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The amastigote forms of *Leishmania* are experts at exploiting host cell processes to establish infection and persist

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Abstract

Leishmania are dimorphic protozoan parasites that live as flagellated forms in the gut of their sandfly vector and as aflagellated forms in their mammalian hosts. Although both parasite forms can infect macrophages and dendritic cells, they elicit distinct responses from mammalian cells. Amastigotes are the parasites forms that persist in the infected host; they infect cells recruited to lesions and disseminate the infection to secondary sites. In this review I discuss studies that have investigated the mechanisms that *Leishmania* amastigotes employ to harness the host cell's response to infection. It should be acknowledged that our understanding of the mechanisms deployed by *Leishmania* amastigotes to modulate the host cell's response to infection is still rudimentary. Nonetheless, the results show that amastigote interactions with mammalian cells promote the production of anti-inflammatory cytokines such as IL-10 and TGF-ß while suppressing the production of IL-12, superoxide and nitric oxide. An underlying issue that is considered is how these parasites that reside in sequestered vacuolar compartments target host cell processes in the cytosol or the nucleus; does this occur through the release of parasite molecules from parasitophorous vacuoles or by engaging and sustaining signaling pathways throughout the course of infection?

Keywords

Protozoan; *Leishmania*; Macrophages; Dendritic cells; Amastigote; Superoxide; Nitric oxide; IL-12

1. Introduction

Leishmania parasites are dimorphic organisms that live and replicate in the gut of sandflies as flagellated forms (promastigote) or as aflagellated forms (amastigotes) in mammalian cells. In the mammalian host these parasites preferentially infect phagocytic cells, primarily macrophages and dendritic cells. It is, however, well documented that within lesions and in the skin, other cell types including neutrophils and fibroblast cells have also been shown to be infected (Bogdan et al., 2000a;Laskay et al., 2003). Several factors determine the outcome of the interactions between mammalian cells and *Leishmania* parasites. One of these factors is the *Leishmania* species. A startling example of an outcome that is species-dependent is the development of morphologically distinct parasitophorous vacuoles (PVs). Whereas parasites of the *Leishmania mexicana* complex (*L. mexicana*, *Leishmania amazonensis and Leishmania pifanoi*) reside in communal PVs that become increasingly distended, parasites of the *Leishmania donovani* complex (*L. donovani*, *Leishmania infantum* and *Leishmania chagasi*) for the most part reside in tight individual PVs from where daughter cells segregate into their

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own PVs. Such differences in the outcome of the interactions between parasites and macrophages is a powerful reminder that *Leishmania* spp. are diverse organisms with significant differences at the genetic level, which result in differences at multiple points in their interactions with hosts cells. The reader is encouraged to consult a recent review by McMahon and Alexander (2004) for a more extensive discussion of the diversity of *Leishmania* and the host immune response to these organisms.

Both the promastigote and the amastigote forms of *Leishmania* can initiate infections. When infections are initiated with promastigotes, these parasites transform within PVs into amastigotes over a period of 24 to 72 h. Thereafter, in infected cells and infected hosts, infections are sustained by amastigote forms. Amastigotes can persist in cells for many days if not weeks. There are differences in the host cell response following interactions with either promastigote or amastigote forms. For example, studies with several *Leishmania* species have shown that whereas macrophages produce superoxide in response to infection with promastigotes, much lower levels of superoxide are produced in response to infection with amastigotes (Pearson et al., 1983;Channon et al., 1984;Pham et al., 2005). Such differences in the host response are to be expected since these parasite forms express stage-specific molecules that can serve as virulence factors. A notable example of a stage specific virulence factor is the lipophosphoglycan (LPG) molecule which is expressed on the surface of the promastigote form, but is minimally expressed on the amastigote form (Igoutz and McConville, 2001). LPG, especially the variant expressed by *L. donovani* parasites, has been shown to exert a dominant effect on numerous processes in the host cell in infections initiated with promastigotes (Descoteaux and Turco, 2002). The majority of studies that have investigated the host cell response to *Leishmania* have evaluated early macrophage responses initiated by promastigotes. Some reports, though fewer in number, have assessed infections that were initiated with amastigote forms. Since these two parasite forms have been shown to elicit differing host cell response, this review will focus primarily on host cell events modulated by amastigote forms of *Leishmania*. Wherever possible, the most recent studies that delve into the mechanisms that determine the host response to infection with amastigotes will be considered. The reader is encouraged to consult several excellent recent reviews in which infections initiated with promastigote forms were considered (Basu and Ray, 2005;Gregory and Olivier, 2005;Olivier et al, 2005).

2. Parasite entry mechanisms

The signaling pathways that mediate the host cell responses activated by infection with amastigotes are undoubtedly dependent on the internalization receptor(s) engaged by *Leishmania* amastigotes. There is general agreement that amastigotes gain entry into cells by phagocytosis, however it is known that amastigotes can infect non-phagocytic cells as well. Although the mechanism of entry of these parasites into non-phagocytic cells is poorly understood, the implication is that these parasites can exploit multiple mechanisms to gain entry into cells. For infection of phagocytic cells, several studies have shown that internalization of *Leishmania* amastigotes, especially in the in vivo setting, is mediated by Fc and complement receptors (Guy and Belosevic, 1993;Peters et al., 1995;Love et al., 1998;Kima et al., 2000). Phagocytosis mediated by Fc and complement receptors has been studied extensively and many of the signaling intermediates that are recruited upon the engagement of each of these receptors are known (Underhill and Ozinsky, 2002). Mosser and colleagues have shown that, similar to the case with other opsonized particles, internalization of amastigotes through the Fc receptor results in macrophage release of anti-inflammatory cytokines such as IL-10 (Kane and Mosser, 2001;Miles et al., 2005). Given the detailed understanding of Fc receptor signaling, the finding by Lodge and Descoteaux (2007) that even in the absence of antibody opsonization, *L. donovani* amastigotes were internalized through a Rac1-dependent mechanism, was a surprise. Whereas Rac1 has been shown to be required for Fc receptor

mediated phagocytosis, many reports have shown that it is not activated during phagocytosis mediated by CR3 (Caron and Hall, 1998;Underhill and Ozinsky, 2002). Based on that knowledge, a requirement for Rac1 in the entry of non-opsonized amastigotes into macrophages would therefore suggest that even when amastigotes are internalized by phagocytic cells, these parasites appear to engage unconventional interactions that might condition the early host cell response to infection.

Interestingly, Morehead et al. (2003) had earlier found that internalization of *L. amazonensis* amastigotes into Chinese hamster ovary cells was Rac1-dependent only when parasites were opsonized with antibodies and entry was Fc receptor-mediated. Otherwise, under non-opsonic conditions parasite entry was Rac1-independent. These are contradictory observations that can be explained by the fact that different cell types and *Leishmania* species were used in both studies. Nonetheless, these results might be an indication that the uptake of amastigotes involves complex interactions that can yield unexpected outcomes. One cautionary note to bear in mind while evaluating such studies comes from a recent study that showed that contrary to the previous studies that had found that Rac1 was only involved in Fc receptor-mediated entry, other receptors, notably CR3, can also engage Rac1 (Hall et al., 2006).

Another molecule that has been implicated in the internalization of *Leishmania* amastigotes is phosphatidylserine (PS). Wanderley et al. (2006) and de Freitas Balanco et al. (2001) showed that *L. amazonensis* amastigotes obtained from infected tissue display PS on their surface. Acquisition and display of PS is described as apoptotic mimicry by these parasites. When *Leishmania* parasites were internalized via the recently described PS receptors on macrophages (Fadock et al., 2000), the macrophages were induced to secrete IL-10 and TGFβ (Fig. 1). One of the intriguing aspects of that observation was that cytokine production by macrophages was dependent on the density of PS on parasites. PS density on parasites was in turn dependent on ill-defined characteristics of parasite lesions from where the tissue-derived parasites were obtained. This would suggest a scenario in which parasites in a host exhibit varying surface characteristics, which would in turn result in the elicitation of varied host cell responses.

A novel internalization strategy not previously implicated in the internalization of *Leishmania* parasites was recently described by Rodriguez et al. (2006). They found that the promastigote forms of *L. chagasi* can be internalized via a caveolin-mediated mechanism. Caveolin-mediated entry involves the participation of lipid rafts enriched with cholesterol that direct particles into intracellular compartment that may sometimes exhibit delayed interactions with lysosomes (Pelkmans et al., 2001). It is interesting that Lodge and Descoteaux (2006), using *L. donovani* amastigotes, found that infection perturbed lipid rafts. Although this would seem contradictory to the involvement of lipid rafts in the *L. chagasi* study, it nonetheless suggests some interaction of amastigotes with lipid raft structures that might ultimately affect intracellular signaling.

Dendritic cells are another important host cell for *Leishmania* parasites. They have been implicated in the dissemination of *Leishmania* parasites (Moll et al., 1993). The dendritic cell designation actually includes cells of different origin including lymphoid, myeloid and plasmacytoid (Shortman and Liu, 2002). Generation of dendritic cells from mouse bone marrow cells or human peripheral blood usually results in the expansion of dendritic cells of myeloid origin. These cells differ phenotypically and functionally from skin dendritic cells, which include Langerhans cells and dermal dendritic cells (Shortman and Liu, 2002). *Leishmania* amastigote entry into dendritic cells, which occurs without induction of maturation, has been characterized as ‘silent entry’ (Bennett et al., 2001;Brandonisio et al., 2004). Although amastigote entry does not induce dendritic cell maturation, infected dendritic cells can be induced to undergo maturation in response to exogenous stimuli (Prina et al., 2004). This would suggest that the parasite’s effects on the cell are mostly localized.

The silent entry of amastigotes into dendritic cells might depend on the receptor engaged by these parasite forms. Colmenares et al. (2002) identified the C-type lectin, ICAM-3-grabbing nonintegrin (DC-SIGN) as the putative receptor on dendritic cells for *L. pifanoi* and *L. infantum* amastigotes. These molecules are parasite species-restricted since another C-type lectin; L-SIGN was shown to be a receptor for *L. infantum* and not *L. pifanoi* (Colmenares et al., 2004). Internalization of particles by these C-type lectins does not result in activation of dendritic cells. Apparently, amastigotes avoid the activation of dendritic cells, not by elaborating virulence factors that deactivate host cell processes, but by selective interaction with a surface receptor that does not result in cell activation. Uptake of antibody opsonized amastigotes resulted in the induction of dendritic cell maturation (Prina et al, 2004).

3. Modulation of signaling in response to infection

Beyond the parasite's interactions at the host cell membrane, it engages signaling pathways that help condition the host cell's responses. Studies that have assessed the early response of macrophages to amastigote infection by assessing global changes in phosphorylation have shown that amastigote infection deactivates or silences the macrophage. However, studies that have focused on the parasite's modulation of specific signaling pathways have yielded mixed and somewhat contradictory results. In light of observations from other systems that had shown that changes in tyrosine phosphorylation regulates several biological processes in cells, studies aimed at evaluating the effect of *Leishmania* infection on macrophage signaling assessed global changes in tyrosine phosphorylation after infection with *Leishmania* amastigotes. Martiny et al. (1999) showed that infection with *L. amazonensis* amastigotes resulted in reduced levels of tyrosine phosphorylation of several proteins tracked by Western blotting. This was in contrast to a previous study by Martiny et al. (1996) that had shown that infection with the promastigote form of *L. amazonensis* induced the appearance of tyrosine phosphorylated proteins. Another study that used *L. amazonensis* amastigotes as well, found that the amount and the pattern of protein tyrosine phosphorylation observed during amastigote uptake by macrophages was reduced compared with that observed during IgG-erythrocyte phagocytosis (Love et al., 1998). Furthermore, a comparable reduction in overall tyrosine phosphorylation was not found during the uptake of heat-killed amastigotes, implying that the actions of live amastigotes must have led to the widespread dephosphorylation.

Although it was not determined what mechanisms were engaged by *L. amazonensis* amastigotes that resulted in the widespread tyrosine dephosphorylation, several recent studies have shown that *Leishmania* infection induces the activation of phosphotyrosine phosphatases of which the Src homology 2 domain containing tyrosine phosphatase (SHP-1) has been the most extensively studied (Forget et al., 2001). Although most of the in vitro studies that have assessed the role of SHP-1 were initiated with promastigote forms of *L. major* and *L. donovani*, in vivo studies where amastigotes are exclusively found, have established a prominent role for these phosphatases in the conditioning of the host cell response by infecting parasites. Since the involvement of SHP-1 during parasite infection has been more fully addressed in the context of parasite suppression of nitric oxide release in response to IFNγ, it will be considered below.

A few studies have investigated the effect of *Leishmania* amastigote infection on mitogen activated protein (MAP) kinases. MAP kinases are important signal transduction intermediates that are activated in response to stress and other stimuli, which then mediate several cellular processes, including cell division, differentiation, cell survival/apoptosis, gene expression, motility and metabolism. Since it is likely that infection alters some of these processes, it is instructive to assess the functional state of MAP kinases in infected cells. One of the molecules that were dephosphorylated by *L. amazonensis* amastigote infection in the study by Martiny et al. (1999) was the extracellular signal regulated protein kinase 1 (ERK1). The authors showed

that there was a steady decrease in ERK as the infection progressed. Unfortunately, it was not determined whether the loss in ERK phosphorylation was the result of selective degradation of this molecule or the action of a phosphatase activated by infection.

A recent study by Cameron et al. (2004) also found that in *L. mexicana* amastigote-infected cells activated with lipopolysaccharide (LPS) there was selective degradation of the MAP kinases JNK and ERK. By using mutant parasites in which the cysteine peptidase b gene was deleted (Δcpb) as well as inhibitors to cysteine peptidases, they proposed that parasite cysteine peptidases mediated the degradation of these MAP kinases. That study did not specifically address the phosphorylation status of the MAP kinases. In a more recent study, Yang et al. (2007) reported that infection with *L. amazonensis* amastigotes results in the activation of the MAP kinase ERK1/2. These authors showed that activation of ERK1/2 was required for the parasite-induced secretion of IL-10 by the infected macrophage. The observation in this later study that infection with *L. amazonensis* amastigotes results in ERK1/2 activation obviously contradicts the results of Martiny et al. (1999) and somewhat the study of Cameron et al. (2004) where infections with *L. amazonensis* and *L. mexicana* amastigotes were assessed, respectively. Such discrepancies are difficult to reconcile, although they might reflect the fact macrophages from different anatomical sites (peritoneal cavity [Martiny et al. study] versus bone marrow [Yang et al. study]) might respond differently. It is also likely that the conditions of parasite culture might influence the outcome of experimental infections. Nonetheless, it does appear that infection with amastigotes modulates MAP kinase signaling that might result in suppression of pro-inflammatory signals while promoting the secretion of IL-10. Hopefully future studies will help clarify which other biological processes are affected by the parasite's effects on MAP kinases.

4. Suppression of superoxide production

Phagocytic uptake of particles mediated by some opsonic and non-opsonic receptors results in superoxide production (Underhill and Ozinsky, 2002). However, several studies have shown that unlike the situation with promastigote forms, macrophage uptake of amastigote forms of several *Leishmania* species does not activate superoxide production (Pearson et al., 1983;Channon et al., 1984;Pham et al., 2005). Furthermore, amastigote infection can suppress superoxide production elicited by otherwise potent activators of superoxide production (Pham et al., 2005). Superoxide is the product of the multisubunit nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex. This complex contains the membrane-bound cytochrome b558, which is composed of at least two polypeptides ($gp91^{phox}$ and $p22^{phox}$) and two non-identical heme groups that are associated with $gp91^{phox}$ (Nauseef, 2004). The $gp91^{phox}$ subunit is synthesized as a 58-kDa polypeptide and, after limited glycosylation in the endoplasmic reticulum, becomes a 65-kDa molecule. Thereafter, it traffics through the trans-Golgi network, where it is additionally glycosylated, acquires heme and emerges as a molecule of 91 kDa (Yu et al., 1999;DeLeo et al., 2000;Nauseef, 2004). This processing or maturation of $gp91^{phox}$ increases its affinity for the other membrane resident subunit, $p22^{phox}$. Four additional components of the NADPH oxidase enzyme, p40, p47, p67 and Rac2 are mostly found in the cytosol and associate with the membrane-bound components upon activation (Nauseef, 2004). Assembly of this enzyme complex on the target membrane is essential for the local release of optimal amounts of superoxide. Several intracellular pathogens have evolved strategies that target distinct points in the assembly of the NADPH oxidase complex (Banarjee et al., 2000; Gallois et al., 2001). The absence of superoxide production elicited by infection could be either an example of evasion or an example of parasite-induced suppression. A couple of reports have considered the molecular mechanism(s) that *Leishmania* deploy to suppress superoxide production. In studies with *L. pifanoi* amastigotes, Pham et al. (2005) showed that assembly of the functional NADPH oxidase enzyme did not occur on PVs that harbored amastigote forms, which was in contrast to vacuoles that harbored promastigote

forms. Western blot analysis of gp91^{phox} on PV-enriched fractions isolated from infected cells suggested that only the immature form of gp91^{phox}, p65, which forms an unstable association with p22^{phox}, was present on PV membranes. This finding was reminiscent of observations with succinyl acetone that had shown that when this pharmacological agent was added to cells, it caused a block in gp91^{phox} maturation by increasing heme oxygenase I (HO-1), the rate limiting enzyme in heme degradation (Taille et al., 2004;Otterbein et al., 2000). Pham et al. (2005) then considered whether infection with *Leishmania* amastigotes might similarly induce HO-1. Indeed, HO-1 is induced by *Leishmania* infection. Furthermore, pharmacological compounds that inhibit heme degradation and HO-1 activity reversed the suppression of superoxide release by *Leishmania* amastigote-infected cells.

Lodge and Descoteaux (2006) also found that infection with *L. donovani* amastigotes do not trigger superoxide production by macrophages. However, they proposed that the inability of the NADPH oxidase enzyme complex to assemble on the PV was the result of defective phosphorylation of one of the cytoplasmic subunits, p47. Apparently, infection of macrophages with *L. donovani* amastigote inhibits protein kinase c (PKC) activity, which has been shown to be required for the phosphorylation of p47 (Fontanye et al., 2002). Amastigotes, like promastigotes, have been shown to inhibit PKC activity (Turco, 1999). However, unlike promastigotes where LPG has been shown to mediate the interactions with PKC, no specific molecules on amastigotes have been identified that function similarly in targeting PKC. Future studies will hopefully identify the parasite molecules on amastigotes that target PKC. The different mechanisms proposed for how *Leishmania* amastigotes suppress superoxide activation might be another example of the different outcomes from the interaction of different *Leishmania* species with macrophages. Nonetheless, these studies show that the absence of superoxide production during amastigote infection of macrophages is the result of parasite-induced suppression, via mechanisms that take advantage of existent host cell processes. The positive aspect of these findings is that parasite-induced suppression of superoxide production can be reversed with appropriate pharmacological agents.

5. Suppression of nitric oxide production

Although there is compelling evidence from murine models of leishmaniasis that nitric oxide (NO) plays a significant role in limiting experimental *Leishmania* infections (Murray and Nathan, 1999;Bogdan et al., 2000b), a few reports have shown that *Leishmania* infection of macrophages can block NO production. NO in macrophages is the product of the inducible NO synthetase gene (iNOS), which is induced by IFNγ. As discussed earlier, Wanderley et al., (2006) showed that *L. amazonensis* amastigotes obtained from infected tissue display PS. When these parasites are internalized via PS receptors on macrophages, the macrophages are induced to secrete IL-10 and TGFβ, which in turn block the induction of iNOS, therefore inhibiting the production of NO. It is quite likely that this strategy for parasite uptake can contribute significantly to the persistence of the infection.

Several studies have shown that *Leishmania*-infected macrophages are unresponsive to IFNγ induction of NO production. Binding of IFN-γ to its receptor on the macrophage surface, results in the dimerization of α and ß receptor units and their phosphorylation. This then initiates a signaling cascade that involves the phosphorylation of the receptor kinases, Janus kinases (Jak) 1 and 2, and beyond that the signal transducer and activator of transcription (Stat) 1, which translocates into the nucleus to activate transcription of some IFNγ-responsive genes (Blanchette et al., 2003). Both promastigote and amastigote forms of *Leishmania* have been shown to selectively inhibit IFNγ activation of Jak 1 and 2 as well as Stat1 (Nandan and Reiner, 1995;Blanchette et al., 1999). *Leishmania* parasites apparently target multiple points in the IFNγ signaling cascade. A few reports have shown that infection results in expression of fewer IFNγ receptors on the infected cell surface and reduced phosphorylation of the remaining

receptors (Fig. 1) (Nandan and Reiner, 1995;Ray et al., 2000). Using mice genetically altered to lack SHP-1, Olivier and colleagues have shown that activation of this phosphatase during infection blocks Jak2 signaling (Blanchette et al., 1999;Forget et al., 2001). More recently, Forget et al. (2005) showed that beyond the effects of *Leishmania* infection on the phosphorylation status of signaling intermediates in the IFNγ signaling cascade, infection with these parasites also resulted in proteosome-mediated degradation of Stat1. The authors reported that most of the *Leishmania* species tested (*L. major*, *L. donovani* and *L. mexicana*) exhibited this capacity to induce the degradation of Stat1. Since these parasites reside in PVs with different characteristics, it is uncertain which signals are transmitted from PVs or which parasite proteins access the cytosol to mediate the activities described above. Studies on a parasite-derived protein that has been shown to bind to SHP-1 are discussed below.

There is some evidence that *Leishmania* infection of humans cells results in limited production of nitric oxide (Gantt et al. 2001). The issue of whether limited NO production is a result of parasite evasion or whether parasites actively suppress the production of NO is just as relevant here. In the hamster model of visceral leishmaniasis where the progression of disease is comparable to that observed in humans, it has been shown that there is also defective production of NO in response to *Leishmania* infection (Melby et al., 2001). However, recent studies (Perez et al., 2005) have shown that this defect might be an epigenetic phenomenon, which stems from the fact that expression of the iNOS gene appears to be uncoupled from other IFNγ responsive genes. Given the similarities in the iNOS gene organization in hamsters and humans, it was suggested that a similar epigenetic mechanism might explain the reduced NO production of human cells in response to *Leishmania* infection (Perez et al., 2005).

6. Inhibition of IL-12 production

A T_H1 response is required for control of leishmaniasis (Reiner and Locksley, 1995;Scott, 2003). The development of a T_H1 response is dependent on the presence of IL-12 at the initiation of the immune response (Scott and Trinchieri, 1997). However, given the critical role of IL-12, it is significant that many studies have found that infection of macrophages with either the amastigote or promastigote forms of *Leishmania* does not activate macrophages to produce IL-12 (Carrera et al., 1996;Weinheber et al., 1998; Belkaid et al., 1999). Rodriguez-Sosa et al. (2001) reported that susceptibility of most mouse strains to *L. mexicana* is due to the inability of infected cells to produce IL-12, rather than a defect in IL-12 responsiveness. Carrera et al. (1996) and Belkaid et al. (1999) showed that the suppression of IL-12 is selective since infection induced the production of most other cytokines that were assayed. Furthermore, not only do infected cells not produce IL-12 but *Leishmania* infection suppresses the production of IL-12 by infected macrophages in response to LPS, an otherwise potent activator of IL-12.

IL-12 is composed of two covalently linked glycosylated chains, p40 and p35, which form the biologically active p70 heterodimer (Scott and Trinchieri, 1997). The p35 gene is ubiquitously expressed by most cells whereas the p40 gene is primarily expressed by phagocytic cells, most efficiently in response to microbial agents and their products (Ma and Trinchieri, 2001). There are both positive and negative inducers of IL-12 (Ma and Trinchieri, 2001). Whereas IFNγ is a positive inducer of IL-12 production, phagocytic receptor co-ligation (Fc and complement receptors, for example), engagement of G protein-coupled receptors and IL-10 negatively regulate IL-12 production (D'Andrea et al., 1993;Marth and Kelsall, 1997; Wagoneer et al., 2005). Thus far, it does not appear that *Leishmania* suppression of IL-12 production is mediated by IL-10 or G protein-coupled receptors (Carrera et al., 1996; Kima, unpublished data). Since both parasite forms inhibit IL-12 production, let us first consider the mechanisms that have been proposed for inhibition of IL-12 by promastigote forms. McDowell and Sacks (1999) proposed that given the inhibitory effects of *Leishmania* on stimuli that rely on Jak and Stat signaling, interference with this signaling pathway might be responsible for the inhibition of

IL-12 production. However, no studies have formally shown that IL-12 is produced in the absence of this pathway. Studies with *L. donovani* promastigotes had implicated the engagement of the MAP kinase ERK in the suppression of IL-12 production (Feng et al., 1999). This effect was shown to be mediated by the LPG expressed on the parasite surface. However, there are contradictory observations that have found that infection with *L. donovani* promastigote does not result in the activation of ERK (Prive and Descoteaux, 2000), thus calling into question the role of ERK in the suppression of IL-12 by these parasites. In studies with *L. amazonensis* parasites, we have found that although ERK and the other MAP kinases are activated by infection with the promastigote form, inhibition of the activation of these MAP kinases does not reverse suppression of IL-12 during infection (unpublished data). Instead, inhibition of signaling through phosphoinositol 3 kinase (PI3K) and the downstream kinase protein kinase B (PKB, Akt) relieves IL-12 suppression induced by infection. On this point, there is evidence from other systems that PI3K/Akt signaling suppresses the production of IL-12 (Martin et al., 2003). Activated Akt (phospho-Akt) can translocate into the nucleus where it interacts with targets that are ill defined (Medema et al., 2000).

It is not known whether infections with *Leishmania* amastigotes employ the same mechanism for the suppression of IL-12 as the promastigote form. LPG expressed on the promastigote surface, which had been implicated in the suppression of IL-12, is minimally expressed on amastigotes. Instead, amastigotes express free glycoinositolglycolipids, also called GIPLs, at high levels as lyso alkyl or alkyacyl-GPI anchored lipids, and other lipids acquired from the host (McConville and Blackwell, 1991). Some studies have speculated that these lipids might indeed mediate the ‘avoidance of activation of macrophages’ and possibly the suppression of IL-12 production in cells infected with *Leishmania* amastigotes (Zufferey et al., 2003;Naderer et al., 2004). However, it has been difficult to evaluate the role of these lipids in events within the infected cell. One limitation of the *Leishmania* system is that gene-targeted knockouts are performed in the promastigote form. Since the synthetic pathways utilized in the synthesis of these lipids are also important for the synthesis of GIPLs that are expressed in the promastigote stage, knockout parasites have been found to inefficiently transform into the infective metacyclic stage and are therefore not viable inside macrophages (Zhang et al., 2005). Recently, studies with parasites that were engineered not to express sphingolipids by deletion of the palmitoyl transferase gene ($spt^{-/-}$) (the first enzyme in the de novo SL biosynthesis pathway responsible for synthesis of the sphingo base) provided an opportunity to test the role of these lipids in amastigote survival in macrophages, including the suppression of IL-12 production and macrophage activation (Zhang et al., 2005). However, the authors found that the $spt^{-/-}$ knockout parasites were able to salvage the synthesis of inositol phosphoryl ceramides, which complicated the analysis of the role of this class of lipids in amastigotes. Nonetheless, they concluded that although these lipids might be required for survival in infected macrophages, they are not primarily responsible for the avoidance of activation of macrophages.

Studies by Cameron et al. (2004) using *L. mexicana* amastigotes implicated cysteine peptidase as the effectors of the inhibition of LPS induced IL-12 production by parasite infected cells. LPS induced production of IL-12 is mediated by NF-κB signaling (Murphy et al., 1995). The NF-κB family is composed of five members: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and c-Rel (Ghosh et al., 1998). Engagement of NF-κB signaling results in the translocation of NF-κB dimers into the nucleus where they bind DNA and turn on responsive genes. Cameron et al. proposed that parasite cysteine peptidases expressed within the infected cell partially degraded NF-κB subunits, which resulted in inefficient translocation of these dimers into the nucleus and abolition of their capacity to bind DNA (Fig. 1). The *Leishmania* amastigote strategy described by Cameron et al. for inhibiting NF-κB appears to be a novel. Other pathogens are also able to inhibit NF-κB signaling. For example, *Toxoplasma gondii* parasites

inhibit NF-κB by inhibiting the phosphorylation of p65/RelA, which blocks the translocation of NF-κB dimers into the nucleus (Shapira et al., 2005).

Another study showed that although *L. major* amastigotes induced the degradation of NF-κB subunits in phorbol myristate acetate (PMA)-activated human cells, the inhibitory effect of the parasite is selectively on the p50/p65 heterodimer (Guizani-Tabbane et al., 2004). The translocation of p50/c-Rel to the nucleus in PMA-treated cells infected with amastigotes was unabated. In addition, the authors found that parasite internalization was not required for inhibition p50/p65 translocation. The results of this study implied that parasites can engage an endogenous host cell signaling pathway that mediates the selective degradation of some NF-κB dimers while permitting nuclear translocation of dimers that are inhibitory (May and Ghosh, 1997). More studies are needed in this area.

7. How do *Leishmania* amastigotes alter processes in the host cell cytosol or nucleus many hours or days after parasite internalization?

Most studies that have assessed the host cell response to *Leishmania* infection have mainly evaluated events that occur within the first 24 h of infection. From the point of parasite contact with mammalian cells, parasites are sequestered from the host cell cytosol by a membrane barrier that undergoes a change in composition as the PV matures. It is from within these PVs that parasites engage signaling pathways and target host cell processes. As discussed above, some signaling pathways, which are selectively engaged by the choice of receptors at the initiation of infection, either convey neutral signals into the cell or engage signals that mediate the suppression of selective processes. One question that is still unresolved is how are signals that modulate host cell processes sustained once the parasite PV is formed? We have found that in infections with *L. amazonensis* promastigotes, which were shown to activate PI3K signaling (Ruhland et al., 2007) PVs display the lipid products of PI3K through at least 12 h p.i. The consequence of this is that PVs become sites for the activation of PKB/Akt, a downstream kinase in the PI3K signaling pathway (unpublished data). Although PI3K signaling plays a significant role in promoting infected cell survival at early times after infection, it apparently plays a minimal role in the survival of infections that are older than 24 h (Ruhland et al., 2007). This would imply that other pathways are engaged by amastigote forms that continue to promote cell survival. It is of interest to determine the identities of the signaling mechanisms that promote survival of older infected cells that harbor replicating parasites. Also, it would be of interest to determine whether other signaling pathways can be initiated from molecules expressed on PVs.

In addition to, or in place of, PVs being sites from where signaling is initiated, it is likely that parasite-derived molecules secreted into the PV can access the host cell cytosol and beyond that the nucleus, and target host cell processes therein. Thus far, the best example of a parasite molecule that can access the host cell cytosol is the parasite-derived elongation factor 1α (EF-1α) that localizes in the cytosol of 16-18 h-old *L. donovani* promastigote-infected cells (Nandan and Reiner, 2005). Amastigotes also secrete EF-1α. EF-1α has been shown to bind to and activate SHP-1, which in turn inactivates Jak2. There is only circumstantial evidence that other parasite-derived molecules traverse the PV membrane to gain access to the host cell cytosol. As discussed earlier, the cysteine peptidases have been implicated in the degradation of NFκB subunits and MAP kinases (Cameron et al., 2004). To be in position to perform these activities, these peptidases would have to traverse the PV membrane and access the host cell cytosol. Mottram el al. (2004) recently proposed a model by which these cysteine peptidases might access the cell cytosol. In their model, vesicles containing these peptidases bud off the PV and their contents are eventually released into the cell cytosol. It is not known whether the cargo of such vesicles would be selective. The A2 genes are amastigote-specific genes that are found in *L. donovani* but not *L. major* (Zhang and Matlashewski, 2001;Zhang et al., 2003).

Ectopic expression of A2 in *L. major* enhances the survival of the transgenic parasites in the spleen and confers to A2-expressing *L. major* the capacity to visceralize (Zhang and Matlashewski, 2001). How do the A2 proteins achieve this? Is their localization limited to the PV? Similar queries can be made of the rest of the amastigote-specific genes. Answers to questions such as these will greatly advance our understanding of the biology of *Leishmania* in infected cells

8. Concluding Remarks

Amastigote survival within infected cells is undoubtedly dependent on the outcome of the parasite's interaction with the host cell at multiple points. In the early stages of infection, these parasites must either avoid inappropriate activation of their host cell or elaborate mechanisms that inactivate anti-parasitic effectors at the time of infection. The internalization receptors engaged by the parasite during cell entry could obviously play a determining role here. In the in vivo setting, parasites have to reduce pro-inflammatory signals such as cytokines or chemokines emanating from the infected cell, which can promote anti-leishmanial responses in their vicinity. Alternatively, it would be prudent for the parasite to sequester parasite molecules from accessing antigen presentation pathways and therefore limit T cell recognition of the infected cell (not discussed). Beyond that, parasites must promote survival of the infected cell in a milieu that is rich with immunologic activity. Finally, parasites have to acquire nutrients from the host cell (not discussed). Our current understanding of the mechanisms that *Leishmania* parasites deploy to modulate these processes is incomplete. The evidence that is currently available suggests that *Leishmania* parasites selectively engage and exploit host cell processes from the point of parasite entry and promote the release of anti-inflammatory cytokines. It is not known how they sustain their effects on these processes from within PVs through the course of infection. There is, however, emerging evidence that parasite-derived molecules such as cysteine peptidases might exert functions beyond PVs in infected cells. Studies that demonstrate the existence of parasite molecules beyond PVs and that attempt to establish their function would contribute significantly to our understanding of the biology of *Leishmania* parasites in infected cells.

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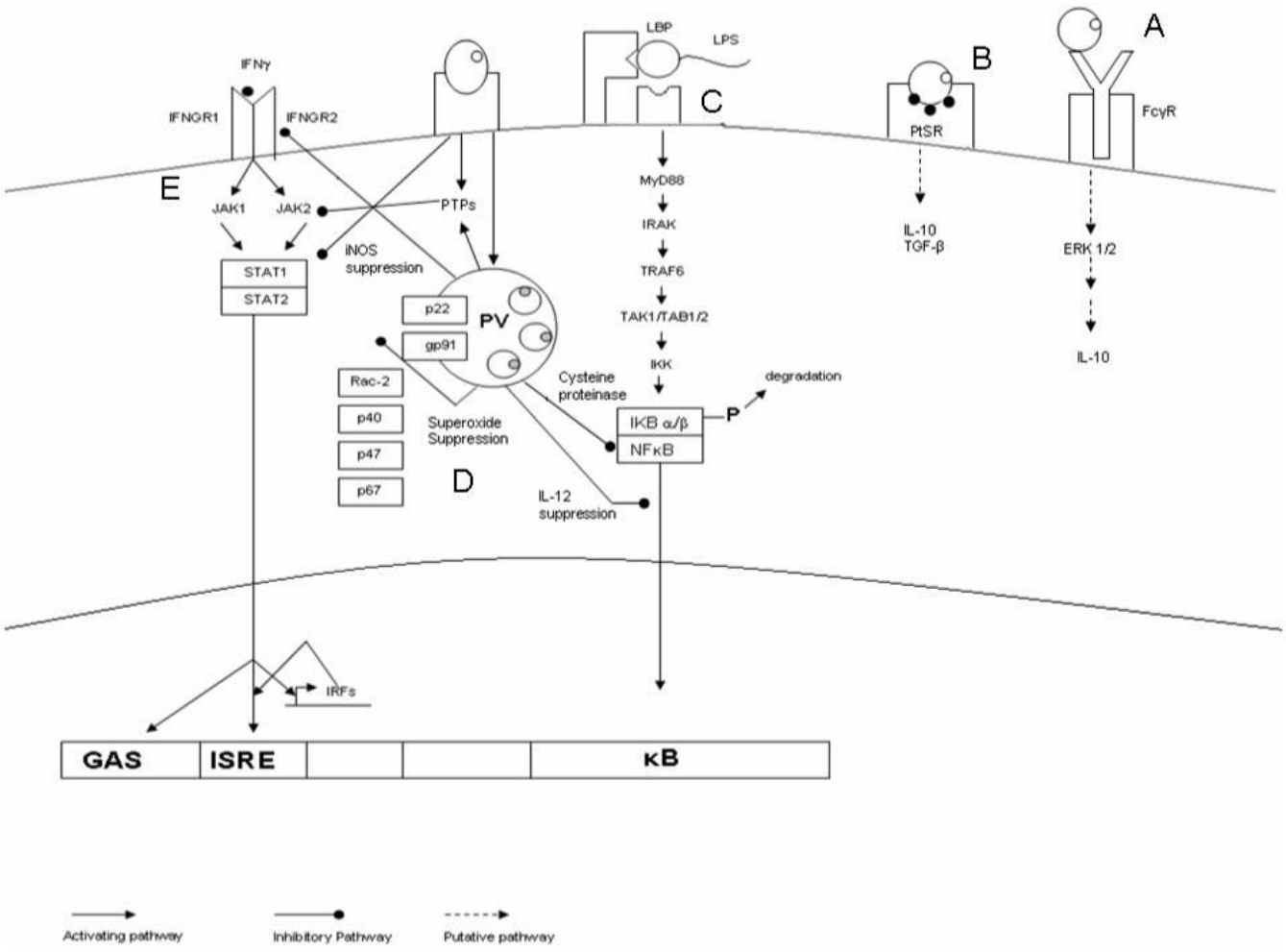

Fig 1.

Schematic diagram showing pathways and processes targeted or engaged by *Leishmania* amastigotes. Amastigotes in tissue are opsonized with antibodies or they acquire and display phosphatidylserine (PS). (A and B) These parasites can be phagocytosed via the Fc receptor (FcγR) or the PS receptor (PtSR), respectively. Engagement of both of these receptors leads to the production of anti-inflammatory cytokines. (C) *Leishmania* amastigotes can suppress lipopolysaccharide (LPS) induced IL-12 production. Either acting through cysteine peptidases or by yet undefined effectors, these parasites degrade NFκB and prevent the translocation of functional dimers into the nucleus, which blocks the secretion of IL-12 in response to LPS. (D) Amastigotes within parasitophorous vacuoles (PVs) inhibit the assembly of the multi-subunit nicotinamide adenine dinucleotide phosphate (NADPH) -oxidase on the PV membrane (PVM) and by so doing they suppress the production of superoxide. (E) *Leishmania* parasites block the response to IFNγ at multiple points, which results in the suppression of nitric oxide (NO) production: upon internalization and during the course of infection, they can induce the activation of phosphotyrosine phosphatases (PTPs) that target Jak2; infection can downregulate the expression of IFNγ receptors (IFNGR1 and IFNGR2); infection can induce the degradation of STAT1.