Deception and manipulation: the arms of *Leishmania*, a successful parasite

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Leishmania spp. are intracellular parasitic protozoa responsible for a group of neglected tropical diseases, endemic in 98 countries around the world, called leishmaniasis. These parasites have a complex digenetic life cycle requiring a susceptible vertebrate host and a permissive insect vector, which allow their transmission. The clinical manifestations associated with leishmaniasis depend on complex interactions between the parasite and the host immune system. Consequently, leishmaniasis can be manifested as a self-healing cutaneous affliction or a visceral pathology, being the last one fatal in 85–90% of untreated cases. As a result of a long host–parasite co-evolutionary process, *Leishmania* spp. developed different immunomodulatory strategies that are essential for the establishment of infection. Only through deception and manipulation of the immune system, *Leishmania* spp. can complete its life cycle and survive. The understanding of the mechanisms associated with immune evasion and disease progression is essential for the development of novel therapies and vaccine approaches. Here, we revise how the parasite manipulates cell death and immune responses to survive and thrive in the shadow of the immune system.

Keywords: *Leishmania*, immunomodulation, apoptosis, innate immunity, acquired immunity

INTRODUCTION

Parasitism is defined as a "non-mutual symbiotic relationship between species, where one species, the parasite, benefits at the expense of the other, the host," Such relationship occurs during leishmaniasis, where the protozoan *Leishmania* spp. takes advantage of its mammalian host in order to survive and thrive.

Leishmania is a genus of trypanosomatid protozoa that combines over 30 species, of which 11 have significant medical and veterinary importance (1). These parasites have a complex digenetic life cycle, with some particularities, requiring a vertebrate host and an insect vector. The alimentary tract of female *Phlebotomus* spp. and *Lutzomyia* spp. sandflies is colonized by the extracellular form of the parasite, the flagellated, and motile promastigote. Within the insect midgut, *Leishmania* undergoes several developmental changes that culminate in the infectious developmental form of the parasite: the metacyclic promastigote. During the insect blood feeding, the parasite infectious forms are released into the mammal host dermis and quickly uptaken by mono and polymorphonuclear (PMN) cells. Ultimately, in the phagolysosome of macrophages, promastigotes will differentiate into the non-motile amastigote form and multiply. The cycle is completed when the sandfly takes another blood meal, recovering free amastigotes or infected cells (1–3).

Leishmaniasis is endemic in 98 countries, 72 of which are developing nations and 13 correspond to the least developed ones, being considered by the World Health Organization as a Neglected Tropical Disease (4, 5). Over 350 million people reside in areas with active parasite transmission (6). Annually, an estimated 1.5–2 million develop symptomatic disease, and approximately 50,000 die, mostly children (4, 7). Climate changes and population mobility can contribute to the increase of the vector activity and, consequently of the disease incidence (8, 9). The infection caused by *Leishmania* spp. can lead to different clinical manifestations depending on complex interactions between the parasite and the host immune response. The disease is normally divided into three main categories: cutaneous, mucocutaneous, and visceral. Cutaneous leishmaniasis is the most extensively studied form of the disease, usually appearing as a self-healing skin ulcer or dermal granuloma that may need several months or years to heal (10). In some cases, these ulcers can become chronic (11). While most *Leishmania* species cause lesions confined to small areas of the skin, a few, such as *L. braziliensis*, cause diffuse lesions that may even spread to mucosal tissues leading to the mucocutaneous form of the disease (12). Finally, visceral leishmaniasis, the most severe leishmaniasis form, is caused by *Leishmania donovani* and *Leishmania infantum*. It is characterized by fever, cachexia, hepatosplenomegaly and hypergamaglobulinemia and, when untreated, can be fatal (13). In endemic countries, *Leishmania* has gained prominence as an opportunistic pathogen in HIV positive and other immunocompromised patients (8, 14). Leishmaniasis is also a major veterinary concern, as dogs are the main reservoir for the parasite in South America and southwestern Europe (15).

There is no human vaccine available at the moment. Nonetheless, prevention of infection through vaccination seems to be a viable option, since in endemic areas the majority of infected persons do not develop clinical symptoms and previous infection leads to robust immunity against the parasite (16). In the absence

of vaccines, control of the disease relies on prophylaxis and treatment, reviewed elsewhere (17, 18). Treatment options are limited, present significant toxicity and require, with the exception of oral miltefosine, administration in ambulatory conditions (18). Drug resistance is also a growing limitation of some anti-leishmanial therapies (19). Therefore, it is essential to develop novel treatment options and vaccine strategies. Such goal has its cornerstone on the solid knowledge of the details of parasite infection. For this, different strategies that *Leishmania* uses to manipulate the immune system to establish infection will be revised here.

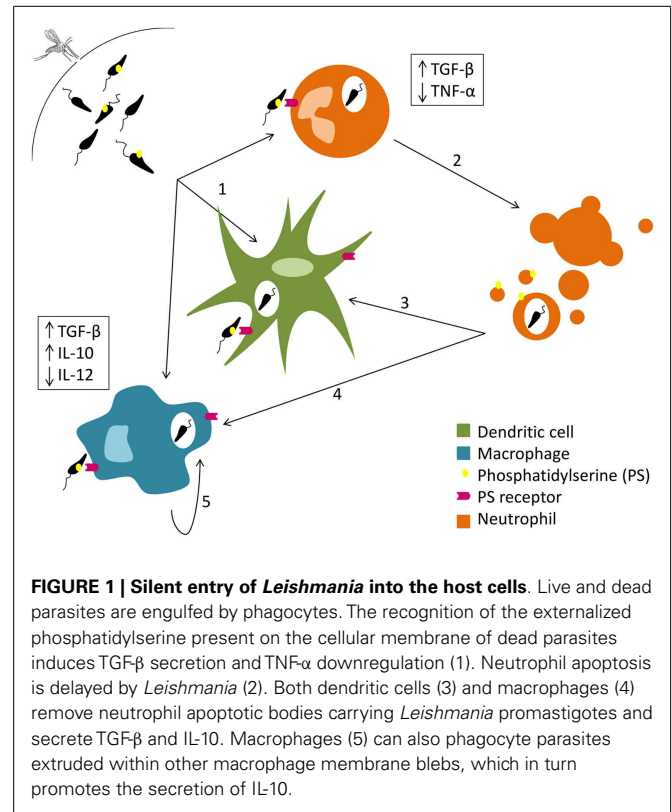## PLAYING WITH DEATH TOWARD THE ESTABLISHMENT AND MAINTENANCE OF INFECTION

Apoptosis, or programed cell death, is a physiological and essential process for the maintenance of general cellular homeostasis. In immunology, this mechanism is indispensable for elimination of autoreactive immune cells (20, 21) and control of the proliferative response (22, 23). Programed cell death also plays a key role in the resolution of infections produced by intracellular pathogens (24). However, and as a result of the continuous host-microbe co-evolutionary process, *Leishmania* developed strategies for using apoptosis to its own benefit.

DEAD PARASITES ARE ESSENTIAL FOR THE SURVIVAL OF FREE PROMASTIGOTES

Parasite cell death, reviewed elsewhere (25–27), seems to be very relevant for the deception of the initial immune response. Some authors described that the presence of apoptotic parasites is essential for successful infection of mice susceptible to cutaneous leishmaniasis. Indeed BALB/c mice did not develop disease after intradermal infection with purified virulent non-apoptotic parasites (28, 29). The need for dead parasites in the infective inoculum is related with the exposure of phosphatidylserine (PS) in the outer leaflet of the parasite cytoplasmic membrane. The exposure of this phospholipid enables a silent invasion, inducing the production of anti-inflammatory cytokines such as TGF-β (30, 31). In fact, a recent study shows that the administration of a PS-targeting antibody after C57Bl/6 mice intradermal infection with *L. amazonensis* promastigotes renders the animals more resistant to the infection (32). Thereby, and as represented in **Figure 1**, the inoculation of equal proportions of dead and live parasites in the mammalian host may allow the silent entry of *Leishmania* into the first cells recruited to the inoculation site (28, 33).

MODULATING APOPTOSIS OF NEUTROPHILS AT THE INOCULATION SITE

It is accepted that macrophages are the cells predominantly infected in leishmaniasis. However, they are neither the first nor the only to be recruited to the site of inoculation. Several evidences support the early recruitment of neutrophils to the inoculation site. Two hours after natural infection of C57Bl/6 mice with *L. major*, neutrophils are predominantly found (34). Such granulocyte infiltration was also seen upon intradermal infection of either BALB/c or C57Bl/6 mice with *L. infantum* and *L. major*, respectively (35, 36), as well as after subcutaneous infection with *L. amazonensis* or *L. major* promastigotes (37–39). Furthermore in a murine air pouch model, *L. major*, and to a lower extent *L. donovani*, predominantly induced the recruitment of neutrophils 6 h

FIGURE 1 | Silent entry of *Leishmania* into the host cells. Live and dead parasites are engulfed by phagocytes. The recognition of the externalized phosphatidylserine present on the cellular membrane of dead parasites induces TGF-β secretion and TNF-α downregulation (1). Neutrophil apoptosis is delayed by *Leishmania* (2). Both dendritic cells (3) and macrophages (4) remove neutrophil apoptotic bodies carrying *Leishmania* promastigotes and secrete TGF-β and IL-10. Macrophages (5) can also phagocyte parasites extruded within other macrophage membrane blebs, which in turn promotes the secretion of IL-10.

after infection (40, 41). Interestingly, the air pouch system revealed that *L. major* derived extracellular vesicles induced the same type of cellular recruitment as parasites (40). These studies preceded the description of Wilson et al. who saw neutrophils infiltration 1 h after intradermal inoculation of *L. donovani* promastigotes in hamsters (42). Although the role of neutrophils during infection is not consensual, several evidences support the capacity of *Leishmania* to modulate their life span. Traditionally, neutrophils show a relatively short life span (43), but *Leishmania* can successfully delay their programed cell death for up than 24 h, potentially benefiting from the protection of a safe intracellular niche (44). However, other studies show induction of neutrophil apoptosis after parasite intake (35). These contradictions may be due to differences in the genetic background of the animal model used (BALB/c versus C57Bl/6), as well as in the parasite inoculation route (45). The delay in the natural apoptotic process of infected neutrophils was related to an inhibition of the pro-caspase-3 processing (44), and the consequent diminishment of caspase 3, a well-known apoptosis executer in neutrophils (46). Moreover, a recent publication clarified the mechanisms by which *L. major* contributes for the neutrophil apoptosis inhibition, showing that the key event is the activation of the extracellular signal-regulated kinases (ERK1/2) survival pathway (47). Sarkar and colleagues showed that the parasite upregulates ERK1/2 phosphorylation, leading to the delay of neutrophil apoptosis (48). Also, this work unveiled additional players of the apoptotic machinery responsible for neutrophil life span enhancement. Among these the anti-apoptotic proteins, Bfl-1 and Bcl-2 were upregulated, preventing the release of cytochrome *c*

from the mitochondria and the downstream activation of caspases. Additionally, processing of the pro-apoptotic Bid was inhibited and the Fas expression reduced, preventing apoptosis triggering (48). This delay of neutrophil death may be essential for the arrival of a sufficient number of antigen-presenting cells (APCs), namely macrophages, and dendritic cells (DCs), to the inoculation site.

"TROJAN HORSE" STRATEGY

After being infected, dying neutrophils secrete different chemotactic factors for macrophages (49, 50); cells that then remove apoptotic neutrophils by phagocytosis and secrete the anti-inflammatory cytokine TGF-β (50). High amounts of IL-10 and low amounts of interleukin (IL)-12 may also contribute for the silent entry of *L. major* into macrophages (51) as shown in **Figure 1**. The parasite can, therefore, arrive to its primary host cell unnoticed and proceed with the infection process, using the so-called "Trojan horse" strategy (52). TGF-β seems to be essential for the establishment of infection not only by *L. major* but also by *L. amazonensis*, although conclusions about the exploitation of the "Trojan horse" strategy in this case cannot be withdrawn (50, 53). DCs have also been related with this tactic. Ribeiro-Gomes et al. recently described in a mouse model of intradermal infection with *L. major* that skin resident DCs uptake apoptotic infected neutrophils and, as a consequence, the activation of *Leishmania*-specific CD4^{+} T cells is prevented somehow (35). Other authors suggested that free parasites silently enter into host cells taking advantage of nearby neutrophil apoptotic bodies with exposed PS (54).

BUYING TIME BY PROLONGING THE LIFE OF MACROPHAGES

When promastigotes reach macrophages, its definitive cellular host, a new step of the infective process begins with their differentiation into amastigotes. Therefore, inhibition of apoptosis may be once more essential for *Leishmania* to protect its niche, enabling the differentiation into the amastigote form that is fully adapted to the phagolysosome. Extensive data exists concerning the capacity of the parasite to increase the life span of infected macrophages. The first description was made by Moore and Matlashewski, who reported that *L. donovani* infection of murine bone marrow-derived macrophages (BMM) represses macrophage apoptosis through a mechanism dependent on the secretion of TNF-α (55). Since then, numerous studies addressed this issue, unveiling some intracellular mechanisms that could explain the death delay. External ATP is known to trigger death in macrophages when injured or stressed, by its binding to purinergic receptors of the P2X family (56, 57). Interestingly, Kolli et al. showed that *L. amazonensis* releases nucleoside diphosphate kinase (NdK), preventing ATP-induced cytolysis of J774 macrophages (58). Further studies are, however, required to access the relevance of NdK in the context of infection. The ERK1/2 pathway also plays a role in the prevention of macrophage apoptosis. Kamir and colleagues described a protein produced by *L. major* that shows structural homology with the human macrophage inhibiting factor (MIF) and exerts similar effects. Indeed this MIF ortholog induced ERK1/2 kinases activation in a CD74-dependent manner, subsequently resulting in the inhibition of macrophage apoptosis *in vitro* (59). The mitochondrial apoptotic pathway is also modulated by *Leishmania*.

BMM infected with *L. major* showed enhanced survival that was related with the prevention of cytochrome *c* release by mitochondria (60), observation possibly explained by the involvement of an anti-apoptotic signaling pathway (61). Ruhland and colleagues showed that *L. major* block macrophage apoptosis through the phosphatidylinositol 3′-kinase (PI3K)/protein kinase B (Akt) signaling pathway. Briefly, Akt phosphorylates the pro-apoptotic Bad, deactivating it, and preventing the release of mitochondrial cytochrome *c* (62), which avoids downstream activation of the effector caspase-3 (60). Similar results were obtained with DCs (63, 64). More recently, it was also shown that apoptosis triggered by oxidative burst is prevented by *L. donovani*. Although infected macrophages were capable of ROS production, a complete abrogation of the downstream caspase cascade was observed due to thioredoxin mediated selective induction of suppressors of cytokine signaling (SOCS) proteins (65). A direct responsibility of a parasitic protein was not addressed in these studies, but we cannot exclude the role of phosphoglycans since there are studies that relate them with apoptosis delay in *L. infantum*, *L. major*, and *L. donovani*-infected macrophages (66, 67). Notwithstanding, the parasites capacity to delay macrophage apoptosis is yet to be shown *in vivo*.

Although parasites delay macrophage death, they cannot prevent it. However, when an infected macrophage dies, *Leishmania* is able to escape. A recent study showed that *L. amazonensis* amastigotes are transferred from cell to cell when the donor host macrophage delivers warning signals of imminent apoptosis (**Figure 1**). Interestingly, that transfer happens without full exposure of the parasite to the extracellular milieu: the parasites are extruded from the host macrophages within membrane blebs rich in phagolysosomal membrane components, which are in turn phagocytized by nearby macrophages that will then secrete the infection promoting cytokine IL-10 (68).

REMOVAL OF EFFECTOR T CELLS BY APOPTOSIS

Modulation of cell death is also used by parasites as a way to directly alter the acquired immune response by elimination of effector cells. Felix de Lima et al. showed that apoptosis levels in both peripheral blood and spleen T lymphocytes from *L. infantum* naturally infected dogs are higher in comparison to control animals. The authors concluded that immunosuppression associated with chronic infection is due to accelerated rates of T cell apoptosis, which in turn contributes to white pulp disorganization in the spleen and diminished T cell levels in peripheral blood (69, 70). Furthermore, active human cutaneous leishmaniasis caused by *L. braziliensis* was associated with increased apoptosis of CD8^{+} and CD4^{+} T cells (71). Interestingly, all of these studies linked T cells apoptosis with active disease. However, the mechanisms are yet to be unveiled. The death receptors apoptotic pathway may be involved, as Fas and FasL expression in human splenic lymphocytes is increased in acute disease (72). Furthermore, the correlation between T cell apoptosis and pathophysiological states was further accessed using mouse infection models. In fact infection of susceptible, but not resistant mice with *L. donovani* induced apoptosis of splenic CD4^{+} T cells after *in vitro* stimulation (73). In this case, the mechanisms involved in apoptosis induction, start to be disclosed. Reckling et al. showed that the pro-apoptotic Bcl-2 family member

Bim possibly has a role in T cell apoptosis in a mouse model of infection with *L. major* (74). Moreover, in another mouse model infected with *L. donovani*, authors concluded that T cell apoptosis could be related with downregulation of PKC and ERK1/2 activities. Ser/Thr phosphatase seems to have a major role in the initiation of this process by dephosphorylation of key molecules of different T-lymphocyte signaling pathways (75).

Table 1 resumes the topics described above, overviewing the modulation of apoptosis by *Leishmania* in different cell types.

OVERCOMING THE IMMUNE LEISHMANICIDAL MACHINERY

Leishmania is one of the few intracellular pathogens that can live and replicate inside the harsh environment of a mature phagolysosome. Apart from this parasite, only *Coxiella brunetti* resides during its entire replicative cycle inside that cellular compartment, as reviewed by Voth and Heinzen (76), while other intracellular pathogens that preferentially infect macrophages escape the phagocytic pathway (77). *Leishmania* must, therefore, cope with different effector molecules from the innate immune response in order to survive.

AVOIDING CELL LYSIS AND TAKING ADVANTAGE OF OPSONIZATION

The first challenge *Leishmania* encounters in the mammalian host is the complement system (78). Traditionally, promastigote complement resistance is associated with two *Leishmania* glycocalyx components (79): lipophosphoglycan (LPG) and the metalloprotease leishmanolisin (GP63). *Leishmania major* parasites deficient for both these molecules demonstrated high complement sensitivity (80–82). LPG avoids the ultimate step of the complement cascade through prevention of the attachment of the C5b-C9-complex to the parasite surface (83, 84). On the other hand, GP63 inactivates C3b preventing the formation of the C5 convertase complex (85, 86). Albeit, Dominguez et al. showed that under physiological conditions 85–100% of *L. donovani*, *L. infantum, L. major*, and *L. amazonensis* promastigotes are killed by complement after 2.5 min in human blood (87). Yet, it was also published that as soon as 1 min after *L. amazonensis* and *L. donovani* contact with human blood, infected granulocytes were easily found (88). Therefore, it is essential for the parasite to escape the complement onslaught by quickly entering a phagocytic cell.

Once again *Leishmania* glycocalyx components are used to subvert the innate immune system enhancing the phagocytosis of the parasites. Both GP63 and LPG can directly interact with the host cell surface through binding to the fibronectin receptor and the mannose/fucose receptor, respectively (89–92). Moreover, iC3b, the cleavage product of C3 by GP63, can function as an opsonin (85), and LPG interacts with the early inflammatory C-reactive protein, which triggers phagocytosis (93, 94). Interestingly, iC3b is a ligand of the complement receptor 3 (CR3) (95), and this interaction is directly related with the downregulation of IL-12 production by macrophages (96). The mechanism by which this downregulation happens is not known; however, we may not exclude a toll like receptor (TLR) inhibition since C5a, another complement component, has a negative impact on the TLR-4 induced IL-12 synthesis (97). This may ultimately contribute for the silent entry of the parasites into the host cells.

Table 1 | Apoptosis modulation during *Leishmania* infection.

Cell type	Alteration of apoptosis related molecules	Outcome	Key player	References
Neutrophils	Phosphorylation of ERK1/2 Upregulation of BX-1 and Bcl-2 Inhibition of Bid and pro-caspase 3 processing Prevention of mitochondrial cytochrome *c* release Downregulation of Fas expression	Apoptosis inhibition	Unk	(48)
Macrophages	Decrease of extracellular ATP Phosphorylation of ERK1/2 Activation of PI3K/Akt signaling pathway Deactivation of Bad Induction of SOCS proteins Inhibition of pro-caspases 3 and 7 processing	Apoptosis inhibition	NdK Lm1740MIF Unk Thioredoxin	(58) (59) (62) (65)
Dendritic cells	Upregulation of BX-1 and Bcl-2? Prevention of mitochondrial cytochrome *c* release? Inhibition of pro-caspases 3 and 7 processing	Apoptosis inhibition	Unk	(63, 64)
T cells	Upregulation of Bim? Deactivation of ERK1/2 Downregulation of Bcl-2 Increase of mitochondrial cytochrome *c* release Upregulation of pro-caspase 3 processing	Induction of apoptosis	Unk Ser/Thre phosphatase	(74) (75)

Akt, protein kinase B; ERK, extracellular signal-regulated kinases; MIF, macrophage inhibiting factor; NdK, nucleoside diphosphate kinase; SOCS, suppressors of cytokine signaling; Ser/Thr, serine/threonine; Unk, unknown.

TOWARD A SUCCESSFUL DIFFERENTIATION: ALTERATIONS DURING THE PHAGOLYSOSOME MATURATION PROCESS

After promastigote entry into the host cell, *Leishmania* needs to differentiate to the amastigote form. Since promastigotes cannot survive in the harsh environment of the phagolysosome (low pH, hydrolases), a delay of phagolysosomal fusion was considered essential for the parasite differentiation process (98). Such delay has been described for *L. major*, *L. infantum*, and *L. donovani* via mechanisms that may or may not involve LPG (98, 99). However, with *L. mexicana* and *L. amazonensis*, this was not proved (100–103). For these parasites, the large parasitophorous vacuoles found in macrophages dilute the hydrolytic enzymes upon lysosome fusion to a level below their effectiveness, allowing promastigotes to differentiate without any requirements of fusion delay (100). In the case of *L. donovani*, it was shown that LPG impairs the association of synaptotagmin V to phagosome membranes, inhibiting the recruitment of the vesicular proton-ATPase and preventing their acidification, allowing promastigote to amastigote differentiation (104). *Leishmania donovani* LPG was also associated with retention of the small GTPase Cdc42 at the phagosome membrane, leading to F-actin accumulation around the phagosome and presumably interfering with vesicle trafficking and phagosome maturation (105, 106).

ROLE OF GP63 IN THE DEFENSE AGAINST ANTIMICROBIAL PEPTIDES

Inside a phagolysosome, fully differentiated or not, *Leishmania* has to deal with other components of the innate immune system: the antimicrobial peptides (AMPs). AMPs are structurally diverse cationic proteins with intrinsic antimicrobial activity, playing normally by disruption of cell surface membranes resulting in osmotic lysis of the pathogen. They can be found both intra and extracellularly, and most of them are constitutively produced and secreted (when applicable) (107, 108). Some human AMPs present activity against *Leishmania*. For example, Kulkarni et al. showed that cathelicidin, an intracellular AMP present in macrophage lysosomes, can kill up to 50% of *L. major* and *L. amazonensis* parasites (109). The same group showed in a different study that α-defensins, produced by neutrophils, also kill *L. major* parasites (110). GP63 play a key role in the defense against these peptides, as it was shown that gp63 KO promastigotes were efficiently killed in a dose dependent manner by AMPs (109).

COPING WITH REACTIVE OXYGEN AND NITROGEN SPECIES (ROS AND RNS)

Once inside the host cell, ROS and RNS are the cellular major arms against *Leishmania*. NO$^{\bullet}$ is synthesized by nitric oxide synthase (NOS) during the conversion of L-arginine to L-citrulline, while O_2^- and other reactive oxygen species (ROS) are generated by the membrane-bound NADPH-dependent oxidases (NOX). These reactive species contribute for the generation of others as $ONOO^-$, $NO_2^{\bullet}$, and nitrogen trioxide (111). Although NO is considered the most relevant microbicidal molecule, ROS are also associated with disease susceptibility since NOX deficient mice are more susceptible to *L. donovani* and *L. major* infection (112, 113). However, unlike what happens with inducible NOS (iNOS) KO mice, NOX deficient mice eventually control the infection (112–114). Therefore, the parasite needs to somehow neutralize

these reactive species and/or prevent their production to avoid a certain death by oxidative stress. The inflammatory cytokine TGF-β produced by infected phagocytes shifts the L-arginine metabolism toward the production of L-ornithine through the activation of arginase (115, 116). This metabolic shift leads to a decrease in NO secretion favoring intracellular *Leishmania* growth (117). Glycocalyx components can also play a role in the protection of *Leishmania* parasites from ROS. A genetic rescue of a *L. amazonensis* GP63 deficient strain increased its intramacrophage survival potential, which was probably related with inhibition of ROS generation (118, 119). In turn, LPG not only prevents ROS generation through inhibition of NOX recruitment to the phagosome membrane, but also directly scavenges these reactive species (81, 120). Glycosylinositolphospholipid (GILP), another component of the glycocalyx, may also be important during the amastigote form, suppressing macrophage iNOS expression and, consequently, NO production (121). Finally, we cannot disregard the intrinsic antioxidant machinery of *Leishmania*, whose most important components are trypanothione synthase and trypanothione reductase. The last one is essential for the fight against ROS and NOS, once disruption of the trypanothione reductase gene renders the parasites susceptible to intracellular killing by macrophages (122). A recent publication shows that *L. donovani* activates multiple own enzymatic mechanisms for the detoxification of ROS and NOS (123). Some of these enzymes have already been associated with protection against reactive species, including the *L. infantum* peroxiredoxins LicTXNPx and LimTXNPx, *L. major* pteridin reductase, and *L. donovani* superoxide dismutase (124–126).

Table 2 discusses the different ways by which components of the *Leishmania* glycocalyx prevents parasite killing by innate immune response.

MODULATING THE IMMUNE RESPONSE THROUGH ALTERATION OF CYTOKINE AND CHEMOKINE SIGNALING AND PRODUCTION

Cytokines are cell signaling mediators, which affect cell function in an autocrine, paracrine, or endocrine manner. Interference with the normal cytokine production is a powerful weapon that the parasite can use for the modulation of immune function. It is generally accepted that production of IL-12 by macrophages and DCs is associated with resistance against *Leishmania*. This cytokine induces naive T cells maturation toward an IFN-γ producing Th1 phenotype (resistant to infection), which in turn induce macrophage M1 activation and elimination of parasites (127, 128). Th2 cytokines, namely IL-4 regarding cutaneous leishmaniasis and IL-10 and TGF-β in the case of visceral disease, have been related with disease susceptibility and progression by induction of an M2 macrophage phenotype (129–131). Therefore, parasites seem to modulate the immune response toward a Th2 phenotype. However, this Th1/Th2 straight polarization seems only to be observed in some murine models, and cannot be fully applicable to human diseases (132). The Th1/Th2 paradigm (reviewed elsewhere) (133, 134) states that Th1 and Th2 cells counter-regulate each other. That would imply that *Leishmania*-induced polarization of the immune response toward a Th2 phenotype would suppress a Th1 immune response.

Table 2 | Glycocalyx components: overcoming innate immune leishmanicidal machinery.

Glycocalyx component	Species	Protective role	Mechanism	References
LPG	*L. major*	Inhibition of complement-mediated lysis	Prevention of attachment of the C5b-C9-complex	(83)
	L. donovani	Promotion of phagocytosis to escape the extracellular milieu	Interaction with C-reactive protein and direct binding to phagocytes receptors	(91, 93, 94)
	L. mexicana			
	L. donovani	Delay of phagolysosome maturation process	Inhibition of the recruitment of vesicular proton-ATPase	(104)
	L. donovani	Reduction of leishmanicidal reactive species	Inhibition of ROS generation	(81, 120)
	L. major		ROS scavenging	
GP63	*L. major*	Inhibition of complement-mediated lysis	Inactivation of C3b	(85, 86)
	L. infantum			
	L. major	Promotion of phagocytosis to escape the extracellular milieu	The C3b inactivation product functions as an opsonin Direct binding to phagocytes receptors	(85, 89, 92)
	L. infantum			
	L. donovani			
	L. major	Prevention of antimicrobial peptide mediated lysis	Proteolytic degradation of the antimicrobial peptides	(109)
	L. amazonensis	Reduction of leishmanicidal reactive species	Inhibition of ROS generation	(119)
GILP	*L. major*	Reduction of leishmanicidal reactive species	Suppression of iNOS expression and NO production	(121)

GILP, glycosylinositolphospholipid; iNOS, inducible nitric oxide synthase; LPG, lipophosphoglycan; NO, nitric oxide; ROS, reactive oxygen species.

However, what is observed in human disease is a peculiar mixed cytokine response, variable, depending on the infective species (132, 133, 135).

LEISHMANIA MODULATES TLR SIGNALING

Toll like receptors recognize a variety of pathogen-associated molecular patterns (PAMPs), from proteins to nucleic acids. Upon engagement, TLRs mediate the activation of different transcription factors, such as nuclear factor-κB (NF-κB) and interferon-regulatory factors (IRFs), leading to the production of inflammatory cytokines (136, 137). Induction of cell mediated immunity (138–140) and promotion of NO production (141) are other two known TLR triggered responses against *Leishmania* infection. Nevertheless, the parasite developed strategies that interfere with TLR associated signaling cascades subverting the traditional pro-inflammatory responses. *Ex vivo* experiments suggest that TLR-2 performs a minor role in initiating the synthesis of pro-inflammatory cytokines, namely IL-12, during mice infection with *L. infantum* (142). Chandra et al. showed that *L. donovani* can shift TLR-2 responses toward a Th2 immune response, with downregulation of IL-12 production in macrophages, through MAP kinase inactivation (143). The crosstalk between TLR-2 and CCR-5 (which expression is dependent on the expression of the first one) was also described as relevant in *L. donovani* infection, promoting parasite internalization and inducing a Th2 immune response (144). Moreover, the interaction between TLR2 and LPG was shown do decrease TLR-9 expression leading to a lesser inflammatory profile (145). Nevertheless, the interplay between *Leishmania* and TLRs is highly complex and needs further clarification, once there are several reports showing that LPG-TLR interactions can also result in increase of anti-leishmanial responses by effector cells (146).

The capacity of *Leishmania* to interact with regulatory proteins of the host may also be relevant for TLR signaling modulation. As an example, *L. donovani* exploits a host negative TLR regulator, the deubiquitinating enzyme A20, to inhibit the TLR-2-mediated pro-inflammatory gene expression, consequently suppressing IL-12 and TNF-α production (147). It was also described that *L. donovani*, along with *L. mexicana* and *L. major*, uses the macrophage tyrosine phosphatase SHP-1 to inactivate kinases involved in TLR signaling (148). As happens with TLR-2, *Leishmania* exploits host TLR regulators to deal with TLR-4 activation. Gupta et al. showed that *L. donovani* parasites alter the ubiquitination pattern of TRAF3, preventing its degradation, which is required for the effective cytosolic translocation of the TLR-4-anchored multiprotein complex. As a consequence, NF-κB is silenced leading to a downregulation of IL-12 and TNF-α production (149). Furthermore, *L. amazonensis* amastigotes can suppress TLR-4 activation on DCs via rapid degradation of intracellular signaling proteins (JAK/STAT, NFκB, and IRF) leading to a decrease in IL-12 production (150). The deubiquitinating enzyme A20 also has a role in the inhibition of the TLR-4-mediated pro-inflammatory response. However, in this case, the regulation is an indirect consequence of active disease promoted by the high levels of TGF-β that infected cells produce (151). Another "macrophage imbalance" mediated by TLR-4 signaling manipulation was described by Shweash et al. These authors reported that *L. mexicana* promastigotes are able to prolong and enhance PGE$_2$, NO, and arginase production through TLR-4, and consequently achieve the reduction of macrophage released IL-12 (152). Finally, *Leishmania* can impair TLR signaling through prevention of receptor ligand interaction. Here, the player is ectoin-like serine peptidase inhibitor, produced by *L. major*, which inhibits neutrophil elastase and consequently prevents TLR-4 activation (153, 154). Ultimately, TLR-4 signaling inhibition in macrophages induces an

Table 3 | Strategies of TLR signaling modulation by *Leishmania*: an overview.

TLR	Species	Key player	Mechanism of modulation	Reference
TLR 2	*L. donovani*	Unk	Shift to Th2 immune response	(143)
	L. donovani	Deubiquitinating enzyme A20	Inhibition of TLR-mediated pro-inflammatory gene expression	(147)
	L. donovani			
	L. mexicana	SHP-1	Inhibition of TLR-mediated pro-inflammatory gene expression	(148)
	L. major			
	L. major	LPG	Downregulation of TLR-9 expression	(145)
TLR-4	*L. amazonensis*	Unk	Degradation of intracellular signaling proteins	(150)
	L. donovani	Deubiquitinating enzyme A20/SHP-1	Inhibition of TLR-mediated pro-inflammatory gene expression	(151)
	L. major	Ecotin-like serine peptidase inhibitor	Shift to Th2 immune response	(154)
	L. mexicana	Unk	Enhancement of PGE_2, NO, and arginase production	(152)

LPG, lipophosphoglycan; NO, nitric oxide; PGE_2, prostaglandin E_2 SHP, sarcoma homology 2 domain phosphatase-1; Th, T helper; TLR, toll like receptor; Unk, unknown.

M2b phenotype that correlates with higher IL-10 levels and a Th2-type immune response (154). **Table 3** collects the data discussed above.

INFLUENCING CHEMOKINE PRODUCTION

As an intracellular pathogen, *Leishmania* depends on the initial recruitment of host cells for successful establishment and perpetuation of infection. Chemokines are small proteins that induce and regulate the migration of immune cells, and their expression is known to be modulated by *Leishmania* spp. (41, 155). Several studies reported the upregulation of numerous chemokines (RANTES/CCL5, MIP-1α/CCL3, IP-10/CXCL10, MCP-1/CCL2, MIP-1β/CCL4, MIP-2/CXCL1, and IL-8/CXCL8) after *L. major*, *L. donovani*, *L. tropica*, *L. infantum*, and *L. panamensis* inoculation (156–161). Interestingly, few of these chemokines attract neutrophils, which can be another *Leishmania* mediated immune modulation strategy. Although neutrophils may be a possible vehicle for *Leishmania*, facilitating infection, it was described that exacerbated neutrophil recruitment is associated with parasite killing (162). On the other way, it was also shown that skin lesions of *L. major* infected mice mainly contained Th2 cell-attracting chemokines, such as CCL7 (163, 164). The absence of Th1 cell-attracting chemokines in these lesions may reflect the downregulation of the expression of genes linked with Th1 trafficking, such as the ones coding for CXCR3 chemokines (165). Last but not least, it was described that *Leishmania* may also profit from malnutrition to impair chemokine secretion and to establish infection (158, 166). Interestingly, differential expression of chemokines induced by distinct parasite strains leads to various infection and disease outcomes. As an example, human infection with *L. mexicana* may lead either to a self-healing cutaneous form or to a non-healing cutaneous disease, associated with the increased expression of CCL2 and CCL3, respectively (167). This differential chemokine expression was also seen in human infection with *L. panamensis* (168), and may be related with parasite virulence, once in a mouse model infected with two strains of *L. braziliensis* (highly virulent versus less virulent) a differential chemokine expression profile was observed (169). Elaboration of these studies would be of great interest, particularly regarding the parasite virulence factors responsible for the induction of the chemokine profiles seen in non-healing/severe pathologies, which will unveil new parasite immunomodulatory players.

INTERFERING WITH CYTOKINE PRODUCTION

Although cytokines are important throughout the whole *Leishmania* infectious process, they are fundamental during the acquired immunity phase. IL-12 is mainly produced by APCs, particularly by DCs (170), and is related with important cytokines that mediate very different outcomes of *Leishmania* infection, such as IFN-γ, IL-10, and IL-4. Therefore, the interference with IL-12 is a recurrent phenomenon in *Leishmania* infection. *Leishmania major* was found to deplete cholesterol, inhibiting the assembly of an IL-12-inducing CD40 signalosome and modifying the cell effector functions (171). Others have reported that *L. major* infection directly down-regulates IL-12 production through a CD40 signaling-regulation (172). Furthermore, *L. mexicana* and *L. donovani* were also found to impair LPS-induced IL-12 production by BMM through cysteine proteinase mediated NF-κB degradation (173, 174). Others have correlated IL-12 downregulation with *Leishmania* evasion mechanisms, probably through PI3K/Akt signaling pathway modulation (175–179). In a recent study, *Batf3*^{-/-} mice, that lack the major IL-12 producing and cross-presenting subsets CD8α^{+} and CD103^{+} DCs, showed enhanced susceptibility to *L. major* infection partially due to reduced IFN-γ and increased IL-4 and IL-10 secretion (180). IFN-γ is released by Th1 cells triggering the leishmanicidal activity of macrophages via expression of the inducible NO synthase which, in turn, leads to the killing of intracellular *Leishmania* (181). Thus, several reports on prevention of IFN-γ secretion and/or action by the parasite exist. Ray et al. showed that infection of macrophages with *L. donovani* causes a decrease in the phosphorylation of the IFN-γR-α subunit, which consequently affects the receptor expression (182). Furthermore, GP63 was related with reduction of IFN-γ producing cells in BALB/c mice infected with *L. amazonensis* (183). Finally, our group reported that the non-secreted *Leishmania* protein LmS3arp is also associated with downregulation of IFN-γ production by splenocytes (184). It was described that regulatory T cells (Tregs) may have a role in the downregulation of IFN-γ, in a murine model infected with *L. amazonensis* (185). However, it

is yet to be unveiled whether and how parasites are able to control these cells. Furthermore, the role of Tregs in infection progression and pathology diverges, depending on the infecting *Leishmania* species. While Tregs are associated with disease exacerbation and parasite persistence, in the infection context with *L. donovani* and *L. major*, respectively, *in vivo* experiments with *L. amazonensis* shown that Tregs aid in disease resolution (185–188). Additionally, Ehrlich et al. demonstrated *in vivo* that both the transfer of Tregs to chronically infected animals with *L. panamensis*, and their treatment with rIL-2/anti-IL-2 Ab complex for Treg expansion contributed for disease amelioration, showing the protective role of Tregs in *L. panamensis* infection and a possible immunotherapeutical role of these cells (189). The immunosuppressive IL-10 has long been associated to visceral disease pathogenesis (190), being not only important in the establishment of infection but also during parasite persistence through the direct inhibition of Th1 cell development, preventing the resolution of the infection (191). In fact, IL-10 receptor blockade or IL-10 KO mice renders animals resistant to *L. donovani* infection (192, 193). The major source of IL-10 in both cutaneous and visceral leishmaniasis is controversial. Some works proposed T regs and Th2 lymphocytes as the main IL-10 producers (190, 194–197), while others claim that Th1 lymphocytes are the main IL-10 source (190, 194, 198–200). Notwithstanding, the parasite can also promote IL-10 production by other cells. For instance, *L. braziliensis* amastigotes and promastigotes induce the secretion of this cytokine by PBMCs (201). This IL-10 secretion was shown to be mediated by phagocytosis of opsonized parasites in an *in vivo* model of low dose infection with *L. major* (202) and also with *L. amazonensis* and *L. mexicana* (203, 204). The *Leishmania* secreted protein *Li*TXN1 is also involved in the promotion of IL-10 production by spenocytes (205). Apart from IL-10, IL-4 also induces Th2 responses (206) and is particularly involved in the promotion of cutaneous leishmaniasis. Tabatabaee et al. suggested that *L. major* secrete immunosuppressive factors that promote IL-4 production by lymphocytes (207). This cytokine was shown to interfere with the synergy of IFN-γ/FasL that contributes to macrophage activation and killing of intracellular *L. major* (208). There is, however, some contradictory studies showing that IL-4 promotes IL-12 production by bone marrow-derived DCs (BMDC) and resistance to the disease (209, 210). Hurdayal et al. clearly showed that DC specific IL-4 receptor alpha (IL-4Rα)-deficient BALB/c mice became hypersusceptible to *L. major* infection, due to a decrease in IL-12 and an increase in IL-10 production by DCs (211). These contradictory observations with IL-4 might be possibly explained by the fact that a low infection dose with *L. major* induces a Th2 response in C57BL/6 mice, whereas high doses induce a Th1 response, both dependent on IL-4 production by lymphocytes (212). Considering the fact that, in average, sandflies transmit not more than 1000 parasites per bite, an induction of Th2 response might be expected in a real situation (213).

Other cytokines have been studied in the context of *Leishmania* infection. IL-17, for instance, has been involved in the outcome of cutaneous leishmaniasis (214–216). Although there are not many studies showing *Leishmania* modulation of this cytokine, some clues exist about how this can happen. Castellano et al. showed that *L. amazonensis* antigens possibly induce a decrease in the

percentage of $CD3^+CD4^+IL-17^+$ human cells, at least in cases of HIV/*Leishmania* co-infection (217). Interestingly, patients with signs of active disease present lower levels of Th17 cytokines (218, 219). Yet, more studies are needed to discover whether *Leishmania* can directly modulate IL-17 production or if it acts on other interlinked cytokines such as IL-6 and IL-23 (201, 216). IL-1β was also shown to influence the clinical course of leishmaniasis, and is strictly related with inflammasome activation, a general but powerful antimicrobial strategy in innate immunity (220). A recent study showed that *Leishmania* can prevent caspase-1-dependent IL-1β activation through a C-type lectin (SIGNR3) mediated signaling process, which consequently favors parasite persistence (221). The parasite key player responsible for this signaling modulation is, however, yet unknown. Finally, IL-13, IL-21, and IL-27 may also have a role in leishmaniasis, either preventing or inducing pathology (222–225).
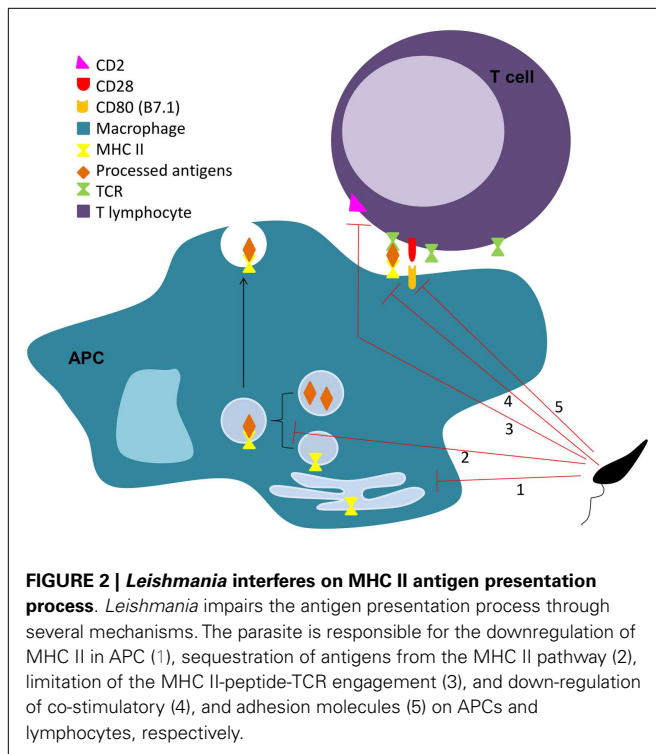
IMPAIRING CELLULAR FUNCTION

Leishmania is able to control the acquired immunity through the impairment of effector cells function. Antigen processing and presentation by APCs is necessary for the efficient priming of effector T cells which, in turn, will generate a directed and specific immune response (226). Through phagocytosis of parasite debris or intracellular parasite degradation, APCs process and present *Leishmania* antigens (227). Both major histocompatibility complex (MHC) I and MHC II antigen presentation are related with *Leishmania* elimination, although only the second one is essential for complete parasite clearance (212, 228). *Leishmania* can interfere with antigen processing and presentation, consequently modulating once again the immune function.

LEISHMANIA INTERFERES WITH ANTIGEN PRESENTATION BY PROFESSIONAL CELLS

In 1987, Reiner et al. described that *L. donovani* decreases macrophage expression of both MHC I and MHC II molecules (229). Others have also reported a *L. major* related downregulation of MHC molecules in DCs (230), which can be mediated by direct parasite internalization of these molecules (231–233). Interestingly, *L. donovani* extracellular vesicles were shown to inhibit MHC-II expression in human monocyte-derived DCs (234). Furthermore, both *L. pifanoi* and *L. amazonensis* amastigotes interfere with the macrophage antigen processing process by sequestration of antigens from the MHC II pathway, through a mechanism involving targeted vacuolar fusion (235, 236). However, prevention of surface-expressed MHC class II-peptide complexes is not the only way by which the parasite impairs antigen presentation (**Figure 2**). *L. donovani* was shown to interfere with BMM antigen presentation by modulating the capacity of surface MHC class II-peptide complexes to engage the T cell receptor (TCR) (237). An increase in the infected cell membrane fluidity by cholesterol depletion and ceramide generation may justify this inefficient engagement (238, 239). Adhesion molecules are also important in the process of antigen presentation. They help during the initiation of contact between APCs and T cells, required for the subsequent formation of the immunological synapse. Bimal et al. reported that particularly $CD4^+$, but also $CD8^+$ T cells, from patients with active visceral leishmaniasis caused by *L. donovani* express

FIGURE 2 | *Leishmania* interferes on MHC II antigen presentation process. *Leishmania* impairs the antigen presentation process through several mechanisms. The parasite is responsible for the downregulation of MHC II in APC (1), sequestration of antigens from the MHC II pathway (2), limitation of the MHC II-peptide-TCR engagement (3), and down-regulation of co-stimulatory (4), and adhesion molecules (5) on APCs and lymphocytes, respectively.

less CD2 than the ones from healthy subjects (240). *In vitro* and *in vivo* studies must, however, be performed to confirm that this downregulation of CD2 in CD4$^+$ T cells is caused directly by the parasite. Co-stimulatory molecules are necessary for the full activation of T cells by APCs, which expression can be downregulated by *Leishmania*. For instance, Kaye et al. showed that BMM infected with *L. donovani* expressed lower levels of co-stimulatory molecules B7.1 and heat stable antigen than the non-infected controls (241). Mbow et al. also reported that Langerhan cells of BALB/c mice infected with *L. major* showed a down-regulation of B7.1 expression (242).

LEISHMANIA-INDUCED CELLULAR ANERGY AND EXHAUSTION

The lack of co-stimulatory molecules on APCs, particularly in DCs, can be a consequence of another immune modulation strategy used by *Leishmania*, the inhibition of cell maturation/activation. The induction of cellular unresponsiveness or anergy is the ultimate weapon that *Leishmania* uses in the fight against the immune system. Impairment of APC function was reported by our group. Briefly, BMDC infection with *L. infantum* promastigotes counteracts LPS-triggered activation. Parasites avoided the upregulation of transcription and surface expression of CD40 and CD86 co-stimulatory molecules on BMDC, through activation of the PI3k/Akt pathway and the impairment of NF-κB transcription factor (243). This DCs activation/maturation arrest was also described for *L. amazonensis* infection on mice and human cells (150, 217, 231, 244). *Leishmania* has also been associated with T cell exhaustion (245). Gautam et al. described that IFN-γ production by CD8$^+$ effector cells was absent in active human visceral leishmaniasis. These cells expressed elevated levels of Cytotoxic T Lymphocytes Antigen 4 (CTLA-4) and programed

death protein 1 (PD1) (246), negative regulators of T cell activation associated with T cell anergy and exhaustion (247). Similar results were also reported by Esch and colleagues, regarding not only CD8, but also CD4 T cells (248). This topic was recently reviewed by our group regarding *Leishmania* and other parasitic infections (249).

CONCLUSION

Remarkable progresses were made in the past years in the knowledge of immunomodulation by *Leishmania*. As a result of a long parasite-host co-evolutionary process, this organism can escape or fight the immune system using diverse and complex strategies. However, the knowledge produced is sometimes dispersed and contradictory, reflecting several variables such as infecting species and different infection models. Notwithstanding, it is now clear that the parasite can modulate cell death, alter the maturation process of the phagolysosome, modulate cytokine, and chemokine production by host cells, and impair cell function, in order to silently enter in host cells and successfully differentiate and infect. Furthermore, *Leishmania* released material seems to have by itself some immunomodulatory potential. Therefore, the study of the parasite exoproteome may contribute for the discovery and characterization of the yet unknown arms that the parasite uses to achieve victory against the immune system. The unraveling of the agents responsible for this modulation will help us to define the requirements for infection and disease. This will ultimately become the cornerstone that will contribute to develop novel strategies to fight the disease. Although not discussed in this review, but not less important, the pressure that the parasite exerts in the host cells metabolism is now an area of growing interest. The nascent field of immunometabolism will also contribute significantly for the full understanding of the infectious process.

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