Published in final edited form as:
Adv Appl Microbiol. 2013 ; 82: 155–184. doi:10.1016/B978-0-12-407679-2.00005-3.

Mechanisms of Immune Evasion in Leishmaniasis

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Abstract

Diseases caused by *Leishmania* present a worldwide problem, and current therapeutic approaches are unable to achieve a sterile cure. *Leishmania* is able to persist in host cells by evading or exploiting host immune mechanisms. A thorough understanding of these mechanisms could lead to better strategies for effective management of *Leishmania* infections. Current research has focused on parasite modification of host cell signaling pathways, entry into phagocytic cells, and modulation of cytokine and chemokine profiles that alter immune cell activation and trafficking to sites of infection. Immuno-therapeutic approaches that target these mechanisms of immune evasion by *Leishmania* offer promising areas for preclinical and clinical research.

Diseases caused by *Leishmania* are a major global health problem as over 12 million people currently suffer from leishmaniasis, with an incidence rate of approximately 2 million annually, according to recent estimates (www.who.int/tdr). It is transmitted by sandflies and presents a wide range of clinical manifestations that depend on the specie and strain of *Leishmania*, degree of virulence, and the immunological state of the host. Cutaneous leishmaniasis (CL) manifests as localized skin lesions that may resolve but can become chronic, leading to severe tissue destruction and disfigurement. Lesions could disseminate in immunocompromised patients giving rise to the diffuse cutaneous leishmaniasis (DCL) (Desjeux, 2004). This disease is caused by *Leishmania major* (the Middle East and Mediterranean Region), *Leishmania mexicana* (Central America), and *Leishmania amazonensis* (South America). *Leishmania tropica* and *Leishmania aethiopica* also cause CL in the Old World. Mucocutaneous leishmaniasis caused by *Leishmania braziliensis* is endemic in South America and is clinically characterized by the involvement of the nasal and oropharyngeal mucosa with extensive tissue destruction due to inflammation. Visceral leishmaniasis (VL), the most severe form of leishmaniasis, is caused by *Leishmania donovani* and *Leishmania chagasi* in the Old and New worlds, respectively. Clinical manifestations include hepatomegaly and splenomegaly due to parasite infiltration of the liver and spleen and if left untreated, it is almost always fatal (www.who.int/tdr) (Alexander, Satoskar, & Russell, 1999; Awasthi, Mathur, & Saha, 2004; Desjeux, 2004).

Chemotherapeutic and prophylactic approaches to the management of the various forms of leishmaniasis have been problematic due to the tendency of the parasite to persist within host cells and subsequent failure to achieve a sterile cure. Such latent or chronic forms of the disease present obvious dangers when the hosts' immune system is compromised. The key

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to effective management of *Leishmania* infections therefore depends on a thorough understanding of the immunological basis for parasite persistence within host cells. Interestingly, macrophages which are the primary immune cells involved in the eradication of *Leishmania* in a mammalian host are the targets for the parasite. How *Leishmania* is able to survive and thrive in this hostile environment as well as capitalize on host defense mechanisms to favor the establishment of disease will be addressed in this review.

1. IMMUNITY TO *LEISHMANIA*

Successful elimination of *Leishmania* depends on the coordinated action of various players of the immune system. From promastigote entry into the blood stream after a sandfly bite to its final mammalian cellular target as an amastigote, the battle between disease establishment and parasite eradication will partly be decided by the ability of *Leishmania* to evade host immunity. Components of host defense important for the eradication of *Leishmania* include both elements of the innate and adaptive immune system.

1.1. The Complement System

It has long been known that the complement system plays a significant role in the eradication of promastigotes in the blood stream of an infected host (Rezai, Sher, & Gettner, 1969). Recent studies have shown that susceptibility of *Leishmania* parasites to complement-mediated lysis in vitro is directly related to the concentration of serum complement (Moreno et al., 2007). Although the classical pathway of the complement system is activated by *Leishmania*, complement-mediated destruction of parasites is generally amplified by the alternate pathway (Hoover, Berger, Nacy, Hockmeyer, & Meltzer, 1984). Amastigotes from *L. donovani* are more resistant than *L. tropica* amastigotes to lysis by complement, suggesting that some *Leishmania* spp. might actively resist this process (Hoover et al., 1984). It should be noted that while unfractionated promastigotes are highly susceptible to lysis by complement, infective metacyclic promastigotes are generally more resistant (Puentes, Sacks, da Silva, & Joiner, 1988).

1.2. Cells of Innate Immunity

Neutrophils are the first host cells to reach the site of *Leishmania* infection within a few hours of inoculation by a sandfly bite (Müller et al., 2001; Pompeu, Freitas, Santos, Khouri, & Barral-Netto, 1991). They have been shown to actively engulf *Leishmania* promastigotes (Mollinedo, Janssen, de la Iglesia-Vicente, Villa-Pulgarin, & Calafat, 2010; Pearson & Steigbigel, 1981) and produce an array of microbicidal factors against *Leishmania* such as nitric oxide (Charmoy et al., 2007), neutrophil elastase (NE) (Ribeiro-Gomes et al., 2007), platelet activating factor (Camussi, Bussolino, Salvidio, & Baglioni, 1987), and neutrophil extracellular traps (Guimarães-Costa et al., 2009). Neutrophils generally have a protective role in most forms of *Leishmania* infections (de Souza Carmo, Katz, & Barbiéri, 2010; Novais et al., 2009), although outcomes are dependent on the *Leishmania* strain, the genetic background of the host and the apoptotic or necrotic state of the neutrophils (Afonso et al., 2008; de Souza Carmo et al., 2010; Filardy et al., 2010; Novais et al., 2009; Ribeiro-Gomes et al., 2004). In vivo depletion of neutrophils during *L. major* infection results in an increase in parasite load in resistant mice (C57BL/6 and C3H/HeJ mice), but susceptible mice (BALB/c mice) show a reduction in parasite load (Ribeiro-Gomes et al., 2004; Tacchini-Cottier et al., 2000).

Early on during *Leishmania* infection, natural killer (NK) cells are also recruited to the infected site after neutrophil recruitment (Müller et al., 2001; Thalhofer, Chen, Sudan, Love-Homan, & Wilson, 2011). They are the primary source of early IFN-γ that favors the Th1 differentiation of $CD4^+$ T cells (Scharton & Scott, 1993) and restricts early parasite

dissemination (Diefenbach et al., 1998; Laskay, Diefenbach, Röllinghoff, & Solbach, 1995). NK cells can also mediate direct parasite lysis through its cytotoxic activity (Lieke et al., 2011) and subsequently contribute to cytokine-mediated inducible nitric oxide synthase (iNOS) induction in *Leishmania*-infected macrophages (Prajeeth, Haeberlein, Sebald, Schleicher, & Bogdan, 2011). Studies using NK cell deficient mice have shown the importance of these cells in the containment of *L. donovani* infection but they are dispensable in models of cutaneous leishmaniasis (Kirkpatrick & Farrell, 1982; Satoskar et al., 1999). In humans, NK cell population has been shown to decrease in cases of progressive disease (Pereira et al., 2009; Peruhype-Magalhães et al., 2005), while sites of healing lesions show an infiltration of $CD56^+$ NK cells (Pereira et al., 2009), which suggests a protective role for NK cell population in human leishmaniasis.

Natural killer T (NKT) cells, a specialized subset of T lymphocytes involved in innate immunity to pathogens, also play important roles in the immune response during the early stages of *Leishmania* infection (Amprey et al., 2004; Ishikawa et al., 2000), although these roles vary depending on the specie of *Leishmania* and strain of mice. *Leishmania* surface glyco-conjugates and glycoinositol are recognized by CD1d restricted NKT cells (Amprey et al., 2004; Mattner, Donhauser, Werner-Felmayer, & Bogdan, 2006) which contribute to hepatic clearance of *L. donovani* in BALB/c mice through the induction of IFN-γ (Amprey et al., 2004), but exacerbates the disease in C57BL/6 mice (Stanley et al., 2008). In *L. major* infection, protection by NKT cells seems to be organ specific, where they contribute to parasite control in skin lesions and the spleen but not in the lymph nodes (Mattner et al., 2006). In humans, an increase in iNKT cell frequency in the bone marrow of VL patients is observed as the disease progresses, which decreases following treatment (Rai, Thakur, Seth, & Mitra, 2011).

1.3. Cells of Adaptive Immunity

Lymphocytes are involved in adaptive immune responses to *Leishmania* infection, primarily through the elaboration of cytokines that activate or dampen the antiparasitic activity of macrophages. It is well known that T cells play a major role in immunity to the various forms of *Leishmania*. Generally, IFN-γ-producing Th1 cells are essential to the resolution of infection with *L. major*, where they induce nitric oxide production in macrophages. On the other hand, susceptibility is associated with the production of cytokines produced by Th2 cells such as IL-4 and IL-13. Indeed, genetically resistant (such as C57BL/6) or susceptible (such as BALB/c) mice to *L. major* infection are characterized by their ability to produce Th1 or Th2 cytokine profiles respectively. This is also the case in visceral leishmaniasis caused by *L. donovani*, in mice and humans where IFN-γ-producing Th1 cells protect against severe disease (Kushawaha, Gupta, Sundar, Sahasrabuddhe, & Dube, 2011). In human cutaneous leishmaniasis IFN-γ production by CD8+ T cells seems to contribute to disease resolution (Mary, Auriault, Faugère, & Dessein, 1999; Nateghi Rostami et al., 2010). However, as demonstrated by *Leishmania* infection models using CD4 or CD8 deficient mice, while CD4 T cells are required for resolution of disease (Chakkalath et al., 1995), the role of CD8 T cells in immunity to cutaneous or visceral leishmaniasis seems to depend on the model used (Belkaid, Von Stebut, et al., 2002; Erb, Blank, Ritter, Bluethmann, & Moll, 1996; Gomes-Pereira, Rodrigues, Rolão, Almeida, & Santos-Gomes, 2004; Huber, Timms, Mak, Röllinghoff, & Lohoff, 1998; Tsagozis, Karagouni, & Dotsika, 2005).

Studies using mouse models that affect the activity and migration of regulatory T cells show that they play key roles in regulating the immune response to *L. major* infection (Liu et al., 2009; Suffia, Reckling, Salay, & Belkaid, 2005; Yurchenko et al., 2006). On the one hand, they dampen the effector response of CD4+ T cells partly mediated through IL-10 expression by Tregs. This results in parasite persistence even in resistant C57BL/6 mice, which has implications in the case of disease reactivation. On the other hand, by allowing

parasite persistence, Tregs contribute to the maintenance of long term immunity to *L. major* (Belkaid, 2003; Belkaid, Piccirillo, Mendez, Shevach, & Sacks, 2002). This balance between effector and regulatory T cells could potentially be exploited by *Leishmania* parasites to evade host immune responses.

Other immune cells are also involved in controlling immunity to *Leishmania*. B cells seem to play a detrimental role in the early stages of *L. donovani* infection, as shown by significantly reduced parasite burdens in B cell deficient mice (Smelt, Cotterell, Engwerda, & Kaye, 2000). Marginal zone B cells in particular have been shown to dampen the cytotoxic activity of antigen specific CD8 T cells as well as the frequency of IFN gamma producing CD4+ T cells (Bankoti, Gupta, Levchenko, & Stäger, 2012), thus contributing to increased parasite loads. Recent studies on myeloid-derived suppressors cells show that they contribute to resistance to *L. major* in an NO dependent manner, despite their ability to suppress T cell activation (Pereira et al., 2011). During *L. major* infection, dendritic cell subsets as well as their differentiation state play diverse roles in modulating the adaptive immune and affecting the outcome of the disease (Kautz-Neu et al., 2011; Wiethe et al., 2008).

1.4. Cytokines and Chemokines

As shown by a variety of cytokine and chemokine knockout mouse models, cytokines and chemokines play a huge role in immunity to a host of infectious diseases including *Leishmania* infections. While some of these secreted protein immune-modulators are involved in the activation and differentiation of immune cells important in parasite clearance (such as IL-12, TNF-α, IFN-γ) (Mattner et al., 1996; Swihart et al., 1995), others could either dampen the immune response against *Leishmania* or activate and differentiate immune cells that will ultimately favor the persistence of the parasite (such as IL-4, IL-10, IL-13, TGF-β) (Kopf et al., 1996). Cytokines are produced by immune or infected cells and exert their function by activating other cells to release molecules that inhibit or favor the growth of *Leishmania*. Chemokines and chemokine receptors are involved in trafficking of immune cells to inflammatory sites. Indeed, many researchers have suggested their use in the immuno-prophylaxis or therapy of leishmaniasis (Gupta, Majumdar, et al., 2011).

2. MECHANISMS OF IMMUNE EVASION

Like any successful pathogen, *Leishmania* has developed strategies to evade host immune mechanisms in order to survive within the host. A significant number of virulence factors discovered in *Leishmania* are directed against circumventing the host immune response. The ability of *Leishmania* to maintain a chronic infectious state within its host depends to a large extent on its immune evasion potential. Indeed, the ongoing battle between the robust immune response mounted by a host and the counter evasion strategies by the parasite will ultimately decide the fate of the disease. The mechanisms of immune evasion by *Leishmania* species include the following:

2.1. Modification of the Complement System and Phagocytosis

Once the female sandfly injects *Leishmania* promastigotes into the mammalian host, it becomes imperative for the parasite to escape or deactivate the host complement system before entering in the macrophages. *Leishmania* is able to evade the host's complement system using a variety of mechanisms. Unlike noninfective procyclic promastigotes, infective metacyclic promastigotes prevent the insertion of the C5–C9 membrane attack complex making them highly resistant to complement-mediated lysis (Puentes, Da Silva, Sacks, Hammer, & Joiner, 1990). This parasite stage-specific difference in complement evasion is due to a developmental modification resulting in the elongation of

lipophosphoglycan (LPG) in metacyclic *Leishmania* (Sacks, Pimenta, McConville, Schneider, & Turco, 1995). Among the various stages of *Leishmania*, metacyclic promastigotes show the highest expression of protein kinases, which phosphorylate complement proteins C3, C5, and C9, resulting in the deactivation of classical and alternative complement pathways (Hermoso, Fishelson, Becker, Hirschberg, & Jaffe, 1991). The activity of *Leishmania* glycoprotein 63 (GP63), a metalloproteinase found abundantly on the surface of metacyclic promastigotes, is pivotal for resisting complement lysis. GP63 cleaves the C3b to an inactive form, C3bi on the surface membrane of the parasite, thereby hindering the formation of C5 convertase (Brittingham et al., 1995).

C3bi also serves as an opsonin, facilitating the uptake of the parasite by binding to complement receptor 3 (CR3) and transiently to CR1 on the surface of macrophages (Kane & Mosser, 2000; Mosser & Edelson, 1985). Attachment via CR3 rather than CR1 is advantageous to the parasite as CR3 ligation even in the absence of *Leishmania* inhibits the production of IL-12 (Marth & Kelsall, 1997). Thus *Leishmania* is able to exploit CR3-mediated phagocytosis, facilitating a 'silent entry' into macrophages thereby evading the host's protective immune response (Da Silva, Hall, Joiner, & Sacks, 1989; Wright & Silverstein, 1983). To further support the role of CR3 in mediating host susceptibility to *Leishmania*, a recent study using CR3 deficient BALB/c mice showed an increased resistance to *L. major* infection (Carter, Whitcomb, Campbell, Mukbel, & McDowell, 2009).

Although macrophages are involved in the eradication of *Leishmania*, they are the primary host cells targeted by the parasite for survival and proliferation. As such *Leishmania* possess a variety of cell surface molecules that enable them localize to macrophages. LPG on the surface of *Leishmania* promastigotes can facilitate binding to mannosyl/fucosyl receptors (Blackwell et al., 1985), complement reactive protein (Culley, Harris, Kaye, McAdam, & Raynes, 1996), and CR4 (Talamás-Rohana, Wright, Lennartz, & Russell, 1990) on macrophage membranes. Furthermore, *Leishmania* glycosylinositol phospholipid (GIPL) and GP63 have important roles in the attachment of parasites to macrophages (Brittingham et al., 1995; Suzuki, Tanaka, Toledo, Takahashi, & Straus, 2002). *Leishmania* amastigotes, unlike promastigotes, can be internalized using phosphatidyl serine and Fc receptors although additional receptors may also participate in the entry process (de Freitas Balanco et al., 2001; Guy & Belosevic, 1993; Weingartner et al., 2012). Recently, our group demonstrated that the phosphoinositide 3-kinase gamma (PI3-Kγ) signaling pathway is exploited by *L. mexicana* to facilitate parasite entry into macrophages and progression of disease. Further, therapy using the PI3-Kγ specific inhibitor AS-605240 protects against cutaneous leishmaniasis caused by *L. mexicana* (Cummings et al., 2012).

2.2. Alteration of Toll-Like Receptor Pathways

Toll-like receptors (TLRs) expressed on the cells of the innate immune system are critical for recognition of pathogen-associated molecular patterns. Initial interaction of the parasite with different TLR's dictates the outcome of the infection (Faria, Reis, & Lima, 2012). TLR2 on host macrophages recognizes *Leishmania* promastigote-derived LPG (de Veer et al., 2003; Flandin, Chano, & Descoteaux, 2006) and amastigote-specific antigens (Srivastava et al., 2012). LPG interaction with TLR2 results in the induction of TNF-α (Flandin et al., 2006), IL-12 (Kavoosi, Ardes-tani, & Kariminia, 2009), NO (Kavoosi, Ardestani, Kariminia, & Alimo-hammadian, 2010), and reactive oxygen species (ROS) (Kavoosi et al., 2009). TLR2 agonists which activate this TLR2 signaling pathway has been utilized in inducing a host protective immune response resulting in parasite clearance from *L. donovani* infected macrophages (Bhattacharya et al., 2010).

To subvert this inflammatory response *L. major* recruits suppressors of the cytokine signaling family proteins, SOCS-1 and SOCS-3, which negatively regulates TLR2 induced

cytokine induction (de Veer et al., 2003). Another mechanism of TLR2-mediated suppression by *Leishmania* involves the activation of host de-ubiquitinating enzyme A20 by *L. donovani* promastigotes resulting in the impairment of TLR2-mediated release of IL-12 and TNF-α as the parasite interferes with the ubiquitination of TRAF6 (Srivastav, Kar, Chande, Mukhopadhyaya, & Das, 2012). On the other hand, *L. amazonensis* capitalize on TLR2 signaling to facilitate the establishment of infection, by increasing the expression of double stranded RNA dependent protein kinase (PKR) and IFN-β. IFN-β increases superoxide dismutase 1 levels which inhibits superoxide-dependent parasite killing and augments parasite replication (Khouri et al., 2009; Vivarini et al., 2011). Furthermore, a similar study with TLR2-deficient mice showed an essential role of TLR2 in lesion development during *L. braziliensis* infection (Vargas-Inchaustegui et al., 2009). These findings suggest multiple mechanisms employed by different species of *Leishmania* to alter TLR2 signaling and enhance parasite establishment.

TLR4 plays a critical role in shaping the host immune response during *Leishmania* infection. Studies with TLR4$^{-/-}$ mice demonstrate the role of TLR4 in the control of *L. major* infection (Kropf, Freudenberg, Modolell, et al., 2004, Kropf, Freudenberg, Kalis, et al., 2004). Glyco-sphingophospholipid (GSPL) and proteoglycolipid complex (P8GLC) from *Leishmania* induce TLR4, promoting a strong antiparasitic immune response (Karmakar, Bhaumik, Paul, & De, 2012; Whitaker, Colmenares, Pestana, & McMahon-Pratt, 2008). There is a strong TNF-α response upon P8GLC-TLR4 engagement in macrophages infected with *Leishmania pifanoi* amastigotes and in vivo treatment with P8GLC showed enhanced parasite clearance in TLR4-competent infected mice compared to TLR4$^{-/-}$-deficient infected mice (Whitaker et al., 2008). Similar results were obtained using GSPL treatment in *L. donovani* infection (Karmakar, Paul, & De, 2011).

Leishmania has devised mechanisms to alter TLR4 signaling pathways to favor establishment of infection. During *L. donovani* infection, TLR4-mediated macrophage activation is suppressed through the release of TGF-β that activates the ubiquitin editing enzyme A20 and Src homology 2 domain phosphotyrosine phosphatase 1 (SHP-1) (Das et al., 2012). *Leishmania major* utilizes its inhibitors of serine protease (ISP) to prevent NE-mediated TLR4 activation, inhibiting *Leishmania* uptake and killing by host macrophages (Faria et al., 2011; Ribeiro-Gomes et al., 2007). On the other hand, *L. mexicana* capitalizes on TLR4 signaling to inhibit the production of IL-12 by infected macrophages and promotes parasite establishment (Shweash et al., 2011).

Other TLRs involved in infection with *Leishmania* include TLR3 and TLR9. Endogenous TLR3 binds to double stranded RNA and has been shown to be activated during *Leishmania* infection. A virulent strain of *Leishmania guyanensis* and *Leishmania vianna* which harbor *Leishmania* RNA virus (LRV1) has been shown to activate the TLR3-TRIF–dependent pathway essential for increased pro-inflammatory mediator expression after macrophage infection (Hartley, Ronet, Zangger, Beverley, & Fasel, 2012). Interestingly, TLR3-mediated immune responses rendered mice more susceptible to infection showing increased footpad swelling and parasitemia along with increased metastasis (Ives et al., 2011).

2.3. Surviving in the Phagosome

To survive inside the macrophage, *Leishmania* must resist the harsh conditions created by the phagocyte, including acidic pH, elevated temperature, and increased oxidative/nitrosative stress. Upon phagocytosis, *Leishmania* promastigotes are internalized into endosomal compartments, where they transform into amastigotes. In order to escape the hostile environment of the phagolysosome, promastigotes transiently prevent fusion of the phagosome and lysosome thereby delaying or inhibiting endosomal maturation as observed by the late expression of Rab7 and LAMP-1 (Olivier, Gregory, & Forget, 2005;

Scianimanico et al., 1999). LPG on *Leishmania* promastigotes inhibits endosome maturation by inducing periphagosomal F-actin accumulation (Holm, Tejle, Magnusson, Descoteaux, & Rasmusson, 2001). LPG also prevents acidification of the phagosome by interfering with the V-ATPase pump, which allows promastigotes to differentiate into resistant amastigotes (Vinet, Fukuda, Turco, & Descoteaux, 2009). Scavenging on host sphingolipids is another survival strategy employed by *Leishmania* amastigotes for counteracting the acidic environment of the phagolysosome (Ali, Harding, & Denny, 2012). In order to dilute the leishmanicidal effect of nitric oxide, *Leishmania* regulates the lysosomal trafficking (LYST) protein resulting in the formation of large parasitophorous vacuoles (Wilson et al., 2008).

Leishmania amastigotes require iron for metabolism and replication, therefore acquisition of iron is critical for its survival within the macrophage phagolysosome (Huynh & Andrews, 2008). In murine macrophages, Nramp1 functions as an efflux pump that translocates Fe^{2+} from the phagolysosome into the cytosol thereby restricting its availability to the parasite. *Leishmania* counteracts this effect by the activation of its own iron transporters, LIT1 and LIT2 which effectively compete with the host's iron sequestering mechanism (Kaye & Scott, 2011). Arginine is also an essential growth factor required by intracellular *Leishmania* amastigotes for the synthesis of polyamines, but is also used by macrophages for microbicidal nitric oxide production (Iniesta, Gómez-Nieto, & Corraliza, 2001; Kropf, Herath, Weber, Modolell, & Müller, 2003). As demonstrated by studies using gene deficient mutants of *Leishmania*, the parasite encoded arginase enzyme not only facilitates growth by supplying essential nutrients to the parasite through the polyamine pathway, but also attenuates the iNOS-dependent killing mechanism of infected macrophages by competing for available intracellular arginine (Gaur et al., 2007; Reguera, Balaña-Fouce, Showalter, Hickerson, & Beverley, 2009). These virulence factors expressed by *Leishmania* amastigotes enable the parasite to evade the host's cellular immune response and survive in the phagosome.

2.4. Defective Antigen Presentation and Co-stimulation

Virulent stages of *Leishmania* have the capacity to attenuate T cell-mediated immune responses by regulating the expression of leishmanial antigen loaded major histocompatibility complex (MHC) molecules on antigen presenting cells. They accomplish this by antigen sequestration or interference with the loading of antigens onto MHC class II molecules (Fruth, Solioz, & Louis, 1993; Kima, Soong, Chicharro, Ruddle, & McMahon-Pratt, 1996; Prina, Lang, Glaichenhaus, & Antoine, 1996). Studies on presentation of the *Leishmania* antigen, *Leishmania* homolog of receptors for activated C kinase (LACK), by infected macrophages showed a transient yet strong LACK-specific T cell response when infected with stationary or log phase *Leishmania* promastigotes. On the other hand, murine macrophages infected with either *Leishmania* metacyclic promastigotes or amastigotes showed a weak or absent LACK-specific T cell activation respectively (Courret et al., 1999). Membrane lipid rafts are important platforms for antigen presentation as it concentrates MHC class II molecules into microdomains, which allow efficient antigen presentation at low peptide densities. *Leishmania donovani* increases the fluidity of lipid rafts in macrophages resulting in defective antigen presentation and diminished T cells responses (Chakraborty et al., 2005). MHC class II, but not class I molecules have been shown to be important in host resistance against *Leishmania* (Huber et al., 1998; Locksley, Reiner, Hatam, Littman, & Killeen, 1993), and only MHC II molecules appear in the parasitophorous vacuole of an infected macrophage (Lang et al., 1994). MHC II molecules, located in specific organelles called megasomes, are endocytosed by *Leishmania* amastigotes and degraded by cysteine proteases (De Souza Leao, Lang, Prina, Hellio, & Antoine, 1995). These examples highlight the ability of *Leishmania* to evade recognition by T lymphocytes by interfering with macrophage antigen presentation.

Co-stimulatory molecules such as B7-1, B7-2, and CD40 expressed on macrophages are also critical for setting up antiparasitic T cell responses (Alexander et al., 1999; Bogdan, Gessner, Solbach, & Röllinghoff, 1996). In *Leishmania*-infected macrophages, there is a reduced expression of B7-1 even after lipopolysaccharide (LPS) stimulation, possibly mediated by parasite-induced prostaglandins (Saha, Das, Vohra, Ganguly, & Mishra, 1995). Similarly, during *L. major* infection B7-1 expression is down-regulated in epidermal cells from susceptible mice (Mbow, DeKrey, & Titus, 2001). Indirect evidence seems to support the notion that *Leishmania* increases the expression of B7-2 to favor the establishment of disease. In the same study by Mbow et al. (2001) B7-2 expression was higher in epidermal cells from susceptible mice than in resistant mice after *L. major* infection. In another study with human leishmaniasis caused by *L. mexicana*, B7-2 expression was higher in monocytes from patients with active DCL, while no change was observed in the expression of CD40 and B7-1 (Carrada et al., 2007). Furthermore, antibody blockade of B7-2, but not B7-1 led to an increased T cell response resulting in diminished parasite load (Murphy, Cotterell, Gorak, Engwerda, & Kaye, 1998; Murphy, Engwerda, Gorak, & Kaye, 1997). This is further supported by studies with *L. major* infected B7-1 knockout, B7-2 knockout, and B7-1/B7-2 double knockout BALB/c mice (Brown et al., 2002). Signaling via CD40-CD40L interactions, critical for the induction of anti-leishmanial responses (Campbell et al., 1996; Kamanaka et al., 1996), is also impaired by *L. major* (Awasthi et al., 2003).

2.5. Alteration of Host Cell Signaling

In order to survive inside macrophages armed with a host of microbicidal factors, *Leishmania* interferes with cell signaling cascades involved in their synthesis. One factor critical for early protective response against *Leishmania* (Murray & Nathan, 1999) is reactive oxygen species which is activated by the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex (De Leo, Ulman, Davis, Jutila, & Quinn, 1996). In *L. donovani*-infected macrophages, phosphorylation of the p67 and p47 subunits of NADPH oxidase is blocked due to impaired protein kinase C (PKC) signaling (Bhattacharyya, Ghosh, Sen, Roy, & Majumdar, 2001) which favors parasite survival (Olivier, Brownsey, & Reiner, 1992; Turco et al., 1987). *Leishmania* promastigote LPG deactivates PKC by interfering with the binding of Ca^{2+} and diacyl glycerol to PKC and obstructing the insertion of PKC into the membrane (Descoteaux & Turco, 1999). Recent studies in *L. mexicana*-infected macrophages show that the ability of parasite-derived LPG to regulate the oxidative burst via PKC-α activity is related to the susceptible/resistant phenotypes observed genetic strains of mice (Delgado-Domínguez et al., 2010). Interestingly, amastigotes, devoid of LPG, have also shown similar attenuation in PKC activity (Olivier et al., 1992) suggesting the existence of LPG independent mechanisms such as *Leishmania*-induced ceramide (Ghosh et al., 2001) and IL-10 induction (Bhattacharyya, Ghosh, Jhonson, Bhattacharya, & Majumdar, 2001). GP63 which cleaves the PKC substrate, myristoylated alanine rich C kinase (MARCKS) and MARCKS-related protein (Olivier, Atayde, Isnard, Hassani, & Shio, 2012), and GIPL (Chawla & Vishwakarma, 2003) are virulence factors that are involved in the inhibition of PKC activity.

IFN-γ-mediated activation of the infected macrophage is critical for the destruction of intracellular *Leishmania* parasites. IFN-γ signals through the JAK/STAT pathway and is critical for the induction of nitric oxide, but this pathway is suppressed by *Leishmania* (Nandan & Reiner, 1995). Interaction of *Leishmania* promastigotes with macrophages activates SHP-1 which shows a strong and increased interaction with JAK2 resulting in its deactivation (Blanchette, Racette, Faure, Siminovitch, & Olivier, 1999). Further studies show that JAK2 inactivation by SHP-1 results in diminished nitric oxide production which favors parasite survival (Blanchette, Abu-Dayyeh, Hassani, Whitcombe, & Olivier, 2009). Further in *Leishmania* infected macrophages, STAT1 inactivation occurs and translocation

of STAT1α to the nucleus is significantly diminished through a mechanism independent of SHP-1 activity (Forget, Gregory, & Olivier, 2005). In both *L. major* and *L. mexicana* infected macrophages treated with IFN-γ, phosphorylation of STAT1α is reduced, but dominant negative STAT1β phosphorylation is increased only in *L. mexicana* infected macrophages which points to species specific differences in the regulation of the JAK/STAT signaling pathway (Bhardwaj, Rosas, Lafuse, & Satoskar, 2005). Furthermore, quenching of membrane cholesterol has recently been shown to be a mechanism by which *Leishmania* impairs with IFN-γ signaling, and re-association of the signaling assembly could be restored by liposomal delivery of cholesterol together with IFN-γ (phospho-JAK1, JAK2, and STAT1) (Sen, Roy, Mukherjee, Mukhopadhyay, & Roy, 2011).

The MAPK signaling pathway (ERK1/2, JNK, and p38MAPK), required for the production of various effector molecules including cytokines and chemokines, is exploited by *Leishmania* as another immune evasive mechanism. This complex signaling pathway could either be activated or suppressed leading to the induction of gene products that favor parasite survival. While *L. donovani* inhibits EKR1/2, p38MAPK, and JNK activation in naïve macrophages leading to the suppression of pro-inflammatory cytokine production (Privé & Descoteaux, 2000), *L. amazonensis* activates ERK1/2 leading to the production of IL-10, a cytokine that contributes to parasite growth (Yang, Mosser, & Zhang, 2007). Mechanisms behind MAPK inactivation by *Leishmania* have been extensively studied. Studies with LPS activated macrophages show that *Leishmania* amastigotes can block the activation of EKR1/2 via the activation of ecto-protein phosphatase (Martiny, Meyer-Fernandes, de Souza, & Vannier-Santos, 1999). In PMA-activated macrophages, *Leishmania* amastigotes deactivated MAP kinase activity (ERK1/2), c-FOS and iNOS expression by activating cellular phosphotyrosine phosphatases (Nandan, Lo, & Reiner, 1999). Studies with SHP-1 deficient infected macrophages confirmed the pivotal role played by this phosphatase in the regulation of ERK1/2 signaling (Forget, Gregory, Whitcombe, & Olivier, 2006). *Leishmania* can also deactivate ERK1/2 by inducing ceramide generation in susceptible host macrophages (Ghosh et al., 2002). Interestingly, *L. mexicana* amastigotes block LPS induced IL-12 production by relying on their own cysteine proteinases for degrading the ERK1/2 and JNK but not p38MAPK (Cameron et al., 2004). Studies with CD40 ligand/antibody, showed that *Leishmania* interferes with the strength of CD40 cross-linking resulting in the reciprocal regulation of ERK1/2 and p38MAPK which governs the production of IL-10 and IL-12 in *Leishmania*-infected macrophages (Mathur, Awasthi, Wadhone, Ramanamurthy, & Saha, 2004). Moreover, *Leishmania* can redirect CD40-regulated immune responses via the reciprocal activation of MAPK phosphatases (MKP), MKP-1 and MKP-3 which reveal a novel parasite-devised immune evasion strategy (Srivastava, Sudan, & Saha, 2011).

Leishmania also regulates other important transcription factors such as AP-1 and NF-kB which have been shown to be important in immunity against the parasite. One study using RelA (NF-kB p65 subunit) knockout infected macrophages show higher intracellular parasite load and reduced NO levels compared to WT (Mise-Omata et al., 2009), establishing the importance of NF-kB in resistance to the disease. *Leishmania* specifically reduces the overall expression of RelA (Calegari-Silva et al., 2009). *Leishmania mexicana* promastigotes rely on the virulence factors GP63 and cysteine peptidase activity to cleave the RelA-p65 subunit into the smaller RelA-p35 subunit which activates specific chemokines that favor parasite multiplication (Abu-Dayyeh, Hassani, Westra, Mottram, & Olivier, 2010; Gregory, Godbout, Contreras, Forget, & Olivier, 2008). *Leishmania mexicana* amastigotes degrade the RelA-p65 subunit though cellular tyrosine phosphatases upon activation by parasite's cysteine peptidase (Abu-Dayyeh et al., 2010). *Leishmania major* amastigotes favor IL-10 induction by selectively inhibiting p65–p50 complex thus allowing selective nuclear translocation of p50–p35 complex (Guizani-Tabbane, Ben-Aissa, Belghith,

Sassi, & Dellagi, 2004). *Leishmania* also attenuates the activity of AP-1 (composed of dimmers of Fos and Jun family members) in infected macrophages in order to regulate the production of pro-inflammatory cytokines IL-1β, TNF-α, and IL-12 (Abu-Dayyeh et al., 2008; Contreras et al., 2010). Previous studies have shown that *Leishmania* interferes with the nuclear translocation of AP-1 which might be due to parasite-induced ceramide generation (Ghosh et al., 2002). Recent reports further show that *Leishmania*-derived GP63 enters the nucleus of the host macrophage to cleave c-Jun and c-Fos subunits of AP-1 rendering them inactive (Contreras et al., 2010). Thus by using multiple mechanisms *Leishmania* is able to interfere with the activity of transcription factors thereby subverting the host immune response.

Other host signaling targets of *Leishmania* include the mammalian mechanistic target of rapamycin (mTOR), which is cleaved by the *Leishmania* protease GP63 leading to the inhibition of mTOR complex 1 (mTORC1) and concomitant activation of 4E-BP1 to promote parasite survival (Jaramillo et al., 2011). Recently, *L. donovani* has been shown to utilize the mTOR signaling pathway to regulate IL-12 and IL-10 production in infected macrophages (Cheekatla, Aggarwal, & Naik, 2012).

2.6. Modulation of Cytokines and Chemokines

By interfering with host cell signaling pathways, many of which are still yet to be completely understood, various species of *Leishmania* possess the capability to modulate the cytokine profile of infected host cells to favor dissemination of the parasite and prevent its eradication. Pro-inflammatory cytokines (such as IL-12), which are essential for the generation of a successful immune response against *Leishmania*, are generally suppressed, facilitating a ‘silent entry’ of the parasite into host macrophages (Belkaid et al., 2000; McDowell & Sacks, 1999; Reiner, Zheng, Wang, Stowring, & Locksley, 1994; Weinheber, Wolfram, Harbecke, & Aebischer, 1998). Conversely, IL-10 which is essential for parasite survival and disease progression is induced by *Leishmania* in infected monocytes and macrophages (Chandra & Naik, 2008; Meddeb-Garnaoui, Zrelli, & Dellagi, 2009). Leishmania also expresses cytokine orthologs that modulate host immune cells. The human macrophage migration inhibitory factor ortholog, produced by *L. major*, has been shown to inhibit macrophage apoptosis and could contribute to parasite persistence and evasion of immune destruction (Kamir et al., 2008).

Chemokines and chemokine receptors play a major role in immunity to *Leishmania* by coordinating the recruitment and activation of anti-leishmanial immune cells (Oghumu, Lezama-Dávila, Isaac-Márquez, & Satoskar, 2010). *Leishmania’s* attempt to alter the chemokine expression profile in the infected tissue microenvironment will therefore contribute to their ability to evade the host’s immune system. For over a decade, *Leishmania* has been known to induce or inhibit the expression of chemokines to control recruitment of immune cells. In human cutaneous leishmaniasis, the diffuse form of the disease has been associated with lower levels of the macrophage chemotactic factor CCL2 (MCP-1), which is higher in the localized form of the disease (Ritter et al., 1996). Since CCL2 also induces antiparasitic activity in macrophages (Mannheimer, Hariprashad, Stoeckle, & Murray, 1996), inhibition of this chemokine by *Leishmania* could potentially facilitate its survival within the host. The *Leishmania* virulence factor LPG can also inhibit migration of monocytes across the endothelial wall by inhibiting the synthesis of CCL2 and the expression of cell surface adhesion molecules including E-selectin, ICAM-1, and VCAM-1 by endothelial cells (Lo et al., 1998). Expression of chemokine receptors CCR4 and CCR5 as well as integrin VLA-4 activity, all involved in macrophage adhesion, has also been shown to be inhibited during *Leishmania* infection (Pinheiro et al., 2006).

The ability of *Leishmania* to selectively recruit immune cells that will facilitate parasite survival was demonstrated by Katzman and Fowell (2008). This study showed that *L. major* can induce the expression of the Th2 attracting chemokine CCL7 at the dermal infection site, allowing the accumulation of IL-4 producing but not IFN-γ producing effector T cells. In contrast, ovalbumin with complete Freund's adjuvant (OVA/CFA) immunized dermis permitted the accumulation of both populations of effector T cells, suggesting that *L. major* actively modulates the local tissue environment in an attempt to evade the hosts' immune response. *Leishmania* promastigotes themselves secrete a chemotactic factor for PMNs, which serve as the host cell in the very early stages of infection (van Zandbergen, Hermann, Laufs, Solbach, & Laskay, 2002). At the same time they can induce the expression of IL-8 by these cells so as to recruit more PMNs which aid in the establishment of infection and proliferation of the parasite. On the other hand, they have the ability to inhibit the expression of IP-10, a chemokine that recruits and activates NK and Th1 cells, which are important in parasite eradication (van Zandbergen et al., 2002).

In a chronic murine VL model, *L. donovani* inhibited dendritic cell migration to T cell areas of the spleen due to a loss in CCR7 expression. Further, treatment of infected mice by adoptive transfer of CCR7 expressing dendritic cells significantly reduced parasite burdens (Ato, Stäger, Engwerda, & Kaye, 2002). Although mechanisms behind the process are still not fully understood, the induction or suppression of chemokine/chemokine receptor expression is a major way *Leishmania* evades the host immune system to favor establishment of disease.

2.7. Modification of T Cell Responses

Th1 cells play a vital role in the elimination of *Leishmania* through the secretion of IFN-γ and CD40L which activate macrophages to produce nitric oxide, a leishmanicidal factor. Recent studies have shown that some secreted factors from *L. major* possess immunosuppressive properties, could dampen lymphoproliferative capabilities and skew T cell polarization toward a susceptible Th2 phenotype (Tabatabaee, Abolhassani, Mahdavi, Nahrevanian, & Azadmanesh, 2011). Similarly, extracts of *L. amazonensis*, a causative agent of CL in the New World, promote a Th2 type immune response in the host, thereby enhancing infection (Silva et al., 2011).

The ability of *Leishmania* to induce the activity of regulatory T cells that suppress anti-*Leishmania* immune responses has been well established in cutaneous and visceral animal models of leishmaniasis (Belkaid, 2003; Belkaid, Piccirillo, et al., 2002; Mendez, Reckling, Piccirillo, Sacks, & Belkaid, 2004). This has also been shown in humans (Ganguly et al., 2010; Katara, Ansari, Verma, Ramesh, & Salotra, 2011; Rai et al., 2012). Tregs generally function as immune-regulators of cell-mediated immune responses, preventing pathology due to uncontrolled effector T cell activity. While this presents apparent advantages to the host, it could very well be capitalized by *Leishmania*, thereby preventing complete eradication of the parasite by Th1 cells. Tregs are retained at the site of infection where they secrete IL-10 and TGF-β which down regulate Th1 and macrophage activity, rendering the area of infection, an immune privileged site (Peters & Sacks, 2006).

In conclusion, current research that focuses on mechanisms of immune evasion by *Leishmania* could shed light on promising targets for therapeutic intervention. This is especially important as medication currently employed in the therapy of *Leishmania* presents problems due to toxicity, patient compliance, and drug resistance. Recent advances in the use of immuno-therapeutic approaches targeted at circumventing the parasites' attempt at host immune evasion include the use of TLR agonists that induce strong cell mediated immune responses. The TLR 4 agonist monophosphoryl lipid A has been used in combination with *Leishmania* antigens or other agonists to protect against cutaneous or

mucocutaneous *Leishmania* infection models (Aebischer et al., 2000; Coler & Reed, 2005; Raman et al., 2010) and some formulations are currently being used successfully in clinical trials (Llanos-Cuentas et al., 2010). Activation of the TLR2 pathway has been shown to reduce parasite burdens in *L. donovani*-infected macrophages (Bhattacharya et al., 2010). TLR2 agonist Pam3Cys and CPG-ODN 2006 have been used in combination with miltefosine in the immunotherapy of experimental visceral leishmaniasis (Gupta, Sane, Shakya, Vishwakarma, & Haq, 2011; Shakya, Sane, Shankar, & Gupta, 2011).

Other promising immuno-therapeutic approaches involve the use of chemokines or chemokine receptor agonists (Gupta, Majumdar, et al., 2011), blockade of co-stimulatory molecules (Murphy et al., 1998, 1997), reconstitution of membrane cholesterol to facilitate re-association of membrane signaling assemblies (Sen et al., 2011), and inhibitors of cell signaling molecules (Cheekatla et al., 2012; Cummings et al., 2012), all mechanisms of which are regulated by *Leishmania* to evade or exploit host immune responses. While this presents an area for extensive future research, current advances have made significant impact and present weighty implications in the management of leishmaniasis.

Acknowledgments

This work was supported by National Institutes of Health grants R03AI090231, RC4AI092624, R34AI100789, R21AT004160 and R03CA164399 awarded to A.R.S and National Institute of Dental and Craniofacial Research Training Grant T32DE014320 awarded to S.O.

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