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Review

Secreted virulence factors and immune evasion in visceral leishmaniasis

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ABSTRACT

Evasion or subversion of host immune responses is a well-established paradigm in infection with visceralizing leishmania. In this review, we summarize current findings supporting a model in which leishmania target host regulatory molecules and pathways, such as the PTP SHP-1 and the PI3K/Akt signaling cascade, to prevent effective macrophage activation. Furthermore, we describe how virulence factors, secreted by leishmania, interfere with macrophage intracellular signaling. Finally, we discuss mechanisms of secretion and provide evidence that leishmania use a remarkably adept, exosome-based secretion mechanism to export and deliver effector molecules to host cells. In addition to representing a novel mechanism for trafficking of virulence factors across membranes, recent findings indicate that leishmania exosomes may have potential as vaccine candidates. *J. Leukoc. Biol.* 91: 887–899; 2012.

Introduction

Parasites of the genus *Leishmania* have coevolved with their mammalian and insect hosts for many thousands of years. It is thus not surprising that they became adept at subverting host immune responses to persist, replicate, and spread. *Leishmania donovani* together with *Leishmania infantum* and *Leishmania chagasi* are the causative agents of VL in humans and are transmitted by the bites of *Phlebotomine* sand flies. As soon as leishmania promastigotes are injected into the dermis of the human host, an intricate network of parasite-host interactions is initiated. This is made more complex by the fact that upon internalization by host macrophages or neutrophils, the parasites transform from the extracellular promastigote to the intracellular amastigote form.

It is becoming increasingly evident that mechanisms of disease pathogenesis and macrophage-leishmania interactions vary significantly, depending on the infecting species. VL has the highest morbidity and mortality of all leishmaniases, and there has been significant recent progress in research centered on VL and its causative agents. Hence, a review of recent advances in the understanding of VL pathogenesis is timely.

Although more than 100 years have passed since *L. donovani* was identified as a causative agent of VL [1], molecular mechanisms of disease have been focuses of intensive investigation, only during the past two to three decades. Despite this relatively brief interval, much has been learned about the disease's pathogenesis. A great deal of evidence supports a model in which leishmania escape host immune responses by actively suppressing critical macrophage activities.

Macrophages play key roles in the immune system's first line of defense, and their major functions are microbial killing and initiation of adaptive immune responses to invading microbes. To carry out these functions, macrophages must become activated. Upon ligation of their PRRs (or stimulation with IFN-γ secreted by NK or T_H1 cells), macrophages undergo activation and produce proinflammatory cytokines such as TNF, IL-1β, and IL-6, which in turn, leads to initiation of a local antimicrobial response [2]. In addition, macrophage activation triggers the production of ROIs and nitrogen intermediates, neutral proteases and lysosomal hydrolases, and the production of antimicrobial proteins and peptides [3, 4]. Ideally, ingested pathogens are internalized in phagosomes, which in turn, fuse with lysosomes, where the intruders are readily digested. Antigens derived from digested pathogens are then presented to T cells, initiating the adaptive immune response. In summary, intact functional properties of activated macrophages are critical for resistance to intracellular infections, as typified by leishmania.

Abbreviations: CR=complement receptor, EF-1α=elongation factor 1 α, Elk-1=E-26-like transcription factor 1, GP63=glycoprotein 63, HSP=heat shock protein, k/o=knockout, LPG=lipophosphoglycan, MoDC=monocyte-derived DC, mTOR=mammalian target of rapamycin, MVB=multivesicular body, PP2A=protein phosphatase 2A, PTP=protein tyrosine phosphatase, PV=parasitophorous vacuole, ROI=reactive oxygen intermediate, SAcP=secreted acid phosphatase, SHP=Src homology-2 domain-containing tyrosine phosphatase, SLA=soluble leishmania antigen, TCPTP=T cell protein tyrosine phosphatase, VL=visceral leishmaniasis

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Despite this arsenal of defense mechanisms, leishmania appear to survive quite well within macrophage phagolysosomes (a.k.a., PV). This implies that these microbes have developed strategies to efficiently evade or subvert macrophage microbicidal effector mechanisms. Inhibition of macrophage activities is brought about by a range of leishmania-derived virulence factors. Several leishmania cell surface molecules have been implicated as virulence factors. The best-studied example is LPG, a molecule that is highly abundant on the surface of *L. donovani* promastigotes. LPG has been shown to interfere with macrophage signaling through PKC and MAPK cascades [5–7] and generation of NO in response to IFN-γ [8]. Importantly, in addition to surface-exposed virulence factors, which may also be released by shedding [9], leishmania have been shown to actively secrete a great repertoire of molecules [10, 11]. This finding opens up a diverse array of possible interactions of parasite and host molecules, taking place at various interfaces. For example, promastigotes could secrete molecules that prime macrophages even before the organisms are ingested. Another possible scenario, which will be discussed extensively in this review, is that internalized amastigotes could secrete molecules that are transported across the PV membrane to interfere with signaling pathways in macrophage cytosol, thereby preventing macrophage activation. Recent technological advances have made it possible to globally analyze the secretome of leishmania [11]; however, very few of these molecules have been fully characterized. In addition, our research group has recently described a novel, exosome-based pathway for secretion and delivery of leishmania-derived molecules to host cells.

In this review, we have focused on characterized virulence factors secreted by leishmania and provide evidence for their roles in subverting host immune responses. Furthermore, we discuss exosome release as a major mechanism of secretion by leishmania and how the delivery of these virulence factor-loaded bullets to host cells represents an effective warfare strategy for these pathogens.

LEISHMANIA DISRUPT MACROPHAGE CELL SIGNALING

Effects on host kinases

In the past two decades, there have been numerous reports showing that infection with *L. donovani* or treatment with purified *L. donovani* molecules results in altered macrophage cell signaling. One of the first reports published in 1987 by McNeely and Turco [12] showed that LPG purified from *L. donovani* potently inhibited the activity of PKC, isolated from rat brain. Since then, it has been established that leishmania differentially regulate the activities of specific PKC isoforms in macrophages, affecting PKC-mediated intracellular signaling and downstream functions. In particular, infection with *L. donovani* inhibited the activity of Ca^{2+}-dependent PKC-β but enhanced Ca^{2+}-independent PKC-ζ activity [13]. Inhibition of PKC activity by *L. donovani* was shown to attenuate PKC-dependent oxidative burst and protein phosphorylation [5]. Moreover, LPG from *L. donovani* was shown to delay phagosome maturation by inhibiting PKC-α-dependent depolymerization of periphagosomal F-actin [14, 15], and infection with *Leishmania major* inhibited PKC-dependent expression of genes for c-fos and TNF-α [16]. Thus, modulation of PKC activity represents an important mechanism by which leishmania limits microbicidal activity.

The dsRNA-activated protein kinase (PKR) has been shown recently to become activated in response to *Leishmania amazonensis* infection, supporting parasite proliferation through up-regulation of IL-10 production [17]. The authors also linked *L. amazonensis*-induced PKR activity to expression of the ROS scavenger SOD1 [18], providing a possible mechanism for prevention of parasite killing.

Another major pathway, which can be manipulated by leishmania, is the IFN-γ-regulated JAK2/STAT1 signaling. We and others have shown that *L. donovani*-infected macrophages are defective in their ability to phosphorylate JAK1, JAK2, and STAT1 upon stimulation with IFN-γ [19, 20]. Disruption of the JAK2/STAT1 pathway has been shown to depend on activation of the host PTP SHP-1 [19]. Furthermore, nuclear translocation of the transcription factor STAT1α was compromised in *L. donovani*-infected macrophages [21]. Similarly, nuclear translocation of the transcription factors AP-1 and NF-κB in response to IFN-γ stimulation was attenuated in macrophages infected with *L. donovani* [22].

More recently, the PI3K/Akt signaling pathway has been implicated in leishmania pathogenesis [23, 24]. Importantly, PI3K has been implicated as a negative regulator of IL-12 production by macrophages [25–27], which is crucial to the balance between T_H1 and T_H2 responses [28, 29]. It is well established that promastigotes of various leishmania species fail to induce IL-12 production by macrophages in vitro and in vivo [30–32]. After infection with *L. major* metacyclic promastigotes, $p85^{(-/-)}$ mice on a *L. major*-sensitive BALB/c background showed a resistant phenotype and an enhanced parasite-specific T_H1 response [25]. This indicates that activity of host PI3K is needed for parasite survival. Ruhland et al. [23] found that infection of RAW264.7 macrophages with promastigotes of *L. major*, *Leishmania pifanoi*, or *L. amazonensis* resulted in a rapid and pronounced phosphorylation of Akt, which was blocked by the PI3K inhibitor LY294002. The authors also provided evidence that leishmania prevent the induction of caspase-3 and inhibit the proapoptotic molecule Bcl-2-associated death promoter through activation of the PI3K/Akt pathway [23]. Hence, activation of PI3K by leishmania is also important for host cell survival, which is needed for pathogen replication, persistence, and spread. Consistent with these findings, phosphatase and tensin homologue, a negative regulator of the PI3K pathway, was found to be essential for effective clearance of in vivo infection with *L. major* [33]. Furthermore, inhibition of the Akt substrate GSK-3β with LiCl resulted in increased susceptibility to *L. major* infection in $p85^{(-/-)}$ mice [34], underscoring the importance of GSK-3β as a downstream effector in leishmania pathogenesis. Importantly, it was reported recently that activation of the PI3K/Akt pathway by *L. amazonensis* promastigotes led to inhibition of macrophage IL-12 production [24]. Moreover, the regulation of the IL-10/IL-12 axis in leishmania infection has been linked

recently to the mTOR pathway [35]. However, in this study, the activation of mTOR was only partly dependent on PI3K, as inhibition with wortmannin was not sufficient to reverse the mTOR-mediated increase of IL-10 secretion by infected cells. Additional investigations will be needed to clarify the role of mTOR in leishmania-induced PI3K signaling.

Two recent, elegant studies have contributed further to establishing the importance of PI3K activation in leishmania pathogenesis. Using p110$\delta^{(-/-)}$ mice, Liu and coworkers [36, 37] showed that lack of this PI3K isoform resulted in robust resistance to infection with *L. major*, which was a result of impaired expansion of regulatory T cells. Our own results support a role for the PI3K/Akt pathway in susceptibility to VL. We found that infection of macrophages with *L. donovani* promastigotes or amastigotes induced activation of Akt, which was inhibited by wortmannin treatment [38]. In addition, our data indicate that induction by *L. donovani* of IL-10 production by myeloid cells is mediated through PI3K-dependent inactivation of GSK-3β, leading to reciprocal activation of the transcription factor CREB [38]. Taken together, these data firmly establish a role for the PI3K/Akt pathway in promoting leishmania pathogenesis. It remains to be elucidated, however, how leishmania targets this pathway.

MAPKs are also targeted by leishmania. For example, in naïve, murine bone marrow-derived macrophages, *L. donovani* prevented the activation of the MAPKs ERK1/2, p38, and JNK, as well as the degradation of IκB-α [39]. Consistent with this, activation of p38 MAPK was found to attenuate *L. donovani* infection in macrophages [40]. Inhibition of ERK1/2 kinases by *L. donovani* amastigotes was accompanied by inhibition of the transcription factor Elk-1 and c-*fos* mRNA expression [41]. It has been demonstrated that the dephosphorylation and inactivation of ERK1/2 MAPKs are results of the activation of host PTPs [22, 41].

Modulation of host phosphatase activities

The first evidence directly linking leishmania pathogenesis to manipulation of host PTP signaling, resulting in a deactivated macrophage phenotype, was presented by our group in 1999 [41]. We showed that infection of murine macrophages with *L. donovani* amastigotes resulted in attenuation of activation and signaling through MAPK, resulting in defective induction of c-FOS and iNOS. In the same study [41], we showed that leishmania infection brought about an increase in PTP activity, including the specific activity of SHP-1 toward MAPK. Moreover, inhibition of PTPs with sodium orthovanadate prior to infection prevented induction of the deactivated macrophage phenotype [41]. These findings were confirmed subsequently in another study, where macrophages infected with *L. donovani* promastigotes displayed an increase in SHP-1 activity, coinciding with a decrease in IFN-γ-induced JAK2 tyrosine phosphorylation and enhanced binding of JAK2 to SHP-1 [19]. Also, the PTP inhibitor peroxovanadium was shown to ameliorate murine visceral and cutaneous leishmaniasis in vitro and in vivo, consistent with a role for SHP-1 [42]. However, much of these findings were collected using largely nonspecific chemical phosphatase inhibitors, which may target not only host but also leishmania phosphatases, which may be important for virulence.

More direct evidence for a role for SHP-1 in leishmania pathogenesis was obtained using phagocytes and mice deficient in SHP-1 (motheaten viable). In these mice, increased resistance to cutaneous leishmania infection was observed, coupled with enhanced expression of iNOS and inflammatory responses [43]. The enhanced inflammatory response to *L. major* was characterized further by increases in proinflammatory cytokine secretion (TNF-α, IL-1β, IL-6), chemokine and chemokine receptor expression, and neutrophil recruitment [44]. However, a subsequent study by Spath et al. [45] questioned the validity of using footpad infection in the viable motheaten mouse model to investigate immune responses to leishmania. They argued that the severe background inflammatory response in these mice is a significant, confounding variable associated with increased footpad swelling and macrophage activation. In an attempt to circumvent this, Spath et al. used a model in which leishmania parasites were inoculated into the rump instead of the footpads, which had been used as sites for inoculation in many previous studies. In addition, they used parasite burden as a measure of infection rather than lesion pathology. Their results showed no difference in parasite survival between *L. major*-infected WT and SHP-1-deficient mice [45]. Moreover, their in vitro studies with peritoneal macrophages isolated from viable motheaten mice likewise showed no difference from controls, with the caveat that these macrophages display residual (5–10%) SHP-1 activity [45]. Taken together, these findings suggest that WT levels of SHP-1 activity are not a general requirement to support *L. major* infection of all macrophage types.

More recent investigations have indicated that activation of SHP-1 prevents IFN-γ-dependent NO production in *L. donovani*-infected macrophages, not only through inactivation of JAK2 but also through inactivation of ERK1/2 and prevention of nuclear translocation of NF-κB and AP-1 [22]. In SHP-1-deficient mice, ERK1/2, and SAPK/JNK (but not JAK2, MEK, or p38) were identified as the main kinases negatively regulated by SHP-1, as the absence of SHP-1 led to their selective activation [46]. Activation of ERK1/2 was linked to enhanced NO expression in response to IFN-γ in these mice, and this was dependent on the activation of the transcription factor AP-1, which was mainly responsible for the transcription of iNOS. This implies that SHP-1 plays a general role in negative regulation of signaling events leading to iNOS expression and NO generation [46] and provides a mechanism to explain how leishmania restricts the production of NO via activation of SHP-1.

Several other host PTPs have been shown to play a role in leishmania pathogenesis. PTP1B and the T cell PTP were shown to be activated and post-translationally modified in *Leishmania mexicana*-infected macrophages [47]. In addition, gene expression profiling revealed recently that *L. donovani* infection markedly up-regulated the expression of MAPK-directed phosphatases MKP1 and MKP3 and PP2A [48]. Up-regulation of MKP1 resulted in p38 deactivation, whereas increased MKP3 and PP2A deactivated ERK1/2, with possible effects on inhibition of iNOS expression [48]. In summary,

modulation of host kinase and phosphatase activities appears to play complex roles in promoting leishmania survival.

The obvious and critical question that follows from this is what are the mechanisms by which leishmania modulate host kinase and phosphatase activities. Bearing in mind, the fact that the vast majority of kinases and phosphatases is cytosolic, interactions with leishmania molecules, which are putative activators or inhibitors, likely take place in the cytosol of the infected cell. This implies that leishmania molecules somehow gain access to this subcellular locale. One obvious, possible mechanism is the secretion of molecules by extracellular promastigotes/amastigotes, followed by macrophage uptake or by amastigotes inside PVs, followed by trafficking across the PV membrane to enter the cytosol. Recent studies focused on secretion and trafficking of leishmania effectors in macrophages are beginning to provide answers to this puzzle, and these are reviewed below.

RELEASE AND DELIVERY OF EFFECTOR MOLECULES

Mechanisms of secretion in *L. donovani*

Numerous reports to date have highlighted the phenomenon of secretion of virulence factors by microbial pathogens as a hallmark of disease establishment and progression (e.g., [10, 47, 49, 50]). Molecules secreted by pathogens (bacterial, protozoan, fungal, or helminth) are present or active at the interface between pathogen and host cells, representing a highly potent means to regulate host responses and disrupt host cell functions. Most pathogen-secreted effector molecules characterized to date are proteins, although secretion of lipids has been reported [51, 52]. Mechanisms of secretion by prokaryotic pathogens have been characterized in considerable detail, but relatively little is known about secretion by eukaryotic organisms. In general, two major secretion mechanisms have been characterized in eukaryotes: classical or ER/Golgi-dependent secretion and nonclassical or ER/Golgi-independent secretion [53]. Notably, a special feature of secretion by leishmania and other trypanosomatids is the polarized delivery of secretory material to the flagellar pocket prior to release [54].

The classical eukaryotic secretion pathway is based on the presence of an N-terminal secretion signal peptide on the soluble secretory protein. This signal peptide directs the protein to the ER, where the signal peptide is cleaved from the mature protein. In the following, the protein is transported to the Golgi apparatus and ultimately, release into the extracellular space by fusion of Golgi-derived secretory vesicles with the plasma membrane. Several studies have been published demonstrating that the classical secretion pathway is operational in leishmania [55–57]. The majority of identified N-terminal secretion signal peptide-containing proteins in leishmania, however, includes surface proteins, for instance, members of the proteophosphoglycan family [57]. To what extent, if at all, the classical pathway contributes further to the leishmania secretome remains to be clarified.

A variety of mechanisms has been proposed to participate in nonclassical protein secretion by eukaryotes, including lysosomal secretion, plasma membrane shedding, release in exosomes, and secretion through plasma membrane transporters [58]. The hydrophilic acylated surface protein B of *L. major* was shown to use a nonclassical secretion pathway [59]. Of interest here, the secreted virulence factor EF-1α from *L. donovani* was found to lack a classical N-terminal secretion signal peptide [10]. A global proteomic analysis of the *L. donovani* secretome revealed that with few exceptions, secreted proteins lack a classical secretion signal peptide [11]. Additional study of these secreted proteins led to the discovery that leishmania use an exosome-based secretion mechanism to release and deliver effector molecules to host cells and modulate their phenotypes [60, 61]. These findings identified a novel and potentially highly powerful virulence strategy for eukaryotic pathogens. The role of exosomes as vehicles for leishmania-derived virulence factors is considered later in this review.

The leishmania secretome

Global analysis of pathogen secretomes has been facilitated greatly by recent advances in high-throughput mass-spectrometric technologies, and the secretomes of a variety of single-celled pathogens have been published (reviewed in ref. [62]). Earlier studies on secreted proteins were limited to characterization of proteins with enzymatic activity present in parasite culture supernatant, proteins that displayed strong antigenic properties, or proteins that could be predicted to be secreted based on the presence of a classical N-terminal secretion signal peptide. Using these approaches, several candidate virulence factors secreted by leishmania were identified including secretory chitinase [63], SAcP [64], EF-1α [10], and silent information regulator 2 [65], among others. Given the potential importance of secretion of effector molecules by leishmania, ideally, the objective should be to identify the secretome to the greatest extent possible to fully comprehend functionality of this complex system.

The first in-depth proteomic analysis of the leishmania secretome was performed by Silverman et al. [11]. In this study, the authors used a stable isotope labeling by amino acids in culture-based approach to identify 358 proteins in *L. donovani* promastigotes. Of these, at least 151 were considered to be bona fide secreted. Surprisingly, bioinformatic analyses revealed that only two of the 151 proteins contained a classical N-terminal secretion signal peptide, suggesting that leishmania mainly uses nonclassical mechanisms for protein export. In a recent study published by Cuervo et al. [66], the released/secreted proteins of *Leishmania viannia braziliensis* promastigotes were analyzed using a combination of two-dimensional gel electrophoresis and MALDI-TOF/TOF mass spectrometry. The authors were able to identify 42 secreted proteins, with similar general functions as described in ref. [11]. Similar to Silverman et al. [11], Cuervo and coworkers [66] found that only two proteins in their exoproteome were predicted to be secreted through the classical pathway. In another recently published study, *L. infantum chagasi*-excreted/secreted proteins were predicted using a genome-based approach [67]. This study was based on screening the annotated *L. infantum* genome for genes, whose predicted protein products have an N-terminal secretion signal peptide and lack transmembrane

domains and membrane anchors. The authors identified 181 proteins, which were predicted to be secreted according to their parameters, and experimentally validated the secretion of five of these proteins by promastigotes and amastigotes. Likewise, a genome-based method for the identification of conserved, secreted proteins in trypanosomatids was published recently, revealing 45 predicted, secreted proteins [68]. The inclusion criteria used here were the presence of a classical N-terminal secretion signal peptide, the presence of a peptidase cleavage site, and conservation of candidates in closely related trypanosomatids. Taken together, these findings indicate that the classical secretion pathway is operational in leishmania. However, as it is clear from several other studies [10, 11, 66], most protein secretion by leishmania occurs via nonclassical pathways and focusing exclusively on classically secreted proteins, can only provide a very limited picture of the global secretome.

Given the plethora of candidate virulence factors that have been identified in the secretome, it will be a challenging task to characterize their properties and functions and establish their roles in leishmania pathogenesis. Moreover, a large number of hypothetical proteins are awaiting detailed functional analysis, and the dynamics of the secretome throughout the different leishmania life-cycle stages and the operational secretory mechanisms will need to be addressed. Study of secreted virulence factors is of high importance, as extracellular factors represent a source of antigens for development of vaccines, and proteins involved in secretory pathways are potential targets for drug development.

Secreted virulence factors interfere with macrophage signaling

As discussed above and summarized in **Fig. 1**, targeting of host signaling pathways contributes to the deactivated phenotype

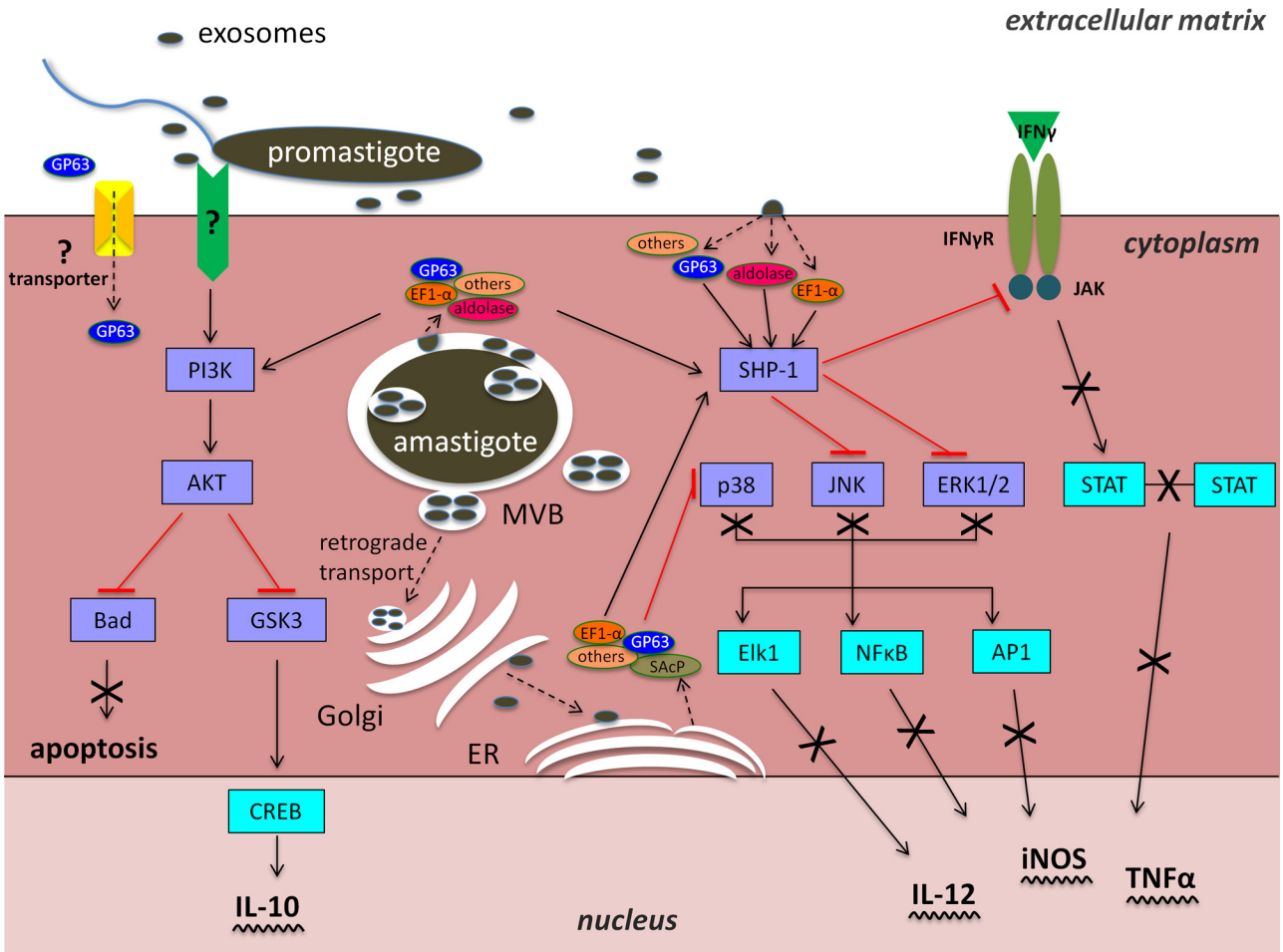

Figure 1. Leishmania-secreted effector molecules target and disrupt macrophage intracellular signaling. Leishmania promastigotes enter macrophages through facilitated phagocytosis and activate MAPK and PI3K signaling cascades. Molecules released by leishmania, extracellulary or intracellularly, may access macrophage cytoplasm through membrane transporters. Exosomal cargo proteins may be released into the cytoplasm by formation of multivesicular bodies, followed by retrograde transport through Golgi and ER or by fusion of the exosomal membrane with the plasma membrane or the membrane of the PV. Once in the host cytoplasm, leishmania-secreted effectors, such as EF-1α, GP63, aldolase, and SAcP, target host kinases and phosphatases, such as SHP-1 and PI3K, modulating their activities. This prevents full macrophage activation and optimal expression of antimicrobial effector mechanisms.

seen in leishmania-infected macrophages. Much remains to be learned, however, about the mechanisms leishmania uses to influence these pathways. One likely possibility is that leishmania secrete effector molecules that target critical host regulators. In support of this hypothesis, several leishmania-secreted proteins have been detected in the cytosol of infected macrophages, including EF-1α, fructose-1,6-bisphosphate aldolase, the secreted form of GP63, and SAcP [10, 47, 50, 69]. Here, we provide evidence for the bona fide secretion of these virulence factors by leishmania and their impact on host immunitiy.

EF-1α

In pursuit of a leishmania-derived effector that might regulate host cell SHP-1, we discovered that *L. donovani* EF-1α actively binds to and activates macrophage SHP-1 [10]. This finding was somewhat surprising, given that EF-1α is a highly conserved, ubiquitously expressed regulator of the rate and fidelity of protein translation. Nevertheless, the SHP-1-binding and -activating properties of *L. donovani* EF-1α were highly specific, as mammalian EF-1α was shown to have no such properties. Taken together, these findings suggested that EF-1α may represent a novel virulence factor. In support of this, iNOS expression in response to IFN-γ was shown to be attenuated in RAW267.4 cells pretreated with purified *L. donovani* EF-1α [10]. Considering that SHP-1 is a cytosolic protein, we sought to identify the subcellular locale in which the interaction of EF-1α and SHP-1 takes place in infected macrophages and found that leishmania EF-1α is present outside of the PV, inside host cytosol. This important finding indicated that EF-1α is released or secreted by *L. donovani* and translocated to macrophage cytosol to interact with target proteins. The absence of an N-terminal secretion signal peptide in the predicted amino acid sequence for leishmania EF-1α suggested that secreteion occurs via nonclassical secretion mechanisms [70]. Proteomic analysis revealed that EF-1α is part of the *L. donovani* promastigote secretome [11]. Moreover, a proteomic study investigating *L. donovani* exosomes showed that EF-1α is part of the cargo of these microvesicles [61]. In summary, these findings provide evidence that leishmania EF-1α is secreted within exosomes and delivered to macrophage cytosol, where its interactions with host SHP-1 promote a deactivated macrophage phenotype.

Fructose-1,6-bisphosphate aldolase

When performing GST-SHP-1 affinity chromatography to identify a leishmania-derived SHP-1 activator, another protein, fructose-1,6-bisphosphate aldolase (hereafter referred to as aldolase), was identified as a SHP-1 ligand [50]. Aldolase not only potently bound SHP-1 but also activated the phosphatase, and expression of leishmania aldolase in RAW267.4 macrophages attenuated iNOS induction by IFN-γ [50]. It was also found that aldolase localized in the cytoplasm of infected macrophages [50]. Interestingly, aldolase was detected as part of the secretome of *L. donovani*, and although it lacks an N-terminal secretion signal peptide [11], it was also shown to be released as cargo of *L. donovani* exosomes [61]. Taken together, these results implicate that leishmania aldolase is secreted and transported to host macrophage cytosol, where it interacts with host SHP-1 to prevent macrophage activation. Whereas the mechanism by which aldolase activates SHP-1 remains to be identified, the fact that there is more than one leishmania-derived SHP-1 activator may reflect the importance of the target [50]. Preliminary results suggest that aldolase and EF-1α cumulatively activate SHP-1 via interacting at independent binding sites on SHP-1 (unpublished results). This implies that multiple activators of SHP-1 may be needed to fully optimize the effect.

GP63

The zinc metalloprotease GP63 (also known as leishmanolysin or major surface protease) is an abundant surface protease of leishmania, as well as other trypanosomatids, and has been recognized as a virulence factor for more than two decades (for a recent review, see ref. [71]). The amino acid sequence of GP63 is highly conserved and contains an N-terminal secretion signal peptide that directs GP63 proteins into the ER and the classical secretory pathway of leishmania [72–74]. Approximately 75% of all synthesized GP63 molecules are located on the cell surface [75] and associated with lipid rafts [76]. The majority of surface GP63 molecules is anchored to the membrane via GPI anchors [77]. In addition to this surface-associated portion, several studies demonstrated that a significant amount of GP63 is released by promastigotes into the extracellular space [72–74]. In GPI mutant *L. mexicana*, it was found that GPI-anchored and unanchored GP63 seem to be trafficked via different pathways, as glycosylation was required for secretion of unanchored GP63, but GPI anchor addition or trafficking was independent of glycosylation [74]. Using a GP63-deficient variant of *L. amazonensis*, McGwire et al. [73] found two different forms of extracellular GP63, one that is released from the cell surface and another that is apparently directly secreted. Release of the cell surface form was inhibited by inactivation of proteolytic activity of the enzyme, suggesting that release involves autoproteolysis [73]. Further supporting the notion that release potentially occurs through different mechanisms, some of the *L. chagasi*-released GP63 molecules exist as membrane-bound vesicles or large, aggregated micelles [72]. Moreover, GP63 was found recently to be part of the cargo of exosomes secreted by *L. donovani* [61], suggesting that despite the presence of an N-terminal secretion signal peptide, GP63 can be secreted via nonclassical pathways. In summary, the protein GP63 exists as an intracellular, surface, or extracellular form, and it can be released or actively secreted, making it a readily accessible virulence factor to interact with the host.

Several functions have been proposed for GP63, and they are likely to vary in the different life-cycle stages of leishmania. GP63 is an endopeptidase that can hydrolyze a range of peptides, and common protein substrates include casein, gelatin, albumin, hemoglobin, and fibrinogen [78]. Early studies in search of a function for GP63 showed that it binds and breaks down the complement component C3 [79], suggesting a role in evasion of complement-mediated lysis. In addition, GP63 mediates binding of uninternalized promastigotes to host cells,

possibly via the host receptors CR3 and CR1, which implies that it may facilitate phagocytosis by macrophages [72]. More recent research provided evidence that GP63 plays very diverse roles in leishmania pathogenesis. For example, several reports showed that GP63 mediates the interaction of leishmania with the host extracellular matrix (ECM). Efficient migration of promastigotes through the ECM was shown to be enhanced by GP63 [80]. Release of internal GP63 from *L. chagasi* promastigotes was increased at 37°C, suggesting it may facilitate close contact between parasite and host macrophages upon parasite entry [81]. Leishmania promastigotes and amastigotes can directly bind to fibronectin, present in the ECM, using surface-localized and secreted GP63 [82]. In turn, the degraded forms of fibronectin can cause decreased production of ROIs by macrophages and increase the accumulation of parasites in infected macrophages [82]. Some lines of evidence have indicated that GP63 promotes intracellular survival of amastigotes in macrophages [83–86], but this effect of GP63 remains controversial. Furthermore, GP63 mediates evasion of the innate immune response by suppression of NK cells [87] and degradation of antimicrobial peptides [56].

Several studies supported the existence of mechanisms of macrophage suppression by direct interaction of GP63 with signaling proteins present in the host cytosol. It was shown that GP63 can cleave epitopes on MHC-1 molecules within infected macrophages, thereby preventing antigen presentation to T cells [88]. Furthemore, the PKC substrate myristoylated alanine-rich C-kinase substrate-related protein was degraded by GP63, providing a mechanism by which leishmania interferes with PKC signaling [89, 90]. Two other important signaling proteins were shown to be cleaved and thereby, inactivated by GP63, namely, c-Jun, which is a central component of the transcription factor AP-1, and the NF-κB p65 RelA subunit [91, 92]. In fibroblasts, exposure to *L. major* resulted in degradation of the phosphorylated adaptor protein p130Cas, the protein tyrosine-phosphatase PEST, cortactin, TCPTP, and caspase 3 [93]. The authors showed that GP63 was the major catalyst of proteolysis during infection and that the cleavage of these protein targets was independent of the presence of intact parasites, suggesting a mechanism of transfer of functional GP63 into the intracellular space [93]. In this study, protein degradation by GP63 mediated the activation of MAPK p38, emphasizing the importance of GP63 in pathogenesis [93]. The same group demonstrated that *L. mexicana* GP63 modulates the activities of host PTPs SHP-1, PTP1B, and TCPTP by cleavage, again independent of internalization of parasites [47]. Interestingly, by confocal microscopy, GP63 was detected in the cytosol of leishmania-infected macrophages in punctuate structures. Furthermore, uptake of GP63 by macrophages and the resulting cleavage of PTPs were dependent on intact host membrane lipid raft domains [47]; however, this was only true for cleavage of SHP-1 and PTPB1 but not TCPTP. These findings suggest a model in which leishmania promastigotes release GP63 molecules, which are then taken up by host macrophages in a lipid raft-dependent manner and translocated to host cytosol, where they cleave host target proteins, most notably, PTPs, thereby promoting a proparasitic phenotype. The fact that the cleavage of TCPTP by internalized GP63 occurred, despite disruption of lipid rafts, implies that there are additional mechanisms for GP63 delivery to macrophages, which may or may not be based on exosomes. The mechanisms of GP63 access to host cytosol continue to be a focus of intense interest.

SAcP

SAcP are enzymes implicated in dephosphorylation of organic substrates [64] and are released from leishmania promastigotes, as well as amastigotes, from the flagellar pocket into the extracellular space [94]. Similar to GP63, SAcP exists as membrane-bound (GPI-anchored) and soluble form, the latter of which is released from the cell [95]. The role of SAcP in leishmania pathogenesis has not been characterized extensively. It has been suggested that *L. amazonensis* PKC activity may modulate its interaction with macrophages via SAcP [96].

In our recent proteomic analysis of the *L. donovani* secretome, SAcP was detected in conditioned medium of promastigotes by Western blotting but was not identified as part of the secretome, as determined by mass spectrometry [11]. The latter result implied that the intracellular abundance of SAcP must be negligible, with nearly all of the synthesized protein being secreted. This result was confirmed by another study, in which SAcP was shown to be highly abundant in *L. donovani* amastigote and promastigote supernatants but absent in total cell lysates from these parasites [69]. Furthermore, SAcP was found in a proteomic analysis of the *L. braziliensis* exoproteome, and the release of SAcP into culture medium over time was shown to be linear, as determined by enzymatic assays [66]. Regarding the mechanism of secretion, controversial data exist, as our own predictions using the SignalP algorithm failed to show the presence of an N-terminal secretion signal peptide [11], whereas others did predict the presence of such a peptide [69, 97]. A Western analysis of exosomes secreted by *L. donovani* promastigotes in vitro demonstrated the presence of SAcP in these vesicles [61], suggesting that SAcP can be secreted via nonclassical pathways. Interestingly, by immunofluorescence microscopy, SAcP was detected recently in small foci throughout the infected macrophage, outside of the amastigote-containing PV, suggesting that it is translocated to the host cytoplasm [69]. In summary, these findings indicate that SAcP is secreted by promastigotes as well as amastigotes, likely by nonclassical means, and it can gain access to host cell cytosol, where it dephosphorylates host proteins.

Transport of effector molecules across the phagolysosomal membrane

Detection of leishmania proteins in macrophage cytosol, where they interact with macrophage proteins raises the question of how these molecules gain access to this cellular compartment. Considering that leishmania interacts with macrophages, extracellularly or intracellularly, there are at least two possible scenarios for translocation of leishmania molecules to host cytosol: extracellular molecules are trans-

ported across the plasma membrane, or intracellular leishmania release molecules into the lumen of the PV, which are then transported across the PV membrane. In Scenario One, internalization of extracellular molecules could occur passively through transporter/carrier proteins or lipid rafts, as has been shown for GP63 [47], or actively through phagocytosis, macropinocytosis, or endocytosis. In the case of exosome-encapsulated molecules, fusion of the vesicle membrane with the plasma membrane and release of cargo into host cytosol are likely possibilities. In the case of Scenario Two, molecules could be transported across the PV membrane by transporter proteins. Alternatively, leishmania exosomes may fuse with the PV discharging their cargo or be reverse-endocytosed out of the PV as individual vesicles or as MVBs, followed by retrograde trafficking through the Golgi to release molecules into the cytosol.

Observations regarding the export of molecules from leishmania PVs are limited. Clearly, amastigotes that reside within PVs for many days, if not weeks, must access the host cytosol to scavenge essential nutrients. Previous work has shown that *L. mexicana* PVs acquire macromolecules from the host cell cytosol [98]. Infection of macrophages with the intracellular bacterium *Mycobacterium tuberculosis* resulted in the formation of bacteria-containing phagosomes with permeable membranes, allowing the access of molecules as large as 70 kDa to the mycobacterial phagosome [99]. It seems likely that there must be mechanisms by which pathogen-derived molecules can travel in the opposite direction [70]. Mottram et al. [100] have proposed recently a model in which *L. mexicana* cysteine peptidases are trafficked from the PV to host cytosol via the host endocytic network.

Clearly, more research focused on how leishmania virulence factors gain access to macrophage cytosol is a high priority. Notably, recent evidence has suggested that exosome-based secretion is a major mechanism by which leishmania-derived virulence factors are released and delivered to the macrophage cytoplasm.

Exosomes as vehicles for the secretion and delivery of leishmania effectors

Vesicle-based secretion is a major nonclassical mechanism of secretion used by a wide range of cell types. Exosomes are a discrete population of secreted vesicles and are 50–100 nm in diameter. The generation of exosomes is achieved by invagination of the endosomal membrane, resulting in formation of MVBs. Release of exosomes into the extracellular space occurs upon fusion of the MVB membrane with the plasma membrane [101]. Exosome biogenesis in mammalian cells was shown to involve several proteins, such as ceramide and Rab GTPases [102, 103]. However, little is known about the general mechanisms of exosome biogenesis and release in lower eukaryotes.

Secretion of vesicles of various types is a well-described phenomenon in prokaryotic as well as eukaryotic cells (for current reviews, see refs. [104–106]). A great variety of molecules, such as membrane-associated and soluble proteins, phospholipids, and even different RNA species, has been shown to comprise cell-specific exosomal cargos. However, the release of exosomes by eukaryotic pathogens containing putative virulence factors has only just begun to be appreciated (reviewed in ref. [107]). We were recently able to demonstrate that protein secretion by *L. donovani* involves the release of bona fide exosomes [61]. Importantly, we showed that *L. donovani* exosomes and their cargo are delivered to macrophage cytosol. We observed that following internalization into phagosomes, leishmania release vesicles into the cytoplasm of infected macrophages. Additionally, we found that extracellular exosomes could be taken up by naïve cells. Ultrastructural analyses showed exosomes and what appeared to be MVBs budding from the PV membrane into the cytoplasm of leishmania-infected macrophages. Furthermore, Western blotting showed that the exosomal markers HSP70 and -90, which we had been shown previously to be cargo of leishmania exosomes, were present in the cytosolic fraction of infected macrophages [61]. We have also found the leishmania effectors EF-1α and GP63 were present in the cytosol of exosome-treated macrophages (unpublished results). In summary, these data support a model in which exosome release by leishmania serves as a general mechanism for protein secretion and for the delivery of cargo to host cell cytosol.

When analyzing the proteome of *L. donovani* exosomes, it was intriguing to see that all proteins previously shown to access the cytosol of leishmania-infected macrophages (EF-1α, aldolase, GP63, SAcP, HSP70, and HSP90) [10, 47, 50, 61, 69] were cargo of these exosomes [61]. In total, analysis of the *L. donovani* exosome proteome revealed 329 proteins, with up to 70% protein overlap with the *L. donovani* secretome. This suggested that exosome release is the primary mechanism of protein export from leishmania. Interestingly, our data showed that leishmania exosome release and cargo can be modified in response to changes in environmental conditions. Heat shock (from 26°C to 37°C) induced an increase in the number of exosomes released by promastigotes in vitro, whereas changes in pH from 7.5 to 5.5 had no effect on the net number of released exosomes but instead, affected the relative enrichment of specific proteins in these vesicles. Functional annotation of the proteins that were released within exosomes under infection-like conditions (37°C and pH 5.5) revealed that phosphatase activity was enriched under these conditions [61]. A very recent study supported these findings by demonstrating that heat shock induced an increase in release of vesicles by *L. mexicana* [108]. This likely reflects a sophisticated exosome-packaging system used by leishmania, which responds to specific environmental conditions [61].

Two important questions to address were whether exosomes play a role in leishmania pathogenesis by modulating host macrophage phenotypes in vitro and affect disease outcome in vivo. Indeed, it became evident that leishmania exosomes had effects on innate as well as adaptive host immune responses. Pretreatment of human monocytes with *L. donovani* exosomes resulted in induction of IL-10 and suppression of IL-8 and TNF-α secretion in response to IFN-γ [60]. Furthermore, secretion of the cytokines IL-12p70, TNF-α, and IL-10 and expression of the surface molecule HLA-DR by mature MoDCs were generally attenuated in response to exosome treatment,

suggesting that these cells may be limited in their capacity to induce T cell responses [60].

Interestingly, exosomes collected from avirulent HSP100 k/o *L. donovani* [109], which lack several effector proteins present in WT exosomes, induced secretion of pro- and anti-inflammatory cytokines by monocytes and MoDCs. Moreover, treatment of MoDCs with HSP100 k/o exosomes and subsequent coculture with naive $CD4^+$ T cells resulted in a pronounced differentiation of these cells into IFN-γ-producing cells [60]. This implies, on the one hand, that HSP100 may have a function in exosome biogenesis and packaging and on the other, that the protein composition of exosomes influences the phenotype of exposed myeloid cells. These findings may also explain the loss of virulence associated with HSP 100 null mutants of *L. major* [110].

In C57BL/6 mice, vaccination with *L. donovani* WT exosomes, and subsequent challenge with promastigotes resulted in disease exacerbation, as determined by enhanced parasite load and modified splenocyte cytokine production [60]. These results indicate that *L. donovani* exosomes promote a proparasitic phenotype in vivo and are consistent with their generally immunosuppressive effects in vitro.

In summary, secretion of exosomes appears to be a major mechanism by which leishmania export secreted virulence factors, such as EF-1α and GP63, into host cytoplasm, where they interact with host signaling molecules, such as SHP-1 and others, to subvert host immune responses. The evidence stongly supports a role for exosomes in the pathogenesis of VL.

POTENTIAL OF AN EXOSOME-BASED VACCINE

Drug toxicities, limited efficacies of specific agents, and the emergence of drug resistance have combined to create an urgent need for a safe and effective vaccine for the prevention of VL. Despite decades of research, to date, no standardized vaccine has passed clinical trials and is available in the clinic. In the recent past, leishmania vaccine research has focused on the use of attenuated strains [111, 112]. Furthermore, several antigens have been identified and characterized as potential vaccine candidates [113]. Interestingly, it was shown that vaccination with SLAs, entrapped in positively charged liposomes, elicited a strong, protective T_H1 response and elimination of 90% of parasites from liver and spleen of challenged BALB/c mice [114]. The lack of safe T_H1 adjuvants is thought to be a main barrier to the development of safe and effective vaccines for leishmaniasis; therefore, these lipid adjuvants have attracted significant interest.

Given that leishmania exosomes are de facto liposomes, carrying leishmania antigenic cargo, we hypothesized that vaccination with exosomes may induce protective immunity against infection. To our surprise, vaccination of C57Bl/6 mice with *L. donovani* exosomes exacerbated disease in these mice upon subsequent challenge with promastigotes [60]. In contrast, vaccination with exosomes from HSP100 k/o *L. donovani* neither exacerbated disease nor induced protection against challenge with WT organisms. Furthermore, in this system, exosomes from HSP100 k/o leishmania potently induced differentiation of naive $CD4^+$ lymphocytes into IFN-γ-producing T-cells, whereas exosomes from WT leishmania did not have this effect [60]. Based on these findings, we hypothesized that exosomes from HSP100 k/o leishmania, in combination with a T_H1 adjuvant, might have significant vaccine potential.

Adjuvants represent critical components in vaccine development, as they both stimulate and direct immune responses with appropriate profiles to promote protection against disease targets [115]. The novel lipid T_H1 adjuvant CAF01, composed of a dimethyldioctadecylammonium bromide/D-(+)-trehalose 6,6′-dibehenate lipid formulation, was demonstrated recently to provide protection in mice against bacterial challenge when administered along with *M. tuberculosis* or *Chlamydia muridarum* antigens [116–118]. We have recently been investigating the effectiveness of CAF01 administration in conjunction with *L. donovani* exosomes. Our initial data show that treatment of resistant C57Bl/6 mice with CAF01 plus exosomes from WT or HSP100 k/o *L. donovani*, followed by challenge with virulent WT organisms, resulted in lower parasite loads when compared with control treatment groups (**Fig. 2A**). Moreover, production of the cytokines IFN-γ, TNF-α, IL-6, IL-10, and IL-17 by spleen cells from CAF01/HSP100 k/o exosome-treated mice was enhanced significantly (Fig. 2B). These findings suggest that a vaccine strategy based on modified leishmania exosomes, combined with the defined CAF01 T_H1 adjuvant, merits further detailed study. Notably, mammalian exosomes have recently gained special attention as potential vaccine candidates in cancer and infectious diseases [119–122]. DCs pulsed with *L. major* were shown to secrete exosomes capable of inducing protective immunity against cutaneous leishmaniasis in BALB/c mice [119]. Our results and these recent findings suggest that the development of an exosome-based vaccine for immunoprophylaxis in leishmaniasis is feasible.

CONCLUDING REMARKS

Leishmania have evolved sophisticated mechanisms to evade or subvert host immune responses and establish chronic infection. Evidence accumulated during the past one to two decades has defined a model, in which leishmania disrupt macrophage activation by targeting host signaling pathways that transmit information to the nucleus. From the studies summarized in this review, it has become clear that a range of effector molecules secreted by leishmania plays key roles in this process. It had long been speculated that translocation of secreted proteins to host cytosol is a prerequisite for interaction of leishmania with host signaling molecules, but until recently, no such mechanism for translocation had been described. The evidence reviewed here clearly identifies exosome secretion as a discrete mechanism by which leishmania release and deliver candidate virulence factors to host cytosol, thereby promoting a proparasitic phenotype. This novel and effective mechanism defines a new paradigm in the molecular pathogenesis of in-

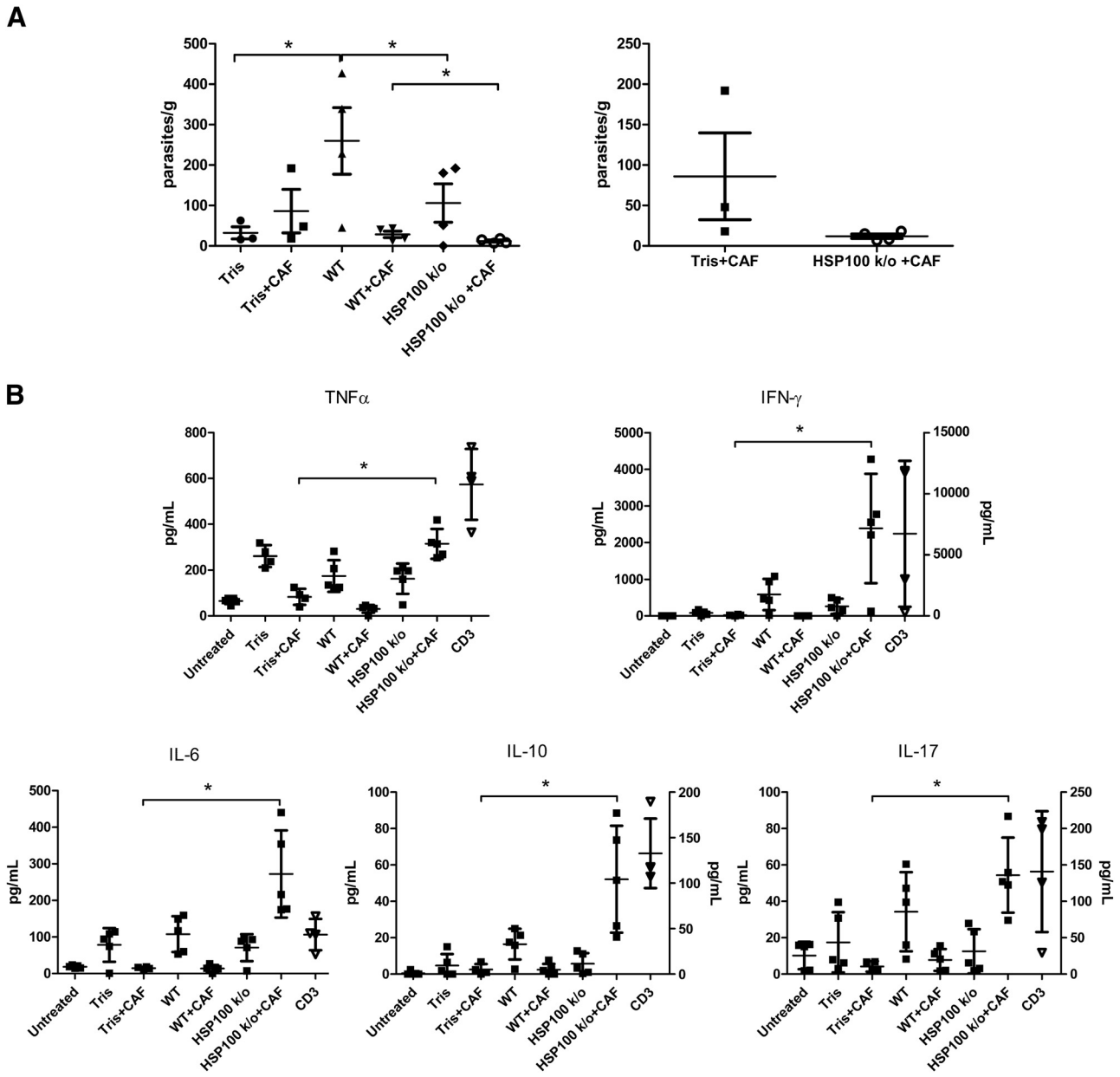

Figure 2. Leishmania exosomes harvested from HSP100 k/o organisms are protective when combined with a T_H1 adjuvant. Experiments were performed as described previously in ref. [60]. Five-month-old, female C57Bl/6 mice were injected s.c. in the right hind leg (three to four mice/treatment group) with 15 μg exosomes isolated from *L. donovani* strain 1SR, either wild-type (WT) or HSP100 k/o, resuspended in 200 μl 10 mM Tris/0.25 M sucrose. For treatments with CAF01 (CAF) adjuvant, exosomes were resuspended in 100 μl Tris buffer and an equal volume of CAF solution. Three mice were treated with Tris buffer alone and three with Tris buffer plus CAF01 alone, and three were left untreated. After 2 weeks, the mice were treated identically again, and 3 weeks later, they were challenged or not with WT *L. donovani* stationary-phase promastigotes via the tail vein. Spleens were harvested after 1 month of infection and single cell suspensions generated using cell strainers. (A) Parasite loads were determined by limiting dilution assay. (B) For cytokine profiling, splenocytes were plated in 96-well round-bottom plates and treated or not with 10 μg/mL SLA (black squares) or 10 μg/mL mouse anti-CD3 (open triangles) in precoated wells. Supernatants were harvested after 48 h and analyzed with the BD cytometric bead array mouse Th1/Th2/Th17 cytokine kit (BD Biosciences, Mississauga, ON). The data shown are from a single experiment. Significant differences between Tris/CAF and HSP100 k/o exosomes/CAF-treated mice were calculated using an unpaired, two-tailed Student's *t* test. $^*P \leq 0.05$.

fection, which likely is not restricted to leishmania. Given the complex cargo of exosomes, future research should focus on the mechanisms by which these molecules are selectively packaged into these vesicles. Furthermore, studies focused on the properties and functional activities of leishmania exosomes and their cargo are likely to generate important, new knowledge, which may identify novel targets for rational drug design and candidates for vaccine development.

AUTHORSHIP

U.L. was involved in selection of the topic and preparation of the manuscript and figures. J.M.S. was involved in design, performance, and data analysis of the vaccination experiments and in preparation of figures. D.N. was involved in manuscript preparation. W.R.M. was involved in design of vaccination experiments and data analysis. J.C. contributed the HSP100 k/o leishmania. L.J.F. was involved in experimental design and performance of proteomic analyses. N.E.R. was involved in overall experimental design, data analysis, and preparation of the manuscript.

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