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Subversion Mechanisms by Which *Leishmania* Parasites Can Escape the Host Immune Response: a Signaling Point of View

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INTRODUCTION

Leishmaniasis, caused by protozoan parasites of the *Leishmania* genus, is an infection encountered in tropical and subtropical regions of the world. *Leishmania* parasites are propagated by different species of the sandfly vector (genus *Phlebotomus* or *Lutzomya*), depending on the region. Old World species of *Leishmania*, such as *L. donovani* and *L. major*, cause pathology from southern Europe to Africa, the Middle East, and throughout southern Asia, whereas New World species (e.g., *L. mexicana*, *L. amazonensis*, and *L. chagasi*) are found throughout South and Central America and as far north as the southern states of the United States. Clinical manifestations differ widely, depending on the *Leishmania* species. The majority of mortality results from the visceral form of leishmaniasis, caused by *L. donovani* or *L. chagasi*; 90% of annual cases are reported in Bangladesh, Brazil, India, Nepal, and Sudan. The most common manifestations are cutaneous lesions; 90% of new cases of cutaneous leishmaniasis occur in Afghanistan, Brazil, Iran, Peru, Saudi Arabia, and Syria, and they are caused principally by *L. major* and the *L. mexicana* subgenus (64, 164). The Viannia subgenus is encountered solely in the Americas and is responsible for the clinical form named mucocutaneous leishmaniasis, which is characterized by facial disfigurement. In 2000, it was estimated that more than 12 million individuals were infected by the various *Leishmania* species in 88 countries, with an estimated 1.5 to 2 million clinical cases (164).

Two distinct developmental stages of *Leishmania* are recognized. Promastigotes are found within the sandfly and have an elongated shape and long flagellum. Promastigotes can be further classified as procyclic promastigotes, which multiply in the gut of the sandfly, or as the infective metacyclic promastigotes, which are found in the mouth parts and anterior gut and do not divide. These differentiate into round or oval amastigotes, which lack flagella, once in the host.

In its mammalian host, the lifestyle of *Leishmania* is that of an obligate intracellular pathogen infecting the hematopoietic cells of the monocyte/macrophage lineage, which it enters by phagocytosis. Since this cell type is specialized for the destruction of invading pathogens and priming of the host immune response, *Leishmania* has had to evolve a range of sophisticated mechanisms to subvert normal macrophage function. These include preventing the activation of deadly antimicrobial agents such as nitric oxide (NO) and also inhibition of many of the cytokine-inducible macrophage functions necessary for the development of an effective immune response. This enables the parasite to evade the innate immune response and to divide within the phagolysosome of the infected macrophage, from where it can spread and propagate the disease within the host. In this review, we focus on the molecular mechanisms whereby *Leishmania* can subvert host surveillance by altering the macrophage signal transduction machinery, thereby modulating the macrophage environment in its favor. It should be noted that studies generally use only one, or sometimes two,

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species of *Leishmania* at a time and a single developmental stage, and this gives the impression that the mechanisms revealed apply to all species. However, given the diversity of pathologies caused by *Leishmania* spp., it is inevitable that significant differences exist in the mechanisms of host cell manipulation. These differences may account for some of the conflicting results described in this review; our understanding is now at a stage where more studies directly comparing the effects of different species on macrophages would be extremely useful.

Parasite Surface and Secreted Molecules

In addition to being distinguished by morphology and location, the various developmental stages of *Leishmania* parasites can be distinguished by their surface molecule composition. Procyclic promastigotes are covered by a 7-nm-thick glycocalyx. The glycocalyx of metacyclic promastigotes is even thicker, at least 17 nm, but it is almost completely absent from amastigotes (123). This jacket comprises glycoproteins and other glycosylated species, which are anchored to the surface membrane by a distinctive glycosylphosphatidylinositol (GPI) linkage (reviewed in detail in reference 45). The dominant surface molecule of promastigotes is lipophosphoglycan (LPG). Its structure varies between *Leishmania* species, but it is composed principally of repetitive units consisting of a disaccharide and a phosphate, linked to the membrane by a GPI anchor. *Leishmania* species differ markedly by the presence of glycan side chains, as well as by their composition and positioning on the LPG core structure. LPG of *L. major*, for example, is highly branched, whereas that of *L. donovani* is not (102). Furthermore, the structure of LPG differs between procyclic and metacyclic promastigotes, being significantly longer in the latter, and is almost completely absent from amastigotes (103, 123). As discussed below, studies using purified LPG or mutant parasite strains defective in LPG production have shown that LPG plays many important roles in parasite survival and modulation of the immune response, and differences in LPG structure and distribution are important for the different properties of the different developmental stages of *Leishmania*.

Another important surface molecule is the glycoprotein gp63 (promastigote surface protease). This is a zinc-dependent metalloprotease with a wide range of substrates, including casein, gelatin, albumin, hemoglobin, and fibrinogen (104). While around 10-fold less abundant than LPG, gp63 is still found throughout the promastigote surface (100, 123). However, its shorter length means that it is essentially buried under a sea of LPG. Like LPG, gp63 is down-regulated in the amastigote form (141). This reduced expression may be counteracted by the absence of LPG on the amastigote surface, meaning that gp63 is no longer masked and may therefore play an important role in amastigote survival and modulation of the host response (102, 123).

The most abundant promastigote surface molecule is glycosylinositol phospholipid (GIPL), a class of GPI-linked glycolipids. These molecules are 10 times more abundant than LPG, but their small size keeps them close to the parasite membrane, so it is unclear what role they play in interaction with the host (45, 101). Unlike LPG, which is continually shed, GIPL has a long half-life and so is believed to play a protective role at the promastigote surface (127).

The completion of the *L. major* database has revealed putative 65 cysteine peptidases; some of these clearly have roles in promoting parasite survival and disease progression, particularly during *L. mexicana* infection (for a recent review, see reference 109). At least 50% of the cysteine peptidase activity is localized within the lysosomes of the parasite, so is unlikely to pay a direct role in modulating macrophage signaling (69). However, it is also clear that a significant number of cysteine peptidases are released following amastigote death and as a result of their unusual intracellular trafficking pathway (17, 69). As described below, these released proteases can modulate macrophage activity by acting directly on the host cell surface or following entry into the macrophage endoplasmic reticulum from the phagosome.

This list is far from exhaustive, and it is likely that other surface molecules play important roles in modulating specific aspects of the host immune response. In particular, amastigotes express some poorly characterized, β-mercaptoethanol-activated metalloproteases (141), and they are even able to incorporate membrane lipids from the host cell (100). Furthermore, other secreted molecules, such as proteophosphoglycans and acid phosphatases, have been directly linked to parasite survival and pathogenicity (89). This review will discuss the role of some of these molecules in subversion of the host defense response.

Initial Interaction and Phagocytosis

Having entered their mammalian host during the blood meal of a female sandfly, *Leishmania* promastigotes must first evade complement-mediated lysis until they are engulfed by a macrophage. *L. major* procyclic promastigotes cannot resist complement action, whereas the metacyclic form, which is specialized for transmission to the host, can fully avoid complement-driven lysis (128). This difference in complement resistance has been shown to depend upon branched LPG on the parasite surface. LPG is longer on the surfaces of metacyclic promastigotes and seems to prevent the attachment of C5b-C9 subunits of the complement complex, which are for cellular lysis. *L. donovani* promastigotes, however, prevent C5 convertase formation by fixing the inactive C3bi subunit on their surfaces (128). The surface glycoprotein gp63, a protease, has been reported to protect *L. amazonensis* and *L. major* against cellular lysis by converting C3b into C3bi, thus favoring parasite opsonization and internalization (16). It has also been proposed that a surface protein kinase of *L. major* may phosphorylate members of the complement system and thereby inactivate the cascade (63).

Parasite surface molecules also play an important role during attachment to the macrophage. In vivo opsonization of *Leishmania* metacyclic promastigotes by C3b and C3bi permits the interaction with the macrophage complement receptor 1 (CR1) and CR3, respectively. However, since C3b is rapidly converted to C3bi by gp63, it appears that CR3 is the more important receptor, and interaction with CR1 is only transient (73). Attachment via CR3 rather than CR1 is advantageous to the parasite, since it will not trigger the oxidative burst during phagocytosis (108). Promastigotes can also attach to the mac-

rophage via the mannose-fucose receptor, which binds to mannan residues of LPG (10). LPG can also interact with C-reactive protein (CRP), an early inflammatory product, and thus triggers phagocytosis via the CRP receptor (31) without leading to the macrophage activation that is usually seen following CRP receptor-mediated phagocytosis (12). Furthermore, gp63 and LPG interact with fibronectin receptor and CR4, respectively (15, 155), although LPG appears to play only a minor role during attachment and internalization (reviewed in reference 38). More recently, a number of *Leishmania* surface molecules that play a role during the initial *Leishmania*-macrophage interaction have been identified, although the macrophage receptors for these molecules are not yet clear. For instance, Chiang and Sefton (27) have identified an ICAM-related molecule, ICAM-L, that may be necessary for the interaction between the parasite and the murine macrophage cell line J774. A recent study has reported that the greater infectiousness of metacyclic promastigotes is due in part to elevated surface phosphatidylserine (157), while blocking antibodies against another parasite surface molecule, GIPL, have been shown to inhibit attachment of *L. major* (154). Amastigotes can also be internalized in an Fc receptor-dependent fashion following opsonization with specific antibodies (57). The large number of receptors implicated suggests a degree of redundancy among parasite-macrophage interactions, although it appears that several interactions are necessary for internalization (15).

Following their attachment to the macrophage, *Leishmania* promastigotes are internalized to the relatively benign environment of the endosome, where they begin to differentiate into amastigotes. Unlike amastigotes, promastigotes are vulnerable to degradation by the acidic and hydrolytic environment of the phagolysosome. They must therefore retard endosome maturation and phagosome-endosome fusion, a process dependent on LPG (39). This retardation has been observed by the absence or delayed arrival of late endosomal markers such as rab7 and LAMP-1 (142) and may be related to the LPG-dependent accumulation of F-actin (67). The mechanism is not completely understood, but it has been shown to depend upon calcium presence (156) and protein kinase C (PKC) inhibition (66). Furthermore, LPG appears to change the shape of membranes, leading to steric repulsion between the phagosome and the endosome (107). The delay in phagolysosome maturation provides a window during which promastigotes can differentiate into the more resistant amastigotes. This is consistent with our earlier observation that parasite survival and infectiousness in different strains of mice correlate with macrophage phagocytic activity (120). Interestingly, while LPG is important for the survival of promastigotes in the early stages of uptake, it is not expressed by amastigotes, indicating that its role is transient and confined to only the early stages of infection.

Another survival strategy used by *Leishmania* parasites is the inhibition of the hydrolytic enzymes and other destructive molecules that are secreted into the phagolysosome. Two newly discovered *Leishmania* molecules, named peroxidoxins LcPxn1 and -2 (4), and a superoxide dismutase (54) are believed to deplete nitrite derivatives and reactive oxygen intermediates (ROI), which are the most important microbicidal small molecules. Furthermore, there is evidence that LPG itself can enhance promastigote survival by neutralizing vacuolar ROI (25). LPG may also protect against lysosomal enzymes, perhaps by its strong negative charge and galactose-mannose repeating units (41). The proteolytic activity of the surface molecule gp63 is optimal at the acidic pH found in phagolysosomes, supporting the suggestion that it targets lysosomal enzymes (143). However, its role is questionable, as parasites with mutations in the six gp63 genes are still capable of survival, differentiation, and replication within macrophages (70).

INHIBITION OF MACROPHAGE FUNCTIONS

Sequestering itself insides the cells of the host allows *Leishmania* to escape many of the immune responses that would otherwise be directed against it. However, it is also necessary to inhibit numerous macrophage functions, particularly those involved in immune surveillance and macrophage activation, at either the protein or gene expression level. One study of 245 macrophage genes showed that 37% were repressed at least twofold following in vitro infection with amastigotes (18), although larger-scale microarray studies have suggested that promastigotes induce and repress to similar extents (26, 136). However, these studies, and our unpublished observations following infection in vivo, all show reduced expression of numerous genes with important roles in various immune, cell physiological, and signaling functions. We describe some of these functions, and how they are affected by *Leishmania*, in detail in the following paragraphs.

Microbicidal Free Radical Production

Two types of microbicidal molecules are recognized for their efficacy against *Leishmania*: NO (86) and ROI (110). NO is critical for parasite clearance, since mice lacking inducible nitric oxide synthase (iNOS) (also called NOS2) are unable to control infection, and macrophages derived from these mice are incapable of eliminating promastigotes in culture (162). Infected macrophages or macrophages incubated with purified LPG or GIPL *Leishmania* surface molecules lose their ability to induce iNOS or to generate NO in response to gamma interferon (IFN-γ) and/or lipopolysaccharide (LPS) (126, 127). However, it seems that IFN-γ and LPG can synergize to generate NO when administered simultaneously to naive macrophages (126, 127). This suggests that contact between the parasite and the macrophage prevents the macrophage from responding to subsequent exposure to IFN-γ produced by lymphoid cells. Inhibition of NO production may result from the production of interleukin-10 (IL-10) and/or transforming growth factor β (TGF-β), inactivation of the JAK/STAT pathway, activation of phosphotyrosine phosphatases, and/or ceramide production, as discussed below.

In contrast to mice deficient for NO production, mice deficient for the generation of ROI can ultimately control the infection, after an initial period of increased susceptibility (111), indicating that ROI play a less important role in parasite clearance. However, ROI generation is also inhibited by *L. donovani* infection (19, 117, 118). Inhibition appears to be dependent on the surface molecules LPG and gp63 (38, 148) and has been shown to involve abnormal PKC activity (118).

Antigen Presentation

In addition to repressing the microbicidal activities of the host macrophage, *Leishmania* inhibits the ability of the host cell to display parasite antigens to other components of the immune system (134). This appears to be related to the infectiousness of the parasite: macrophages infected with insect-adapted, procyclic promastigote cultures are initially able to present the parasite LACK antigen, but they lose this ability as the parasite begins to differentiate (30). Furthermore, macrophages infected with the infectious, metacyclic form of promastigotes present very little LACK antigen, and amastigote-infected cells present none at all (30).

Some studies have shown that *L. donovani* inhibits antigen presentation by repressing major histocompatibility complex (MHC) class II gene expression, both basal and particularly following stimulation with IFN-γ (35, 133, 134). In contrast, macrophages infected with *L. amazonensis* have been shown to express normal levels of MHC class II (84, 124). Antigen presentation may be inhibited in this case by interfering with the loading of antigens onto MHC class II molecules (50, 124) or by sequestration of the MHC class II molecule and/or antigens within the phagolysosome (79, 85). De Souza Leao and colleagues demonstrated a third level of inhibition, at least for the case of *L. amazonensis*, when they observed direct endocytosis of MHC class II molecules by amastigotes themselves, followed by cysteine peptidase-dependent degradation (40). Consistent with the phagosomal location of the *Leishmania*, MHC class II appears to be more important than class I for resistance, although class I does have a role to play, at least in some situations (158). Mice defective in MHC class I presentation are resistant to infection with *L. major*, but MHC class $II^{-/-}$ mice are susceptible (68, 88).

Antigen presentation depends upon cellular communication through costimulatory molecules such as B7/CD28 and CD40/CD40L. It has been demonstrated that B7-1 of *L. donovani*-infected macrophages could not be further expressed in response to LPS stimulation (75) and that this inactivation process was prostaglandin dependent (137). It seems that inhibition of CD40/CD40L ligation is responsible for the absence of iNOS and macrophage microbicidal activities (72, 147) and that cure of *L. major* infection depends upon an active CD40/CD40L ligation (23, 62). Recent findings suggest that p38-dependent signaling triggered by CD40 interaction is altered in infected macrophages, and this may lead to diminished iNOS expression (2). In contrast, repression of MHC class II gene expression appears to involve a cyclic AMP-independent mechanism (83).

Repression of Cytokine Production

Leishmania prevents the activation of an effective immune response by inhibiting production of a number of cytokines, particularly those involved in the inflammatory response (IL-1 and tumor necrosis factor alpha [TNF-α]) or in T-lymphocyte activation (IL-12). LPS-induced IL-1β secretion has been reported to be inhibited in *L. donovani*-infected (134, 135) and LPG-exposed (47) macrophages. LPG seems to repress IL-1β transcription by acting through a promoter repression sequence (60). In contrast, IL-1α transcription is induced by *L. major*, but this is not reflected in increased secretion, indicating that a downstream repression mechanism counteracts the induction (61). Interestingly, the induction of IL-1α appears to be Myd88 dependent, suggesting a role for Toll-like receptors. TNF-α production is also repressed in infected macrophages treated with LPS (36). More recently, this has been shown to involve IL-10 and PKC inhibition (8).

The capacity of *Leishmania* to infect macrophages without inducing proinflammatory cytokines, and then to inhibit their induction in response to various agonists, probably represents a survival mechanism whereby the parasites can inhibit a harmful inflammatory reaction. Nevertheless, these studies have been mainly performed in an in vitro context. Recent studies performed in vivo have clearly demonstrated that proinflammatory cytokines (IL-1, IL-6, and TNF-α) as well as various chemokines, a family of cytokines responsible for recruitment of inflammatory cells to the site of infection, were induced in the early stages of *L. donovani* and *L. major* infection (98). Of interest is that *L. major* promastigotes were shown to be better activators of proinflammatory events than *L. donovani*, as shown by a greater, transient recruitment of inflammatory cells. This may reflect the different pathologies caused by the two strains. In addition, both species recruit a heterogeneous population of host inflammatory cells, including neutrophils and monocytes/macrophages (98). This is of particular interest from a host defense point of view, since neutrophils have been recently shown to be important for controlling *L. major* infection (46, 87).

The cytokine IL-12 plays a critical role in the regulation of cellular immune responses. It is essential for T-lymphocyte activation and subsequent IFN-γ secretion leading to macrophage activation and production of microbicidal molecules. It is therefore not surprising that *Leishmania* has developed the ability to inhibit IL-12 production. This has been shown for promastigotes of *L. donovani* and *L. major* (24), *L. mexicana* amastigotes (163), and the phosphoglycan portion of LPG (122) in vitro. IL-12 inhibition has been also reported to occur in *L. major*-infected mice (5). The intracellular mechanism is still unclear. Macrophage complement receptors and Fcγ receptor, which are known to interact with *Leishmania* during phagocytosis, have been shown to repress IL-12 (93, 152). Furthermore, Piedrafita et al. showed that *L. major* LPG-mediated IL-12 repression was independent of the NF-κB transcription factor family, despite IL-12 being NF-κB responsive (122). Instead, repression may result from increased ERK1/2 phosphorylation (44); however, that report is problematic, because it contrasts with the well-documented dephosphorylation of ERK during infection (discussed below). A very recent report has shown that the abilities of *L. mexicana* amastigotes to degrade NF-κB and to repress IL-12 are both dependent on cysteine peptidase B activity (22). While this is purely correlative, there is no reason to assume that promastigotes of Old World species and New World amastigotes employ the same mechanisms, especially given that both species and developmental stages differ markedly in both cysteine protease and LPG type and expression.

Induction of Immunosuppressive Molecules by *Leishmania* Infection

In addition to inhibiting the functions of their host macrophages, *Leishmania* parasites can induce the production and/or

secretion of various immunosuppressive signaling molecules, such as arachidonic acid metabolites and the cytokines TGF-β and IL-10. These affect numerous different cell types, directly and indirectly, thus distorting the normal immune response and favoring parasite survival.

TGF-β production is induced by several *Leishmania* species in vitro and in vivo (reviewed in reference 13). Augmentation of TGF-β secretion correlated with retarded iNOS expression and reduced NK cell activity in lymph nodes (140, 151). This is consistent with the idea that TGF-β inhibits macrophage microbicidal action and the production of IFN-γ by NK cells, although the exact role of NK cells during leishmaniasis is somewhat controversial (74, 80, 90, 138, 139, 161). A recent study demonstrated that *L. chagasi* induces TGF-β production in the immediate environment of the infected human macrophage, and this may permit the local inhibition of immune responses (51). Interestingly, at least for the case of *L. chagasi*, the increased production appears to be a result not of increased gene expression but of cleavage of pro-TGF-β by amastigote cysteine proteases to produce active TGF-β (51, 145). Interaction between the macrophage and phosphatidylserine motifs on the amastigote surface has also been proposed to trigger this induction (49).

IL-10 is another anti-inflammatory cytokine produced by *Leishmania*-infected macrophages in vitro, apparently via interaction with the Fcγ receptor (153). Its production may be responsible for the suppression of macrophage microbicidal activity involving NO, production of several cytokines (IL-1, IL-12, and TNF), and expression of costimulatory molecules such as B7-1/2 (reviewed in reference 32). Its importance in vivo is illustrated by the observation that transgenic mice constitutively expressing IL-10 are unable to control *Leishmania* infection (73). As for TGF-β, IL-10 is apparently induced following recognition of amastigote surface phosphatidylserine residues by the macrophage (49).

Prostaglandin E2 (PGE2) seems to be generated by *Leishmania*-infected macrophages and to favor parasite survival and progression (43, 98, 131, 132). This arachidonic acid metabolite has been reported to cause inhibition of macrophage proliferation and to suppress production of TNF-α, IL-1, and reactive oxygen intermediates (6). A recent study reports that PGE2 induction in *L. donovani*-infected macrophages depends upon PKC activation and cyclooxygenase-2 expression (98). Interestingly, one study has correlated increased visceralization of *L. donovani* in malnourished mice with increased PGE2 production in the lymph nodes (1).

It is therefore clear that *Leishmania* parasites are capable of modulating numerous macrophage functions in order to promote survival within the host. While we have seen that the parasite surface coat is responsible for triggering many of these effects, we have not directly addressed the intracellular mechanisms by which the signals are communicated. Some of the intracellular signaling pathways that are modulated by *Leishmania* are discussed in the next section.

LEISHMANIA-INDUCED ALTERATION OF HOST CELL SIGNALING

A cell transmutes a stimulus on the outside of the plasma membrane into changes in the cell's physiological program by means of intracellular signaling pathways. These are usually triggered by the ligation of an external ligand, such as a cytokine or a *Leishmania* surface molecule, to a receptor on the cell surface. This ligation causes activation of the receptor, commonly by phosphorylation and/or conformational changes, resulting in activation of second messengers within the cytosol. These second messengers are often protein kinases, which then phosphorylate other kinases to continue a cascade that ultimately results in the activation of effector molecules, such as transcription factors or actin filaments, and causing a change in the cell's behavior. It should be emphasized that the activity of an intracellular pathway is normally determined by a balance of both positive and negative regulation. Activation of a given kinase cascade will often result in the activation of its opposing phosphatases, in a classic example of negative feedback. Many prokaryotic and eukaryotic pathogens, including *Leishmania*, have evolved various strategies to exploit host cell signaling regulatory mechanisms by distorting this balance between positive and negative influences. In the following paragraphs, we describe some of the most important observations made to date concerning modulation of host cell signaling by *Leishmania* infection (summarized in Fig. 1).

Ca^{2+}- and PKC-Dependent Pathways

One of the first second messengers that was reported to be modulated by the *Leishmania* parasite is calcium (Ca^{2+}). *Leishmania* infection was shown to augment the intracellular Ca^{2+} concentration of phagocytes (41, 117). The surface molecule LPG seems to chelate Ca^{2+} (41) and to contribute to the rapid elevation of intracellular Ca^{2+} concentration (91); however, a causal link between these two properties has not been established. The increased intracellular calcium concentration appears to be a result of augmented Ca^{2+} uptake by infected cells in response to depletion of intracellular Ca^{2+} stores (91). While cells infected with *L. donovani* or *L. mexicana* have shown altered Ca^{2+}-dependent responses, such as chemotaxis and production of ROI (14, 117), it is hard to establish a clear sequence of events linking the two. For example, inhibition of ROI production may also be due to a significant reduction of the inositol-1,4,5-triphosphate (IP_3) (117). This may be due to the action of a *Leishmania* acid phosphatase responsible for dephosphorylation of IP_3 (33), or the elevated Ca^{2+} concentration itself could activate a host IP_3 phosphatase as part of a calcium-dependent cascade (116).

A number of downstream regulatory enzymes require Ca^{2+} in order to be fully active (for example, the serine/threonine phosphatase calcineurin [also called PP-2B or PPP3]). Elevated intracellular Ca^{2+} concentrations are therefore consistent with increased calcineurin activity (91). Some isoforms of PKC are also Ca^{2+} dependent, so it is perhaps surprising that there is reduced PKC activity in *L donovani*-infected macrophages, and this contributes to parasite survival (37, 106, 118). Promastigote LPG has been shown to cause this inhibition (37); however, amastigotes, which lack LPG, can also inhibit PKC in infected human monocytes, indicating the availability of alternative mechanisms (118). LPG-mediated repression may be explained by the observations that it interferes with binding of regulators, including Ca^{2+} and diacyl glycerol, and can also block PKC membrane insertion (reviewed in refer-

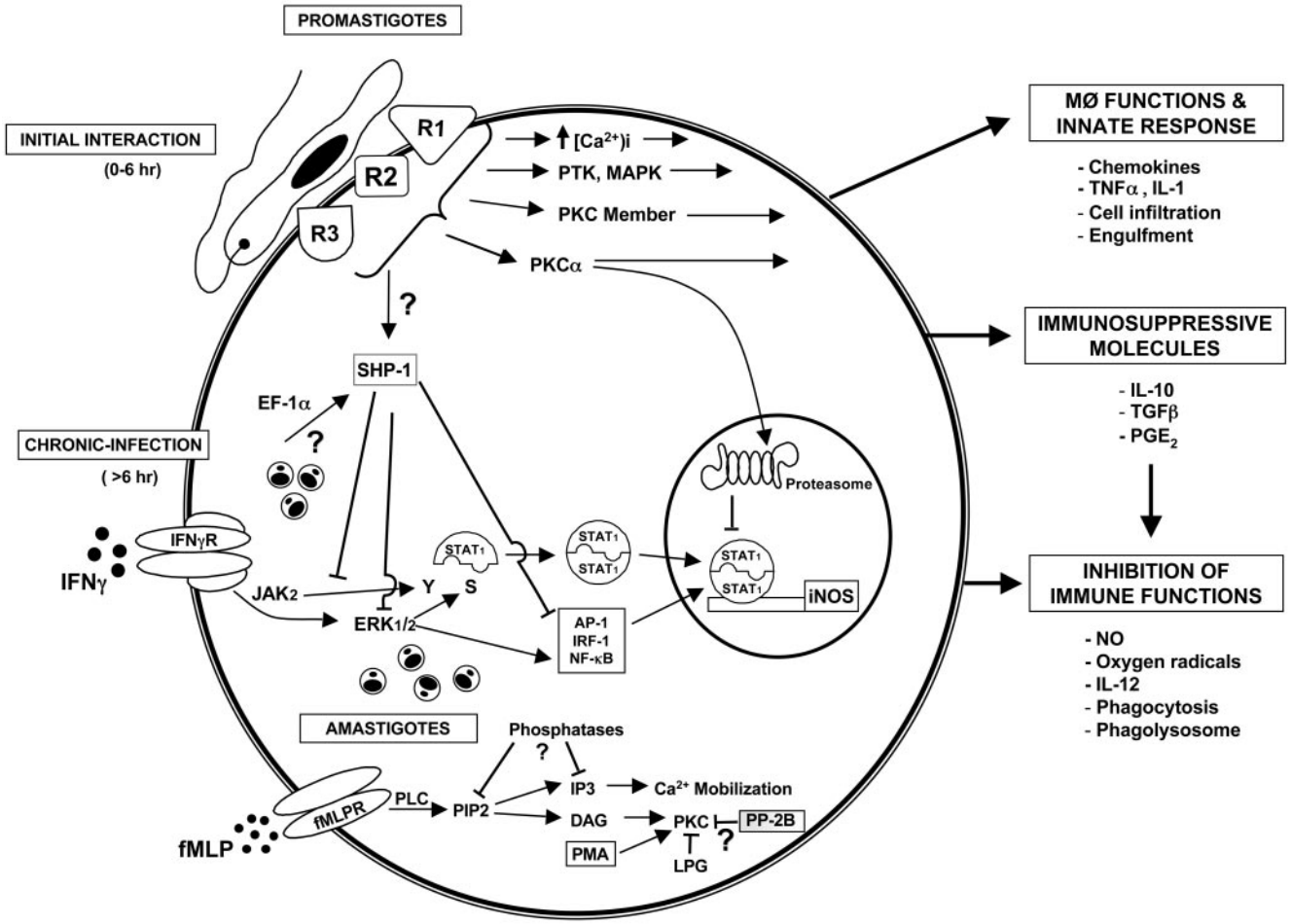

FIG. 1. Signaling events leading to the induction or inhibition of macrophage functions during *Leishmania* infection. *Leishmania* internalization within the macrophage is a receptor-mediated event, and this initial host-pathogen interaction is responsible for the rapid activation and deactivation of several signaling pathways leading to macrophage functions (e.g., phagocytosis, chemokine secretion, and prostaglandin secretion). SHP-1 negatively affects JAK2, Erk1/Erk2 MAP kinases, NF-κB, IRF-1, and AP-1, thus inhibiting IFN-γ-inducible macrophage functions (e.g., nitric oxide, IL-12 production, and immunoproteasome formation). STAT1α degradation by proteasome is PKCα dependent. Other phosphatases (e.g., IP_3 phosphatase and calcineurin) and surface parasite molecules (e.g., LPG) play a pivotal role in the alteration of various second messengers (e.g., PKC, Ca^{2+}, inositol lipids, and inositol phosphates), regulating important phagocyte functions (e.g., NO and superoxide production). Mϕ, macrophage; DAG, diacyl glycerol; PLC, phospholipase C; fMLPR, formyl peptide receptor; PIP2, phosphatidyl inositol 4,5-biphosphate.

ence 38). Interestingly, reduced sensitivity of PKC to diacyl glycerol was first demonstrated biochemically in cells that had been infected with *L. donovani* amastigotes, and it correlated with reduced generation of oxygen radicals (118). Various species of *Leishmania* and purified gp63 have been reported to inhibit MARCKs-related protein, a substrate of PKC associated with the cytoskeleton and involved in vacuole dispersal (28, 29). GIPL, another *Leishmania* surface glycolipid, may also inactivate PKC, but this process needs further investigation (105).

Interestingly, inhibition of Ca^{2+}-dependent isoforms of PKC, in particular PKCβ, is accompanied by induction of calcium-independent PKCξ (9). Both inhibition and activation are apparently a result of increased ceramide synthesis following infection (52). This ceramide has also been proposed to contribute to activation of phosphotyrosine phosphatase (PTP) and inhibition of mitogen-activated protein (MAP) kinases (discussed below).

One study has also demonstrated that addition of recombinant IL-10 favors macrophage PKC inhibition during *L. donovani* infection and that addition of anti-IL-10 antibodies prior to infection prevented inhibition of PKC (8). This is surprising, since PKC modulation was previously shown to be a direct result of interaction between the macrophage and the parasite (9, 49, 98). However, it is very possible that IL-10 secreted from infected macrophages inhibits the PKC activity of neighboring, naive macrophages, thus weakening their ability to take part in an immune activation and perhaps making them more prone to subsequent infection.

JAK2/STAT1- and IFN-γ-Mediated Signaling

A number of the macrophage functions suppressed by *Leishmania* infection (e.g., NO and MHC class II) are IFN-γ inducible. It is therefore not surprising that *Leishmania* can inhibit JAK2/STAT1 signaling, which is the principal pathway down-

stream of the IFN-γ receptor. Infected macrophages are defective in their ability to phosphorylate JAK1, JAK2, and STAT1 upon IFN-γ stimulation (11, 114). In the case of *L. donovani* promastigotes, this inactivation process has been shown to depend upon PTP activation, especially activation of the PTP SHP-1 (11) (Fig. 1). One study has also shown that negative regulation of the IFN-γ receptor contributes to JAK2/STAT1 pathway inactivation in *L. donovani*-infected cells (130). However, this does not seem to be the case following *L. mexicana* amastigote infection, where IFN-γ-dependent regulation of MHC class I is not affected by infection, indicating that the primary signaling lesion lies downstream of the IFN-γ receptor (78).

MAP Kinase Family

Inhibition of MAP kinases following *Leishmania* infection has been reported by two independent studies, following stimulation with LPS or phorbol myristate acetate (94, 113). In the first study, the use of *L. amazonensis* amastigotes was shown to rapidly alter ERK1 MAP kinase phosphorylation in response to LPS (94). In the second, the authors used *L. donovani* amastigotes to demonstrate that ERK1/2 MAP kinase inactivation was accompanied by the inhibition of the transcription factor Elk-1 and c-*fos* expression (113). In both studies, it was suggested that PTPs are responsible for the dephosphorylation of ERK1/2 MAP kinases. However, Martiny et al. found that the PTP in question was derived from the parasite (94), whereas Nandan et al. suggested an endogenous macrophage PTP (113). Our recent data support the latter study by showing that the macrophage PTP SHP-1 dephosphorylates and inactivates ERK1/2, as macrophages deficient for SHP-1 showed normal JAK2 and ERK1/2 MAP kinase activities and IFN-γ-dependent NO generation (G. Forget et al., submitted for publication). Ghosh et al. reported that infection of macrophages with *L. donovani* results in increased synthesis of ceramide (52). This appears to result in reduced phosphorylation of ERK1/2, apparently via activation of an endogenous phosphatase, with resultant repression of NF-κB and AP1, reduced NO generation, and enhanced parasite survival (52, 53).

It seems that other MAP kinase family members are also inactivated following *Leishmania* infection. For example, the p38 MAP kinase has distorted activity in *L. major*-infected cells following stimulation with a specific anti-CD40 antibody, which mimics the phagocyte–T-lymphocyte interaction (2). The authors of that study suggested that p38 inactivation contributes to the inhibition of iNOS induction and NO generation.

Ceramide-mediated inactivation of ERK1/2 MAP kinases and resulting inhibition of the transcription factors AP-1 and NF-κB have been also proposed to explain the absence of NO generation by *L. donovani*-infected cells (53). These studies are supported by the observation that all three MAP kinases subfamilies (ERK1/2, p38, and JNK) are not induced when *L. donovani* is phagocytosed by naive macrophages (125). This is due in part to LPG, at least in the case of ERK1/2, as LPG-deficient promastigotes do trigger ERK1/2 phosphorylation (125). However, this does not necessarily mean that LPG is directly responsible for the inactivation of these kinases but might mean merely that, in the absence of LPG, the interaction of *Leishmania* with its host cell is modified. Using LPG-deficient *L. donovani* promastigotes, we have observed that the PTP SHP-1 was still induced by these parasites, resulting in inactivation of signaling and transcription factors related to JAK2 and MAP kinase signaling pathways (M. Olivier et al., unpublished data). The observation that ERK1/2 is activated in the absence of LPG (125) may be a secondary effect of other, LPG-independent processes, such as chemokine and PGE2 secretion. Finally, it seems that p38 activation is important for the control of *Leishmania* infection, as the parasite survival rate is diminished in macrophages subjected to treatment with anisomycin, which activates p38 (71). This is not surprising, since the use of any compounds that could augment signaling pathways leading to macrophage activation should demonstrate protective action against *Leishmania* infection. Using the PTP inhibitor peroxovanadium, we demonstrated, in vitro and in vivo, that modulation of host cell signaling by targeting negative regulatory molecules can confer almost full protection against the development of cutaneous and visceral leishmaniasis (99, 119).

A very recent study has proposed another potential mechanism, in which *L. mexicana* amastigotes cause the rapid, cysteine peptidase-dependent degradation of ERK and JNK but not p38 (22). At first sight this new report contrasts remarkably with the earlier studies, in which ERK1/2 phosphorylation, but not abundance, was affected by infection. However, those studies all used promastigotes of *L. major* or *L. donovani* or amastigotes of *L. donovani*, and the only other report of ERK phosphorylation following infection with New World *Leishmania* (*L. amazonensis*) amastigotes did not address total ERK abundance (94). It is therefore possible that a marked difference in the mechanism of ERK inactivation between *Leishmania* species has been revealed. The choice of mechanism may depend on divergent evolution between New and Old World strains or on another factor. Direct comparative studies involving a range of species, differing in geographical origin and clinical outcome, are necessary to resolve this question.

SHP-1 Protein Tyrosine Phosphatase

Leishmania can also activate various molecules that inhibit intracellular signaling cascades. An important negative regulatory molecule is the PTP SHP-1 (Src homology 2 domain-containing tyrosine phosphatase, also called SHPTP-1, HCP, and PTP1C), which is expressed principally in hematopoietic cells but also in smooth muscle (92) and epithelial cells (3). This phosphatase contains two SH2 domains in its N-terminal portion, a phosphatase domain conserved in a central position and a C-terminal tail (167). SH2 domains of SHP-1 play a dual role that consists of substrate recognition and PTP autoregulation. These domains specifically bind proteins that are phosphorylated on a tyrosyl residue, followed by three to six specific amino acids that are part of a conserved motif (121, 146). In the case of SHP-1, its two SH2 domains recognize the target protein by the presence of an immunoreceptor tyrosine-based inhibitory motif with the consensus sequence I/V/LxYxxL/V (20).

SHP-1 is responsible for the negative regulation of many signaling pathways in all hematopoietic cell types, by acting in a variety of fashions. For instance, SHP-1 can bind to receptors

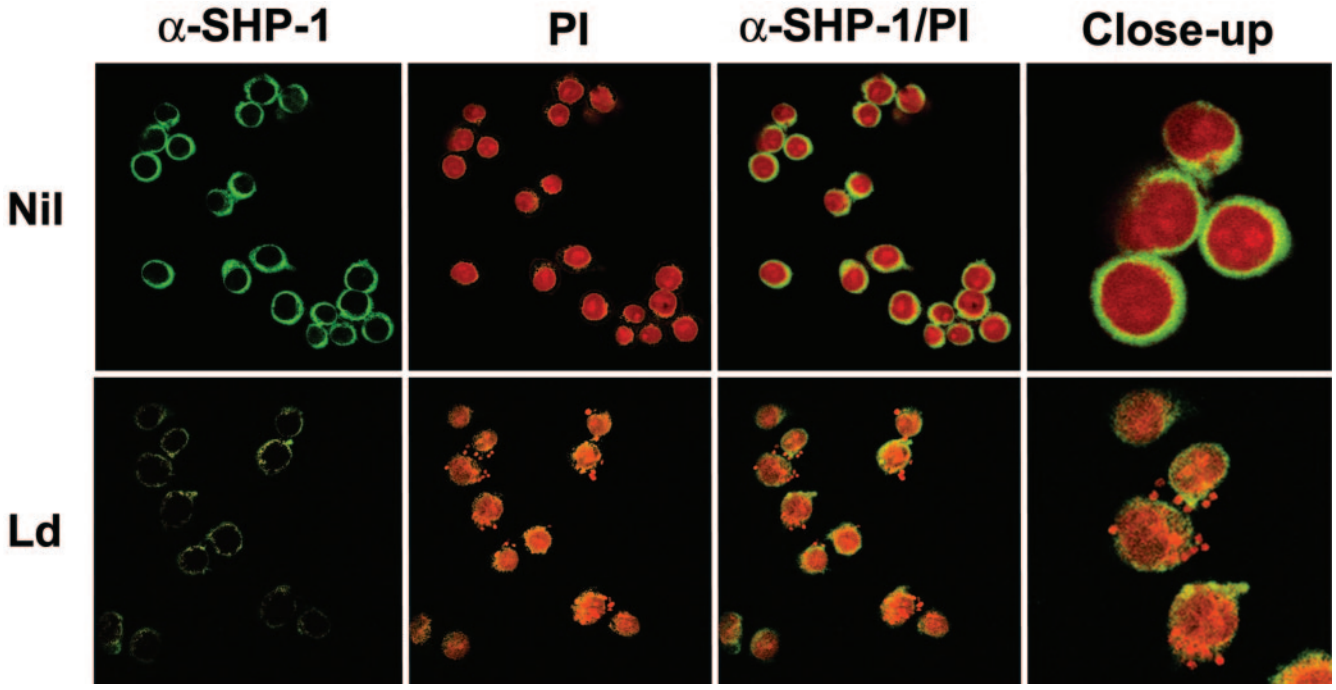

FIG. 2. Activation of SHP-1 in *Leishmania*-infected macrophages. Naive macrophages or macrophages infected for 30 min with *L. donovani* were stained with propidium iodide (PI) (red) or antibody specific for SHP-1 (green). SHP-1 is spread evenly throughout the cytoplasm in control cells but adopts a more punctate distribution following infection. Foci of SHP-1 are also visible in the nuclei of infected cells. Nil, no infection; Ld, *L. donovani* infection.

and dephosphorylate them directly; it can also associate with a receptor and dephosphorylate other members of the receptor binding complex. The PTP can also interact with other cytosolic proteins and tyrosine dephosphorylate them or their associated proteins (48). The majority of documented SHP-1 effects are the result of the inhibition by dephosphorylation of various kinases and their signaling pathways. SHP-1 plays a vital role in limiting the activation of the JAK/STAT pathways following cytokine receptor stimulation. Dephosphorylation of JAK1/2, TYK2, and STAT1α, -2, -3, -5α/β, and -6 has already been documented (34, 58, 81, 129, 166). In viable motheaten mice, whose SHP-1 phosphatase activity is deficient, increased nuclear translocation of the transcription factor NF-κB has been reported (46, 76, 95), which seems to provoke an exacerbated inflammatory response. SHP-1 has been also reported to activate (82, 165) or repress (113; Forget et al., submitted) MAP kinases, as well as to induce Src kinase activity (144).

Some studies have demonstrated that the inhibition of IFN-γ-dependent phosphorylation cascades following infection is due to activation of host cell tyrosine phosphatases (11, 119). Injection of PTP inhibitors, the bis-peroxovanadium compounds, into mice infected with *L. major* or *L. donovani* results in control of the infection. Both cutaneous lesions and parasitic load were significantly reduced in *L. major*-infected mice, whereas *L. donovani*-infected mice were completely protected (119). That in vitro and in vivo study was the first demonstration that modulation of host PTP by the parasite plays a pivotal role in the progression of the infection. The bis-peroxovanadium-mediated protection was shown thereafter to depend upon iNOS induction and NO generation, as well as to involve augmented innate inflammatory response that must have played an important role in the containment of the infection (99).

In a parallel study, we have demonstrated that macrophage PTP activity is activated very rapidly, within 5 min of exposure to *L. donovani* promastigotes, and this correlates with a rapid, general tyrosine dephosphorylation of high-molecular-weight proteins (11; Forget et al., submitted). SHP-1 is the most important component of this PTP activation (Fig. 2) (Forget et al. submitted). Furthermore, SHP-1 directly associates with JAK2 following *L. donovani* infection, and this interaction could explain in part the inability of JAK2 signaling to be triggered in response to IFN-γ stimulation (11). More recently, using immortalized macrophage cell lines deficient for SHP-1, we have firmly established that this PTP is responsible for inactivation of JAK2 and ERK1/2 MAP kinases. Protection of these kinases in SHP-1-deficient macrophages allows these cells to respond to IFN-γ stimulation by generating NO, even when they are infected (Forget et al., submitted). Corroborating our findings, another group has reported that SHP-1 interacts strongly with MAP kinases upon *L. donovani* infection (113). They showed that infected macrophages did not respond to phorbol myristate acetate, an artificial PKC activator, as reflected in inhibition of ERK1/2 phosphorylation, Elk-1 inactivation, and c-*fos* mRNA expression. A striking difference between these two studies is that the inhibition of ERK was observed 17 h postinfection, whereas interaction between JAK2 and SHP-1 and consequent down-regulation of JAK2 were observable within 1 h. Differences between cell lines and their status of differentiation may partially explain this discrep-

ancy. However, the capacity of the parasite to rapidly inhibit the host cell functions is essential for its survival. In addition to our studies using promastigotes, amastigotes of *L. amazonensis* have also been shown to rapidly inactivate ERK1/2 (94). Comparing the capacity of *L. donovani infantum* promastigotes and amastigotes to trigger host PTP activity, we found that both can rapidly and similarly induce this negative regulatory activity in macrophages (I. Abu-Dayyeh and M. Olivier, unpublished data). Recently, it has been proposed that the elongation factor EF-1α of *L. donovani* could be responsible for the later induction of SHP-1, observed 16 h postinfection (115). However, SHP-1 is activated so rapidly that a receptor-mediated process must also be necessary in the early stages (11, 94). The particular receptors involved may be revealed by a strategy using small interfering RNAs against different negative regulatory receptors of the immunoglobulin family.

Over the last few years, we have firmly demonstrated that SHP-1 plays a crucial role in the progression of *Leishmania* infection. Using the SHP-1-deficient, viable motheaten mice, we showed that *L. major*-infected animals did not develop footpad swelling and that the parasitic load was significantly reduced (46). Histochemical and in situ hybridization analyses of the infected footpads showed that, in contrast to the case for wild-type animals, iNOS gene expression and various signaling molecules (STAT1, NF-κB) known to be involved in that expression were not altered or diminished in SHP-1-deficient mice. In fact, infection resulted in increased NO generation, which must have contributed to killing of the parasite. This was in fact supported by the finding that aminoguanidine, an inhibitor of iNOS, completely reversed the protection from *L. major* infection observed in SHP-1-deficient animals (46). In addition, we recently obtained similar in vivo results concerning the role of SHP-1 in the control of visceral leishmaniasis conferred by *L. donovani* infection. Of interest, using a pathogenomic approach, we found that a majority of the host genes involved in signaling and the immune response that were repressed following infection in vivo were negatively regulated by PTP SHP-1 (Olivier et al., unpublished data).

The SOCS Family

A new group of negative regulatory proteins has recently been discovered. The suppressors of cytokine signaling (SOCS) family is now recognized to play an important role in the inhibition of signaling induced by several cytokines, principally through its action on the JAK/STAT pathway. The first member to be discovered was cytokine-inducible SH2-containing protein (CIS). The CIS gene is an early gene that is induced in response to stimulation of cytosine receptors, and its overexpression has been shown to inhibit IL-3-mediated signaling (42, 112, 150, 168). It now seems that eight members constitute this family of negative regulator: CIS itself and SOCS1 to -7 (65, 96, 149). Proteins from this family contain two important conserved sites, a central SH2 domain and a 40-amino-acid SOCS box motif towards the C terminus (77).

SOCS genes are not expressed in naive macrophages. Their transcription is induced in response to various cytokines, hormones, and growth factors, such as IFN-γ, that activate the JAK/STAT pathways (reviewed in reference 56). Once transcribed, the SOCS proteins will inhibit the JAK/STAT pathways in a negative feedback loop. Their modes of action vary, but as an example of how this type of regulator works, it has been reported that CIS seems to binds to phosphorylated JAK via its SH2 motif. This may then block binding and thus phosphorylation of STAT (97).

One study has shown that SOCS3 is induced in *L. donovani*-infected human phagocytic cells (7). SOCS3 mRNA expression was induced in a transient fashion by live or heat-killed parasites, but it was not induced by LPG alone and was not dependent on phagocytosis or production of proinflammatory cytokines (TNF-α or IL-1), stimuli that are known to trigger SOCS expression (7). While the data are convincing, our earlier studies were unable to show induction of any SOCS family members in *L. donovani*-infected macrophages (Olivier et al., unpublished data). Furthermore, the functional consequences of SOCS3 induction have not been addressed; further studies showing the role played by this inhibitor family in subversion of macrophage functions by *Leishmania* will be of great interest.

Proteasome-Mediated Protein Degradation

Regulation of second messengers and in particular of some transcription factors depends upon the ubiquitin-proteasome proteolytic pathway, which is responsible for the degradation of a great number of cellular and foreign proteins. This plays a key role not only in the regulation of the cell cycle and division and stress response but also in the immune responses and inflammation, among others (reviewed in reference 55). As the target transcription factor of the JAK2/STAT1 pathway, STAT1α plays an important role in the induction of many IFN-γ-inducible genes, which are repressed by *Leishmania*. Consistent with this role, STAT1α phosphorylation (114) and DNA binding activity (130; Forget et al., submitted) are markedly reduced in *L. donovani*-infected cells. Various studies in different contexts have shown that the use of proteasome inhibitors could stabilize STAT phosphorylation levels. However, none have clearly addressed the hypothesis that proteasome action was directed against STATs. Some have reported that proteasomes were affecting receptor-JAK stability or JAK activity (21, 59, 159, 160, 169). In a recent study, we have observed that the inability of IFN-γ to activate STAT1α nuclear translocation in *Leishmania*-infected cells is the consequence of rapid and sustained STAT1α protein diminution that begins very early after the interaction between the pathogen and the host cell (Forget et al., submitted). Importantly, the same results were obtained following infection with a variety of different *Leishmania* species (*L. major*, *L. donovani*, *L. mexicana*, or *L. braziliensis*). This phenomenon seems to be specific to STAT1α, since STAT3 levels were unchanged. Using a PTP inhibitor and SHP-1-deficient macrophages, we were able to show that PTPs were not involved in *L. donovani*-mediated STAT1α inactivation. Proteasome inhibitors were shown to rescue STAT1α protein degradation. Our study further revealed that protein kinase Cα (PKCα)-dependent signaling could be implicated in this proteasome-mediated STAT1α inactivation process. Together, these results argue for a direct role of the proteasome pathway in the specific proteolysis of STAT1α in macrophages infected with *Leishmania*, representing a new mechanism whereby pathogens could subvert microbicidal actions.

CONCLUSION

Intracellular parasites living within the harsh environment of phagocytes have developed strategies permitting their rapid physiological adaptation, escape from first-line defense systems, and the capacity to inhibit several functions of their host cells. This review has addressed some of the mechanisms by which *Leishmania* achieves this by manipulating signaling pathways of the host macrophage. Such strategies to manipulate the host immune response are by no means unique to *Leishmania*. There is increasing evidence that a great number of unicellular and pluricellular pathogens have also evolved means to inactivate or exacerbate immune cell functions, contributing to their survival and the development of pathogen-specific pathologies. It is also likely that mechanistic details will differ, sometimes markedly, between promastigotes and amastigotes and also between different species of *Leishmania*. These differences are likely to be of great importance in explaining the widely different clinical manifestations of leishmaniasis. However, it is clear that the common trait of these modulations is the manipulation of the host cell signaling system. As signaling pathways can be pharmacologically manipulated, a better knowledge of their role and the mechanisms whereby they regulate host immune cell functions and pathogen growth should permit the development of new therapies to control infectious agents.

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