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MINIREVIEWS

Toll-Like Receptors and Leishmaniasis▽

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More than 10 million people around the world are currently affected by *Leishmania* sp. (33). Infection with this protozoan parasite continues to be a problem in underdeveloped countries and is a continuous worry in developed countries due to the possibility of the disease afflicting tourists returning from countries where the organism is endemic (82). Leishmaniasis is a neglected infectious disease, and it affects poor and marginalized populations. It is distributed, in its visceral, mucosal, and cutaneous forms, throughout more than 90 countries in Africa, the Americas, Asia, and Europe (5). It constitutes a serious public health problem and causes significant morbidity and mortality. In recent years, economic globalization and the increase in travel have extended the distribution of the disease to developed countries. Approximately 350 million people live in areas where there is active parasite transmission of zoonotic and anthroponotic leishmaniasis. Zoonotic transmission occurs in rural and periurban environments, whereas anthroponotic transmission occurs in urban environments (7).

From a strictly biological point of view, de Almeida et al. described humans as just one of the actors in the leishmaniasis drama (23). Also involved are the parasite, the insect, and other hosts.

Several immunological studies have increased our understanding of the adaptive response in leishmaniasis. Thus, this disease has been used as a model of Th1 and Th2 responses. Unfortunately, there are still several aspects of the initial steps of *Leishmania* infection in humans that are largely unknown.

BEFORE INFECTION

Leishmania is a genus of protozoan flagellates belonging to the family Trypanosomatidae and order Kinetoplastida (35). Two distinct developmental stages of *Leishmania* are recognized, the promastigote and the amastigote. Promastigotes are found within the sandfly and may be further classified as procyclic promastigotes, which multiply in the gut of the sandfly, or as infective metacyclic promastigotes, which are found in parts of the mouth and the anterior gut. The amastigotes live inside lysosomal vacuoles present in phagocytes of the vertebrate host, which are ingested by the female phlebotomine sandfly when it takes an infected blood meal (68). At this time, members of the subfamily Phlebotominae are the main vectors of *Leishmania* sp.; the New World species belong to the genus *Lutzomyia* (84).

An infected sandfly bites a new host during a blood meal and then regurgitates more than 100 metacyclic promastigotes (89). During this phase of the infection, an instantaneous and nonspecific immune response is initiated against *Leishmania* factors, saliva products, and the sucking organ of the insect, which is called the "proboscis" (19).

POTENTIAL ANTIGENS

The *Leishmania* surface is covered with molecules that vary depending on the development stage of the parasite (31). The procyclic promastigotes are covered with a 7-nm-thick glycocalyx, and the infective metacyclic promastigotes are covered with a glycocalyx layer that is at least 17 nm thick. This covering is almost completely absent from the amastigotes, suggesting that the promastigote cell coat is a stage-specific structure that may be lost after phagocytosis and after intracellular transformation (69). The glycocalyx consists of glycoproteins and related products. The latter components are composed of members of a family of glycoinositol phospholipids (GIPLs) and lipophosphoglycans (LPG) (49, 50). The whole structure is species specific and constitutes the first antigens to come into contact with the host's immune system (51, 52, 80).

LPG is the main promastigote molecule and is composed of repetitive units of disaccharide and phosphate, which are linked to the external surface of the plasma membrane by the GPIL anchor. The structure of LPG is different in different species of *Leishmania* and also in different promastigotes of the same species; LPG is significantly more commmon in the metacyclic form and is rare in the amastigotes. These differences in the LPG may explain the increasing resistance of metacyclic promastigotes to complement-mediated lysis (51). Recently, Spath et al. demonstrated that *Leishmania* phosphoglycan$^{-/-}$ cells were unable to survive in activated macrophages but retained the ability to persist indefinitely in the

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mammalian host without inducing disease in nonactivated macrophages (87). Later, the same outcome was reported in a model of *Leishmania* LPG$^{-/-}$ cells, revealing that the previous findings may be related to LPG (86).

Glycoprotein gp63 (promastigote surface protease) is derived from a 63-kDa glycoprotein, even though isoforms with different molecular masses have been found (91). It is a zinc-dependent metalloprotease that plays some role in amastigote survival associated with some modulation of the host's response (21, 48, 53, 56).

Many other interesting and important surface molecules have been described. Nevertheless, the scarcity of these structures suggests that they contribute less to the general surface architecture of *Leishmania*. Thus, other products not described above may be less important in the immune response, at least according to the majority of researchers.

LPG OR gp63

The roles of the LPG and gp63 pathogen-associated molecular patterns appear to complement each other, and these molecules appear to be the trigger of the immune response against *Leishmania* (93). Some studies have demonstrated that LPG might inhibit the fusion of the phagosome with the lysosomes, a weapon lethal to the parasite. There is evidence that the interaction of the parasitophorous vacuoles with the endocytic organelles is very limited. In contrast, vacuoles formed around a *Leishmania* mutant lacking the cell surface LPG fused extensively with endosomes and lysosomes, promoting complete destruction of the parasite (25, 26). A previous study has also shown that gp63 can inhibit some degradative phagolysosomal enzymes (85).

Although both antigens may be considered important inhibitors of macrophage activation, other factors are involved in the inhibition of this activation, including (i) alterations in the cyclooxygenase and lipooxygenase pathways (72), (ii) suppression of macrophage expression of class I and class II major histocompatibility complex gene products (45, 73), (iii) defective regulation of calcium-dependent signaling (66), (iv) altered activation and translocation of protein kinase C (67), and (v) activation of the Src homology 2 domain containing tyrosine phosphatase-1 (60). Thus, we concluded that the mechanisms involved in amastigote survival in the macrophage are highly complex and not exclusively related to external proteins.

LPG, GPIL, and gp63 are thought to be the first substrates to encounter the innate immune system. It is recognized that the presence of *Leishmania* in a tissue induces the production of cytokines by antigen-presenting cells (APCs). Interleukin-12 (IL-12) is one of the principal cytokines responsible for the predominant Th1 response and the success of the cellular response by the adaptive system in individuals with leishmaniasis. IL-4 produced by CD4^{+} T lymphocytes (the predominant Th2 response) appears to be responsible for an unfavorable response to *Leishmania* clearance in the tissues (20, 88). Although Th1 and Th2 patterns have been well described and cytokine production and function have been well established, the link between *Leishmania* and the production of cytokines is still not well understood. The expression of IL-12 by APCs, such as dendritic cells (DCs), is conditioned by the recognition of parasite antigens (16).

DCs are potent APCs and induce T-cell activation, like that which occurs with IL-12, IL-10, and gamma interferon (IFN-γ), by cytokine synthesis (23). IL-12 is the main cytokine involved in the initial response, activating NK cells and a Th1 immune response. Not only DCs but also the macrophages are important cells after activation, and they are mediated mainly by IFN-γ. Activated macrophages produce tumor necrosis factor alpha (TNF-α) that synergizes with IFN-γ in the induction of inducible nitric oxide synthase (iNOS) production and further *Leishmania* destruction (6).

Despite this general understanding, the exact mechanism of APCs and the development of the ideal immune response are still not known. It is in this context that the role of the Toll-like receptors (TLRs) must be understood.

OVERVIEW OF TLRs

The TLR signaling pathway is one of the first defensive systems against invasive microorganisms (62). TLRs are transmembrane proteins that confer specificity to the innate immunity cells by recognition of every known category of pathogen that causes human disease. The TLR family consists of 11 members (TLR1 to TLR11) which have specificity for different pathogens and produce different cytokines (38).

TLRs are located on either the plasma membrane or internal membranes of macrophages, DCs, and NK cells. T and B lymphocytes also express TLRs. Cytoplasmic signaling domains of TLRs are separated from the ligand-recognizing extracellular or luminal domains by a single membrane-spanning domain. This extracellular domain contains multiple repeats of a leucine-rich motif (76, 77, 83). Among the TLRs located on internal membranes, such as lysosomes, TLR3, TLR7, TLR8, and TLR9 are the receptors that have been described thus far. TLR1, TLR2, TLR4, TLR5, and TLR6 are located on plasma membranes. It is noteworthy that TLR1 and TLR6 are anchored to the TLR2 using the same signaling pathway despite the fact that they have a different ligand-recognizing extracellular domain (30, 41, 61, 64).

After recognition of specific pathogen antigens, TLRs trigger NF-κB, which then proceeds to the nucleus and promotes the transcription and further synthesis of proinflammatory cytokines (13). These specific pathogen antigens are called pathogen-associated molecular patterns and, in general, are surface molecules or internal structures (RNA, DNA, proteins, enzymes) produced only by microbes and not by host cells. This enables the innate immune system to distinguish between self and nonself.

TLR signaling cascades lead to the phosphorylation of IκB (pre-NF-κB), which targets this protein for degradation, leading to the release of NF-κB dimers (29, 34). Between the activation of TLR and the release of NF-κB, there is an important step that is mediated by adaptor molecules. MyD88 is the most common adaptor molecule for the activation of NF-κB and is present in most TLRs (Fig. 1). Other examples are mitogen-activated protein kinases (JNK or p38), as described by Jono et al. (40).

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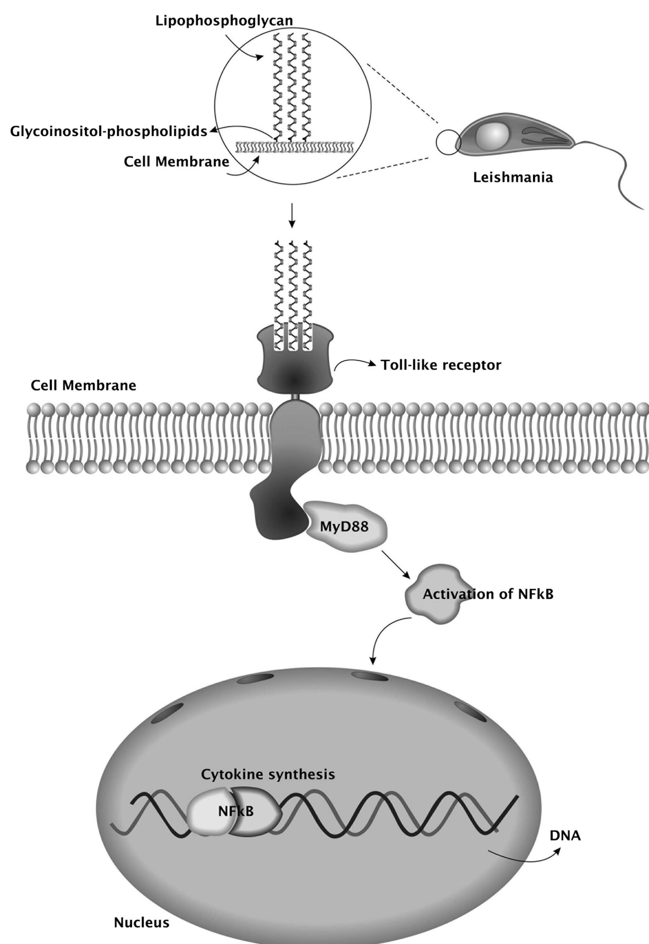

FIG. 1. TLR and MyD88 pathway in *Leishmania* infection. Suggested specific pathogen antigens are activators.

TLR PATHWAY AND LEISHMANIASIS

Several studies have demonstrated that different receptors mediate the uptake and phagocytosis of *Leishmania* sp. by macrophages, although the initial signaling events are unknown. Since recent studies suggested the role of TLRs in the recognition of *Plasmodium*, *Toxoplasma*, and *Trypanosoma*, several groups around the world have also initiated research into the role of TLRs in leishmaniasis (1, 18, 42, 58, 78, 92). The main studies published in this area are shown in Table 1.

The first study evaluating TLRs and the MyD88-dependent pathway in *Leishmania* infection was performed by Hawn et al. in 2002 (36). This study evaluated experimental *Leishmania major* infection and cytokine expression from macrophages in MyD88$^{-/-}$ mice. These authors showed that there was less mRNA expression of IL-1α in the MyD88$^{-/-}$ group and also showed that the levels of IL-1α promoter activation in the MyD88$^{+/+}$ group were similar to the levels obtained with lipopolysaccharide (control group). Unfortunately, *Leishmania* possesses several mechanisms that are capable of decreasing the expression of cytokines. This study evaluated only in vitro cytokine promoter activation and not TLR activation or in vivo cytokine production. In view of these facts, the authors considered it important to study TLRs in knockout animal models.

One year later, Muraille et al. demonstrated the importance of the MyD88 pathway in C57BL/6 mice, a model of the dominant Th1 response with a tendency to cure the cutaneous ulcers caused by *Leishmania*. MyD88$^{-/-}$ mice had a greater number of cutaneous lesions than wild-type C57BL/6 mice (MyD88$^{+/+}$). The number of lesions of the MyD88$^{-/-}$ mice was close to that of BALB/c mice, which have a dominant Th2 response and a tendency to have an increased number and severity of lesions (59). Increased levels of IL-4 and decreased levels of IFN-γ and IL-12(p40) were also demonstrated. A similar study was performed by Debus et al. using C57BL/6 mice (MyD88$^{-/-}$), and they obtained the same results. They therefore evaluated a group of mice with a monoclonal anti-

TABLE 1. Published studies evaluating the TLRs and pathways in *Leishmania* infection

Authors	Reference	Model	Species	Pathway or TLR	Outcome
Hawn et al.	36	BALB/c	*L. major*	MyD88$^{-/-}$	Decreased IL-1α promoter activation
Muraille et al.	59	C57BL/6 (B6WT)	*L. major*	MyD88$^{-/-}$	Increased number of lesions and IL-4 levels, decreased levels of IFN-γ and IL-12
Debus et al.	24	BALB/c	*L. major*	MyD88$^{-/-}$ and IL-4^{-}	Inhibition of IL-4, increased IFN-γ levels
de Veer et al.	28	C57BL/6	*L. major*	MyD88$^{-/-}$	Absence of TLR2 increased the number of lesions
Becker et al.	14	Cell culture	*L. major*	TLR2	LPG could activate NF-κB by TLR2 ligation
Kropf et al.	43	C57BL/10ScN	*L. major*	TLR4$^{-/-}$	Absence of TLR-4 increased the number of lesions
Kropf et al.	43	C57BL/10ScN	*L. major*	TLR4$^{-/-}$ but IL-12	Leishmaniasis control was TLR4 and IL-12 dependent
Lange et al.	56	BALB/c	*L. major*	TLR9	Vaccine decreased the number of lesions by the TLR9 pathway
Antoniazi et al.	10	C57BL/10ScN	*L. major*	TLR4^{-} and TLR4^{+}	Little variation of chemokine levels
Flandin et al.	32	Cell culture	*L. donovani*	TLR3	TLR3 was activated by double-stranded RNA
Li et al.	47	BALB/c	*L. major*	TLR9	IL-18 improved the Th1 response, probably via TLR9
De Trez et al.	27	C57BL/6	*Leishmania* sp.	MyD88	Pathway improved DC maturation
Ribeiro-Gomes et al.	74	C57BL/6, BALB/c	*L. major*	TLR4	Neutrophil elastase activated TLR4
Schleicher et al.	79	C57BL/6, BALB/c	*L. infantum*	TLR9$^{-/-}$	Cytokine production from DCs was dependent on TLR9

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body against IL-4, which enhanced the Th1 response with higher IFN-γ levels (24).

Despite these studies, it remained unclear which MyD88-dependent TLR was involved. Debus et al., who performed the first study mentioned above, described experiments with TLR2- and TLR4-deficient mice that displayed a normal Th1 response, thus creating some controversy (24).

LPG, TLR2, TLR3, TLR4, AND TLR9

We have described the importance of LPG and GPIL in the immune response. de Veer et al. attempted to demonstrate the relationship of LPG and GPIL to MyD88 and TLR2 (28). The LPG was associated with an increased TNF-α level independent of TLR4. This NF-κB activation by LPG was mediated by TLR2. The study mentioned above concluded that TLR2 is an activator of NF-κB mediated by LPG but that other structural products of *Leishmania* could also activate other TLRs. Becker et al. obtained the same result in vitro using NK cells and suggested that three molecules of LPG aggregate with one molecule of TLR2, thus corroborating the theory regarding this TLR (14). These data invalidate the concept of unresponsiveness of TLR2 and TLR4 in leishmaniasis (24).

Beyond the studies that have demonstrated the importance of TLR2, studies on TLR4 have also yielded promising results. Debus et al. and de Veer et al. have demonstrated that LPG and GPIL have no effect on TLR4 (24, 28). A later study demonstrated that TLR4 was required for efficient parasite control, probably due to the activity of iNOS (44). The activation of iNOS leads to NO synthesis and *Leishmania* death. In the absence of TLR4, the more intense activity of arginase increases the formation of urea and reduces the formation of NO (10). In studying the activation of systemic inflammation through TLR4 by pancreatic elastase, Ribeiro-Gomes et al. determined the role of neutrophil elastase. This enzyme produced by neutrophils induces the leishmanicidal activity of macrophages through TLR4 activation (74). The last studies confirmed the benefits of TLR4 activation in terms of *Leishmania* death, a concept that has been demonstrated for other parasites and bacteria (15, 53, 54, 55, 65).

Mice with a mutation in the TLR4 gene were unable to heal *Leishmania* cutaneous lesions (57, 70). These results were later confirmed by the same group, using an experimental model that was responsive to IL-12 (43). IL-12 is a very important cytokine in the immune response against *Leishmania*. This cytokine develops a Th1 pattern and can be activated by TLR9. In view of this, Li et al. demonstrated the production of proinflammatory cytokines, especially IL-12, via TLR9 in *L. major*-infected mice (47). In humans, TLR9 is present only in plasmacytoid DCs and B lymphocytes. The importance of this receptor in the acute phase of the disease has not been well established yet. Nevertheless, recent studies have demonstrated that it may enhance the effect of vaccination against *Leishmania* (46, 71). In visceral leishmaniasis, NK cells are associated with a good prognosis, and TLR9 is required for the activation of these cells as it is essential for the production of IL-12 by DCs (79).

Recently, some evidence has shown that TLR3 can also contribute to the recognition of *Leishmania* (32). This receptor, like TLR7, TLR8, and TLR9, is located in intracellular endosomal membranes, recognizes double-stranded RNA, and triggers NF-κB and the production of IFN-γ (4). Besides the localization of TLR3, it induces cytokine production by means of a signaling pathway similar to that of the TLR from the plasma membrane. Thus, MyD88 is an adaptor protein that is shared by all the known TLRs (37). However, TLR3 also uses a MyD88-independent pathway to NF-κB and production of IFN-γ (32). Double-stranded RNA is not a structure found in *Leishmania*, but a possible explanation may be the presence of the RNA virus found inside the parasite (63). However, Old World *Leishmania* species, such as *Leishmania donovani*, do not harbor *Leishmaniavirus*. It is possible that double-stranded regions of natural cellular RNA components from eukaryotic cells (human or *Leishmania*), such as rRNA or heterogeneous nuclear RNA or tRNA, can trigger signaling by the TLR3 present in internal membranes under pathophysiological conditions (75, 81, 83). A previous study failed to detect double-stranded RNA structures in *Leishmania* that could activate TLR3, as recently demonstrated for *Schistosoma* eggs in DCs (2). Structures other than double-stranded RNA that are recognized by TLR3 may be present in *Leishmania*. Further study is required to confirm these findings.

de Veer et al. demonstrated that the production of TNF-α and the activation of NF-κB that occurred in experiments with *L. major* were the same as the production of TNF-α and the activation of NF-κB that occurred in experiments with other *Leishmania* species, such as *Leishmania mexicana*, *Leishmania aethiopica*, and *Leishmania tropica* (28). The MyD88-dependent TLR pathway is involved in the induction of DC maturation, which was demonstrated previously with *L. major* by Muraille et al. (59). The same experiment was performed with *L. donovani*, and the response was similar (27).

USE OF TLR

Immunotherapy represents a promising therapeutic approach to treatment of active leishmaniasis since no significant drug has emerged over the past 40 years (8, 9, 11). Vaccine adjuvants and immunotherapy for leishmaniasis have yielded promising results (3, 39, 90). The adjuvants seem to improve the efficacy of vaccine therapy and immunotherapy by stimulation of cellular immunity through TLRs (3). TLR4 activation during immunotherapy for leishmaniasis has been associated with an increase in the cure rate in animal models (17), demonstrating the relationship between TLR and immunotherapy for *Leishmania* infection (12, 22). The therapeutic effects of TLR activation in immunotherapy are associated with the expression of high levels of IFN-γ and early expression of IL-12.

Although this immunological review of the role of TLRs in leishmaniasis has brought some light to the controversy, further study needs to be undertaken in humans, as the human immune system is different from that of the mouse. Despite the fact that isolated human cells have been studied, the *Leishmania* species or strain used in almost all research projects was not the species or strain most commonly found in American tegumentary leishmaniasis. Our knowledge of the innate immune response improved rapidly over the last decade, and this has brought new therapeutic prospects to light, including the

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use of TLRs for immunotherapy of neglected diseases caused by intracellular parasites.

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