Update on *Leishmania* Mediators That Promote Immune Evasion

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Abstract Parasites of the *Leishmania* genus are the causative agents of the complex disease called leishmaniasis. Clinical manifestations in this disease range from cutaneous lesions, disseminated cutaneous lesions, mucocutaneous lesions, and visceral disease. Even though immune responses are elicited in response to infection, infection with several *Leishmania* species is not self-limited. The mechanism(s) by which parasites avoid the lethal effects of the immune response is the subject of intense research. This research includes studies on the identity and targets of the parasite mediators that are deployed to limit effectors of the immune response. This review will profile some of the parasite mediators for which there has been recent progress and the processes that they target.

Keywords *Leishmania* • Immune evasion • Tryparedoxin peroxidase • ROS • RNS • Type 1 IFN

Introduction

The survival of *Leishmania* in the mammalian host is dependent on the outcome of multiple host–parasite interactions throughout the duration of the infection. *Leishmania* lesions are a milieu that is rich in immunologic activity: chemokines produced by infected or bystander cells induce cell recruitment to that site; cytokines released there activate cells in the lesion. In light of the fact that *Leishmania* persist in lesions, it can be inferred that either the immune effectors in lesions are not sufficiently antiparasitic or parasites deploy many strategies to limit the effect of those responses.

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A. Satoskar and R. Durvasula (eds.), *Pathogenesis of Leishmaniasis: New Developments in Research*, DOI 10.1007/978-1-4614-9108-8_2,

Both anti-inflammatory and proinflammatory cytokines and chemokines are produced during *Leishmania* infections. There is active research aimed at determining how *Leishmania* parasites contribute to the development of those immune responses. Several recent reviews have considered the impact of elicitation of cross regulatory mediators on *Leishmania* infections (Nylén and Sacks 2007; Alexander and Brombacher 2012; Kima and Soong 2013). Going forward, it is of interest to determine whether elicitation of responses with counter-regulatory effects occurs fortuitously or whether it is a *Leishmania* strategy for survival.

There is convincing evidence that intracellular signaling pathways that transmit responses to cytokines or to stimuli that result in cytokine production are modulated by the infection. The reader is referred to several excellent reviews that have provided detailed summaries of those studies (Ben-Othman et al. 2009; Gregory and Olivier 2005; Gupta et al. 2013; Kima 2007). Examples of the signaling mediators that are modulated by infection include the MAP kinases, signal transducers and activators of transcription (STATs), and nuclear factor kappa b (NF-κB). The effect on the infection process is that infected cells are refractory to most immunologic effectors in their vicinity. Our understanding of the parasite mediators that target many of these host processes is still incomplete. However, several recent studies have provided valuable insight into parasite mediators that are elaborated during infection. The identity of those parasite molecules and their targets in the host will be the focus of this review. An issue that is raised, for which there is limited evidence, is the schemes by which molecules that are synthesized by *Leishmania* parasites within vacuolar compartments gain access to their host targets. For parasite molecules with targets in the infected cell cytosol, they have to be exported across the parasitophorous vacuole (PV) membrane; for parasite molecules whose targets are in the host cell nucleus, an additional membrane, the nuclear membrane, has to be overcome; some parasite molecules have targets outside the infected cell.

Parasite mediators that detoxify the respiratory burst. Macrophages are the primary hosts of *Leishmania* parasites. The respiratory burst in macrophages is composed of several oxygen and nitrogen reactive compounds. Generation of these compounds occurs in response to distinct stimuli. However, once the response is initiated, oxygen and nitrogen radicals can synergize to form more potent species. Reactive oxygen species (ROS) including superoxide radical anion and H_2O_2 are primarily produced by the NADPH oxidase (NOX) and dual-oxidase (DUOX) family of enzymes (Nauseef 2008). They are produced in response to innate stimuli such as phagocytosis. NOX-2 (out of five variants) is the most important in macrophages. This enzyme is composed of five subunits that assemble at the site of phagocytosis and subsequently on the limiting membrane of phagosomes that harbor internalized particles. In contrast to ROS, nitric oxide (NO), the initial reactive nitrogen species (RNS), is produced in response to immune stimuli, specifically cytokines. NO production in macrophages is the product of the inducible nitric oxide synthetase (iNOS) that acts on arginine in the presence of oxygen to catalyze the release of NO. NO can be subsequently converted to nitrogen oxide or peroxynitrite, which are potent oxidative species. ROS and RNS can cause oxidative

damage to biomolecules such as lipids, proteins, and DNA, which leads to loss of membrane integrity, defective replication, and eventually cell death.

Both the promastigote and amastigote forms of *Leishmania* are internalized by phagocytosis. It should be expected therefore that ROS is produced during parasite internalization. However, several studies with the amastigote forms of *Leishmania* have shown that uptake of amastigote forms is silent (Channon et al. 1984; Pham et al. 2005; Lodge and Descoteaux 2006). The situation with promastigote forms is more controversial as species differences in superoxide production have been reported (Lodge et al. 2006). These observations imply that *Leishmania* amastigotes elaborate a mechanism to prevent superoxide production during parasite uptake. Details on the proposed mechanisms by which *Leishmania* parasites limit superoxide production have been discussed in several reviews (Kima 2007; Moradin and Descoteaux 2012).

Thioredoxins and peroxiredoxins: These are members of a family of proteins that are characterized by their active site motifs, containing either one or two cysteinyl residues. These thiol groups are essential for (1) the reduction of protein disulfides (thioredoxins), (2) protein de-glutathionylation (glutaredoxins), or (3) the reduction of H_2O_2 (peroxiredoxins). Peroxiredoxins use a redox-active cysteine residue (peroxidatic Cys) to reduce a broad spectrum of substrates, including H_2O_2, organic hydroperoxides, and peroxynitrite ($ONOO^-$) (Hanschmann et al. 2013). Upon reduction of the peroxide, the peroxidatic Cys-SH is oxidized to sulfenic acid (Cys-SOH). Peroxiredoxins return to their reduced state upon reduction of the disulfide by an appropriate electron donor. *Leishmania* lack catalase and selenium-glutathione peroxidases. Instead they have a unique oxidoreductase of the thioredoxin superfamily, known as tryparedoxin (TXN). The peroxidoxins of *Leishmania* and other trypanosomatids are commonly referred to as tryparedoxin peroxidases (TXNPxs) (Flohé 2012). Two TXNPxs have been described in *Leishmania* that are localized in the parasite mitochondrion and cytosol (Castro et al. 2004; Jirata et al. 2006; Barr and Gedamu 2003). Overexpression studies and other approaches have revealed some substrate differences between these two variants; cTXNPx preferentially inactivates H_2O_2 and the organic hydroperoxide *t*-bOOH (Castro et al. 2010). In contrast, overexpression of *Li*mTXNPx in promastigotes did not ensure any significant resistance to exogenously added H_2O_2, but sheltered parasites when exposed to *t*-bOOH. The functions for the TXNPx molecules have thus far been linked to their localization within the parasite. Current views are that the mitochondrial variant detoxifies oxidative radicals that are produced primarily during electron transport (Castro et al. 2008). The cytosolic variant functions in response to parasite stress from changes in the parasite's environment such as temperature increase in the mammalian host in contrast to the sand fly gut.

In light of the fact that the mitochondrial and cytosolic TXNPxs are within the parasite, it would appear that the surface of the parasite is still vulnerable to the effects of oxygen radicals unless there are other molecules that are exported to the parasite surface or secreted from the parasite. The generation of superoxide in phagosomal compartments occurs at the limiting membrane from where oxygen

radicals diffuse. ROS diffuse poorly across membranes; translocation across membranes occurs through channels and transporters (Bienert et al. 2006). NO that is produced by the cytosolic enzyme, iNOS, reacts with oxygen to form peroxynitrite and other species that diffuse more readily across membranes (Denicola et al. 1998). Based on an inability to recover peroxidase activity in the supernatant fluid from infective promastigotes, Romao et al. (2009) concluded that it is unlikely that cTXNPx is secreted either from promastigotes or from amastigotes. In studies to identify parasite molecules that are exported from PVs, Kima and colleagues identified a novel TXNPx variant that is released from intracellular parasites and is exported from PVs in the host cell (Kima et al. 2010). Recently, a secreted peroxidase activity was described (Singh et al. 2013) and was proposed to target host molecules in the infected cell cytosol. Although the peroxidase activity of these secreted peroxidases has not been evaluated sufficiently, there is a strong likelihood that a parasite peroxidase is available to detoxify oxygen and nitrogen radicals within the PV and in the host cell.

In addition to their activity on oxygen radicals, peroxiredoxins have been shown to exert other functions that might be just as important in parasite pathogenesis. It was determined in studies where the mTXNPx gene (mtxnpx(−)) was deleted that loss of that peroxidase molecule resulted in reduced virulence (Castro et al. 2011). However, there was no difference in the capacity of the mtxnpx(−) parasites to resist oxidants that were generated throughout the parasite's life cycle or to which parasites were exposed. Interestingly, complementation with a peroxidase defective but otherwise identical mutant restored wild-type infectivity to the genetically ablated parasites. They deduced that the protein conferred thermotolerance to the parasite by stabilizing other critical parasite molecules; basically it acted as a chaperone. Besides their peroxidase function, peroxiredoxins have also been shown to modulate host immune responses partly through their interactions with signaling intermediates in the toll-like receptor 4 (TLR4) signaling pathway (Ishii et al. 2012). Could intermediates in the TLR4 pathway be targets of the secreted TXNPxs?

Other ROS/RNS deactivating molecules: There are other molecules that carry out functions to limit the oxidative burst. These include superoxide dismutases (SODs). SODs are metalloenzymes that play a role in parasite defense mechanisms by detoxifying H_2O_2 and O_2. Three types of SODs, Cu/ZnSOD, MnSOD, and FeSOD, have been identified based on their metal cofactor. Two classes of intracellular FeSODs have been identified in *Leishmania*: FeSODA and FeSODB1&2. Expression of FeSODB1 in contrast to FeSODB2 is low in the early logarithmic stage and increases toward the stationary and amastigote stages (Plewes et al. 2003). Evidence of the important roles of these molecules has come from gene deletion studies. Parasites in which a single allele of the FeSODB1 gene was genetically ablated failed to thrive within macrophages (Plewes et al. 2003). The primary location of FeSODB1 and FeSODB2 is the glycosomes. Based on that location it is unlikely that those molecules would be available to react directly with external O_2^- generated from macrophages. Either glycosomes play a significant role in parasite antioxidant defense or the current understanding of the participation of these

molecules in parasite protection is incomplete. FeSODA is localized to the mitochondrion. Overexpression studies showed that it protects parasites from miltefosine-induced oxidative-induced stress (Getachew and Gedamu 2012). Even though FeSODA is released into the parasite cytosol after prolonged exposure to miltefosine, it is not known whether it is released by intracellular parasites under physiological conditions.

Other molecules have been described that have an indirect effect on ROS detoxification. One of these molecules is the ferrous iron transporter LIT1. Expression of this molecule is controlled by iron availability, but its effect on parasite resistance to ROS is through its regulation of SOD activity. It was also suggested that through its effect on iron levels in the parasite, LIT1 expression serves as trigger for *Leishmania* differentiation from the promastigote to the amastigote form (Mittra et al. 2013). Another molecule that plays a role in ROS detoxification is subtilisin. Subtilisin is an unusual Clan SB, family S8 serine protease (Swenerton et al. 2010). In studies to determine the function of subtilisin, parasites that lacked the subtilisin genes were derived (SUB−/−). The authors found that in SUB−/− parasites the expression of TXNPxs was altered. Moreover, SUB−/− *Leishmania* were found to have increased sensitivity to hydroperoxides compared with wild-type parasites in vitro. It was unexplained why SUB−/− parasites were also not able to transform from the promastigote to the amastigote form.

Parasite molecules that block cytokine signaling: As discussed earlier, immune effectors are released into *Leishmania* lesions. Parasites persist in lesions in part because they modulate the response of infected cells to cytokines and other effectors. Just a handful of *Leishmania* molecules have been implicated in the targeting of signaling intermediates. Some of those *Leishmania* molecules that target intermediates in the signaling pathways of cytokines are profiled below.

Casein kinases target the IFNAR1 receptor. Type I IFNs signal via interaction with the heterodimeric receptor complex composed of two chains (IFNAR1 and IFNAR2). Ligand binding activates receptor-associated members of the JAK family of tyrosine kinases, Jak1 and Tyk2. These kinases phosphorylate and activate the STAT proteins, which increase transcription of the IFN-induced genes whose products exert antiviral, immunomodulatory, and antiproliferative effects. The IFNAR1 subunit of this receptor is essential for IFN-α/β signaling. The levels of IFNAR1 are mainly regulated by ubiquitin-dependent endocytosis and ensuing degradation of this chain and the entire type I IFN receptor. Casein kinase 1α (CK1α) is a major bona fide kinase of IFNAR1 that mediates basal phosphorylation, ubiquitination, and turnover of IFNAR1. Several studies implicated CK1α in ligand-independent degron phosphorylation and degradation of IFNAR1 stimulated by ER stress inducers, including VSV. A *Leishmania* orthologue of casein kinase 1 (L-CK1) was recently identified that is capable of increasing IFNAR1 degron phosphorylation in cells (Liu et al. 2009). Infection of either dendritic cells or macrophages with *L. major* modestly decreased IFNAR1 levels and attenuated cellular responses to IFN-α in vitro. Although it was not shown where L-CK1 encounters IFNAR1, it is likely to be outside the *Leishmania* PV. This implies that

this *Leishmania* molecule is exported from PVs by a mechanism that is yet to be described. Type 1 interferon exerts a regulatory effect on IFNγ. In leishmaniasis, type 1 interferon plays a complex role as shown by Soong and colleagues (Soong 2012). Expression of L-CK1 might exert a greater effect on the course of infection than is presently appreciated.

Leishmania molecules that target NF-κB: NF-κB is a family of transcription factors that regulates the expression of a large variety of genes. Most of those genes are involved in processes such as inflammatory and immune responses of the cell, cell growth, and development. NF-κB is activated by extracellular stimuli. There are five protein members of the NF-κB family: RelA (p65), c-Rel, RelB, p105 (NF-κB1), and p100 (NF-κB2). The latter two are produced as precursor proteins that are cleaved into a p50 and p52 subunits. RelA, RelB, and c-Rel contain a transactivation domain, which means that they can activate gene expression. p50 and p52 can bind DNA but lack transactivation domains. NF-κB proteins are bound to inhibitory IκB proteins that retain associated NF-κB molecules in the cytoplasm. There are three main members of the IκB family, IκBα, IκBβ, and IκBε. IκB are in turn regulated by members of the IκB-kinase (IKK) complex, which consists of two kinase subunits, IKK1 (IKKα) and IKK2 (IKKβ), and a regulatory protein, NEMO (IKKγ). Activation of NF-κB can be induced by proinflammatory cytokines and pathogenic components, through the so-called classical activation pathway. Here the IKK2 subunit of the IKK complex induces phosphorylation of IκB proteins, leading to their polyubiquitination and subsequent degradation by proteasomes. The release of NF-κB from inhibition results in their translocation to the nucleus, where they form dimers that bind to specific gene sequences. An alternate pathway of NF-κB activation occurs when IKK1 homodimers phosphorylate NF-κB2 (p100 precursor) and induces its processing to p52. *Leishmania* infection has been shown to inhibit NF-κB-mediated gene activation. At least two parasite molecules have been implicated. The cysteine proteinase, CPb, is a member of the Clan CA family C1 papain-like group of cysteine peptidases. It exists as multiple isoenzymes, which are encoded by a tandem array of similar *CPB* genes. The CPBs of *L. mexicana*, for example, are stage-regulated and the isoforms present differences in their substrate specificity and catalytic properties. Several of the NF-κB family members were shown to be targets of CPb. Specifically, p65 Rel A and c-Rel that are activated by exposure of cells to lipopolysaccharide (LPS), and IκBα and IκBβ were degraded extensively by LmCPb expressed by *Leishmania* amastigotes (Cameron et al. 2004). This effect on NF-κB resulted in the inability of infected cells to secrete IL-12 in response to LPS activation. These studies were confirmed with gene deletion studies of the LmCPb gene. Interestingly, although infection of macrophages with *Leishmania* promastigotes also inhibited IL-12 production in response to LPS, the pattern of degradation of NF-κB was different. Here, p65 Rel A was degraded to a 35 kDa fragment (Gregory et al. 2008; Abu-Dayyeh et al. 2010). The other *Leishmania* molecule that has been implicated in the degradation of NF-κB was shown to be *Leishmania* metalloprotease gp63. The participation of gp63 in NF-κB degradation as well as other processes was recently reviewed (Isnard et al. 2012).

Given that NF-κB is localized in the cytosol of host cells, a mechanism must exist for LCPb and gp63 to gain access to that compartment. Mottram and colleagues proposed that in infected cells, parasites secrete CPb into the PV lumen (Mottram et al. 2004). Then vesicles containing CPb and other proteins pinch off from the PV. The vesicles rupture in the cytosol, thus releasing parasite molecules that can target host molecules in the cytosol. No better model for parasite protein export from PVs has been proposed. Interestingly, a different mechanism has been proposed for how gp63 gains access to the host cell cytosol. GP63 is expressed most abundantly by promastigote forms. Olivier and colleagues showed that gp63 can be delivered to uninfected cells (Olivier et al. 2012). They suggested that through its interactions with macrophage surface molecules, gp63 can be internalized by a lipid rafts-mediated endocytic process. Once these parasite proteins access the cytosol they may target other host molecules. For example, LPb has been implicated in the degradation of the signal transducer and activator of transcription 1alpha (STAT-1α), and activating protein 1 (AP-1) (Abu-Dayyeh et al. 2010). Multiple other targets for gp63 have been described (Olivier et al. 2012). Going forward, it will be of interest to determine the details of these and other schemes by which parasites are exported from PVs. Furthermore, information of other molecules that are exported should shed light on processes that parasites target for survival.

Molecules that target Activated Protein-1 (*AP-1*). Several studies had shown that AP-1 transcription factor is inactivated by *Leishmania* infection. Activated Protein-1 (AP-1) is an important transcription factor that mediates gene regulation in response to physiological and pathological stimuli, including cytokines and growth factors. AP-1 is formed by homodimers of Jun family members (c-Jun, Jun B, and Jun D), or heterodimers of Jun and Fos family members (c-Fos, Fos B, Fra 1, and Fra 2). In studies to determine how *Leishmania* target the function of AP-1, Olivier and colleagues focused on the *Leishmania* surface-expressed metalloprotease GP63 (Contreras et al. 2010). They performed electro-mobility shift assays (EMSA) to assess DNA binding of AP-1 in the presence of lysates from cells infected with parasites that lacked GP63 or normal GP3 expression. AP-1 binding to DNA was impaired in the presence of GP63 expression. Moreover, incubation of lysates with GP63 resulted in degradation of c-Jun, Jun B, c-FOS, and AP-1 subunits. Intriguingly, parasite internalization was not required for GP63 to exert its effect on AP-1. Could bystander uninfected cells be susceptible to their AP-1s being targeted? Based on this observation, the authors deduced that GP63 that are GPI-anchored molecules and that can also be secreted by promastigotes can gain access into cells and traffick to the nucleus where they encounter AP-1. The details of the trafficking of GP63 to the nucleus are unclear. However, they showed a requirement for the presence of GPI-anchored molecules, which would suggest that endocytic vesicles that participate in their uptake might have special trafficking cues. The expectation should be that when GP63 are expressed by amastigote forms, albeit at much lower levels, those molecules will have to engage a different trafficking path to the nucleus.

Concluding remarks. *Leishmania* survival in its mammalian host is dependent on the outcome of numerous host–parasite interactions. These interactions might determine which cytokines or chemokines are released into *Leishmania* lesions. The presence of cytokines in the vicinity of *Leishmania*-infected cells could be of limited value if the parasites elaborate molecules that limit responsiveness to those cytokines. Only a small number of parasite mediators have been described that target host responses. As to be expected there are many outstanding questions about where and how those parasite mediators interact with their host targets to promote *Leishmania* pathogenesis. There are exciting new studies underway on export schemes of *Leishmania* molecules. Hopefully, those studies will shed light on the export of molecules profiled in this review and also the export and function of other molecules that are yet to be identified.

Acknowledgment Studies by P.E.K. were supported by funds from the UF foundation.

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