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Immunopathogenesis of non-healing American cutaneous leishmaniasis and progressive visceral leishmaniasis

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Abstract

The outcomes of *Leishmania* infection are determined by host immune and nutrition status, parasite species, and co-infection with other pathogens. While subclinical infection and self-healing cutaneous leishmaniasis (CL) are common, uncontrolled parasite replication can lead to non-healing local lesions or visceral leishmaniasis (VL). It is known that infection control requires Th1-differentiation cytokines (IL-12, IL-18, and IL-27) and Th1 cell and macrophage activation. However, there is no generalized consensus for the mechanisms of host susceptibility. The recent studies on regulatory T cells and IL-17-producing cells help explain the effector T cell responses that occur independently of the known Th1/Th2 cell signaling pathways. This review focuses on the immunopathogenesis of non-healing American CL and progressive VL. We summarize recent evidence from human and animal studies that reveals the mechanisms of dysregulated, hyper-responses to *Leishmania braziliensis*, as well as the presence of disease-promoting or the absence of protective responses to *Leishmania amazonensis* and *Leishmania donovani*. We highlight immune-mediated parasite growth and immunopathogenesis, with an emphasis on the putative roles of IL-17 and its related cytokines as well as arginase. A better understanding of the quality

and regulation of innate immunity and T cell responses triggered by *Leishmania* will aid in the rational control of pathology and the infection.

Keywords

Pathogenesis; Immune regulation; *Leishmania*; Cutaneous leishmaniasis; Visceral leishmaniasis

Introduction

Leishmaniasis represents a group of neglected tropical diseases caused by infection with protozoan parasites from the genus *Leishmania*. These parasites are widely distributed in 88 tropical and subtropical countries and pose a major public health problem and a risk for people living in or traveling to the endemic areas. It has an annual estimated worldwide incidence of 600,000 and prevalence of 12 million cases. Leishmaniasis is a vector-transmitted disease, and at least 20 species of *Leishmania* are known to be pathogenic for humans [1]. These parasites have a digenetic life cycle, multiplying as flagellated promastigotes in the midgut of sand flies and as non-flagellated amastigotes within mammalian phagocytes. Uncontrolled parasite replication can lead to non-healing cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), mucosal leishmaniasis (ML), or visceral leishmaniasis (VL). The available anti-*Leishmania* drugs are far from satisfactory due to high costs, toxicity, or drug resistance. While prophylactic and therapeutic vaccines are in great need, there are currently no effective vaccines for any leishmaniasis forms. This is partially due to an incomplete understanding of correlates of protective immunity and insufficient information on parasite antigens that elicit protective T cell response and appropriate regulation, since both inadequate and excessive immune responses can contribute to pathogenesis, leading to vaccine failure.

The epidemiology of leishmaniasis is extremely diverse. The outcomes of infection are determined by both the host immune status and the parasite species involved. Subclinical infection and self-healing CL are common. Asymptomatic *Leishmania* infection is detected in endemic areas by a positive, delayed-type hypersensitivity (DTH) skin test (called leishmanin or Montenegro skin test). In some individuals, infections can progress to non-healing skin or mucosal lesions, or other severe forms. Co-infection with other pathogens can also complicate disease severity. Leishmaniasis and human immunodeficiency virus (HIV) are common infectious diseases with overlapping and expanding endemic areas, a situation which has led to an increased occurrence of *Leishmania*/HIV co-infection [2]. For example, while Southern Europe had the most co-infection cases reported to WHO in 2001, the number of co-infection cases in other endemic areas has markedly increased, as 30 % of all VL patients in Northwest Ethiopia are also infected with HIV [3]. These two pathogens promote each other by enhancing pathogen intake, intracellular survival, and disease progression [4], and such cross-interactions markedly complicate diagnosis, treatment, and patient management [2, 3].

Host immune responses to *Leishmania* parasites are regulated by highly complex pathways. Animal models of CL and VL have been instrumental for dissecting the mechanisms

underlying protective immunity and disease pathogenesis. The current paradigm in murine CL models of *Leishmania major* infection is that healing requires sequential events, including the activation of dendritic cells (DC), the production of Th1 differentiation cytokines (IL-12, IL-18, and IL-27), the activation of IFN- γ /TNF- α -producing Th1 cells, and the classical activation of macrophages (M Φ ; reviewed in [5], as illustrated in red in Fig. 1). The biochemical pathways in classically activated M Φ , as well as roles of reactive oxygen species (ROS) and reactive nitrogen intermediates, such as nitric oxide (NO) in parasite killing are well described [6]. However, there is no generalized consensus for the mechanisms of host susceptibility, as different events are preferentially triggered in a given host (humans versus animal models) following infection with different *Leishmania* species (as illustrated in blue in Fig. 1). For example, IL-4, IL-10, and IL-13 have differential roles in host susceptibility to distinctive substrains of *L. major* [5]. Notably, the Th1/Th2 polarization of IFN- γ /IL-4 production observed in murine models of *L. major* infections is not fully applicable to human diseases and to some other *Leishmania* infection models (reviewed in [7]). Much less is known regarding the pathogenic mechanisms of non-healing American CL and progressive VL.

IL-17-producing CD4⁺ T cells (Th17) have recently been defined as a separate effector T cell lineage, important in the maintenance of homeostasis through the production of IL-17A, IL-17F, IL-21, and IL-22 [8, 9]. The discovery of this new CD4⁺ T cell subset explains the effector T cell responses that occur independently of known Th1 and Th2 cell signaling pathways. ROR γ t and ROR α are the two master transcriptional regulators involved in Th17 differentiation, but other transcriptional factors (e.g., STAT3, IRF4, and BATF) also contribute to this process (reviewed in [10]). Ghoreschi et al. [9] have reported two distinct Th17 subsets under in vitro stimulation conditions: the conventional Th17 (or Th17 β) cells generated following TGF- β /IL-6 stimulation and inflammatory Th17 cells generated following IL-6/IL-23/IL-17 stimulation. These Th17 subsets play pivotal roles in autoimmunity and chronic inflammatory diseases [11] and participate in defense mechanisms against certain pathogens including *Leishmania* (see below). These seminal studies open new areas for investigation and validation in humans and experimental animals.

In this review, we focus on the immunopathogenesis of non-healing American CL and progressive VL. The purpose of this review is not to provide a comprehensive summary for the host immune responses against *Leishmania* parasites or protective/deleterious host immunity against *L. major*, as there are excellent reviews covering these areas [5, 12]. Instead, we will summarize recent evidence from human patients and animal studies that reveals the mechanisms of dysregulated, hyper-responses to *Leishmania braziliensis*, as well as the presence of disease-promoting responses to *Leishmania amazonensis* and *Leishmania donovani* parasites or the absence of protective responses. We will highlight immune-mediated parasite growth, as well as the immunopathogenesis of non-healing CL and progressive VL, with emphasis on the putative roles of IL-17 and its related cytokines, as well as macrophage-derived arginase and parasite-derived factors. A better understanding of the quality and regulation of innate immunity and T cell responses triggered by *Leishmania*, especially in the context of HIV co-infection, would aid in the rational control of pathology and the infection.

Non-healing human diseases associated with *L. braziliensis* infection

Parasites in the *Leishmania* (*Viannia*) subgenus (*L. braziliensis*, *Leishmania guyanensis*, *Leishmania panamensis*, and *Leishmania peruviana*) are the most prevalent etiologic agents of human CL in Central and South America. Among these infections, approximately 2–3 % of patients develop disseminated skin lesions, and 3–5 % of patients progress to ML [13]. The main pathogen of ML in the Amazon region is *L. braziliensis*, followed by *L. guyanensis* [14]. ML is a severe and disfiguring form of the disease, usually compromising mucosal regions of the nose, mouth, and pharynx [15, 16]. Pathologically, ML is characterized by excessive T and B cell responses to the parasite. These patients tend to produce elevated levels of anti-*Leishmania* antibodies and excessive amounts of proinflammatory cytokines (e.g., IFN- γ , TNF- α , and IL-6) [15, 17]. Large-scale, gene polymorphism studies indicated that promoter polymorphisms for high production of IL-10 by monocytes and regulatory T (T_{reg}) cells increase the risk of CL lesions [18], whereas polymorphisms for high production of IL-6 and CCL2 increase the risk of ML, but not of localized CL [19, 20]. These studies may indicate a differential link between the host genetic predisposition for dysregulated inflammatory responses and the risk of severe infections. However, how host-, pathogen-, and microenvironment-associated factors lead into ML progression remain largely uncharacterized. Recent studies discussed below have shed some new light on contributors to the infection outcomes.

Roles of IL-17-producing cells

In response to stress, tissue injury, or invading pathogens, host innate immune cells [21] and newly described T cell subsets can produce IL-17 [8, 9]. IL-17-producing cells play a pivotal role in neutrophil recruitment at early stages of infection, as well as in autoimmunity and chronic inflammatory diseases. In human infection caused by *L. donovani*, IL-17 and IL-22 are associated with protection against kala azar [22, 23]. With regard to American CL, four studies have investigated the production of and possible roles for IL-17 in *L. braziliensis* infection. Bacellar et al. [24] first reported in 2009 that lymphocytes obtained from patients with ML and CL produce higher levels of IL-17 than did lymphocytes obtained from uninfected control subjects, implying a role for IL-17 in the pathogenesis of the inflammatory reaction in leishmaniasis. This group also compared cytokine profiles in PBMCs of subclinical infection, patients with CL, and healthy subjects [25]. They found no major roles for regulatory cytokines such as IL-10 and IL-27, but the subclinical group tended to have higher levels of IL-17 than the other two groups. A similar association between IL-17 and protection was found in individuals exposed to *L. donovani* during an outbreak of VL [23] (see below). A possibility of IL-17 in an innate immune response to *L. braziliensis* infection is suggested by the authors, but not thoroughly investigated. Boaventura et al. evaluated the involvement of the IL-17-type response in the inflammatory infiltrate of biopsy specimens from ML patients. They found that IL-17⁺ T cells and neutrophils, as well as IL-17-inducing cytokines (IL-1 β , IL-23, IL-6, and TGF- β), were readily detected in the ML biopsies, and that these staining patterns were coincident with those of neutrophil elastase, myeloperoxidase, and MMP-9 [26]. In models of arthritis, Th17 cells have been identified to be osteoclastogenic and mediate bone desorption [27, 28], so these cells may similarly drive the destructive processes in ML. Therefore, IL-17 may

contribute to ML pathogenesis through several mechanisms, including neutrophil activation, tissue injury, and osteoclast activation (as illustrated in blue in Fig. 1).

More recently, Castellano et al. [29] found that following stimulation with *L. braziliensis* antigens, there was a decreased frequency of IL-17⁺CD3⁺CD4⁺ cells in circulating T cells from an HIV-positive ML patient; however, the biological significance of this alteration remained unclear. Collectively, these clinical studies indicated that the regulated production of IL-17 contributes to infection control, while excessive IL-17 can promote neutrophil influx and tissue damage, thereby increasing the risk of ML. Additional studies will verify this hypothesis and reveal the major producers of IL-17 at early versus late stages of infection. Furthermore, in light of the previous finding of reduced IL-10 receptor expression in ML compared to CL lesions [30], the role of IL-10 in regulating the IL-17-driven inflammatory response needs additional investigation. Since human neutrophils and monocytes can express two IL-8 receptors, CXCR1/IL-8RA and CXCR2/IL-8RB [31], it is important to examine whether the differential expression of these receptors has any functional significance in proinflammatory activities of neutrophils during *Leishmania* infection. Along this line, there is a report of an association of ML with the rare derived G allele at *CXCR1* and for a positive role of neutrophils in preventing this form of the disease [32]. Additional immunological studies are warranted to define the regulation of IL-17 and CXCR1 in neutrophil-mediated responses.

Roles of IFN- γ - and TNF- α -related mediators

IFN- γ and TNF- α are key cytokines in the protective responses against intracellular pathogens, and their gene expression levels are regulated by single nucleotide polymorphisms (SNP) [33, 34]. Analysis of TNF polymorphisms in Venezuelan CL patients clearly indicates a positive association with ML [34]. Matos et al. conducted an IFN- γ SNP study with 78 CL and 58 ML cases and 609 healthy controls in Brazil. They found no association between IFN- γ polymorphism and American CL forms, but reported that gene polymorphisms can influence parasite-induced IFN- γ production [35]. Given that excessive production of IFN- γ and TNF- α is a hallmark for ML, it is also possible that *L. braziliensis* parasites are intrinsically potent in stimulating host responses, favoring cellular recruitment and disease severity. To test this hypothesis, our group examined the response of healthy volunteer PBMCs to *L. braziliensis* and used *L. amazonensis* as an infection control [36]. We observed that while *L. braziliensis* infection induced the production of CXCL10 (also known as IP-10) and IL-10 in human PBMCs and blood monocytes, an enhanced expression of CXCL10 and its receptor CXCR3 was predominantly detected in CD14⁺ monocytes. The chemoattractant factors secreted by *L. braziliensis*-infected cells were more efficient in recruiting uninfected PBMCs (predominantly CD14⁺ cells) than were *L. amazonensis*-infected cells. Also, we found that sera of *L. braziliensis*-infected patients (especially the ML cases) had significantly elevated levels of CXCL10, CCL4, and soluble TNF receptor II [36]. Therefore, *L. braziliensis* parasites are highly competent in stimulating the host to produce multiple inflammatory mediators, and different chemokines may affect the predominance of cell infiltration in distinct clinical manifestations. CXCL10 is secreted mainly by monocytes, fibroblasts, and endothelial cells in response to stimuli (e.g., viral infection, LPS, IL-1 β , and IFN- γ) and involves the recruitment of monocytes, M Φ s, and T

cells to sites of inflammation (see review in [37]). The investigation of CXCR3 ligands (CXCL9, CXCL 10, and CXCL11) and other inflammatory chemokines, in the context of IFN- γ /TNF- α production, in localized versus disseminated lesions has shed new light on the complex regulation of inflammatory responses during *L. braziliensis* infection [38].

Non-healing human diseases associated with *L. amazonensis* and *Leishmania mexicana* infection

Parasites in the *L. L. mexicana* complex (*L. mexicana*, *L. amazonensis*, and *Leishmania pifanoi*) are the etiological agents for a broad spectrum of leishmaniasis in Central and South American countries, including mild and localized CL, DCL, and rarely fatal VL [39]. DCL is a rare, but severe, form and is not limited to patients in South America. In patients with *Leishmania tropica*, *L. major*, or *L. donovani* infections in India and Africa, DCL or disseminated CL is also considered a clinical indicator of HIV co-infection [40, 41]. There is also a case report of DCL that developed in the context of the immune reconstitution inflammatory syndrome in a man with AIDS following initiation of antiretroviral therapy [18]. Generally speaking, patients with DCL share some clinical characteristics. For example, the lesions have a presentation of numerous non-ulcerating nodules and are histopathologically characterized by heavily parasitized M Φ s. These patients often have a negative reaction to the leishmanin skin test and poor antigen-stimulated T cell proliferation in vitro, although some patients remain responsive to other antigens such as tuberculin and lepromin [39]. These profound impairments in cell-mediated immune responses partially explain the relatively poor responses and frequent relapses of DCL patients following anti-*Leishmania* chemotherapy. This antigen-specific T cell anergy is likely due to alterations in the activation of DC and M Φ following infection with *L. amazonensis* (see review in [15, 42] and illustration in blue in Fig. 1).

Impaired host innate immunity

Among the *Leishmania* species that are pathogenic to humans, only the *L. mexicana* complex parasites are capable of forming huge parasitophorous vacuoles that contain numerous amastigotes. Under cell-free and in vitro conditions, *L. amazonensis* parasites (especially the amastigote form) are highly resistant to neutrophil- or M Φ -derived leishmanicidal molecules, or anti-*Leishmania* drugs, compared to other *Leishmania* species [43, 44]. These are indicative of biological features that are unique and intrinsic to these parasites. *L. amazonensis* amastigotes are superior in silent invasion, preventing the activation of NK, DC, and M Φ triggered by exogenous stimuli such as LPS [42, 45]. The intracellular events triggered by *L. amazonensis* infection in M Φ s have been described in detail [45, 46]. The suppression of host cell activation can be initiated at early stages of amastigote–host contact. On one hand, *L. amazonensis* amastigotes can infect human DCs through multiple receptors such as Fc receptors, complement receptors, heparin-binding proteins, and DC-SIGN [47]. Signaling through these receptors can lead to altered DC activation and impaired responsiveness to exogenous stimuli through MAP kinase-mediated mechanisms [48]. On the other hand, *L. amazonensis* amastigotes can expose phosphatidylserine (PS) on their surface, leading to enhanced parasite growth and host suppression through IL-10 and TGF- β production by host cells [49]. Although the origin and

biochemical nature of PS detected on amastigote surface remains undefined, PS exposure may contribute to the suppression of anti-*Leishmania* immunity [50], as reported for other pathogens such as *Toxoplasma gondii* and *Vibrio cholerae* [51, 52]. No doubt, *L. amazonensis* and its related parasites have evolved complex strategies to down-modulate host innate immune responses, leading to T cell malfunction (as illustrated in black in Fig. 1).

The quality of T cell responses to *L. amazonensis* and *L. mexicana*

In a recent report, Campanelli et al. [53] analyzed leukocytes isolated from early skin lesions (<30 days of infection), late lesions (>60 days of infection), and DCL lesions (>10 years). Compared with the early-infection lesions, the late-infection lesions showed a reduced frequency of CXCR3⁺ cells, but an increased frequency of CCR4⁺ and IL-10⁺ T cells. Compared with the CL lesions, the DCL lesions exhibited a reduced frequency of CCR3-, CCR4-, CCR5-, and CCL17-positive cells, but an increased frequency of CCL7-positive cells. These changes correlate with the impaired T cells responses observed by other groups [17, 54], implying a poor quality of T cell responses to *L. amazonensis* antigens. This notion is further supported by a recent study of Macedo et al. [55], which compared the quality of T cell responses induced by promastigote crude antigens of *L. braziliensis* and *L. amazonensis* in PBMCs from healed CL patients. Their multi-parametric flow cytometric studies indicated that *L. braziliensis* antigens induced an important proportion of multi-cytokine-producing CD4⁺ T cells (28 % of the total Th1 response evaluated), whereas *L. amazonensis* antigens mostly (68 %) induced single-positive cells, 57 % of which were IFN- γ single positives. Since the percentages of total IFN- γ -producing CD4⁺ T cells induced by both antigens were comparable, this study emphasizes the importance of Th1 response quality, not just its magnitude, in the study of diverse immunopathogenesis of leishmaniasis.

The role of CD8 cytotoxicity in DCL versus CL patients is less clear. Hernandez-Ruiz et al. [56] compared the overall immune effector functions of CD8⁺ T cells for ten CL and four DCL Mexican patients diagnosed with *L. mexicana* infection. They found that in comparison to the CL group, peripheral CD8⁺ T cells from DCL patients displayed impaired effector functions, as judged by their levels of IFN- γ production in vitro and cytotoxicity against *L. mexicana*-infected autologous M Φ s. This impaired CD8 function correlated with the reduced numbers of CD8⁺ T cells and TUNEL⁺ apoptotic cells in lesions of DCL patients. These authors have suggested a functional exhaustion of CD8⁺ T cells in DCL patients and provided some evidence for restoring their effector functions, following the stimulation of these CD8⁺ T cells with TLR2-specific agonists such as Pam3Cys and *L. mexicana* LPG [56]. This is the first in vitro study for TLR2-mediated restoration of effector functions for CD8⁺ T cells derived from DCL patients. Given that DCL patients lack effective innate and adaptive immune responses for controlling parasite infection and anti-*Leishmania* drugs only achieve transitory remission in DCL patients, this report, together with related studies in patients and mice, highlights the need for comprehensive treatment schemes for patients infected with *L. amazonensis* and *L. mexicana*. Since the number of CD8⁺ T cells tends to increase during the healing process of the skin lesions of DCL patients [57], it is important to further define the overall effector mechanisms of CD8⁺ T cells in human leishmaniasis.

Animal models of *L. braziliensis* and *L. amazonensis* infection

While genetically resistant and susceptible strains of mice are valuable for defining polarized Th1 and Th2 immune responses, as well as the contribution of Th17 and T_{reg}, during *L. major* infection [5], the susceptibility of mice to the New World *Leishmania* species is diverse and complex, depending on the parasite species under investigation. In general, all commonly used inbred strains of mice (including BALB/c mice) are genetically resistant to *L. braziliensis* infection. The gene-targeted deletion of key molecules involved in NO production can increase mouse susceptibility to *L. braziliensis*. Although mice are also generally resistant to *L. guyanensis*, *L. panamensis*, and *L. peruviana*, chronic skin lesions can be successfully induced following infection of BALB/c mice with percoll-purified promastigotes of *L. panamensis* [58]. For parasites in the *L. Viannia* subgenus, hamsters are a more suitable model than mice for the pathological study of localized and metastatic lesions [59]. In contrast, commonly used inbred strains of mice are genetically susceptible to *L. amazonensis* infection (see review by [60]). The deletion of either the *IL-4* or *IL-10* gene has no major effect on mouse susceptibility to *L. amazonensis*; however, deleting functional CD4⁺ T cells or B cells markedly reduces lesion pathology [61, 62], implying a complex, Th2-independent regulation of host susceptibility to *L. amazonensis* infection (see review by [63]).

Self-healing infection caused by *L. braziliensis*

L. braziliensis infection is self-healing in all inbred strains of mice, and small lesions reach their peaks at about 4–6 weeks post-infection. Parasite clearance is primarily due to the efficient activation of DC, MΦ, and T cells at the site of infection and in the draining lymph nodes, and to the intrinsic susceptibility of this parasite to microbicidal activities of the host cell. The interaction of neutrophils with *L. braziliensis*-infected MΦ also promotes parasite killing via TNF-α- and superoxide-associated mechanisms [64]. To further test the notion that *L. braziliensis* infection efficiently triggers innate and adaptive responses, we generated axenic amastigotes of *L. braziliensis* and examined DC and MΦ responses to *L. braziliensis* and *L. amazonensis*. By using fluorescence-labeled promastigotes and amastigotes, we found that *L. braziliensis* (but not *L. amazonensis*) infection-induced DC activation/maturation and IL-12p40 production in both infected and bystander cells, and that DC activation was accompanied by upregulation of the JAK/STAT signaling pathway, especially in regard to the expression of phosphorylated-STAT1 and -STAT3, IFN regulatory factor 1 (IRF-1), and ISG15. Moreover, *L. braziliensis*-infected DCs were highly efficient in priming naïve CD4⁺ T cells to proliferate and produce IFN-γ and IL-17 [42, 65]. These new findings from mouse models aid in our understanding as to how the host mounts immune responses against *L. braziliensis*. However, *L. braziliensis*-infected mice are not good models for studying ML, partially due to the relatively high sensitivity of their promastigotes and axenic amastigotes to NO-mediated killing (Fig. 2a) and to the relative ease of the mouse MΦs to produce NO (in comparison to human or hamster MΦs [66]).

Non-healing CL caused by *L. amazonensis*

In sharp contrast, *L. amazonensis* infection is non-healing in all inbred strains of mice (see review by [60]). Skin lesions become evident after 2–4 weeks and progress continuously for

a few months, and some of these lesions can be ulcerated at late stages. The dermal lesions of *L. amazonensis*-infected mice have a dense cellular infiltrate, composed mainly of parasitized, vacuolated MΦs, and scattered T and B lymphocytes [61, 62]; these histopathological features closely resemble those of non-healing lesions observed in DCL patients [39, 67]. Although promastigotes of *L. amazonensis* can transiently trigger a low level of DC and MΦ activation, amastigote-carrying cells show little signs of activation (Fig. 2b). In general, *L. amazonensis*-infected DCs are poor antigen-presenting cells, and their co-cultured T cells are poorly activated (Fig. 2c) [42, 65, 68]. Therefore, repression of host cell activation by *Leishmania* parasites appears to be species-specific, and *L. amazonensis* amastigote infection may represent an anergic pathogenicity extremity [17]. Yet, lesion pathology in mice relies on activation of pathogenic CD4⁺ T cells that produce low levels of IFN-γ, IL-10, IL-17, and other cytokines/chemokines [68, 69]. On one hand, these low-quality T cell responses are ample for recruiting monocytes/MΦs, but are insufficient for activating them fully. Of note, *L. amazonensis* amastigotes are relatively resistant to NO-mediated parasite killing (Fig. 2a), as well as to neutrophil-mediated parasite killing [44]. *L. amazonensis* may also be unique in sensing both the external and internal arginine pool by regulating two transporter-coding genes, or in utilizing host innate machineries (e.g., the autophagic and lipid metabolic pathways) for their favored intracellular growth [63, 70]. Therefore, *L. amazonensis* parasites are extraordinary in their ability to repress host cell activation, resist host anti-microbial machinery, and utilize host resources for their own growth (as illustrated in blue in Fig. 1).

The implication of the new and interesting findings is that a multi-pronged treatment regimen may be desirable for *L. amazonensis* infection. Thus, for example, an anti-*Leishmania* therapy in conjunction with immune stimulation may be optimal. We therefore tested whether pre-exposure to *L. braziliensis* can stimulate anti-*L. amazonensis* responses. We found that pre-exposure to *L. braziliensis* can partially protect mice against *L. amazonensis* infection by increasing the magnitude of T cell responses [69]. It will be interesting to identify the T cell-stimulating antigens of *L. braziliensis* unique to this species or common to other species. Such antigens would be of value for monitoring human and mouse T cell responses at different stages of infection with *L. braziliensis* and for potential development of prophylactic or therapeutic vaccines against leishmaniasis.

Spectrum and immunopathogenesis of progressive VL in humans

Infection with *L. donovani* and *Leishmania infantum/Leishmania chagasi* can be asymptomatic or subclinical (oligosymptomatic) in a majority of individuals. Less than 10 % of infected people develop active VL or kala azar, which is characterized by fever, fatigue, anorexia, weight loss and cachexia, hepatosplenomegaly, and pancytopenia. The course of disease typically runs over several months and is usually fatal unless the patient receives specific treatment. Asymptomatic *Leishmania* infection is detected by a positive DTH response, whereas subclinical or non-progressing oligosymptomatic infection is usually evident due to a positive serological test with a few mild and non-specific symptoms [71, 72]. Both retrospective and prospective studies have demonstrated that asymptomatic or subclinical infections are six- to tenfold more common than active VL [73]. Individuals who have had an asymptomatic infection have been shown in limited epidemiological and human

challenge studies to be protected from the development of active disease. The immunopathogenic mechanisms of VL in humans are not fully understood. Most information has been gained by comparing parasite antigen-induced immune responses between asymptomatically infected (putatively resistant) individuals and those with active disease (putatively susceptible). This approach makes the tenuous assumption that susceptibility is driven solely by the host immune response and not modified by parasite or environmental factors.

Cytokines, T cell responses, and T_{reg} cells

PBMCs from asymptomatic skin test-positive subjects in VL endemic areas demonstrate strong antigen-induced IFN- γ responses and induction of intracellular parasite killing, whereas PBMCs from active VL patients produce low levels of IFN- γ . Furthermore, low IFN- γ production is characteristic of individuals with subclinical infection that subsequently progressed to VL. While these descriptive studies identify immunological features of active disease, they do not differentiate a permissive host response that led to active disease from a host response that is modulated by the infection. Several pieces of evidence indicate that the production of IFN- γ is insufficient to confer a protective immune response to the visceralizing *Leishmania*. First, there is elevated expression of IFN- γ (and IL-12) in the plasma of patients with active VL [74, 75]. Second, there is a high level of IFN- γ mRNAs in the lymph node [76], spleen [77], and bone marrow [78] of patients with active VL. Third, whole blood stimulation assays indicate IFN- γ production by blood cells during active VL [79]. Lastly, not all *Leishmania*-reactive, IFN- γ -producing T cell clones derived from a subclinically infected individual were able to activate infected M Φ to kill intracellular parasites [80]. Th17 cells and IL-17 have been shown to play a protective role against some, but not all, intracellular pathogens [81], but also may be responsible for the excessive inflammation seen in some pathological states, including ML [26]. Recently, individuals exposed to *L. donovani* during an outbreak of VL in eastern Sudan were found to be most protected against the development of VL when their PBMCs produced IL-17 and IL-22 in response to stimulation with heat-killed parasites [23].

The finding that a robust proinflammatory and type 1 cytokine response does not mitigate progressive VL in humans prompted the consideration that other cytokines known to impair M Φ -mediated killing of *Leishmania* could have a pathological role [82, 83]. IL-4 and IL-13, which play a prominent disease-promoting role in *L. major* infection [84], were found to be increased in the serum of some, but not all, patients with active VL [74, 85–87]. The importance of IL-10 in the pathogenesis of human VL is more strongly supported [82]. Patients with VL have elevated levels of IL-10 in serum or plasma [74, 86–88] and increased IL-10 mRNA expression in the spleen and bone marrow [85]. Circulating levels of IL-10 correlated strongly with parasite loads in the blood of patients with VL [89]. In vitro neutralization of IL-10 in PBMC cultures from patients with VL resulted in enhancement of Th1 cell responses to *Leishmania* antigens, but more notably, neutralization of IL-10 in ex vivo cultured cells from splenic aspirates promoted parasite clearance [90]. T cell IL-10 production in VL may be driven by the proinflammatory cytokine IL-27, as the expression of IL-27's subunits (IL-27-28 and EBI-3) was upregulated in a CD14⁺ M Φ -enriched fraction of splenocytes isolated from patients with active VL [91]. In this same study, increased T

cell expression of IL-21, which can amplify IL-27 production, was identified in the spleens of patients with VL, and treatment of cells with IL-27 and IL-21 significantly enhanced the antigen-induced production of IL-10. Recently, a disease-promoting proinflammatory cytokine response was attributed to increased bacterial translocation from the gastrointestinal tract and endotoxin-mediated immune modulation in patients with VL [92].

T (CD4⁺ CD25⁺ reg Foxp3⁺) cells are a source of IL-10 and contribute to chronic infection in murine *L. major* infection [93]; however, their possible role in VL has been somewhat controversial. CD4⁺CD25⁺ cells that also expressed Foxp3 mRNA and produced IL-10 were also found at increased levels in the spleens and lymph nodes of mice infected with *L. infantum* [94]. Before treatment, CD4⁺CD25⁺ cells were also elevated in the peripheral blood of patients with VL compared to findings post-treatment and when compared to those in endemic controls [95]. However, the CD4⁺CD25⁺ population may also include activated, conventional T cells and will not allow the discrimination of T_{reg} without including the Foxp3⁺ marker. Elegant work by Nylen et al. and Maurya et al. showed that CD25⁺Foxp3⁺ T_{reg} do not accumulate in the spleen [85, 96] and are not a major source of IL-10 in patients with active VL [85]. They proposed that IL-10-producing CD4⁺CD25⁺Foxp3⁺ T cells are likely a major contributor to the pathogenesis of VL [85]. However, recent work by Rai et al. identified an increased accumulation of CD4⁺CD25⁺Foxp3⁺ cells (but not conventional activated CD4⁺CD25⁺Foxp3⁺ T cells) in the bone marrow of patients with VL compared to the patient's peripheral blood or to bone marrow from healthy controls [97]. This finding of increased T_{reg} in the bone marrow [97] but not in the spleen [85, 96] is puzzling and deserves further investigation. In patients with post-kala azar dermal leishmaniasis (PKDL), the expression of mRNAs for Foxp3, CD25, and IL-10 correlated with tissue parasite loads, implying the accumulation of natural T_{reg} cells [98].

Impact of malnutrition

Epidemiologic studies have documented a greatly increased risk for VL in the malnourished host (reviewed in [99]). In fact, one could argue that since VL is a disease of the severely impoverished, rarely does it occur in the well-nourished host. Malnutrition was identified as a risk factor for severe disease and death from VL in both children (OR 5.0) and adults (OR 11.0) [100]. Malnutrition-related VL is particularly evident in displaced and impoverished populations [101, 102], and the recently described movement of transmission into peri-urban slums is likely to lead to an increase in the malnutrition-infection synergism [103]. Although the immunopathogenic basis for the malnutrition-related risk of VL has not been investigated, investigations in a murine model of polynutrient deficiency (deficient protein, energy, zinc, and iron) [104–106], which closely mimicked moderate acute malnutrition, revealed an increased rate of early dissemination following cutaneous infection with *L. donovani* [105]. Notably, this dissemination was related to the loss of lymph node barrier function and early parasite escape from the draining lymph nodes in the polynutrient-deficient mice. Other investigations in this murine model revealed alteration of the innate MΦ response [104, 106] and impaired adaptive immunity (reduced IFN-γ production) in mice vaccinated against *L. chagasi* [107].

Impact of HIV co-infection

The cruel synergism between *L. donovani* and HIV is increasingly evident, as there is an expansion of regions in the world where co-infections occur. It is estimated that HIV increases the risk of VL development in *L. donovani*-exposed populations by several hundred-fold [3], through either decreased resistance to a new primary infection or reactivation of a previous subclinical infection [108]. Co-infection studies in primary human monocyte-derived MΦs, DCs, and tonsillar tissue demonstrated that each pathogen has a detrimental effect on containment of the other—*Leishmania* infection enhances HIV replication via chronic immune activation, and that HIV promotes *Leishmania* infection by suppressing a protective host defense [109–112]. The latter is corroborated by higher levels of *L. donovani* parasitemia in HIV co-infected individuals [113] and low CD4⁺ T cell counts despite suppression of viral load by antiretroviral therapy [114].

Mouse and hamster models of VL

The immunopathogenesis of visceralizing *Leishmania* infection has been most commonly studied in murine models of systemic infection. Mice infected by the intravenous route develop an acutely increasing visceral parasitization, followed by control of parasite replication and reduction in parasite load in the liver over 4–6 weeks and a more chronic infection in the spleen. Although the chronic parasitism does not lead to significant overt morbidity or mortality, the model has been instructive in elucidating immunoprotective and immunopathogenic mechanisms in both the liver and spleen.

Control of hepatic infection in mice

Resolution of hepatic infection is mediated by several critical immunological processes: (1) generation of a type 1 T cell response, (2) effective granuloma formation, and (3) induction of classical macrophage activation (reviewed in [115, 116]). Polarization of T cells to a Th1 phenotype is evident early in the course of infection and maintained by the balance of ongoing stimulation/expansion and increased apoptosis. The critical requirement for IFN- γ -producing Th1 cells is established by demonstration of impaired control of infection in IFN- γ -, STAT1-, and T-bet-deficient mice [117]. IRF-7 and IRF-5, both of which are activated by MyD88-dependent TLR7 signaling, are critical to the generation of effector Th1 responses [118, 119]. Effector T cell migration into the hepatic granuloma is dependent on the expression of CCL19 and CCL21 [120]. Kupffer cells, which make up the primary parasitized phagocyte in the granuloma, present antigens to T cells to initiate the effector response. Neutrophil responses to *L. donovani* contribute to host defense directly through anti-parasitic effector activity and indirectly by modulating the adaptive immune response toward a protective type 1 phenotype [121]. Classical MΦ activation by IFN- γ /TNF- α or other proinflammatory microbial products leads to the generation of ROS and NO. Both ROS and NO contribute to the control of parasite replication in vivo because mice deficient in gp91phox (a component of NADPH oxidase) have impaired early control of infection (even though these mice ultimately resolve hepatic infection), and NOS2-deficient mice have impaired early and late control of infection [122]. Other, yet-to-be-identified MΦ effector mechanisms also are likely to have a role [123].

The generation of an anti-leishmanial hepatic granulomatous response requires the coordinated interaction of multiple cell types, cytokines, and effector molecules [124]. The production of chemokines (CCL2, CCL3, and CXCL10) by Kupffer cells leads to an influx of inflammatory monocytes and neutrophils within the first few days of infection. CD4⁺ and CD8⁺ T cells subsequently accumulate through recruitment and local expansion, leading to the formation of an inflammatory granuloma that generates IFN- γ and TNF- α (reviewed in [115, 116]). A number of other proinflammatory cytokines [IL-2, IL-12, lymphotoxin, and granulocyte macrophage-colony stimulating factor (GM-CSF)] also contribute to granuloma formation and function. Although Th2 cytokines are generally considered to have an immunopathogenic role in some CL models, they play an important role in granuloma formation and protection against *L. donovani*. In mice deficient in IL-4, IL-13, or IL-4R α , there was impaired granuloma maturation and control of hepatic replication of *L. donovani* [125, 126].

Non-resolving splenic infection in mice

L. donovani infection is not as readily controlled in the murine spleen. Following systemic infection, *L. donovani* parasitizes the marginal zone M Φ s (MZM) and marginal metallophilic M Φ s in the splenic marginal zone (MZ). The early production of IL-12 by DCs that have migrated from the MZ into the periarteriolar lymphocyte sheath (PALS) in response to CCL19 and CCL21 was found to be critical for the generation of effector T cells and control of parasite replication early in the course of infection [120]. However, these early responses are inadequate to clear parasites from the spleen (in contrast to the resolution of infection in the liver) because there is a slow increase in parasite burden after several weeks, accompanied by splenomegaly and extensive remodeling of the splenic micro-architecture (reviewed in [115, 116]). Splenic expression of IL-10, but not IL-4, contributes to the maintenance of parasite replication (reviewed in [83]). Moreover, IL-10-deficient mice have shown an increased resistance to experimental *L. donovani* infection [127, 128].

Central to the splenic remodeling is the widely distributed and excessive production of TNF- α (as noted above, moderate levels of TNF- α are required for the protective immune response in the liver), which mediates the loss of MZMs [129] and the depletion of stromal fibroblastic reticular cells (FRC) in the PALS [130]. The FRC make up the splenic conduits that are a critical source of the cytokines CCL19 and CCL21, which mediate CCR7-dependent migration of DCs from the MZ to the PALS [131]. However, the infection-induced depletion of splenic FRC over time has been reported to lead to reduced chemokine expression and less recruitment of CCR7-bearing DCs and naïve T cells into the T cell zone of the PALS [130]. However, DCs isolated from naïve mice were able to migrate into the PALS of *L. donovani*-infected spleens, mediating a reduction in splenic parasite loads, but DCs isolated from infected mice had impaired migration related to reduced CCR7 expression [130]. Thus, the reduced migration of DCs into the PALS is probably a consequence of the suboptimal expression of both the receptor and its ligands. As splenic infection progresses, the white pulp becomes shrunken and disorganized, and there is destruction of follicular DCs and loss of germinal centers. This destruction is associated with neovascularization and infiltration of the white pulp with heavily parasitized M Φ s [132]. The neovascularization process was reversed to some degree by administration of the

broadly acting receptor tyrosine kinase inhibitor, sunitinib, which also enhanced the generation of IFN- γ -producing Th1 cells [132]. In parallel with the remodeling of the white pulp, there was also expansion of the red pulp vasculature, which was dependent on the influx of Ly6C⁺ inflammatory monocytes [133]. The remodeled red pulp in the *L. donovani*-infected spleen also showed enhanced myelopoiesis, evident by a 20- to 30-fold increase in the numbers of colony-forming units-granulocyte, monocyte (CFU-GM) [134]. This increase was in part due to the active proliferation of progenitor cells and was dependent on increased GM-CSF and TNF- α [134, 135]. This profound expansion of splenic myeloid progenitor cells was accompanied by an increase in parasite loads [134], implying that the accumulation of myeloid cells supported parasite replication.

Progressive disease in hamsters

The Syrian hamster model of VL provides an opportunity to investigate immunopathogenic mechanisms in a model of disease that mimics the progressive, fatal disease of humans. Hamsters infected with *L. donovani* were found to develop a remarkably higher visceral parasite burden than mice and suffer cachexia, massive splenomegaly, pancytopenia, and ultimately death [136]. Following a period of relatively silent infection, the splenic parasite burden was reported to increase dramatically, and there was an accompanying increase in splenic cellularity, primarily the result of an expansion of the myeloid population [137]. During the course of infection, hamsters showed disrupted splenic architecture accompanied with lymphoid depletion [138] and the loss of antigen-reactive T cell responses [137, 139, 140]; however, the adherent cell-free T cells isolated from hamsters were able to respond to leishmanial antigens and to transfer parasite-specific DTH reactivity to naïve hamsters [139]. Lymphocytes from the lymph nodes of *L. donovani*-infected hamsters were found to show both cell-cycle arrest and TGF- β -triggered apoptotic death mediated through upregulation of tyrosine phosphatase activity [140]. In the liver, there was heavy parasitization of the Kupffer cells, which are surrounded by an inflammatory infiltrate, but fully organized granulomas did not develop [138]. The uncontained infection was found to occur despite a vigorous type 1 immune response with increased hepatic and splenic expression of IFN- γ , IL-2, IL-12, and TNF- α [141, 142], a response that is associated with control of *L. donovani* infection in mice. The paradoxical expression of IFN- γ in the face of progressive infection is similar to the findings in human VL. However, concomitant with the type 1 response, there was also increased splenic expression of IL-4, IL-13, IL-10, IL-21, and TGF- β [136, 141]. The roles of these cytokines in the pathogenesis of progressive VL have not been defined.

In striking contrast to mouse M Φ s, IFN- γ -activated hamster M Φ s expressed minimal NOS2 and did not generate detectable NO; they were unable to restrict the replication of intracellular *L. donovani* [136, 142]. The reduced transcription of NOS2 mRNA in response to *Leishmania* infection was related to a IFN- γ -hypo-responsive promoter (similar to what was described for the human NOS2 promoter) and was in part due to the absence of a *cis*-element for NF-IL6 (C/EBP β), which is a member of a CCAAT-enhancer binding protein family of transcription factors, in the hamster NOS2 promoter [143]. The inability to control parasite replication in VL was not only related to ineffective classical M Φ activation (low NOS2) but also parasite-induced, STAT6-dependent alternative M Φ activation with

dominant arginase 1 expression and polyamine production [136]. Selective knockdown of either arginase 1 or STAT6 led to a reduced parasite burden in this model. Interestingly, the expression of arg1 and activation of STAT6 required the de novo synthesis of protein, implying that additional co-activators and/or pathways contribute to STAT6-dependent, *L. donovani*-induced arg1 expression. Recently, it was found that IL-10 expressed in the spleens of mice infected with *L. donovani* induced the upregulation of IL-4R α , which was required for arg1 expression [144]. Thus, synergy between the IL-4/STAT6 and IL-10/STAT3 pathways may play a central role in the pathogenesis of VL.

Subversion of macrophage function as a contributor to non-resolving and progressive *L. donovani* infection

It is well described that *Leishmania* parasites can alter the signaling pathways in mouse and human DC and M Φ s (reviewed in [145, 146], as marked in black in Fig. 1). These alterations may play a role in human VL, by subverting the innate immune response and rendering the infected cells less responsive to activating stimuli. For the most part, however, corroborative studies of M Φ s from the site of infection, especially in human VL, are lacking. There are a number of mechanisms by which *L. donovani* escapes M Φ effector activity (see Table 1). Most notably, *L. donovani* inhibits protein kinase C (PKC) and members of the MAP kinase families, and impairs IFN- γ -mediated M Φ activation, largely, but not exclusively, due to broad activation of cellular protein tyrosine phosphatases (PTPs). Several recent findings deserve to be highlighted. Efforts to illuminate the mechanisms of *Leishmania*-mediated activation of cellular PTPs revealed that several *Leishmania* proteins, including EF-1 α [147, 148], fructose-1,6-bisphosphate aldolase [149], and GP63 [150] are exported from the phagolysosome into the cytosol and activate PTPs (reviewed in [146]). Impairment of PKC signaling is known to be critical for *L. donovani* survival in M Φ s (reviewed in [151]). Recently, the role of PKC isoforms in the regulation of cytosolic phosphatases and downstream MAP kinase signaling and cytokine production was defined [152]. Infection of mouse M Φ s with *L. donovani* led to upregulation of uncoupling protein 2 (UCP2), a mitochondrial membrane protein that is a negative regulator of ROS generation [153]. Functional knockdown of UCP2 led to enhanced ROS generation and ROS-mediated inhibition of protein tyrosine phosphatases [153], which are known to subvert M Φ activation pathways that lead to generation of NO and parasite killing [154]. *L. donovani* infection of human M Φ s led to phosphorylation of CREB and inactivation of glycogen synthase kinase-3 β (GSK-3 β), which resulted in IL-10 production [155] and parasite replication. Since IFN- γ expression in infected tissues is characteristic of VL, it is of considerable interest to understand why this response is ineffective in curbing parasite replication. In *L. donovani*-infected murine M Φ s, IFN- γ receptor expression and receptor–ligand interaction were unaltered; however, co-localization of the two IFN- γ receptor subunits was impaired due to infection-mediated increase in membrane fluidity and cholesterol quenching [156]. IFN- γ signaling and parasite killing could be recovered by delivery of cholesterol to the M Φ membrane [156].

Concluding remarks

The emerging evidence from recent studies has uncovered the complex host–*Leishmania* interactions that contribute to delay in healing or non-healing lesions (summarized in Fig. 1). At the early stages of the infection, host, parasite, or vector factors that promote sustained neutrophil influx and altered neutrophil functions can facilitate promastigote infection in the host and parasite spread to other target cells [157, 158]. At the subsequent stages, multiple and complex mechanisms of immune dysregulation act alone or in combination, resulting in persistent infection and/or tissue damage, depending on the involving parasite species.

Firstly, IL-17-producing T cells, as well as IL-17-inducing cytokines (IL-1 β , IL-6, IL-23, and TGF- β), seem to have complex and dual roles, depending on the infection stages and involving parasite species. Their protective roles are likely to be in conjunction with Th1 cell activation. Their detrimental roles are mediated via the regulation of additional waves of neutrophil recruitment, promoting lesion progression and tissue damage, as elegantly illustrated in *L. major*-susceptible mice [159, 160] and suggested in the above-mentioned human infections with *L. braziliensis*. Secondly, host factors that trigger M Φ alternative activation (IL-4, IL-10, IL-13, and TGF- β insulin-like growth factor) or M Φ suboptimal activation (low levels of IFN- γ and TNF- α) can create a microenvironment in favor of amastigote replication [46, 63]. Parasite growth within these M Φ s can be mediated via host arginase-dependent and -independent mechanisms, as illustrated by mouse models of *L. amazonensis* and *L. mexicana* infection and hamster models of *L. donovani* infection [136]. Similarly, parasite-encoded arginase and arginine transporters can serve as virulence factors, subverting M Φ microbicidal activity [70, 161]. Thirdly, host or parasite factors that repress NO or ROS production by infected neutrophils or M Φ s, or repress the activation of DC and T cells, will delay parasite clearance and lesion control [46]. In this regard, recent studies of NO-resistant *L. amazonensis* and *L. braziliensis* isolated from patient lesions have offered new insights into non-healing CL and non-responsiveness of patients to antimony therapy [162–164].

It is clear that hyper- and hypo-immune responses both contribute to non-healing American CL and severe VL, and that the magnitude of these responses are regulated by DC and T_{reg} cells. The major challenge is to define the host and parasite factors that lead to immune dysregulation at early versus late stages of infection. It is particularly important that the function of leukocyte populations be defined within the tissue environment of the infection site to avoid the limitations of in vitro models of infection/response. A better understanding of how different species and stages of *Leishmania* parasites can selectively utilize host innate defense machinery and adoptive immunity for immune evasion, intracellular growth, and immunopathogenesis will lead to the rational design of control strategies for this and other related infections.

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Fig. 1.

The complex factors for the regulation of healing and non-healing leishmaniasis

Fig. 2.

Differential responses of *Leishmania amazonensis* (*La*) and *Leishmania braziliensis* (*Lb*). **a** Kinetics of NO-mediated killing of *La* and *Lb* parasites. CFSE-labeled promastigotes (*Pm*) and axenic amastigotes (*Am*, $1 \times 10^7/\text{ml}$) were exposed to 10 mM NaNO₂ in PBS pH 4.5 at 23 °C. The intensity of CFSE was determined by flow cytometry. For untreated parasites, only the 90-min data are shown. **b** Bone marrow-derived DCs were generated from C57BL/6 mice and infected with promastigotes and axenic amastigotes of *La* and *Lb* at the indicated ratios. At 24 h post-infection, the levels of IL-12p40 in culture supernatants were assayed by ELISA. **c** DC (infected for 24 h) were co-cultured with naïve CD4⁺ T cells ($2 \times 10^6/\text{ml}$) at an 1:10 DC-to-T ratio for 4 days. The levels of cytokines in culture supernatants were assayed by ELISA. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **b** and **c** were adapted from Vargas-Inchaustegui et al. [65]

Table 1*Leishmania donovani*-induced alteration of macrophage signaling

Signaling molecule/pathway	Effect of <i>L. donovani</i> infection [Reference]
MAPK/ERK	Impaired MAP kinase activation and c-Fos and Elk-1 expression [165]
	Inhibition of p38 and ERK1/2 by activation of phosphatases [152]
	Impaired activation of ERK1/2, p38, JNK; degradation of I κ B- α [166]
	Inhibition of ERK phosphorylation by ceramide-mediated activation of PTP [167]
	Inhibition of p38 MAPK and activation of ERK1/2 leading to decreased IL-12 and increased IL-10 production [168]
	Inhibition of ERK phosphorylation by ceramide [169]
	SHP-1-mediated inactivation of ERK1/2 [154]
IFN- γ /STAT1	Reduced co-localization of IFN- γ receptor subunits [156]
	Reduced IFN- γ receptor alpha expression and phosphorylation [170]
	Impaired STAT1 translocation [171]
	Decreased expression of IRF-1 [171]
	Proteasome-mediated degradation of STAT1 [172]
PKC	SHP-1-mediated inactivation of JAK2 [154, 173]
	Inhibition of PKC- α -mediated phagosomal maturation [174]
	Ceramide-mediated dysregulation of PKC activation [175, 176]
	Inhibition of PKC- β but activation of PKC- ϵ [177]
NF- κ B/AP-1	SHP-1-mediated inactivation of NF- κ B and AP-1 [154]
	PPAR- γ -mediated inhibition of NF- κ B activation [178]
PI3K/mTOR	PI3K-dependent, mTOR-mediated upregulation of IL-10 [179]
Akt/GSK-3 β /CREB	Akt-2-mediated inactivation of GSK-3 β leading to CREB induced IL-10 expression [155]

Ld L. donovani, IFN- γ interferon-gamma, PTP protein tyrosine phosphatase, SHP-1 Src homology region-2 domain-containing phosphatase-1, MAPK mitogen-activated protein kinase, ERK extracellular signal-regulated kinases, STAT1 signal transducer and activator of transcription 1, JAK Janus kinase, PKC protein kinase C, PI3K phosphatidylinositol 3-kinases, mTOR mammalian target of rapamycin, NF- κ B nuclear factor kappa-B, AP-1 activator protein 1