytoadherence in *Babesia* is mediated by constant gene conversion of *ves* family genes encoding the variant erythrocyte surface antigen 1 (VESA1) [85].

A puzzling question arises from these observations: if infected erythrocytes pass through the body unchecked since they lack major histocompatibility complex (MHC) expression, overwhelming proliferation of the parasites may cause premature death of the host prior to successful transmission to an arthropod vector? Interestingly, in spite of the fact that malaria parasites sequentially express variant surface molecules exposing the immunodominant antigens to the host immune defences, infection is actually prolonged. Thus, the parasite must undergo antigenic variation and rates of growth that enable the host to control infection while allowing for transmission of the parasite prior to

its death. This mechanism to prolong infection was also evident in merozoites of *Babesia* species. Due to coating of the merozoite surface with glycosyl-phosphatidyl-anchored proteins crucial for initial attachment to the host erythrocyte surface, they are targeted by host-protective antibodies [86]. These surface-anchored proteins (Variable Merozoite Surface Antigens-VMSA) exhibit varying degrees of intra-species antigenic polymorphisms allowing these parasites to evade the host immune system at the population level [75]. Nevertheless, studies involving African children have shown that variant specific immunity, namely, secretion of IgG antibodies directed against *P. falciparum* variant surface antigens (VSA), has been correlated with protection from clinical malaria in Ghana, Kenya, and Tanzania [79, 87, 88]. Thus, VSA antigens have been proposed as excellent candidates for malaria and *Babesia* vaccine development.

5. Humoral Immunity Protects against Challenge Infections

Although B cells have been regarded as minor contributors to protective immunity and resistance to primary infections with Apicomplexa, numerous studies have shown that hosts infected with these parasites are capable of producing protective, parasite-specific immunoglobulins (Ig) of all major classes after an episode of infection and recovery [57, 89–92]. Thus, early work by Rose and colleagues has shown that humoral antibodies, induced by live *Eimeria* infection, can provide excellent passive protection against challenge infections with the same parasite [93, 94]. Likewise, studies on mice infected with *T. gondii* have shown that intestinal IgA antibodies to major surface protein SAG-1 (P30) were produced after peroral infection and found to inhibit infection of murine enterocytes by directly blocking the parasite entry [91]. In addition, Precigout et al. have demonstrated an inhibitory effect of antibodies directed against a 17-kDa merozoites membrane protein on *B. divergens* parasite growth [92]. Furthermore, studies on invasion of red blood cells by *P. falciparum* merozoites have revealed that since RBCs do not express the MHC complex, parasite killing by T lymphocytes is not important. Instead, antibodies specific to merozoite surface molecules (MSP-1) and proteins externalised from the apical complex play a major role in immunity to asexual blood stages [95].

The *Plasmodium* merozoite surface protein 1 (MSP-1) is a 200 kDa multicomponent precursor complex derived by proteolytic processing during erythrocyte invasion. The 42 kDa C-terminal component is cleaved (i.e., secondary processing) to produce soluble 33 kDa and 19 kDa fragments that remain on the merozoites surface [96]. Studies have shown that anti-merozoite antibodies are capable of neutralizing parasites by Fc-dependent mechanisms involving macrophages, thus reducing the parasitemia and clinical disease [87, 97, 98]. In addition, a number of recent studies have shown that children naturally infected with malaria secrete anti-MSP-1 antibodies ($MSP\text{-}1_{19}$ mAb) that block the binding of *Plasmodium* merozoites to the surface of the red blood cells and also inhibit secondary processing of MSP-1. In addition, studies investigating the protective properties of maternally derived IgG and IgM antibodies to the 19 kDa domain of MSP-1 of *P. falciparum* have shown that mothers who have tested positive for anti-MSP-1 (19 kDa fragment) IgG antibodies conferred protection against placental infection and infection in their infants [99].

It has been shown that, in *babesiosis* infection, IgG antibodies produced as a result of live infection can prevent infection of erythrocytes by binding and neutralizing sporozoites before they invade their target cells. Similar observations were reported in chickens where antisporozoite antibodies specific to glycosyl-phosphatidylinositol-anchored *E. tenella* surface antigen 1 (EtSAG1) appeared to inhibit parasite binding and invasion of the host cell [24]. However, it seems that the protective role of these antibodies is limited since it can only neutralize sporozoites from the time the parasites egress and the time they gain access to new cells. Thus, it is hoped that genome-wide fingerprinting techniques [100] will aid in the identification of additional immunoprotective antigens that can be used in combination to induce the maximal inhibitory humoral immune response.

In addition to antigen-specific polyclonal and monoclonal antibodies capable of inhibiting asexual stages, antibodies raised to antigens localized exclusively to gametocyte/zygote stages were also found to be highly immunogenic and capable of providing passive protection in vivo [20, 101, 102]. Early experiments involving immunisation with purified sexual-stage gametes of *P. gallinaceum* in chickens showed that effective transmission-blocking immunity can be achieved by reducing the infectivity of gametocytes and oocyst development [103, 104]. Thus, Pfs25 and Pvs25 proteins expressed on the surface of ookinetes in the mosquito stage of *P. falciparum* and *P. vivax* have been used extensively as candidates for malaria transmission-blocking vaccines, since lowering the density of circulating parasites would not produce sterilizing immunity, instead it would allow individuals to develop long-lasting, naturally acquired immunity to malaria [12, 105, 106].

Work by Wallach and coworkers, aimed at applying transmission-blocking immunity to control infections caused by *Eimeria,* hypothesised that antibodies raised against the gametocyte/zygote stages of development can act to inhibit oocyst development and thereby provide a block in parasite transmission (see Figure 1). A method was developed for purifying *E. maxima* gametocytes from the infected chicken gut mucosa and immunodominant gametocyte antigens, namely, Emgam56, Emgam82, and Emgam230 localized to the WFBs and the oocyst wall of the maturing zygote, were extracted [53, 102, 108]. Passive immunisation experiments showed that there was a good correlation between the intensity of IgG and IgM antibodies binding to gametocyte antigens by Western and ELISA with the ability of those sera to provide passive protection in vivo [109]. The mechanisms by which these antibodies inhibit oocyst maturation are still obscure; however, it is hypothesised that antibodies raised to the immunodominant antigens retard zygote development by interfering with the processing of wall proteins or the wall-hardening processes [53, 110]. In addition, a protective monoclonal antibody

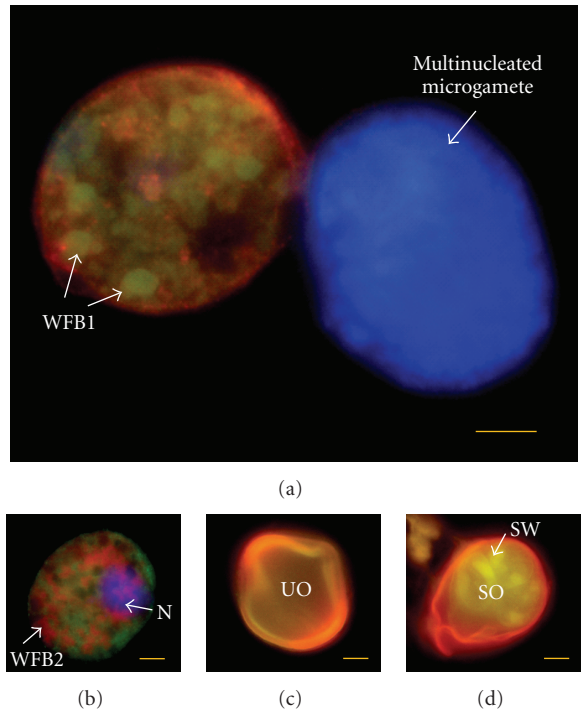

Figure 1: Developing and mature micro- and macrogametes and oocysts harvested from the infected chicken intestine 134 h post infection (p.i.) and double-labelled with a monoclonal antibody raised to antigens confound to WFB1s (E1D8) [107], and a polyclonal affinity purified gametocyte antigens Emgam56, Emgam82, and Emgam230, amongst other molecules (anti-APGA) specific to molecules contained within the wall-forming bodies (1 and 2) [53] and visualised with fluorescein isothiocyanate (green) or revealed with rhodamine-conjugated goat anti-mouse IgG secondary antibody (red). Counterstained with DAPI. Abbreviations: N, nucleus; SO, sporulated oocysts; SW, sporocyst wall; US, unsporulated oocyst; WFB1, wall-forming body type 1; WFB2, wall-forming body type 2. Bars represent 5 μm.

raised against Emgam56 localised to the WFB2 (1E11-11), as well as the inner layer of the oocyst wall, was also found to react strongly with the Stieda body of the sporulated oocysts (M. Wallach, unpublished data). Similar results were reported by Krücken et al. using a monoclonal antibody E2E5 raised to WFB2s of *E. tenella* [101]. The in vitro excystation inhibition assay showed that the antibody E2E5 can significantly interfere with parasite development by impairing sporozoite excystation. It is tempting to speculate that the 1E11-11 monoclonal antibody inhibits or blocks excystation of the sporocyst in a similar manner, thereby reducing the number of infectious sporozoites released in the intestine of infected birds allowing them to develop protective immunity induced by exposure to low doses of parasites.

Jenkins and colleagues have shown that ruminants immunized with a DNA vaccine expressing a gene isolated from *C. parvum* encoding a sporozoite antigen (CP 15/60) were capable of inducing antigen-specific antibodies [111, 112]. In that study, it was found that using various routes of vaccination resulted in differing antibody responses and titres. The authors, therefore, suggested that the route of antigen delivery of any protozoan vaccine requires careful formulation and optimisation of delivery systems.

Finally, in studies carried out by Wallach and coworkers on *Eimeria* [109], it was found that in order to achieve protective immunity using parasite extracts requires the inclusion of the correct antigens and exclusion of the irrelevant ones. Their results indicated that while some parasite-specific antigens induce protective immunity, others actually induce an exacerbation of the infection. Therefore, in the design of any parasitic vaccine, it is crucial that the combination of various antigens maximizes their inhibitory effect on parasite growth and development.

6. The Apicomplexa Are the Manipulators of Host Defence Mechanisms

One of the main defence mechanisms employed by host cells is programmed cell death (apoptosis) ensuring regulated removal of damaged and infected cells [113]. But because the survival and development of intracellular apicomplexan parasites is dependent upon the continuous supply of host cell nutrients and protection from immune attack, the parasites have adapted to extend the life of the infected cells by inhibiting the host cell apoptotic machinery through interference with the intracellular signalling molecules, notably phosphatidylinositol 3-kinase (PI3-K). PI3-K is involved in a variety of functions including cell growth, proliferation, and intracellular trafficking, amongst others [61, 114–117]. *P. falciparum* is a good example of how parasite secreted proteins prevent host cell death to ensure its own development and survival. Sporozoites of *Plasmodium* species are stealthy invaders that first travel to the liver (hepatocyte) cells, where the growth and development of the daughter cells, hepatic merozoites, takes place. Recent results have shown that prior to the establishment of the PV, sporozoites of *P. falciparum* transmigrate through a number of hepatocytes before they anchor to and invade the suitable cell via exocytosis of proteins contained within the apical complex. It has been shown that the thrombospondin-related adhesive-protein- (TRAP-) like protein plays a role in this process. Additionally, the wounding of the hepatocyte induced by invading sporozoites releases growth factors which in turn appear to inhibit PI3-K and block the signalling pathways destined for apoptosis. Leirião and colleagues have shown that once the parasite is established in the hepatocyte, it secretes HGF/MET signalling molecules into the host cell cytoplasm, thereby conferring resistance to apoptosis to ensure survival and maturation of the daughter cells [117]. However, which signalling upstream of PI3-K occurs during *Plasmodium* infection is yet to be determined. Interestingly, upon maturation of merozoites, *Plasmodium* seems to be able to induce host cell death to liberate the motile progeny. Strum et al. have shown that this process involves cysteine proteases [49, 50]. Moreover, similar mechanisms were found to play a role in release of sporozoites from the oocysts [118]. Although work is ongoing to try and elucidate

the mechanisms involved in these processes, it appears that the Apicomplexa have learned to inhibit host cell death during parasite development and subsequently activate it, liberating thousands of new progeny.

T. gondii has also evolved a broad spectrum of adaptations to challenges presented by its life style [119]. Chronic toxoplasmosis is the trademark of the parasite's success and is induced by the slow-replicating bradyzoites safely tucked away in the remodelled PV, the tissue cyst. Like *Plasmodium*, *T. gondii* modulates host cell apoptosis by both inhibiting and triggering the programmed cell death [120]. Chen et al. have shown that Fas/FasL ligand-dependent mechanisms mediate the inflammatory responses induced by the apicomplexan infection [115, 121]. But the parasites have evolved to neutralize granzyme/perforin-mediated killing of infected T cells and natural killer cells (NK) by modifying transcription and posttranscriptional modification of IFN-γ -regulated genes, the major mediators of resistance to *T. gondii* infections. Likewise, del Cacho et al. have demonstrated that *E. tenella* and *E. necatrix* second-generation schizonts first induce NF-$\kappa\beta$ activation to protect the transformed cells from apoptosis, allowing the schizonts to mature and later cause NF-$\kappa\beta$ inhibition to trigger host cell apoptosis to facilitate the release of merozoites [7].

The Apicomplexa have evolved to live in synergy with their infected hosts because they completely depend on it for survival; however, some apicomplexan infections induce a great deal of immunopathology and can lead to host cell death. For example, *Eimeria* and *Cyclospora* both interfere with the absorption of nutrients across the intestinal mucosa and can cause death due to malaise, diarrhoea, and dehydration. Because apicomplexans increase in numbers while, in their hosts, the severity of infection is proportional to the parasite density—the smaller the number, the greater the chance of asymptomatic infection and the greater the chances of the parasite survival. However, the immunological defence of a host can also cause extensive tissue damage and clinical symptoms. Patients with cerebral malaria usually have elevated levels of tumour necrosis factor alpha (TNF-α) and IgE considered to be responsible for fever and tissue lesions to an extent where vital functions of the host fail leading to a coma [122].

7. Are We Losing the Battle against Apicomplexan Parasites?

Despite a great deal of effort and technological advancements in biotechnology, molecular biology, genetics, immunology, and vaccinology, there are no vaccines for humans against malaria and toxoplasmosis at the present time, and it seems that we are losing the battle in the fight against pathogenic protozoans. The current failure to develop a practical vaccine may well be attributed to our inadequate understanding of the mechanisms underlying (1) the naturally acquired immunity against apicomplexans, (2) acquired parasite resistance to most (if not all) antimicrobial compounds used to date, and (3) in the case of arthropod transmitted protozoans, failure to implement adequate vector control programs in tropical and subtropical regions.

The life cycles of the Apicomplexa are complex, thus, it is hoped that a multivalent, multistage vaccine will alleviate the problems caused by these pathogenic protozoans. Although this approach has attracted a great deal of commercial and research interest, the critical issues to be addressed include the identification of stage-specific antigens capable of inducing protective immunity and the delivery methods in a form that will stimulate an adequate protective immune response. The main impediment in the search and selection for immunostimulatory antigens is the lack of in vitro assays to analyse and predict immune responses. The transmission blocking assays, relying on counting the number of oocysts produced, and the inhibition of sporozoite invasion assays have both been used extensively to evaluate parasite inhibition induced by neutralizing antibodies.

Although in vivo experimentation is extremely difficult for malaria, other model systems can be used to dissect the fine details and the effect of neutralizing antibodies. It is very possible that, in the development of an antiprotozoan vaccine capable of inducing only partial immunity, resistant mutants would be selected that are even more pathogenic than existing strains. In the malaria scenario, this could be catastrophic since the parasite would undergo recycling and be transmitted throughout the community leading to an increase in morbidity and mortality. It is, therefore, of great hope that in the battle against these pathogenic protozoan parasites, including *Plasmodium*, *Cryptosporidium,* and *Toxoplasma*, the completion of their genomes and proteomes may provide information needed to design vaccines, assess the effects of immunization on parasite pathogenicity and the selection of unwanted mutants, and in the final analysis control the diseases caused by this class of parasites.

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Review Article

Nonimmune Cells Contribute to Crosstalk between Immune Cells and Inflammatory Mediators in the Innate Response to *Trypanosoma cruzi* Infection

Maria Pilar Aoki,[1] Eugenio Antonio Carrera-Silva,[1,2] Henar Cuervo,[3] Manuel Fresno,[3] Núria Gironès,[3] and Susana Gea[1]

[1] *Departamento de Bioquímica Clínica, Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba 5000, Argentina*
[2] *Department of Immunobiology, School of Medicine, Yale University, New Haven, CT 06520, USA*
[3] *Centro de Biología Molecular Severo Ochoa, Consejo Superior de Investigaciones Científicas (CSIC), Universidad Autónoma de Madrid (UAM), Cantoblanco, Madrid 28049, Spain*

Correspondence should be addressed to Maria Pilar Aoki, paoki@fcq.unc.edu.ar

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Chagas myocarditis, which is caused by infection with the intracellular parasite *Trypanosoma cruzi*, remains the major infectious heart disease worldwide. Innate recognition through toll-like receptors (TLRs) on immune cells has not only been revealed to be critical for defense against *T. cruzi* but has also been involved in triggering the pathology. Subsequent studies revealed that this parasite activates nucleotide-binding oligomerization domain- (NOD-)like receptors and several particular transcription factors in TLR-independent manner. In addition to professional immune cells, *T. cruzi* infects and resides in different parenchyma cells. The innate receptors in nonimmune target tissues could also have an impact on host response. Thus, the outcome of the myocarditis or the inflamed liver relies on an intricate network of inflammatory mediators and signals given by immune and nonimmune cells. In this paper, we discuss the evidence of innate immunity to the parasite developed by the host, with emphasis on the crosstalk between immune and nonimmune cell responses.

1. Introduction

The intracellular protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas disease, which is a health threat for an estimated 10 million people, living mostly in Latin America. More than 25 million people are at risk of the disease. It is estimated that in 2008 Chagas disease killed more than 10.000 people [1]. Although this infection occurs mainly in Latin America, in the past decades it has been increasingly detected in the United States of America, Canada, many European, and some Western Pacific countries. This is now a new worldwide challenge to nonendemic countries [1, 2]. The infective trypomastigote form invades macrophages and other cell types, where it is converted into the amastigote form and replicates. Acute manifestations often include parasitemia, which decays with the onset of immunity. Progression from the acute to the chronic phase coincides with the clearance of parasites from the blood stream and tissues. After years or even decades of primary infection, up to 30% of chronically infected people develop cardiac alterations, and up to 10% develop digestive, neurological, or mixed alterations [1].

Despite nearly a century of research, the most intriguing challenge for understanding the pathophysiology of Chagas' heart disease still lies in the complex host-parasite interrelationship. Different mechanisms have been defined to explain the pathogenesis of human and experimental Chagas disease. Among the mechanisms described, autoimmunity is the one that has received the most experimental evidence but also controversy [3–6]. Nevertheless, there are studies suggesting

that parasite persistence in the host tissues is relevant in the pathogenesis of the disease [7–9]. Both theories recognize the transcendental role of innate immunity during host defense as well as in the development and progression of myocarditis during Chagas disease. Geographical variation in the severity of different forms of the disease indicates the importance of *T. cruzi* genetic variation in addition to host genetic background. Unfortunately, it remains as a neglected disease in the world, and, despite considerable research, effective vaccines and adequate drugs for *T. cruzi* infection are still lacking. In this paper, we discuss the evidence of innate immunity to the parasite developed by the host, with emphasis on the crosstalk between immune and nonimmune cell responses, and its role in sustaining defense as well as injurious processes.

2. Role of Toll-Like Receptors in the Innate Immune Recognition of *Trypanosoma cruzi*

The innate immune response is initiated by pattern-recognition receptors (PRRs), which recognizes pathogen-associated molecular patterns [10, 11]. Different PRRs generally recognize diverse ligand specificities. The broad specificities of the PRRs and their ability to form functional multireceptor complexes allow large combinatorial repertoires. This further diversifies the recognition and signaling of cooperating PRRs and enables the host to detect almost any type of pathogen, discriminate between different microorganisms, and mount a competent immune response. The PRRs most widely investigated are the toll-like receptors (TLRs). This receptor family comprises 10 and 13 functional members in humans and mice, respectively. Besides sensing pathogens, ranging from bacteria to fungi, parasites, and viruses, it is now thought that they recognize endogenous ligands which have an important role in the regulation of inflammation as well as in noninfectious disease [11]. Studies focusing on host innate immunity against *T. cruzi* infection demonstrated that these receptors are crucial for many aspects of microbial elimination, including recruitment of phagocytes to infected tissues and subsequent killing [10–12]. However, it has been reported that, activated to excess, TLRs can mediate pathology [12]. The TLR signaling pathways consist of two cascades: a myeloid differentiation primary-response-gene-88- (MyD88-) dependent pathway and a Toll/IL1R-domain containing adaptor protein inducing IFNβ (TRIF-) dependent (MyD88-independent) pathway. The MyD88-dependent pathway mediates the production of proinflammatory cytokines through all TLRs except for TLR3, while the TRIF-dependent way is indispensable for the induction of type I IFNs through TLR3 and TLR4 [13].

Taking into account that proinflammatory cytokines produced by TLR activation play an important role in the immunopathology of chronic Chagas' cardiopathy, it has been proposed that a single-nucleotide polymorphism in the genes that encode proteins in TLR signaling could play an important role in differential susceptibility to Chagas disease. Thus, it was recently demonstrated that *T. cruzi*-infected individuals who are heterozygous for the MAL/TIRAP S180L variant lead to a decrease in signal transduction upon ligation of TLR2 or TLR4 to their respective ligands, which is associated with lower risk of developing chronic Chagas' cardiomyopathy [14].

TLRs are expressed on different immune cell populations, including macrophages, dendritic cells (DCs), B lymphocytes, specific T-cell subsets, and even on nonimmune cells such as fibroblasts, parenchyma cells, and epithelial cells. The importance of TLRs during *T. cruzi* immune response was initially evidenced by studies performed with professional antigen-presenting cells [15], in which the authors remark the importance of TLR2 as a mediator of the defense mechanisms during the early stages of the host response to infection. Internalization of intracellular parasites by phagocytosis is a key event in the initiation of the immune response, with phagosomal maturation being central to microbial killing and antigen presentation. Regarding the *T. cruzi* entry process, several studies have examined the mechanisms of invasion or internalization of this parasite, being the host cells and the host molecules involved in this interaction still not completely understood. Interestingly, we have recently demonstrated that activation of small guanine-phosphonucleotide-binding proteins Ras-related protein-(Rab-) 5, fusion of early endosomes, and phagocytosis induced by trypomastigotes in macrophages, involved TLR2 but were independent of TLR4 [16] (Figure 1). Signaling through the TLR2 by the parasite-released-antigen Tc52 stimulated the maturation of DCs and strikingly rescued immunized mice from lethal infection [17]. Moreover, the activation of TLR2 leaded to the secretion of chemokines inducing leukocyte recruitment [18]. Thus, parasite antigens and the cytokines locally released may act together to promote DC maturation and subsequent development of protective Th1 response. In our lab we also found that the inoculation of TLR2-synthetic ligand prior to infection *in vivo* improved the survival of lethally infected mice [19]. Noticeably, other authors showed that infected TLR2(−/−) mice produced enhanced levels of cytokines suggesting that, *in vivo*, TLR2 may have a predominant immunoregulatory role during acute infection with *T. cruzi* parasites, at least with the Y strain [20]. However, these authors observed no major difference in parasitemia and mortality between infected TLR2 knockout and wild-type mice. Furthermore, MyD88 knockout mice were more susceptible to *T. cruzi*, with higher parasitemia and greater mortality [21]. Additional studies attributed most of the MyD88-dependent host resistance to the cooperative activation of TLR2 and TLR9 [20] (Figure 1). The activation of TLR9 by *T. cruzi* came from early studies showing that parasite genomic DNA stimulates cytokine responses in professional presenting cells [22].

The study of linkage between *T. cruzi* innate immunity and the generation of adaptive immune response has been scarcely explored. Recently, it was proposed that a weak TLRs activation might contribute to the relatively slow expansion despite strong CD8+ T cell response during acute *T. cruzi* infection. This study was performed evaluating the frequency of parasite-specific CD8 T cells among other parameters. The authors found an earlier but transient

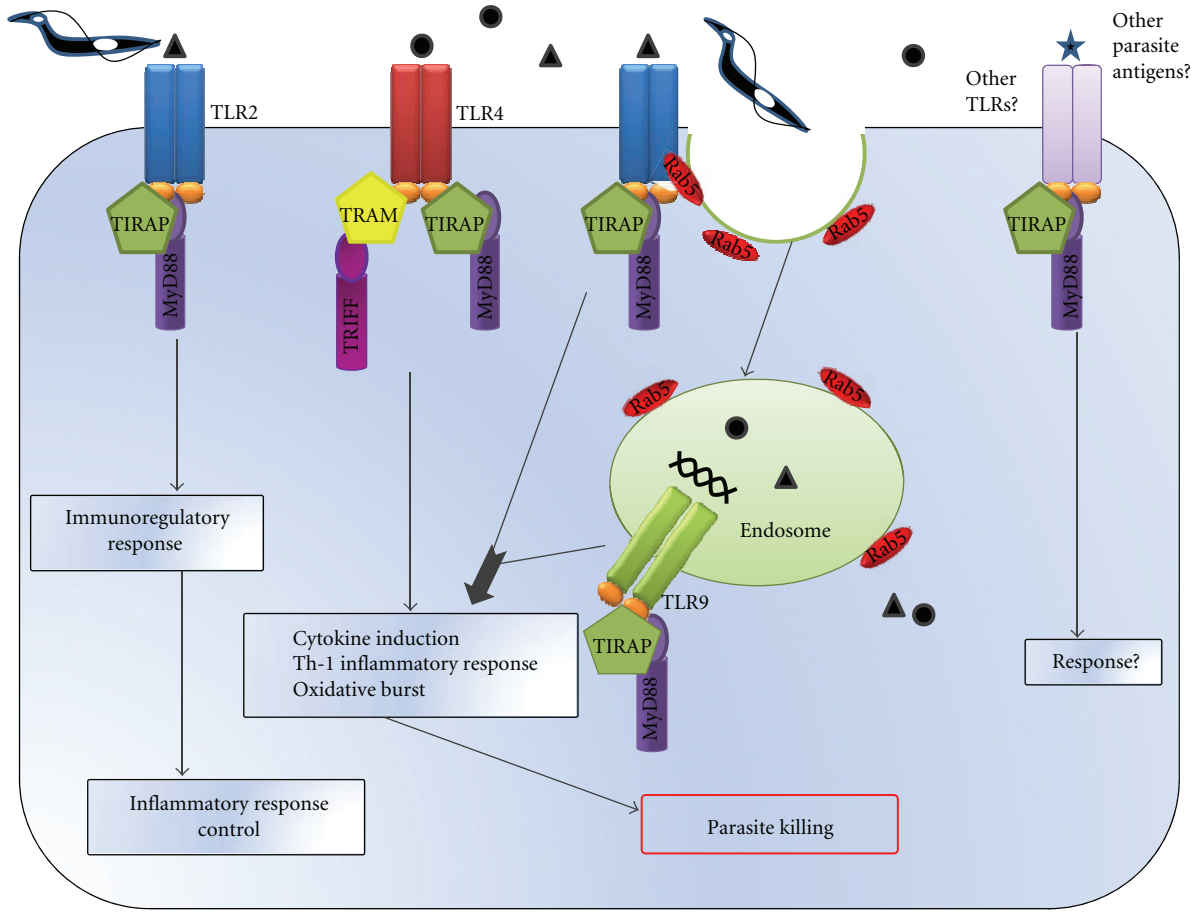

Figure 1: TLR-dependent innate immune responses. *T. cruzi*-derived components are recognized by TLR2, TLR4, and TLR9, triggering the production of proinflammatory cytokines and microbicidal effectors. Thus, parasite antigens and the cytokines locally released act together to promote the development of protective Th1 response, which lead to parasite growth control. Moreover, TLR2 signaling also has immunoregulatory properties essential to hinder the immune response induced by the parasite. Regarding the *T. cruzi* entry process, TLR2 but not TLR4 is involved in the activation of Rab-5, fusion of early endosomes, and phagocytosis of trypomastigotes. Furthermore, it is plausible to think that others unexplored TLRs and/or parasite antigens could be involved in the induction of the innate immune responses against *T. cruzi*.

induction of this cell population by the administration of the combination of TLR9 plus TLR2 agonist concomitantly with the infection [24]. Otherwise, Oliveira and colleagues (2010) found that *T. cruzi*-infected TLR2(−/−), TLR4(−/−), TLR9(−/−) or Myd88(−/−) mice generated both specific cytotoxic responses and IFNγ-secreting CD8+ T cells at levels comparable to wild-type mice, although the frequency of IFNγ+ CD4+ cells was diminished in infected knockout Myd88 mice [25]. Thus, the authors concluded that neither the lack of each TLR2, TLR4, or TLR9 nor the absence of all MyD88-mediated pathways affect the development of cytotoxic function and number of CD8+ T cells, which are crucial effectors against this parasite.

The potent immune response elicited by *T. cruzi* requires the generation of immunoregulatory network in order to prevent or minimize reactivity to selfantigens or an excessive response to the parasite. It has become clear that active suppression mediated by regulatory T-cell (Treg) populations is crucial for the control of the immune response both in human and experimental *T. cruzi* infection [26, 27]. It has been demonstrated that Tregs display an increased level of TLR2, TLR4, TLR5, TLR7/8, and TLR10 expression compared to conventional effector CD4+ CD25− T cells, suggesting that the expansion and function of this regulatory cells may be closely influenced by TLR ligands [28–31]. In line with this, the immunoregulatory role for TLR2 reported during the acute infection could be explained by the fact that the suppressive function of Tregs is directly controlled by the triggering of TLR2 but not TLR4 or TLR9 [32]. TLR2 ligands activate the expansion of Tregs by an indirect effect via antigen-presenting cells or by direct TLR2 triggering of Tregs. Moreover, signaling through TLR2 strongly enhance CD25 expression inducing an increased sensitivity to interleukin (IL) 2. It is believed that the increase in IL2 receptor expression on Tregs [32] and IL2 production by effector T cells temporally abrogate the suppressive capacity of the Tregs *in vivo* [31] (Figure 1). Therefore, it is plausible to think that TLR2 ligands provided by the parasite could first expand Tregs and abrogate their suppressive phenotype. When low numbers of the pathogen are present, as in the

persistent phase of infection, Tregs regain their immune-suppressive phenotype and could be responsible for the pathogen persistence.

3. TLR Ligands from *Trypanosoma cruzi*

T. cruzi display numerous ligands for the TLRs. In 2001, Gazzinelli's team found two potent TLR2 activators; the protozoan trypomastigote surface-highly purified glycosylphosphatidylinositol (GPI) anchors linked to the surface mucin-like glycoproteins, and free GPI anchors named glycoinositolphospholipids (GIPLs) were recognized through TLR2. These parasite ligands trigger IL12, TNFα, and nitric oxide (NO) production by inflammatory macrophages [15, 33]. Regarding other parasite molecules, it was reported that the *T. cruzi* Tc52-released protein induces human DC maturation signaling through TLR2. Tc52 comprises two homologous domains, which contain a glutathione-binding site and a hydrophobic C-terminal region, and is essential for parasite survival and virulence. Authors proposed that Tc52 would be one candidate molecule to design a multicomponent vaccine to control *T. cruzi* infection [17]. On the other hand, Oliveira et al. (2004) observed that *T. cruzi*-derived GIPL ceramide, in high concentration, could activate mouse cells through TLR4 *in vitro.* Furthermore, TLR4-mutated C3H/HeJ mice were highly susceptible to *T. cruzi* infection [34].

In addition, Bafica and colleagues demonstrated that *T. cruzi*-DNA, a TLR9 agonist, stimulated cytokine production by antigen-presenting cells and cooperatively participated in the control of infection [20]. A more recent study identified the ODNs containing CpG motifs in the *T. cruzi* genome responsible for the immunostimulatory activation of TLR9 from mouse and human infected cells, suggesting that the killing of parasites may be required to release agonists of TLR9 [35]. Remarkably, infected double knockout TLR2(−/−)TLR9(−/−) mice developed a parasitemia equivalent to animals lacking MyD88 but did not show the mortality displayed by MyD88(−/−) animals. Authors suggest that TLR9 has a primary role in the MyD88-dependent induction of IL12/IFNγ synthesis during infection.

Summing up, although some parasite ligands have been reported as TLR agonists, it is plausible to think that other molecular patterns from this complex parasite may activate different combination of TLRs on target/effector cells. The combined activation of these receptors would drive the final outcome of host cellular response determining the defense as well as tissue damage.

4. Toll-Like Receptor-Independent Innate Immune Responses to *Trypanosoma cruzi* Infection

As was discussed above, it is well established that the TLR-dependent pathway initiates an effective innate immune responses against *T. cruzi*. However, infection of cells deficient for expression of the TLR adaptor proteins TRIF and MyD88 still produces cytokines in response to this protozoan, suggesting that other TLR-independent pathways also may be activated during the early immune response. In this sense, new families of PRRs have emerged as important components of the innate immune system that sense the presence of this microorganism and drive the host defense to a protective phenotype.

The NOD-like receptors (NLRs) comprise a large family of intracellular PRRs responsible for the recognition of microorganisms independent of TLR signaling [36]. The first and better characterized members of this family are NOD1 and NOD2 [37, 38]. Although these receptors were extensively characterized as PRRs for bacterial and viral infection, little is known regarding their role in the recognition of intracellular parasites, that is, *T. cruzi*. In this regard, Silva and coworkers (2010) recently demonstrated that the effective response required for host resistance to infection was exclusively mediated by NOD1 but not by NOD2 receptor [39]. Despite normal cytokine production in the sera, NOD1(−/−) mice were highly susceptible to this infection, as judged by the high parasite load in spleen and heart tissues and succumbed to the infection in a similar way to Myd88 and nitric oxide synthase (iNOS) knock-out mice. In light of their results, the authors concluded that the NOD1-dependent response may be implicated in host resistance to *T. cruzi* by mechanisms independent of cytokine production (Figure 2).

Strikingly, *T. cruzi* infection is able to activate other innate immune pathways in the absence of TLR signaling, although the sensing molecules that recognize the parasite ligands are still unknown. Studies performed *in vitro* showed that trypomastigotes trigger IFNβ expression in immune and nonimmune cells by engaging a novel TLR-independent pathway that requires both TANK-binding kinase 1 (TBK1) and IFN-regulatory factors (IRF)3 [40] (Figure 2). Although the role of IFNβ in the protection against parasite infection remains controversial [41, 42], it was demonstrated that IFNβ is responsible for resistance of macrophages infected with *T. cruzi* mainly in the absence of MyD88 [43].

Furthermore, the activation of one member of the nuclear factor of activated T-cell (NFAT) family transcription factors NFATc1 mediated IFNγ production by macrophages and DC, developing an effective Th1 response and DC maturation during *T. cruzi* infection in double-knockout mice (Myd88−/− and Trif−/−), despite high sensitivity to the infection [44]. A pivotal signaling for the activation of NFATc1 is mediated by the Ca^{2+} pathway. Previously, it was demonstrated that the parasite increases intracellular Ca^{2+} through interaction of kinins (bradykinin) with the bradykinin B2 receptor, which is another defense mechanism (Figure 2). In infected tissues, trypomastigotes induce a robust secretion of chemokines and plasma extravasations in macrophages via TLR2, thus providing the substrates for the proteolytic generation of kinins, which are also involved in DC maturation and IL12 production [45–48].

5. Innate Immune Response in Nonimmune Target Tissues Elicited By *Trypanosoma cruzi*

It is known that innate immune cells, including macrophages and DCs, play pivotal roles in immune response; however,

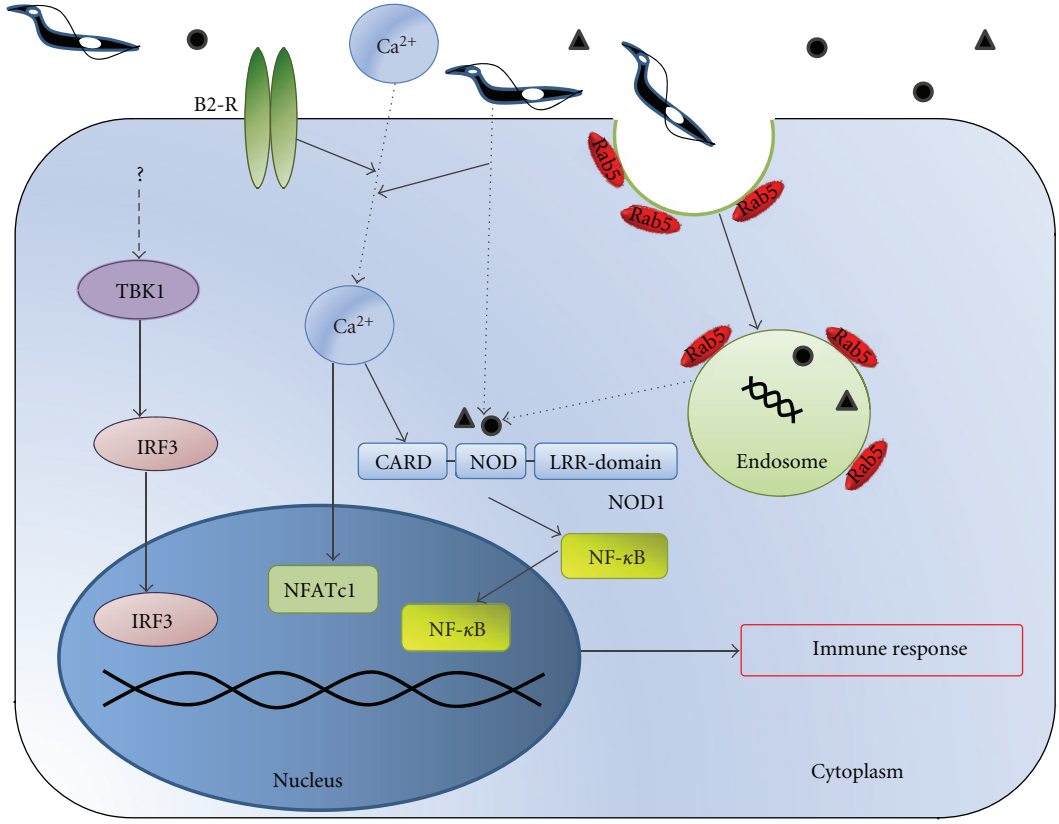

Figure 2: TLR-independent innate immune responses. Infection triggers increased intracellular Ca^{2+} concentration through interaction of bradykinin with the bradykinin B2 receptor (B2-R) among other mechanisms. Target innate immune cells utilize Ca^{2+} to activate the Ca-dependent signaling pathway leading to the activation of NFATc1. Intracellular *T. cruzi* is recognized by NOD1, activating NF-κB. *T. cruzi* is also recognized by unknown molecules leading to the activation of TBK1 and IRF3. Altogether the mechanisms participate in the induction of an effective immune response against the parasite.

nonimmune cells such as parenchyma cells, epithelial cells, endothelial cells, and fibroblasts, among others, also contribute to immunity development [49]. Thus, the outcome of the immune response in a target tissue depends not exclusively on the immune cells but also on the intricate network and signals given by immune and nonimmune cells. Furthermore, although the dominant feature of the innate immune system is to protect the host from infectious agents, it may have other roles in mammalian biology. For example, TLRs on parenchyma cells have been demonstrated to be involved in tissue repair and homeostasis [50, 51].

Accumulative evidence demonstrates that the liver has specific immunological properties and contains a large number of resident and nonresident cells that participate in the regulation of inflammatory and immune responses [52, 53]. Although Kupffer cells are considered the primary cells to respond to pathogen-associated molecular patterns, recent studies provide evidence that multiple populations of nonhematopoietic liver cells, including sinusoidal, endothelial cells, stellate cells, and hepatocytes, express and respond to PRR signaling as well as taking on the roles of antigen-presenting cells [52–54].

Liver cells express a variety of TLRs, which have been shown to participate in hepatic tissue injury and repair, and contribute to the pathogenesis of a variety of liver diseases [52, 55]. However, the action of TLRs on liver cells in host defense against invading pathogens is less clear. The liver is the target of a wide range of microbes including *Listeria*, *Salmonella,* and *Plasmodium* species. However, there are few data related to the implication of *T. cruzi* experimental infection and the relevance of the innate immune response against this parasite in this organ [56, 57].

We have reported a severe hepatic injury in B6 mice infected with Tulahuen *T. cruzi* trypomastigotes. We noted that this mouse strain showed a higher mortality than BALB/c mice, associated with an unbalanced proinflammatory cytokine profile, a decreased TLR2 and TLR4, and an increased TLR9 expression in liver [23]. Supporting our results, it was demonstrated that *T. cruzi*-infected TLR2 knockout mice produced higher levels of proinflammatory cytokines and NO than wild-type mice. These results suggest that TLR2 has an important immunoregulatory role preventing excessive activation of innate immunity and uncontrolled production of proinflammatory cytokines [33]. Furthermore, we also showed that infected BALB/c mice developed a softer environment where the balance between cytokine storm and immunomodulatory signaling given by TLR2 and TGFβ may modulate the inflammatory damage

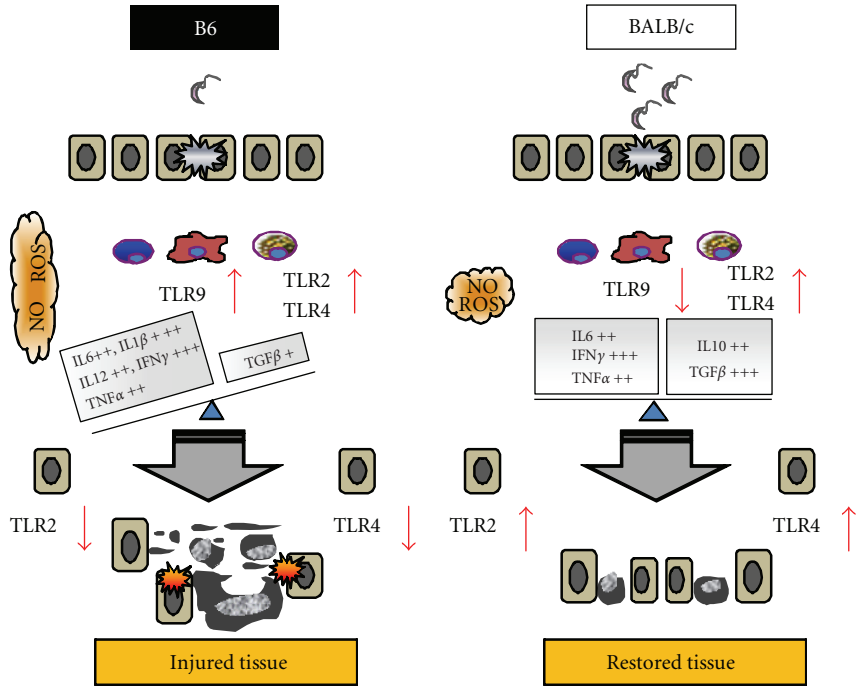

Figure 3: Comparative analysis of hepatic injury, inflammation and TLR expression in *Trypanosoma cruzi*-infected B6 and BALB/c mice. The parasitemia was higher in BALB/c than B6 mice. However infected B6 mouse strain showed stronger and injurious inflammatory environment (increased NO and ROS) associated with high levels of TLR2, TLR4, and TLR9 in hepatic leukocytes. In contrast, BALB/c mice displayed more balanced proinflammatory/immunoregulatory cytokines profile during the acute infection. Furthermore, TLR2 and TLR4 were upregulated in infiltrating leukocytes and hepatocytes as well, while TLR9 expression was low in hepatic leukocytes of infected BALB/c mice. Altogether the results suggested that the strong inflammatory environment elicited in infected B6 mice plus the loss of TLR2 signaling may be responsible for the severity of the hepatic injury and higher mortality of this mouse strain [19, 23].

in the liver [19] (Figure 3). We additionally demonstrated a stronger expression of hepatic iNOS and a higher NO production by liver leukocytes of infected B6 compared to BALB/c mice [19]. Several authors have described that reactive oxygen species (ROS) can induce cell death by either apoptosis or necrosis in liver pathologies [58, 59]. In this sense, an enhanced and sustained nicotinamide adenine dinucleotide phosphate (NADPH) oxidase p47-phox expression and the coexpression of gp91 and p47-phox were found only in liver from infected B6 [19]. Thus, the activation of NADPH oxidase enzymatic complex would be a key player in the liver damage, probably as an instrument contributing to liver apoptosis and necrosis during infection in B6 mice (Figure 3). In addition, we found that while TLR2 and TLR4 expression on hepatic immune infiltrating cells was similar in both mouse strains, TLR9 expression showed a clear difference in hepatic leukocytes. Thus, only leukocytes from infected B6 mice sustained high expression of TLR9 throughout the acute phase. These results support the hypothesis that continuous TLR9 signaling might contribute to excessive and harmful inflammatory response in infected B6 mice. In accordance with our results, a crucial role of TLR9 during *T. cruzi* infection was shown [20]. Interestingly, in hepatocytes we found that TLR2 and TLR4 are differentially modulated in infected BALB/c and B6 mice, suggesting that these innate immune receptors would play a role not only in immune cells but also in liver parenchyma cells (Figure 3). In this sense, it has been postulated that TLR signaling in parenchyma cells would be a key mechanism to prevent death caused by excessive cytokine release [60, 61].

There are increased evidences demonstrating the potential role of TLR-ligands treatment as therapeutic approach and they have shown to be highly effective in the protection against protozoan, among them *T. cruzi* [14, 39, 40]. In our study we further observed that pretreatment with Pam3CSK4, a TLR2/TLR1 agonist, before infection induced a marked reduction of proinflammatory cytokines, nitrite, and transaminase levels and a decrease in the number of hepatic inflammatory foci and consequently in the mortality of infected mice [19]. In this study we postulate that the inadequate integration of signals involving molecular (TLRs, cytokines, NO, and ROS) and cellular (immune and parenchyma cells) components influences the outcome of local immune response during this parasite infection. Moreover, the differential TLR and cytokine modulation in the liver, induced by *T. cruzi* infection, emphasize the importance of local innate immune response in hosts with different genetic background and could contribute to the understanding and the design of novel immune strategies in controlling liver pathologies.

On the other hand, local innate immunity also has a key role in the pathophysiology of several cardiovascular diseases. The heart muscle, initially thought to be a bystander in the immune response to *T. cruzi*, has been found to be an active participant in the innate response, a hypothesis firstly postulated by Postan et al. (1999) [62]. During this infection, cardiomyocytes are actively integrated in the inflammatory response releasing NO, cytokines, and chemokines which, in turn, attract leukocytes to the inflammatory site and control intracellular parasite replication [63–67]. Cardiac cell exposure to proinflammatory cytokines may pre-condition the myocardium environment to temporarily protect cardiomyocytes from growth factor deprivation-induced apoptosis [68]. In fact, we found that *T. cruzi* infection protects isolated cardiac myocytes from apoptotic cell death induced by serum deprivation, and this effect was due to an increase in Bcl-2 molecule. Interestingly, we also found that the infected cardiomyocyte culture pretreated with inactive cruzipain, a major parasite antigen, enhances antiapoptotic protection as well [69, 70]. In a recent study, we explored the nature of the crosstalk between cardiac innate immunity and *T. cruzi* infection. We found that the triggering of TLR2 signaling could be playing an important role in cardiomyocyte protection elicited by *T. cruzi* (Ponce et al., results submitted). Another study indicates that signaling through TLR2 and NF-κB activation also led to the production of IL1β, which mediated the cardiomyocyte hypertrophy observed in Chagas' myocarditis [71].

Adipose tissue has also emerged as an important target for infection, since a significant number of parasites are found within this tissue during the chronic phase of infection [72]. Because the adipocyte act as an active endocrine cell, it is plausible to speculate that these cells may be critically involved in the progression and reactivation of the disease. Adipose tissue contains a number of different cell types. A massive macrophage (F4/80-positive cells) influx was observed in adipose tissue during acute infection. Thus, macrophages and adipocytes combined may be important contributors to systemic inflammation. In adipose tissue, TNFα, IFNγ, and IL1β protein expression were upregulated at least 10-fold compared with noninfected mice. *In vitro* studies with a cell line model for adipocytes (3T3-L1) revealed that the levels of TLR2 and TLR9 but not TLR4 expression were upregulated. In addition, IFNγ, TNFα, and IL1β were also increased under infection [73].

Taken together, the results cited here make it clear that TLR signaling contributes in the beginning and development of the immune response, but the resolution does not depend on individual pathways but on the integration of multiple signals. The combined activation of different PRRs can result in complementary, synergistic, or antagonistic effects that modulate innate and adaptive immunity [11].

6. Microbicidal Activity of Effector Cells and Inflammatory Mediators against *Trypanosoma cruzi*

Arginine and tryptophan metabolism in macrophages depends on cytokine-inducible enzymes and produce mediators involved in microbicidal or suppressive mechanisms in the context of infection. Classical activation of macrophages by Th1 cytokines during infection by intracellular parasites is thought to be protective, whereas alternative activation by Th2 cytokines is involved in the survival of extracellular parasites. Thus, iNOS and arginase have been involved in the regulation of the Th1/Th2 balance during immune processes, and have been used as markers for M1/M2 activation, respectively [74].

Arginase and iNOS metabolize L-arginine, a semi-essential amino acid, to L-citrulline plus NO and urea plus L-ornithine, respectively. Two arginase isoforms have been described in mammals encoded by different genes [75]. Arginase I is cytoplasmic and is highly expressed in liver and alternatively activated macrophages by Th2 cytokines (IL4, IL13) [76, 77] and also by IL10, TGFβ, GM-CSF, and prostaglandin E_2 (PGE_2) [76, 78, 79]. Arginase II is mitochondrial and expressed in a wide variety of tissues and cell types, mainly in kidney [80] and cardiomyocytes [69], and is induced by TLR ligands [81]. The product of arginase activity, L-ornithine, can be metabolized by ornithine aminotransferase giving L-proline, which is required for collagen synthesis and by ornithine decarboxylase (ODC), which results in polyamine synthesis needed for proliferation of all eukaryotic cells.

There are three NOS isoforms: neural NOS (nNOS), endothelial NOS (eNOS), and iNOS, which catalyze the oxidation of L-arginine to L-citrulline and NO. Activated iNOS is found in a diversity of cell types in the immune system [82] and also in cardiomyocytes [83]. The most common inducer for iNOS is IFNγ combined with LPS, but other cytokines such as IL12, IL1β, and TNFα are also able to induce it.

In vitro T. cruzi infection triggers the induction of potent NO-dependent trypanocidal activity in infected cardiomyocytes [65] and macrophages [84–89]. In addition, the interaction of macrophages with apoptotic cells through vitronectin receptor increases TGFβ and PGE_2 release, which promoted parasite proliferation by increasing ODC activity [90].

The *in vivo* role of iNOS in *T. cruzi* infection is still debated, because experiments with iNOS-deficient mice are contradictory [91–93]. However, the administration of iNOS inhibitors to infected mice results in increased parasitemia and mortality, indicating a protective role [94]. On the other hand, when excessive, NO can also have a cytotoxic effect in the host and lead to immune suppression of T cells. In addition, NO production during acute *T. cruzi* infection in rats was inhibited in peripheral blood monocytes, due to the increase of arginase activity [95]. In the mouse model of acute infection, it has been described that the expression of both arginase isoforms and ODC is higher in susceptible BALB/c mice than in C57BL/6 mice [96]. This was associated with an increased parasite burden in BALB/c heart tissue. Interestingly, arginase II was expressed by cardiomyocytes, whereas arginase I was found in infiltrating $CD68^+$ macrophages. These results suggest that infection induces arginase expression, which may not only influence host cell and parasite survival but which might

also downregulate the counterproductive effects triggered by iNOS in the heart during infection. The myeloid-derived suppressor cells (MDSCs), which increase during acute *T. cruzi* infection, also express iNOS and arginase, and they are highly efficient in suppressing activated T cells [95]. It is possible that the induction of iNOS and arginase seen in infected hearts suppresses T-cell activation, allowing parasite replication. In this direction, it is possible that arginase-expressing infiltrated macrophages are MDSCs.

Moreover, the immunization of susceptible BALB/c mice with cruzipain resulted in enhanced anti-inflammatory cytokine secretion, associated with the induction of a $CD11b^+$ $GR1^+$ spleen immature myeloid population that exhibited arginase, but not iNOS, activity [97]. This phenotype is compatible with the MDSC population. Furthermore, cruzipain-stimulated naive macrophages released IL10 and TGFβ and displayed enhanced arginase activity, favoring *T. cruzi* growth [98, 99]. By contrast, the immunization of resistant C57BL/6 mice with cruzipain resulted in the secretion of IL12 and IFNγ and consequently the induction of iNOS messenger and protein expressions as well as high NO production [100]. These findings point up the importance of host genetic background in macrophage response. In another study, macrophages from mice, immunized with a plasmid DNA containing the gene encoding the catalytic domain of *T. cruzi* transsialidase, were able to effectively kill intracellular parasites by a NO-dependent mechanism [101]. Furthermore, CD4 and CD8 T-cell clones are able to produce IFNγ that inhibits parasite replication into macrophages. These results encourage the use of this strategy for developing vaccines against Chagas disease [102].

The inflammatory cytokines also induce the enzyme indoleamine-pyrrole 2,3-dioxygenase (IDO) in macrophages, which converts the essential amino acid L-tryptophan to N-formylkynurenine. During *T. cruzi* infection, there is a systemic activation of IDO, and its inhibition induces an exacerbated parasite load and infection-associated pathology. Further, the authors demonstrated that treatment of *T. cruzi*-infected mice with the IDO downstream metabolite, L-kynurenine, was able to kill the parasite and to improve the survival of lethally infected mice. Moreover, IDO activity was critical to control *in vitro* parasite's replication despite the high production of NO produced by IDO-blocked *T. cruzi*-infected macrophages [103]. In summary, IDO activation and a high iNOS/arginase balance are related to a better outcome of the disease. These evidences suggest that intervention of IDO and iNOS/arginase pathways could be useful in antitrypanosomatid therapeutic strategies for acute infection.

The production of superoxide anion (O2−) by neutrophils and other phagocytes is an important event in innate immune response. This metabolite is the precursor of a range of chemicals referred to as reactive oxygen species. Although these act as microbicidal agents and kill invading microorganisms, there is growing evidence to suggest that myocardium from patients with Chagas disease is exposed to sustained oxidative stress-induced injuries involved in disease progression [104]. The superoxide anion is mainly produced by the multiprotein enzyme complex NADPH oxidase, which is inactive in resting phagocytes but becomes activated after interaction with pathogens and their subsequent engulfment in the phagosome [105]. In response to a pathogen stimulus, the soluble subunits p47phox, p67phox, and p40phox translocate en bloc to the membrane, where they bind flavocytochrome b558. It is clear that inflammatory cytokines are key players in the induction of the oxidative metabolism. Macrophages exposed to IFNγ and TNFα became primed to a state of enhanced responsiveness by the respiratory burst with the induction of membrane and cytosolic components. During *T. cruzi* infection, neutrophils, murine splenocytes [106, 107], blood monocytes, and macrophages produced ROS and destroyed intracellular forms of this parasite [108, 109]. However, ROS are also produced by infected cardiomyocytes, and signal the production of proinflammatory cytokines through the activation of NF-κB, thereby contributing to maintaining the sustained inflammatory state observed in Chagas disease [110].

In our laboratory, we recently demonstrated that cruzipain was able to induce ROS production by splenocytes and macrophage line RAW 264.7. This parasite glycoprotein triggered NADPH oxidase activation and induced the production of several ROS *in vitro*, mainly O2− [111]. As expected, macrophages, derived from cruzipain-immune mice, primed *in vivo* with IFNγ and TNFα, produced more ROS than naive macrophages. This work was the first to report that oxidative stress can be induced by a *T. cruzi* antigen.

It has been proposed that strong oxidants, macrophage-derived peroxynitrites ($ONOO^-$), arising from the reaction of NO with superoxide radical (O2−) participate in cytotoxic mechanisms against *T. cruzi* inside the phagosome. More recently, it was demonstrated that internalization of trypomastigotes by macrophages triggers $ONOO^-$ formation when NO and O2− were produced simultaneously intraphagosomally. This microbicidal mechanism was evidenced by amastigote killing, detected by nitroxidative protein modifications and parasite ultrastructural alterations [112].

Summing up, NO, ROS, and additionally $ONOO^-$ [113, 114] are also very efficient mechanisms in the fight against pathogens. However, these reactive oxygen and nitrogen species are very cytotoxic and, when excessive, can result in tissue damage and promote inflammatory diseases.

7. Concluding Remarks

In this paper we emphasized the importance of the TLR signaling pathway in the innate immune response to the protozoan parasite *T. cruzi*. This parasite has multiple ligands that elicit a potent innate immunity and the subsequent development of adaptive immune response. This activation pathway leads to pro- and antiinflammatory cytokine synthesis. While there is much evidence indicating that MyD88 is a crucial molecule for activation of this type of receptor, other TLR-independent mechanisms in host-parasite interaction are being elucidated. Thus, it has been demonstrated that NLRs which recognize pathogens in the cytoplasm are involved in parasite recognition. Furthermore, several other

mechanisms that induce intracellular Ca^{2+} influx as well as activation of NFATc1 and bradykinin B2 receptor can be activated by this parasite infection. The combined activation of TLRs and other cytoplasmic receptors opens new and interesting viewpoints in our understanding of the synergistic or antagonistic combined action of different PRRs.

The knowledge of the role of TLRs in the pathogenesis of Chagas disease and the identification of new *T. cruzi*-derived TLR ligands is not only important for developing better adjuvant to be used in vaccines, but also new immunotherapy to prevent or minimize Chagas disease pathology. In addition, new pharmacological drugs that disrupt TLR signaling may be attractive when excessive pathology-associated inflammation occurs, as well as in experimental acute infection.

Abbreviations

DC: dendritic cell
GIPLs: glycoinositolphospholipids
GPI: glycosylphosphatidylinositol
IDO: indoleamine-pyrrole 2,3-dioxygenase
IL: interleukin
IRF: IFN-regulatory factors
MDSCs: myeloid-derived suppressor cells
MyD88: myeloid differentiation primary-response gene 88
NADPH: nicotinamide adenine dinucleotide phosphate
NFAT: nuclear factor of activated T-cell transcription factors
NLRs: NOD-like receptors
eNOS: endothelial nitric oxide synthase
iNOS: inducible nitric oxide synthase
nNOS: neural nitric oxide synthase
NO: nitric oxide
NOD: nucleotide-binding oligomerization domain
ODC: ornithine decarboxylase
ODNs: oligodeoxynucleotides
Pam3CSK4: tripalmitoylcysteinylseryl-(lysyl-) 4; TLR2 ligand
PGE2: prostaglandin E2
PRRs: pattern recognition receptors
Rab: Ras-related protein 5
ROS: reactive oxygen species

TBK1: TANk-binding kinase 1
TIRAP/MAL: TIR domain that contains the adaptor protein, also known as MAL
TLRs: toll-like receptors
Treg: regulatory T cell
TRIF: Toll/IL1R-domain containing adaptor protein-inducing IFNβ.

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