



Leishmania exosomes deliver preemptive strikes to create an environment permissive for early infection

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Herein, we review evidence supporting a role for *Leishmania* exosomes during early infection. We suggest a model in which *Leishmania* secreted microvesicles released into the extracellular milieu deliver effector cargo to host target cells. This cargo mediates immunosuppression and functionally primes host cells for *Leishmania* invasion. *Leishmania* ssp. release microvesicles and the amount of vesicle release and the specific protein cargo of the vesicles is sensitive to changes in environmental conditions that mimic infection. *Leishmania* exosomes influence the phenotype of treated immune cells. For example, wild-type (WT) exosomes attenuate interferon- γ -induced pro-inflammatory cytokine production (TNF- α) by *Leishmania*-infected monocytes while conversely enhancing production of the anti-inflammatory cytokine IL-10. The *Leishmania* proteins GP63 and elongation factor-1 α (EF-1 α) are found in secreted vesicles and are likely important effectors responsible for these changes in phenotype. GP63 and EF-1 α access host cell cytosol and activate multiple host protein-tyrosine phosphatases (PTPs). Activation of these PTPs negatively regulates interferon- γ signaling and this prevents effective expression of the macrophage microbicidal arsenal, including TNF- α and nitric oxide. In addition to changing macrophage phenotype, WT vesicles dampen the immune response of monocyte-derived dendritic cells and CD4+ T lymphocytes. This capacity is lost when the protein cargo of the vesicles is modified, specifically when the amount of GP63 and EF-1 α in the vesicles is reduced. It appears that exosome delivery of effector proteins results in activation of host PTPs and the negative regulatory effects of the latter creates a pro-parasitic environment. The data suggest that *Leishmania* exosomes secreted upon initial infection are capable of delivering effector cargo to naïve target cells wherein the cargo primes host cells for infection by interfering with host cell signaling pathways.

Keywords: *Leishmania*, exosomes, immunosuppression, early infection, cytokine, secretion, vesicles

INTRODUCTION

The past decade has seen a significant expansion in our knowledge of *Leishmania* secreted proteins. In the past few years alone, seven reports addressing some aspect of the *Leishmania* secretome have been published (Revest et al., 2008; Silverman et al., 2008, 2010a; DebRoy et al., 2010; Kima et al., 2010; Paape et al., 2010; Hassani et al., 2011). The rationale driving this research has been that these protozoan parasites are likely dependent on secretion of effector molecules into the host extra- and intracellular environments for initiation and maintenance of successful infection. This theory is based on investigations of individual secreted proteins (Vannier-Santos et al., 1995; Hubel et al., 1997; Klein et al., 1999; Joshi et al., 2002; Lieke et al., 2008; Gomez et al., 2009; Halle et al., 2009; Silverman et al., 2010b; Jaramillo et al., 2011) and proteins mechanistically involved in the secretion pathway (Besteiro et al., 2006), which showed reduced virulence after loss of protein function or secretion. It has been known for some time that these eukaryotic organisms use a classical secretion pathway for the proper dissemination of newly translated proteins throughout the cell and it has

been thought that this pathway was the route to the cell surface (McConville et al., 2002). However, of the seven reports on *Leishmania* secreted proteins, only one found that a large proportion of the secreted proteins identified contained an N-terminal secretion signal peptide for classical secretion (Paape et al., 2010). In all of the remaining reports, the authors observed the opposite to be true, with a majority of secretome proteins lacking a secretion signal peptide (Revest et al., 2008; Silverman et al., 2008, 2010a; DebRoy et al., 2010; Kima et al., 2010; Hassani et al., 2011). Three of the seven reports which investigated alternative mechanisms of secretion identified vesicles secreted by *Leishmania* (Silverman et al., 2008, 2010a; Hassani et al., 2011). Moreover, another report that investigated the intracellular localization of secreted proteins found the majority of the proteins examined associated with vesicular structures in the parasite as well as in infected host cells (Kima et al., 2010). Though in the nascent stages of discovery, the role of *Leishmania* secreted vesicles in pathogenesis has been investigated both directly and indirectly (Gomez et al., 2009; Halle et al., 2009; Silverman et al., 2010a,b; Hassani et al., 2011). In this review we

will discuss what is currently known about the role that secreted vesicles play in *Leishmania* pathogenesis.

MICROVESICLE RELEASE BY LEISHMANIA

In eukaryotic cells secreted vesicles are formed either at the plasma membrane, referred to as “PM blebs,” or in the lumen of various subcellular organelles – including endosomes, the trans-Golgi, and lysosomes—which are generally termed “exosomes” upon release from the cell. Due to the mechanism of their biogenesis, both types of vesicles (PM blebs and exosomes) contain cytosol and display the extracellular plasma membrane leaflet (which is also the luminal leaflet of the origin intracellular vesicles) on their outer surface. As a result the vesicles are oriented in the same manner as the cell – a characteristic that may well be central to their function(s) during infection.

Exosomes are formed by invagination of the limiting membrane of intracellular organelles, resulting in the formation of multi-vesicular bodies (MVB; **Figure 1**). Exosomes are released into the extracellular space upon fusion of the MVB with the plasma membrane. These vesicles are 50–100 nm in diameter and

are of a specific density (Keller et al., 2006). One of the seven reports that investigated protein secretion by *L. donovani* concluded that these organisms were releasing *bona fide* exosomes (Silverman et al., 2010a). This conclusion was based primarily on comparison of the vesicles collected from *Leishmania* conditioned medium to mammalian exosomes. *Leishmania* vesicles were found to be nearly identical in morphology and density to mammalian exosomes (**Figure 1**) and the protein content of the *Leishmania* secreted vesicles was found to overlap with the canonical mammalian exosome proteome (Simpson et al., 2008) by more than 50% (Silverman et al., 2008, 2010a). Likewise, homologs of a subset of the proteins known to regulate exosome biogenesis and release were identified in the *Leishmania* exosomes (Silverman et al., 2010a).

Another report on *Leishmania* protein secretion showed the plasma membrane of *L. mexicana* promastigotes to be decorated with vesicles, the number of which increased when the parasites were subjected to a short 37°C heat shock (Hassani et al., 2011). These vesicles were also collected from the *Leishmania* conditioned medium and imaged (**Figure 1**). This showed that they

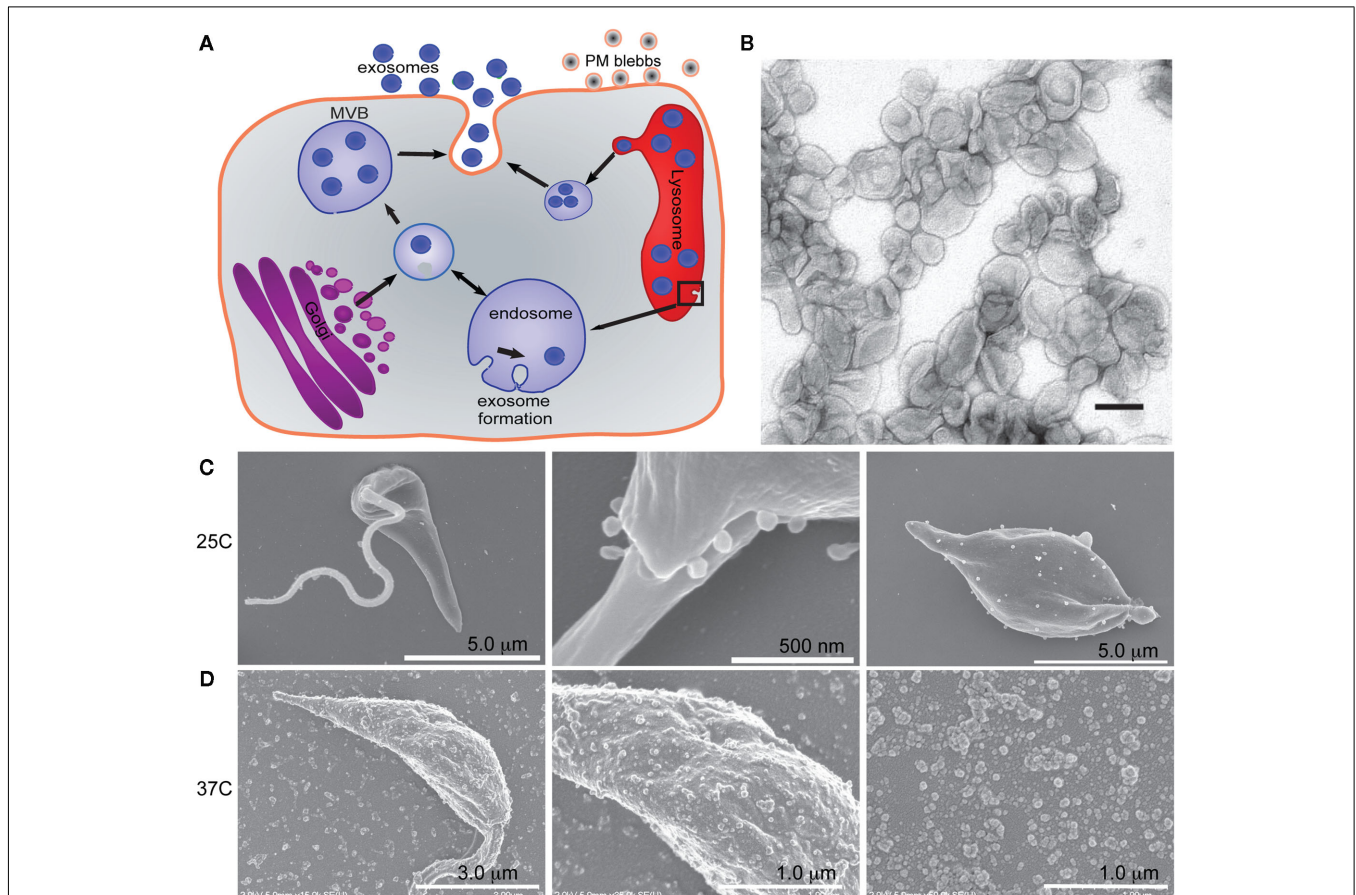


FIGURE 1 | *Leishmania* secrete microvesicles that are likely a combination of exosomes and plasma membrane blebs. (A) Cartoon illustrating the exosome secretion pathway and plasma membrane blebbing. **(B)** *Leishmania* exosomes were isolated and purified in a sucrose gradient prior to imaging by TEM. Scale bar = 100 nm. Reprinted with permission from (Silverman et al., 2010a). **(C)** Scanning electron micrographs of *Leishmania*

promastigotes (left and middle panel) and axenic amastigotes (far right panel) shows vesicles at the flagellar pocket and decorating the cell membrane. Reprinted with permission from (Silverman et al., 2008). **(D)** Scanning electron micrographs of *Leishmania* promastigotes (left and center panels) after a 4 h 37°C heat shock and the vesicles collected from the media during that time (right hand panel). Reprinted with permission from Hassani et al. (2011).

had a similar morphology to the previously described *Leishmania* exosomes (Silverman et al., 2010a) with the exception that they had a markedly larger size range, with some of the vesicles almost 0.2 μm in diameter – fourfold larger than the average exosome (Bobrie et al., 2011). Scanning electron micrographs of *L. donovani* axenic amastigotes after 1 week of axenic culture show similar vesicles decorating the cell surface (Silverman et al., 2008). It is possible that in both of these cases the vesicles were not exosomes *per se*, but possibly PM blebs. PM blebs are formed by budding or shedding from the plasma membrane and are known to be heterogeneous in size and density and to be released under cellular stress (Mathivanan et al., 2010).

No publication to date has unambiguously determined whether *Leishmania* secreted vesicles are of intracellular or plasma membrane origin. We propose that *Leishmania* are capable of secreting both types of vesicles, as mammalian cells are, and that each plays a role in pathogenesis. In fact, given that both PM blebs and exosomes contain cytosol and display the outer leaflet of the plasma membrane on their surface, it is possible that the vesicles formed intracellularly and those formed at the plasma membrane play overlapping roles when interacting with the host.

THE PROTEIN CARGO AND SECRETION OF MICROVESICLES BY LEISHMANIA IS INFLUENCED BY THE HOST ENVIRONMENT

If *Leishmania* secreted vesicles are involved in pathogenesis, one would expect that the mammalian host environment, which is at a higher temperature and lower pH than the gut of the sandfly vector, would affect vesicle release, vesicle cargo or both. Two independent reports now claim to have observed temperature-dependent vesicle release by *Leishmania* (Silverman et al., 2010a; Hassani et al., 2011). It has been shown that vesicle release by *L. donovani* increased threefold when stationary phase promastigotes were exposed to heat shock mimicking human body temperature, 37°C, for 24 h (Silverman et al., 2010a). A more recent report similarly found significant temperature induced secretion of vesicles after only 4 h of heat shock (Hassani et al., 2011). Furthermore, quantitative proteomic analysis found the protein cargo of *Leishmania* exosomes to be both temperature and pH sensitive (Silverman et al., 2010a). For example, functional characterization

of *Leishmania* exosomes released under infection-like conditions (37°C and acidic pH) showed that exosomes harvested at 37°C at neutral pH were enriched in Kinase Activity related proteins and in direct contrast vesicles harvested in acidic pH were enriched in Phosphatase Activity (Silverman et al., 2010a). Likewise, analysis of the brief (4 h) heat shock induced “exoproteome” found a large portion of those proteins to have Catalytic Activity (Hassani et al., 2011). This specific packaging of individual proteins and functional groups, may likely reflect a sophisticated packaging of virulence factors by *Leishmania* in response to specific environments. **Table 1** lists a small selection of the most interesting candidate virulence factors identified in *Leishmania* exosomes based on their increased secretion at 37°C or at acidic pH. Additional study is required to elucidate the role of individual exosomal proteins in *Leishmania* pathogenesis. In any case, the temperature-dependent increase in vesicle release – along with the environment-dependent differences in protein content – support the conclusion that *Leishmania* exosome-mediated protein secretion is an active process subject to regulation by environmental signals.

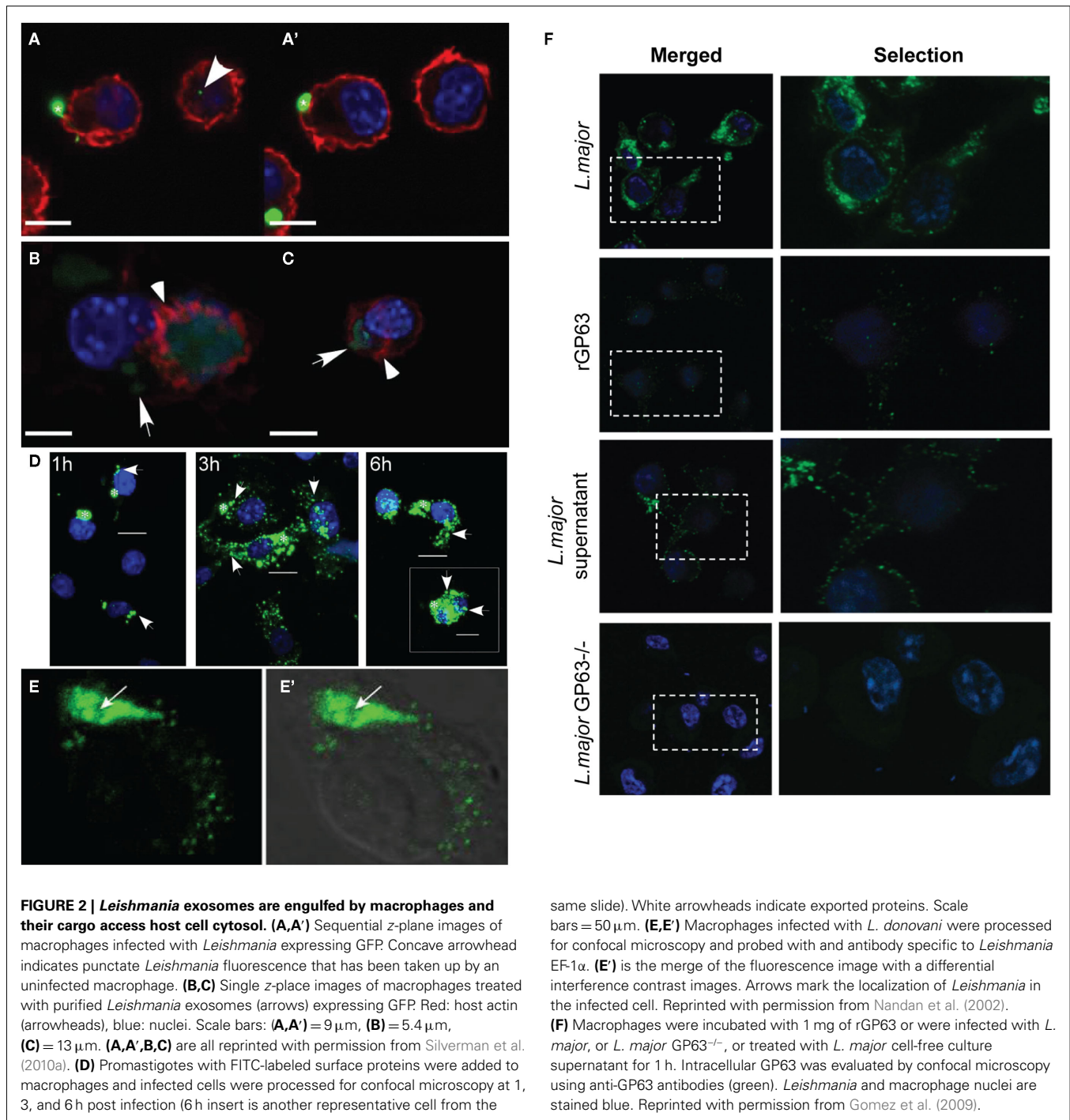
LEISHMANIA SECRETED VESICLES DELIVER CARGO TO HOST CELLS

The specific up-regulation in vesicle release induced by infection-like temperatures suggests that the parasites release vesicles into the extracellular milieu prior to invasion of a host cell [macrophage, neutrophil, or dendritic cell (DC)]. These vesicles could be released from either recently inoculated promastigotes (and might actually be part of the inoculum, after secretion by metacyclic *Leishmania* in the sandfly salivary gland), by free amastigotes after cell rupture, or both. Following release, these exosomes would be free to interact with naïve host cells, either through surface binding, fusion with the plasma membrane, or by endocytosis. As illustrated in **Figure 2**, internalization of *Leishmania* exosomes from the extracellular environment by naïve cells has been directly observed (Silverman et al., 2010a). Furthermore, secretion of vesicular structures by *Leishmania* into the cytoplasmic compartment of an infected host has also been demonstrated (Silverman et al., 2010a).

We have observed exosomal delivery of cargo including GP63 and heat shock protein 10 (HSP10) to macrophages (Silverman et al., unpublished data). Additionally indirect evidence supporting

Table 1 | *Leishmania* secreted vesicles carry candidate virulence factors.

Functional class	GeneDB Accession No.	Protein identity	Enriched in acidic pH	Reference
Immune evasion/ suppression	LmjF10.0460	GP63, leishmanolysin		Joshi et al. (2002), Gomez et al. (2009), Halle et al. (2009)
	LmjF26.0620	Heat shock protein 10	+	Galli et al. (1996); Johnson et al. (2005)
	LmjF26.1240	Heat shock protein 70		Elsner et al. (2007)
Intracellular survival	LmjF15.1040	TRYP1, tryparedoxin peroxidase	+	Reiner and Malemud (1985)
	LmjF11.0350	14-3-3 like protein	+	Porter et al. (2006)
T cell antigens	LmjF35.2210	Kinetoplastid membrane protein-11		Carvalho et al. (2005)
	LmjF28.2740	Activated protein kinase c receptor (LACK)		Carvalho et al. (2005)
	LmjF08.1110	Stress-induced protein sti1		Webb et al. (1997)



the ability of exosomes to deliver effector cargo to naïve cells has been published. Macrophages infected with *L. major* have parasite-derived GP63, a *Leishmania* virulence factor, in their cytosolic compartment and also associated with the inner leaflet of the plasma membrane (Figure 2; Gomez et al., 2009). Notably, GP63 was similarly found in these same subcellular locales when naïve macrophages were incubated with *Leishmania* cell-free culture supernatant (Figure 2; Gomez et al., 2009). Based on our published results, as well as those of Hassani et al. the

culture supernatants used by Gomez et al. clearly must have contained secreted vesicles carrying GP63 (Silverman et al., 2010a; Hassani et al., 2011). As such the cytosolic location of the GP63 after incubation with culture supernatant is very likely to be the result of exosome-mediated delivery of the protein. Likewise, in the immunofluorescence experiments carried out the GP63 fluorescence was punctate, suggesting that host cells engulfed GP63 in a vesicular fashion (Gomez et al., 2009). This punctate fluorescence is very similar to that which was observed in the characterization

of *Leishmania* exosome uptake by macrophages (Silverman et al., 2010a). Furthermore, it was found that treatment with the cholesterol chelator methyl- β -cyclodextrin known to disrupt lipid rafts and exosome release and uptake (Keller et al., 2006) – blocked macrophage uptake of GP63 (Gomez et al., 2009). Taken together, these findings show that GP63 is (1) secreted by *Leishmania* in vesicles, (2) delivered to host cytosol, (3) taken up in a vesicular manner, and (4) that uptake is blocked by reagents which affect vesicle uptake. The data suggest a model in which exosomes are involved in parasite-to-host delivery of GP63.

GP63 is not the only protein cargo of *Leishmania* exosomes to have been found in the cytosol of host cells. Elongation factor-1 α (EF-1 α ; **Figure 2**) and the heat shock proteins 90 and 70 have also been detected in the cytosol of *Leishmania*-infected macrophages (Nandan et al., 2002; Silverman et al., 2010a), suggesting that perhaps these vesicles are also involved in delivery of effectors to host cytosol after infection has taken hold.

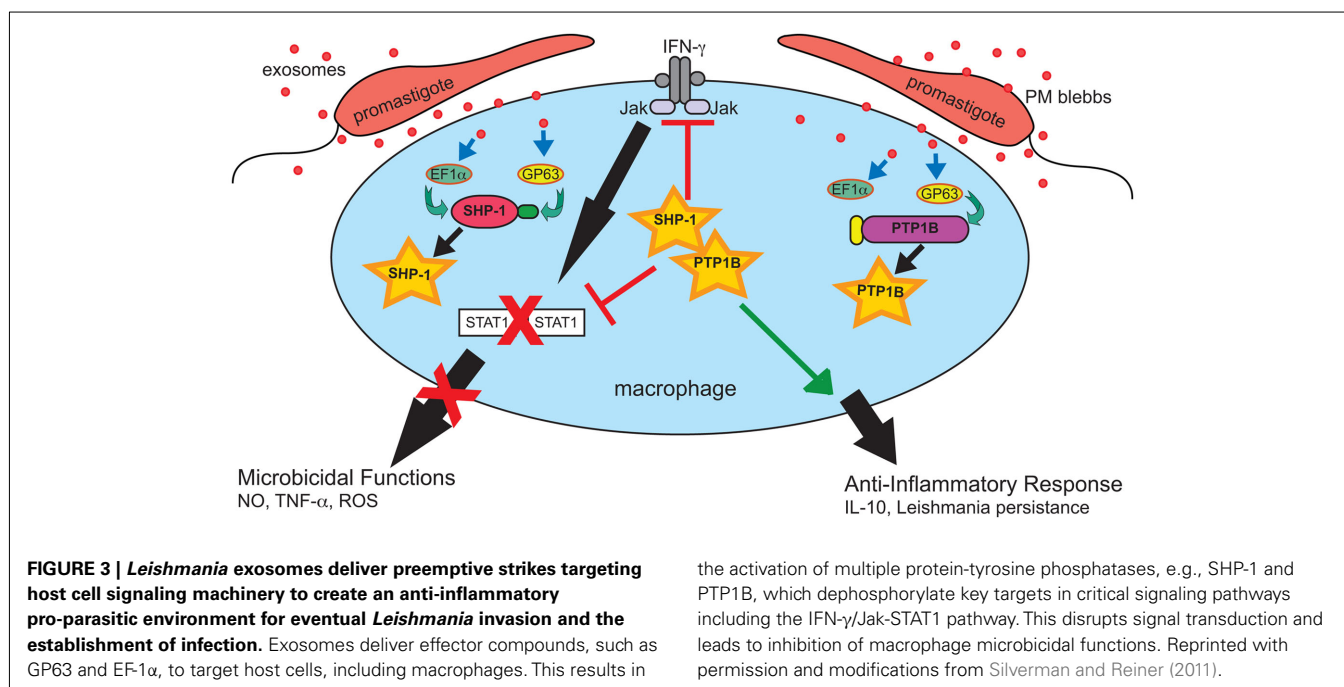
LEISHMANIA SECRETED VESICLES HAVE IMMUNOMODULATORY PROPERTIES

We have proposed a model suggesting that early during infection *Leishmania* secreted vesicles interact with and functionally prime naïve host cells in the local environment for infection. In support of this model, it is now known that *Leishmania* secreted vesicle treatment brings about clear changes in host cell phenotype. *L. donovani* exosomes induced production of the neutrophil chemokine IL-8 by differentiated THP-1 macrophages (Silverman et al., 2010a). A model has recently emerged suggesting that *Leishmania* use neutrophils as Trojan horses to deliver themselves to macrophages via a “silent phagocytosis” pathway, thus avoiding cell activation (van Zandbergen et al., 2004). Exosome induced IL-8 secretion may likely bring about the early recruitment of neutrophils to the site of infection (Peters et al., 2008; Peters and

Sacks, 2009) and in this manner contribute to development of early infection. It is worth noting that incubation of primary human monocytes with *Leishmania* exosomes did not induce IL-8 production (Silverman et al., 2010b) and this discrepancy may be related to specific stages of monocyte/macrophage differentiation. In any case, under appropriate local conditions the induction of IL-8 release by exosomes may promote pathogenesis.

Beyond IL-8, the cytokine IFN- γ is widely considered to be crucial for activating macrophages and clearing leishmanial infections (Nandan and Reiner, 1995). To test the hypothesis that exosomes prime host cells for infection, we treated primary human monocytes with exosomes to infection with virulent *Leishmania* and then subsequently treated those cells with IFN- γ (Silverman et al., 2010b). The results showed that cells that were infected after exosome treatment had blunted responses to IFN- γ for the induction of the proinflammatory cytokines TNF- α and IL-8 (**Figure 3**). In contrast, exposure of monocytes to exosomes prior to infection significantly potentiated IFN- γ -induced IL-10 production (Silverman et al., 2010a). Given that IL-10 is a potent anti-inflammatory cytokine involved in mediating immune suppression during *Leishmania* infection (Murray et al., 2002; Anderson et al., 2008, 2009), enhanced production of IL-10 as a result of exposure to exosomes suggests a significant role for these vesicles in pathogenesis.

Gomez et al. (2009) published a study focused on cell signaling which provides additional support for a model in which *Leishmania* exosomes attenuate IFN- γ -induced macrophage cytokine expression. The opposing functions of protein-tyrosine kinases (PTKs) – activators – and protein-tyrosine phosphatases (PTPs) – deactivators control much of a cell’s biological processes. It has been known for some time that tyrosine phosphorylation in *Leishmania* infected macrophages is generally impaired as a result of *Leishmania* activation of the Src homology-2 domain-containing phosphatase-1 (SHP-1; Blanchette et al., 1999; Nandan et al., 1999,



2002). Gomez et al. (2009) found that, similar to *Leishmania* infection, cell-free culture supernatant from *L. mexicana* and *L. major* was capable of cleaving and activating various host macrophage PTPs including SHP-1, PTP1B, and T cell PTP. Notably, activation occurred almost immediately; cleavage products were detected after only 1 min of exposure to *Leishmania* culture supernatant and were enzymatically active (Gomez et al., 2009). It can be assumed based on subsequent work from this same laboratory, as well as our own work, that this culture supernatant contained secreted vesicles (Silverman et al., 2010a; Hassani et al., 2011). *Leishmania* GP63, cargo of *Leishmania* exosomes, was found to be required and responsible for the cleavage and activation of PTPs (Gomez et al., 2009). This activity, in addition to GP63 cytosolic delivery, was blocked by treatment with methyl- β -cyclodextrin (Gomez et al., 2009), which is known to disrupt both lipid rafts and exosome trafficking (Lancaster and Febbraio, 2005; Keller et al., 2006). Macrophages infected with *Leishmania* have been shown to exhibit reduced phosphorylation of Jak tyrosine kinases and deficient production of nitric oxide (NO) in response to IFN- γ treatment and both of these abnormalities are believed to be brought about as downstream effects of PTP activation (Gregory and Olivier, 2005; Nandan and Reiner, 2005). As illustrated in **Figure 3**, the functional result of this *Leishmania*-induced PTP activity is an impaired IFN- γ signaling cascade required for proper macrophage activation (Nandan and Reiner, 1995; Blanchette et al., 1999; Nandan et al., 1999, 2002; Forget et al., 2005). Gomez et al. (2009) showed these phenotypic changes to be dependent on GP63 as well as host SHP-1 and PTP1B. Taken together, these findings demonstrate that cytosolic delivery of GP63, most likely as exosome cargo, is required to bring about activation of host cell PTPs to bring about negative regulation of macrophage activation in response to IFN- γ .

Interestingly, GP63 is not the only *Leishmania* protein secreted in exosomes known to activate PTPs. Nandan et al. found that *Leishmania* EF-1 α specifically activated SHP-1 *in vitro* and *in vivo*. In addition, this group found that delivery of *Leishmania* EF-1 α to macrophages using the protein delivery system Profect (which is incidentally based on lipid vesicles – similar to exosomes) prevented the induction of iNOS in response to treatment with IFN- γ (Nandan et al., 2002). Nandan et al. also found that *Leishmania* EF-1 α translocated from the phagolysosome into the cytoplasmic compartment of infected cells with a punctuate vesicular morphology (**Figure 2**) and more recent work showed EF-1 α to be a common component of *Leishmania* exosomes (Nandan et al., 2002; Silverman et al., 2010a).

Another interesting finding that has emerged from this work, is that HSP100^{-/-} *L. donovani* produced vesicles apparently lacking GP63 and EF-1 α . Though similar to wild-type (WT) exosomes in terms of morphology and biochemistry, HSP100 knock-out exosomes were stimulatory for cytokine production both in monocyte-derived dendritic cells (MoDCs) and monocytes (Silverman et al., 2010b). This may reflect the inability of these exosomes to activate PTPs with their inhibitory effect due to the absence of GP63 (Gomez et al., 2009; Halle et al., 2009). Moreover, when MoDCs were exosome treated and then co-cultured with naïve CD4⁺ T lymphocytes, the number of IFN- γ producing T cells that emerged was two times greater when the

MoDCs were treated with HSP100^{-/-} vesicles as compared to treatment with WT exosomes (Silverman et al., 2010b). Notably, the enhanced stimulatory capacity of HSP100^{-/-} exosomes did not involve changes in the expression of co-stimulatory markers (CD80, CD86, and HLA-DR) by exosome treated MoDCs when compared with WT exosomes (Silverman et al., 2010b). Presently, the mechanistic basis for these results are not understood although it is highly likely that differences in protein cargo of the vesicles are involved – and likely cargo dependent effects including those of GP63's on PTPs activities.

In contrast to the findings with HSP100^{-/-} exosomes, vesicles harvested from lipophosphoglycan (LPG) 2 null *Leishmania* behaved like their WT counterparts in all of the above experiments (Silverman et al., 2010b). Presumably the LPG2^{-/-} vesicles are lacking in LPG only and contain GP63 and EF-1 α (Spath et al., 2003; Silverman et al., 2010b), which may account for their WT-like properties, although this was not explicitly examined. These findings suggest that the null or inhibitory phenotypes of WT and LPG2^{-/-} exosomes are reflections of the specific protein composition of these vesicles – and potentially reliant on GP63, EF-1 α , or both. Furthermore, the delivery of inhibitory cargo by *Leishmania* exosomes including both packaging and secretion appears to be dependent upon HSP100.

LEISHMANIA EXOSOMES PRODUCE PERMISSIVE ENVIRONMENTS FOR PARASITE INFECTION *IN VIVO*

The results discussed above support a model in which *Leishmania* exosomes deliver virulence factors – GP63, EF-1 α , and others – to host cells and interfere with host cell signaling pathways required to control infection. *In vivo* investigations of the immunomodulatory properties of *Leishmania* exosomes, using mouse models of infection provide further evidence to support this model (Silverman et al., 2010b). Exposure of resistant C57BL/6 mice to WT *L. donovani* exosomes resulted in higher parasite loads in the spleen after subsequent infection and a cytokine profile suggestive of an immune response dominated by suppressor T cells producing both IL-10 and IFN- γ (Silverman et al., 2010b). Th1 cells dually producing IL-10 and IFN- γ have recently been shown to be the dominant suppressor cell in *Leishmania* infections (Anderson et al., 2007; Nylén et al., 2007), and high IFN- γ production has recently been linked to generation of suppressor cell types (Eljaafari et al., 2009). Moreover, the pro-parasitic properties of WT exosomes have been shown to cross *Leishmania* species. Thus, similar to the results for *L. donovani* exosomes, exposure of susceptible BALB/c mice to *L. major* exosomes prior to infection exacerbated disease progression, and this appeared to involve promotion of Th2 polarization (Silverman et al., 2010b). In both of these experimental models, the mice were infected for 4 weeks prior to examining parasite loads and hence provide a window on exosome effects during early infection. Longer term infection studies will be required to examine the *in vivo* effects of exosomes on chronic infection. Nevertheless, these *in vivo* findings indicate that *Leishmania* exosomes are capable of biasing the immune response to make it permissive for infection, perhaps through their generally inhibitory effects on monocytes as well as MoDCs.

In vivo data with PTP knock-out mice further supports this conclusion. *L. major* infection of either PTP1B null mice or SHP-1

null mice resulted in delayed footpad thickening and decreased parasite burden – though only during the early stages of infection, as well as greater leukocyte recruitment (Forget et al., 2001; Gomez et al., 2009). This demonstrates a clear role for these PTPs in creating a parasite permissive environment early during infection. Furthermore, these effects were shown to be GP63 dependent (Gomez et al., 2009). These *in vivo* findings suggest that GP63-dependent cleavage and activation of host PTPs plays a crucial role during the early, stages of the disease, perhaps reflecting the exosome-mediated delivery of GP63 and early priming of target cells for infection.

CONCLUSION

The findings reviewed above support a model in which exosome-mediated delivery of GP63 and EF-1 α to target cell cytosol activates host PTPs, which prevent IFN- γ -induced signaling and blunt the pro-inflammatory cytokine response to infection. The

data strongly suggest that *Leishmania* secreted vesicles are capable of delivering effector molecules to naïve host cells which in turn modulate the immune response in a pro-parasitic manner. *Leishmania* secreted vesicles and their effector cargo are likely essential events to establish early stage infection and set the stage for subsequent events leading to the development of chronic infection.

ACKNOWLEDGMENTS

The authors would like to acknowledge the following individuals for their contributions to studies cited in this review: Ulrike Lambertz, Emily Thi, Devki Nandan, Joachim Clos, Leonard Foster, and Megan Levings. In addition, we would like to thank Martin Olivier for generously allowing us to reprint important figures. Neil Reiner is supported by the Canadian Institutes of Health Research grant MOP-84582 and Judith Silverman is supported by a grant from PrioNet Canada.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 23 September 2011; paper pending published: 11 November 2011; accepted: 23 December 2011; published online: 09 January 2012.

Citation: Silverman JM and Reiner NE (2012) *Leishmania* exosomes deliver preemptive strikes to create an environment permissive for early infection. *Front. Cell. Inf. Microbio.* 1:26. doi: 10.3389/fcimb.2011.00026
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