An exosome-based secretion pathway is responsible for protein export from *Leishmania* and communication with macrophages

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Summary

Specialized secretion systems are used by numerous bacterial pathogens to export virulence factors into host target cells. *Leishmania* and other eukaryotic intracellular pathogens also deliver effector proteins into host cells; however, the mechanisms involved have remained elusive. In this report, we identify exosome-based secretion as a general mechanism for protein secretion by *Leishmania*, and show that exosomes are involved in the delivery of proteins into host target cells. Comparative quantitative proteomics unambiguously identified 329 proteins in *Leishmania* exosomes, accounting for >52% of global protein secretion from these organisms. Our findings demonstrate that infection-like stressors (37°C ± pH 5.5) upregulated exosome release more than twofold and also modified exosome protein composition. *Leishmania* exosomes and exosomal proteins were detected in the cytosolic compartment of infected macrophages and incubation of macrophages with exosomes selectively induced secretion of IL-8, but not TNF-α. We thus provide evidence for an apparently broad-based mechanism of protein export by *Leishmania*. Moreover, we describe a mechanism for the direct delivery of *Leishmania* molecules into macrophages. These findings suggest that, like mammalian exosomes, *Leishmania* exosomes function in long-range communication and immune modulation.

Key words: Exosome, *Leishmania*, Infection, Secretion

Introduction

Leishmania spp. are the causative agents of a group of tropical and sub-tropical infections termed the leishmaniases. These chronic, largely non-resolving infections affect an estimated 12 million people worldwide and 2 million new cases are believed to occur each year (Chappuis et al., 2007). Recent environmental changes such as urbanization, deforestation, and new irrigation schemes have expanded endemic regions and have led to sharp increases in the number of reported cases (Croft et al., 2003). Progress in controlling the leishmaniases requires an improved appreciation of the cell biology of infection. For example, the role of secreted proteins in *Leishmania* pathogenesis is poorly understood and no general mechanism of protein secretion by *Leishmania* has been identified.

In a recent analysis of the secretome of *Leishmania donovani*, we found that only 14% of the proteins contained an N-terminal classical secretion signal peptide (Silverman et al., 2008). This surprising result correlated with our results from a pharmacologic screen investigating the mechanism of secretion, by which classical inhibitors of eukaryotic protein secretion, such as Brefeldin A (BFA), showed no effect on *Leishmania* protein secretion (unpublished data). Surprisingly, the *Leishmania* secretome contained 100% of the proteins (for which there is a *Leishmania* ortholog) previously identified in exosomes secreted by B lymphocytes and dendritic cells (Silverman et al., 2008). Moreover, using scanning electron microscopy (SEM) we detected 50 nm vesicles budding from the flagellar pocket of *L. donovani* (Silverman et al., 2008). These results led us to hypothesize that *Leishmania* use an exosome-based mechanism to actively regulate protein release from the cell.

Exosomes are 30-100 nm organelles now known to be released by numerous mammalian cell types including reticulocytes, B-cells and T-cells, dendritic cells and macrophages (Bhatnagar et al., 2007; Thery et al., 1999; Wang et al., 2008; Wubbolts et al., 2003). These vesicles are formed within endosomes, by invagination of the limiting membrane, resulting in the formation of multivesicular bodies (MVBs). Exosomes are then released into the extracellular environment by fusion of MVBs with the plasma membrane (Keller et al., 2006). Exosome biogenesis and release is an intricate process, involving multiple protein complexes (Keller et al., 2006). This process has been proposed to be dependent upon the endosomal sorting complex required for transport (ESRCT) (de Gassart et al., 2004), although ESRCT dependence remains controversial (Trajkovic et al., 2008).

Bioactive exosomes are released by cells that are infected with either bacteria or viruses (Bhatnagar et al., 2007; Nguyen et al., 2003) as well as by diverse tumor cells (Graner et al., 2009; Liu et al., 2006). To our knowledge, only two non-mammalian cell types have been shown to release exosomes: *Caenorhabditis elegans* (Liegeois et al., 2006) and *Cryptococcus neoformans* (Rodrigues et al., 2008). In addition, some members of the phylum Ascomycota have been shown to export proteins using secreted vesicles, although these have not yet been fully characterized (Albuquerque et al., 2008). Even though much remains to be learned, we are beginning

to appreciate the functions of mammalian exosomes as intercellular signaling and communication devices, and have begun to unravel their complex roles in immune modulation and immune surveillance.

We report here that exosome release is a general mechanism for protein secretion by *Leishmania*. Moreover, we show that these vesicles deliver cargo to and communicate with macrophages. As far as we are aware, this is the first evidence reported for exosome release by a protozoan pathogen as well as the first direct evidence for an apparent broad-based mechanism of protein export from *Leishmania*.

Results

Protein secretion by *Leishmania* involves the release of exosome-like vesicles

To determine whether release of exosomes was the major mechanism of protein export from *Leishmania*, conditioned medium from both *L. donovani* and *L. major* was subjected to differential centrifugation with a final speed of 110,000 ***g*** (Raposo et al., 1996), the characteristic speed for pelleting exosomes. The pellets were found to contain exosomal markers (Stoorvogel et al., 2002) such as heat shock protein (HSP) 70, HSP90 and elongation factor-1α (EF-1α) (Fig. 1A). Additionally, the 110,000 ***g*** pellets had distinct protein profiles when compared with the cells from which they were collected and the cellular debris pelleted before collection of exosomes (Fig. 1B, P4 compared with P1-P3).

We investigated whether these high-speed pellets contained intact vesicles, as opposed to membrane fragments, using three independent approaches: (1) trypsin sensitivity, (2) density gradients, and (3) microscopy. We treated the 110,000 ***g*** pellets with trypsin in the presence or absence of a detergent (Fig. 1C) to determine whether the proteins detected via western blotting (Fig. 1A) were indeed luminal to vesicles. Unlike trypsin treatment alone, which had a minimal effect on the amount of HSP70, HSP90 or EF-1α, the combination of detergent and trypsin completely abolished

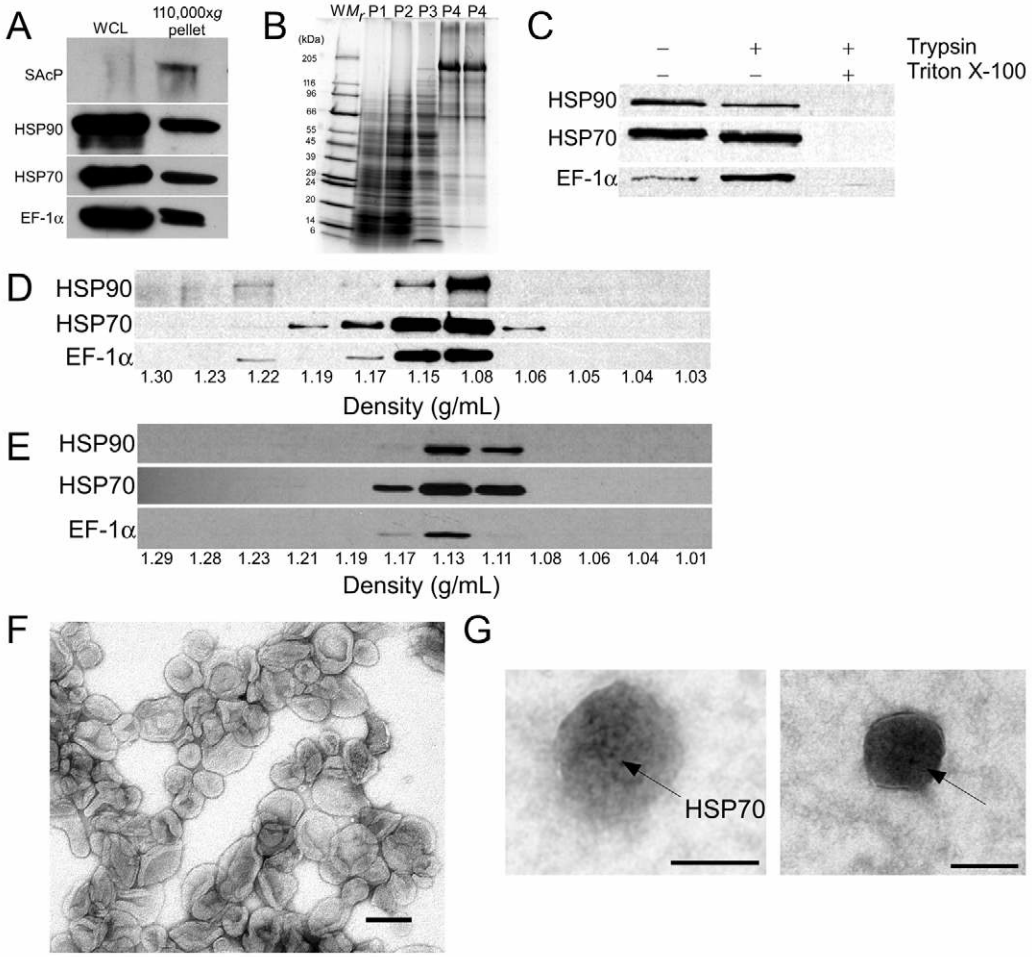

Fig. 1. *Leishmania* conditioned medium contains bona fide exosomes. (**A**) *Leishmania*-secreted microvesicles were harvested following the conventional exosome isolation protocol (Raposo et al., 1996) from conditioned medium. Whole-cell lysates and 110,000 ***g*** pellets were probed for *Leishmania* orthologues of mammalian exosomal markers HSP70, HSP90 and EF-1α. (**B**) To collect exosomes, *Leishmania* conditioned medium was subjected to differential centrifugation. Equal amounts of cellular (P1, 300 ***g***), cell debris (P2 and P3, 700 ***g*** and 15,000 ***g***), and exosome collection speed pellet (P4, 110,000 ***g***) protein were fractionated by SDS-PAGE and detected by Coomassie blue staining. WM_r, wide molecular mass marker. (**C**) *Leishmania* microvesicles carry exosomal markers as cargo. Vesicles were treated with 1 mg/ml trypsin, with or without 0.1% Triton X-100 to disrupt vesicle membranes. *L. donovani* (**D**) and *L. major* (**E**) exosomes migrate to the characteristic exosome-containing density in a sucrose gradient. Pelleted vesicles were floated into a linear sucrose gradient (0.25-2.5 M). After ultracentrifugation, fractions were analyzed by western blotting by probing for exosomal markers HSP70, HSP90 and EF-1α. The data shown in A-E represent at least three independent experiments with similar results. (**F**,**G**) Sucrose-gradient-purified *Leishmania* exosomes processed for TEM (F) or immunoEM (G). Exosomes on grids were probed with *Leishmania* specific anti-HSP70. Arrows indicate HSP70. Scale bars: 100 nm.

exosomal marker signals (Fig. 1C). These results indicate that the 110,000 ***g*** pellets contained intact vesicles in that their cargo was protected from enzymatic degradation by a membrane. Exosomes migrate to a specific density (1.08-1.15 g/ml) in a linear sucrose density gradient. Consistent with this, *Leishmania* secreted vesicles migrated to densities of 1.06-1.17 g/ml (Fig. 1D and 1E). Moreover, examination of the secreted vesicles after sucrose density purification by transmission EM showed them to contain vesicles ranging from 30 to 70 nm in diameter (Fig. 1F-G) with the cup-shaped morphology characteristic of mammalian exosomes (Keller et al., 2006). In addition, we detected the exosomal marker HSP70 within these vesicles by immunoEM (Fig. 1G). Secondary antibody controls confirmed that the gold labeling was specific to HSP70 in the exosomes.

Based upon our observation by SEM of 50 nm vesicles budding from the flagellar pocket of *L. donovani* promastigotes (Silverman et al., 2008), we carried out further ultrastructural analysis of vesicle release and this included both *L. donovani* and *Leishmania mexicana*. This analysis identified 50 nm vesicles and MVBs budding from the plasma membrane of *L. mexicana* (supplementary material Fig. S1), and similar vesicles within the flagellar pocket of *L. donovani* promastigotes (data not shown). Taken together, these biochemical and ultrastructural findings indicate that *Leishmania* release microvesicles that have all the characteristic properties of bona fide exosomes.

Leishmania exosome release at elevated temperature and low pH

Leishmania experience heat shock when they are delivered to a mammalian host (37°C) by the bite of a sandfly (26°C). To investigate heat-shock-induced changes in exosome release, we collected vesicles from stationary phase populations of *L. donovani* promastigotes incubated at either 37°C or 26°C for 24 hours. Populations cultured at the two temperatures were counted and adjusted before exosome collection to ensure equivalent numbers. After collection, exosomes from the two treatment groups were resuspended in equal volumes of lysis buffer. When equal volumes were analyzed, it was clear that exosomes collected from cells at 37°C had a significant increase in exosomal marker proteins (Fig. 2). When an equal amount of protein from the two samples was analyzed, no difference was observed, indicating that the high temperature induced exosome release, as opposed to increased secretion of HSPs per se. To confirm that this finding was not an artifact of a global increase in production of the heat-shock proteins, we probed whole-cell lysates generated from the cells that produced the exosomes and found no difference in HSPs between the two conditions (data not shown). It is well known that incubating stationary phase *Leishmania* at 37°C induces a growth arrest (Barak et al., 2005) and that the lowering of the pH is required for full differentiation into amastigotes. Our results were consistent with this. After a 24 hour exosome collection, the 26°C sample had consistently expanded to more than 6.0×10^7 cells/ml, whereas the population cultured at 37°C was static at 4.5×10^7 to 5.0×10^7 cells/ml (data not shown). To account for the observed 2.2-fold increase in HSP70 release at 37°C (Fig. 2B) each organism at 37°C would have had to release roughly three times the number of vesicles as an organism cultured at 26°C. This also applied when HSP90 was used (Fig. 2C).

In contrast to heat shock, lowering the pH of the medium from 7.5 to 5.5 to reflect conditions in a maturing phagolysosome, had no effect on the number of vesicles released when compared with

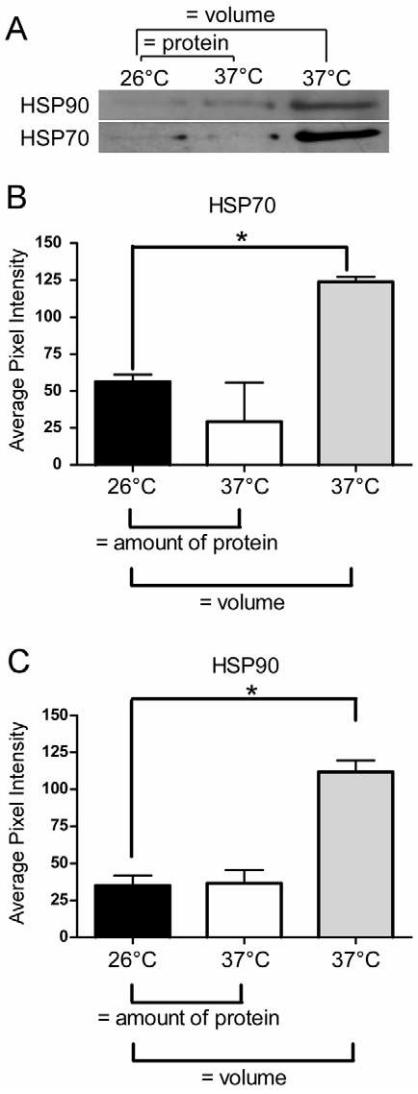

Fig. 2. Heat shock increases exosome release from *L. donovani*. Exosomes were harvested from equal numbers of promastigotes at either 37°C or 26°C for 24 hours. After isolation and purification, exosomes were resuspended in an equal volume and the amount of protein estimated. (**A**) An equal volume of each sample analyzed by western blotting for exosomal markers to illustrate changes in total protein released under the different conditions. As the 37°C sample had greater protein content than 26°C sample, an aliquot of the 37°C sample equal to the amount of 26°C protein analyzed was included as a control. The data shown are representative of at least three similar experiments. The HSP70 (**B**) and HSP90 (**C**) signals were analyzed by densitometry and the values from the three experiments averaged and compared for statistical significance using a paired Student's *t*-test. **P*≤0.005 for HSP70 and **P*≤0.01 for HSP90.

secretion at neutral pH (Fig. 3). Unexpectedly, we reliably detected significantly less of the exosomal marker EF-1α in vesicles harvested from cells incubated at low pH (Fig. 3D). This finding was concordant with a quantitative proteomic analysis (discussed below), where EF-1α had a pH 7.5/pH 5.5 ratio higher than the overall mean indicating relative enrichment at pH 7.5 (Fig. 3D and supplementary material Table S1). Taken together, these results suggest that although an acidic environment did not influence bulk

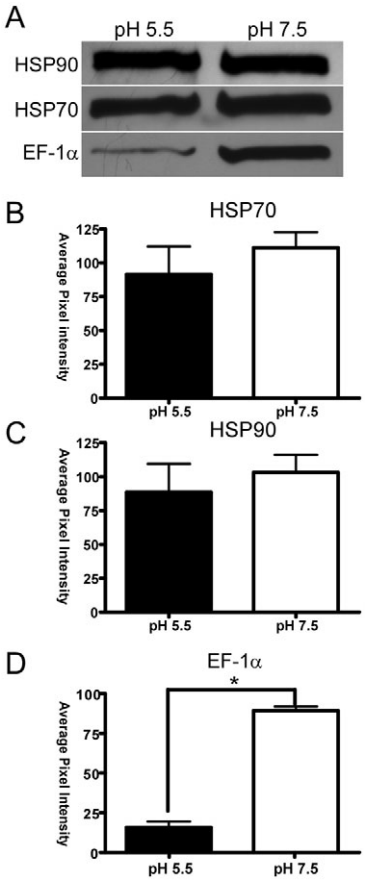

Fig. 3. Changes in pH affect the cargo of *L. donovani* exosomes. Exosomes were harvested from equal numbers of promastigotes cultured at either pH 7.5 or pH 5.5 for 24 hours at 37°C. After isolation and purification, the exosomes were resuspended in equal volumes of PBS. The amount of exosomal protein recovered was estimated and found to be the same under the two conditions. (**A**) An equal volume of each sample analyzed by SDS-PAGE followed by western blotting. The data shown is representative of at least three experiments with similar results. The HSP70 (**B**), HSP90 (**C**) and EF-1α (**D**) signals were analyzed by densitometry and the mean of the respective values from three experiments calculated and compared for statistical significance using a paired Student's *t*-test. **P*≤0.005.

exosome release from *L. donovani* at 37°C, it did affect the cargo of these vesicles.

To control for the possibility that cell death resulting from the heat shock and reduced pH was responsible for the increase in vesicles release, we analyzed the viability of the treated *Leishmania* populations by flow cytometry. We found that increasing the temperature from 26°C to 37°C and 37°C+pH 5 resulted in a slight increase in the number of dead cells, from ~5% at 26°C to ~8% at both 37°C and 37°C+pH 5 (data not shown). Clearly, this 3% increase in cell death could not have accounted for the 100% increase in exosomal protein released brought about by heat shock (Figs 2 and 3).

Proteomic analysis of *L. donovani* exosomes

Two quantitative proteomic analyses of exosomes secreted by *L. donovani* under early-infection-like conditions were conducted. The first analysis compared the proteomes of vesicles collected at 26°C versus 37°C (both at pH 7.5). The second analysis compared vesicles collected in medium at pH 7.5 versus pH 5.5 while holding the temperature constant at 37°C. In both cases, exosomes collected from equal volumes of culture medium were compared.

In six individual 37°C/26°C comparative liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses over 400 proteins were identified in *Leishmania* exosomes. For inclusion in the exosome proteome and the quantitative analysis, a protein must have been identified in at least two of the analyses. Using these parameters, the relative amounts of 233 exosome proteins secreted at 37°C and at 26°C were quantified (Fig. 4A and supplementary material Tables S1 and S2). The ratios ranged from 15 to 0.15, a clear indication that specific enrichment of proteins into vesicles occurred in response to changes in temperature. In every analysis performed, the proteins with the highest 37°C/26°C ratios were α-tubulin and β-tubulin: LmjF13.0280, LmjF33.0792 and LmjF21.1860 (supplementary material Table S1). Furthermore, HSP90 (LmjF33.031) had a ratio of 5, placing it in the top 75th percentile of all 37°C/26°C ratios. Four different HSP70 homologs were identified (LmjF30.2460, LmjF28.2770, LmjF28.1200, LmjF26.1240) all of which had ratios indicating their relative enrichment in the 37°C exosomes (supplementary material Table S2). In addition to illustrating that changes in temperature brought selective enrichment of certain proteins, these quantitative proteomic data are concordant with the findings reported in Fig. 2 showing that high temperature resulted in the secretion of more vesicles.

Using similar parameters for inclusion in the quantitative analysis, 175 exosomal proteins were identified in vesicles collected under conditions of neutral pH versus low pH (Fig. 4B and supplementary material Table S2) at 37°C. α-tubulin and β-tubulin proteins had ratios around 1 (supplementary material Tables S1 and S2), indicating they were present in approximately equal amounts in either condition. This was also true of the heat-shock proteins: HSP90 (LmjF33.0312) had a ratio of 0.9, HSP70 (LmjF26.1240) had a ratio of 1. However, not all heat shock proteins followed this pattern, HSP10 (LmjF26.0620) had a ratio of 0.7, placing it within the bottom 25th percentile (Fig. 4B and supplementary material Table S1). Again, these results agreed with the western blotting results (Fig. 3) indicating that acidic pH influences exosome cargo, but not exosome release.

We amalgamated the two quantitative comparisons to generate a global *Leishmania* exosome proteome (supplementary material Table S1). Proteins that were identified in at least two of ten experiments were included, bringing the subtotal to 329 proteins (supplementary material Table S1). These inclusion parameters are conservative when compared with similar studies in the literature (Gilchrist et al., 2006; Simpson et al., 2008) where for example, only one peptide detected in a single experiment was required for inclusion. The exosome proteome of *L. donovani* accounted for over half (52%; Fig. 4C and supplementary material Table S3) of the total secretome of stationary phase *L. donovani* (Silverman et al., 2008). Considering the strict inclusion parameters imposed on both the secretome and exosome analyses, this is probably an underestimate of their similarity. In fact, several of the individual exosome LC-MS/MS analyses overlapped with the secretome by more than 70%. The high protein overlap between the *Leishmania* secretome and the exosomes suggests that these vesicles are the primary mechanism of protein release from *Leishmania*.

When compared with a recently compiled list of common mammalian exosomal proteins (Simpson et al., 2008), we found that *Leishmania* exosomes contained orthologs for approximately 52% of the mammalian exosome proteome (Fig. 4D and supplementary material Table S4). In addition to characterized orthologs, such as

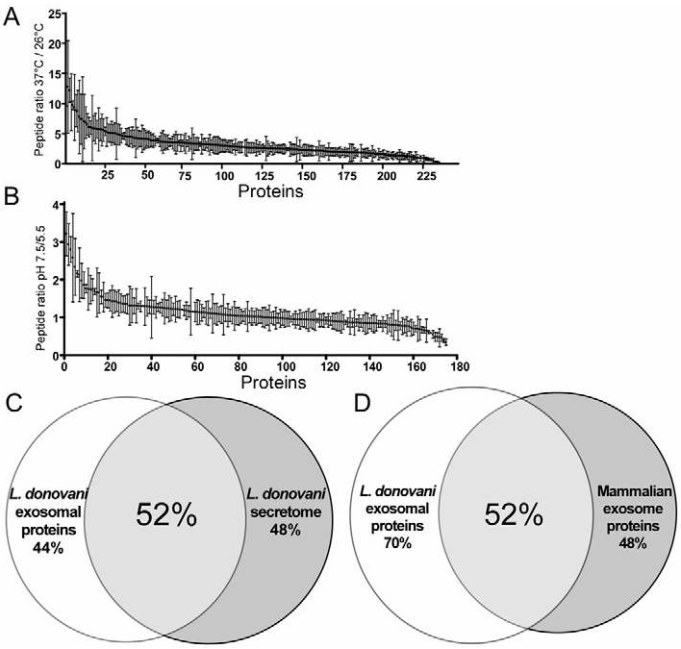

Fig. 4. Quantitative proteomics identifies the cargo and relative abundance of exosomal proteins under infection-like conditions, showing that exosomes account for 52% of the secretome and have high protein overlap with mammalian exosomes. Mean peptide ratios for each protein identity from the LC-MS/MS analyses were calculated. (**A**) 37°C/26°C ratio values, overall mean=3.16, s.d.=2.08; *x*-axis numbers correspond to each protein identity with 233 in total (supplementary material Table S2). The mean and s.e.m. from at least three measurements for each protein are shown. (**B**) pH 7.5/pH 5.5 ratio values, overall mean=1.10, s.d.=0.42, *x*-axis corresponds to the total 175 proteins identified (supplementary material Table S2). The mean and range from at least two measurements for each protein are shown. The protein identities from the two quantitative experiments, 10 independent LC-MS/MS analyses in total, were combined to generate an exosome proteome of *L. donovani* (supplementary material Table S2). (**C**) The 329 exosome proteins compared with the 358 *Leishmania* secretome proteins (supplementary material Table S3). (**D**) *Leishmania* exosome proteins compared with the common mammalian exosome proteins (supplementary material Table S1) recently compiled (Simpson et al., 2008). The mammalian exosome proteins are grouped into categories of proteins with common function, e.g. ribosomal proteins. For comparison, if the *Leishmania* exosomes contain any proteins falling within a category, that category of proteins is considered to be shared between *Leishmania* and mammalian exosomes. To determine the percentage of proteins unique to *Leishmania* exosomes, the total number of proteins falling into each shared category is subtracted from the total number of exosomal proteins (322).

Rab1 (LmjF27.0760) and Rab11 (LmjF10.0910) (Savina et al., 2002), this included three *Leishmania* exosome-associated hypothetical proteins identified by BLAST analysis as similar to mammalian exosomal proteins involved in exosome biogenesis: LmjF23.0990 was similar to the ESCRT-3 subunit Chmp2; LmjF27.1640 to Alix, and LmjF29.0110 to Radixin (Simpson et al., 2008).

Functional annotation of exosomes released at elevated temperature and low pH

Using the results of the quantitative proteomics, we carried out a functional characterization of *Leishmania* exosomes released under either normal culture conditions (26°C) or under infection-like conditions (37°C and acidic pH). The results depicted in supplementary material Fig. S2 show that exosomes were enriched in a wide variety of functions including the process of vesicle-mediated transport, and in intracellular membrane-bound organelles (supplementary material Table S5). Interestingly, for exosomes harvested at 37°C, kinase activity was enriched in vesicles at neutral pH, in direct contrast to the enrichment of phosphatase activity in acidic pH exosomes (supplementary material Fig. S2B). The concentration of specific and sometimes opposing functions within exosomes harvested under different conditions provides additional evidence that *Leishmania* exosome-mediated protein secretion is an active process subject to regulation.

Leishmania exosomes are released into infected macrophages and are taken up by naive cells from the extracellular environment

Based upon our findings that the majority of *Leishmania* protein secretion appeared to be mediated by exosomes, we hypothesized that *Leishmania* expressing green fluorescent protein (GFP) would secrete GFP in exosomes. This turned out to be correct. Wild-type *L. donovani* were transformed with a plasmid encoding GFP (Ha et al., 1996). Western blotting and fluorimetery confirmed the presence of GFP in exosomes harvested from the GFP-Ld (data not shown). As shown in Fig. 5, we found that as early as 2 hours after infection, infected macrophages contained GFP-labeled punctate structures with the morphology of vesicles ~200 nm in diameter (Fig. 5A-D). Exosomes are too small to be resolved individually with a confocal microscope; however, the punctuate structures visible (Fig. 5A-B, concave arrowheads) were of an appropriate size for MVBs containing multiple GFP^+ exosomes. Moreover, we observed actin rings (Fig. 5A-B, convex arrowhead) encircling GFP^+ vesicles (Fig. 5A-B, concave arrowhead) in the processes of blebbing off internalized *Leishmania* (Fig. 5A-B, asterisk) into the infected macrophage. In Fig. 5A, it is evident that the macrophage contains at least three other *Leishmania*-derived vesicles. Fig. 5C also depicts a cell with multiple *Leishmania*-derived vesicles apparently secreted by the internalized *Leishmania*. Fig. 5D shows an infected cell of the left, and an uninfected cell on the right. The uninfected cell contains a GFP^+ vesicle, indicating that the macrophage has engulfed vesicles released by un-internalized *Leishmania* into the extracellular environment.

To examine further the extent to which macrophages take up *Leishmania* exosomes from the extracellular environment, we treated macrophages with GFP^+ exosomes harvested and purified from GFP-Ld under endotoxin free conditions. Similarly to what we observed when we infected cells with GFP-Ld (Fig. 5A-D), macrophage actin rings were seen mediating engulfment of GFP^+ exosomes (Fig. 5E-F, convex arrowhead). Furthermore, the GFP^+ exosomes appeared to be accessing the cytoplasm of the host cell (Fig. 5E, arrow). It is noteworthy that no GFP fluorescence was detectable at time points later than 2 hours, indicating that the internalized *Leishmania* proteins were degraded, or that the exosomal GFP is no longer detectable after it diffuses throughout the comparatively vast host cytoplasm.

We used fluorescent labeling of *Leishmania* surface proteins (such as leishmanolysin GP63, which was consistently present in *Leishmania* exosomes, supplementary material Table S2), as an orthogonal approach to show that *Leishmania* export vesicles into the cytoplasm of infected macrophages (Fig. 6A). This analysis showed that surface proteins released from FITC-*Leishmania* accumulated in the host cell cytoplasm with punctate morphology

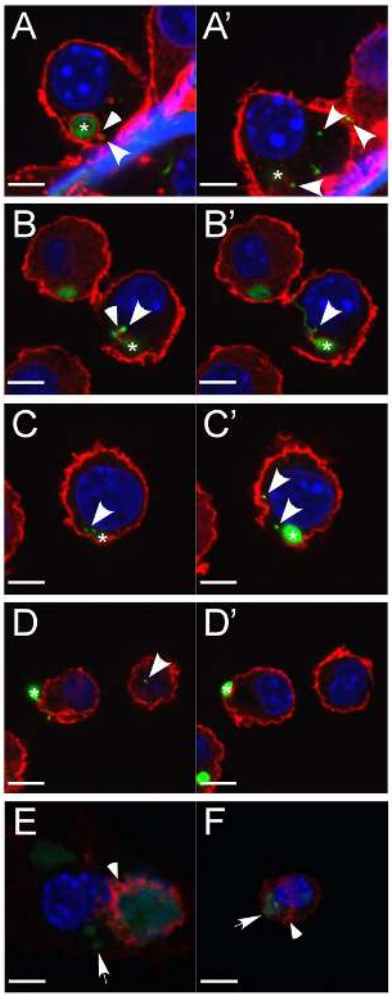

Fig. 5. *Leishmania* exosomes are released into infected macrophages. J774 mouse macrophages were incubated with (**A-D**) GFP-expressing *L. donovani* or (**E**,**F**) endotoxin-free exosomes from GFP-Ld at 50 μg/ml. After 2 hours, cells were processed for imaging by confocal microscopy: red, host actin; blue, nuclei. Single *z*-plane images are shown. Asterisks indicate internalized *Leishmania*. Convex arrowheads indicate actin rings. Concave arrowheads indicate exosome containing MVBs. Concave arrows indicate internalized exosomes. Scale bars: 5.4 μm (A,A′); 7 μm (B,B′); 6 μm (C,C′); 9 μm (D,D′); 5.4 μm (E); 13 μm (F).

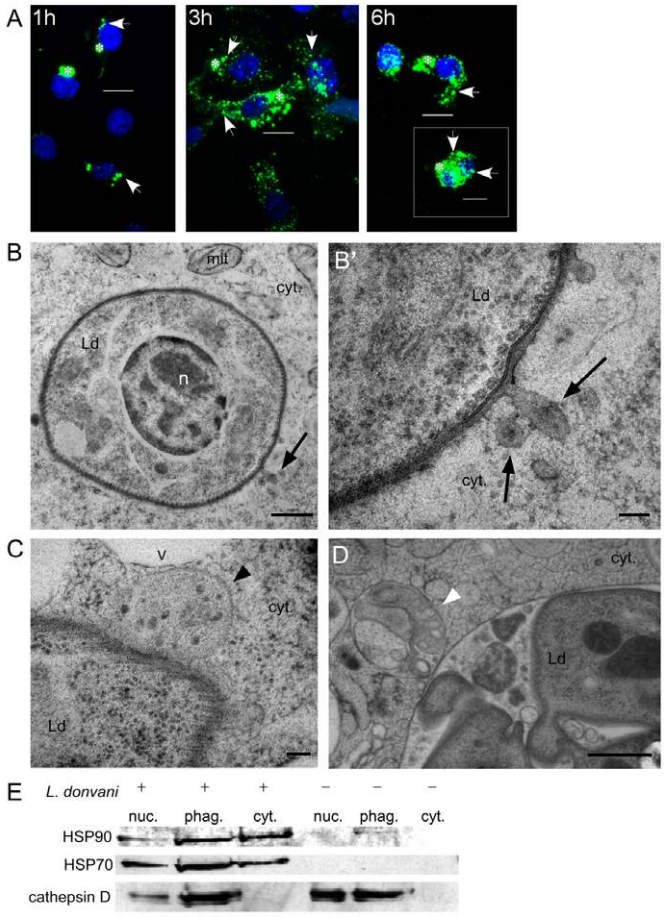

Fig. 6. *Leishmania* exosomal markers are delivered to the cytoplasm of infected macrophages. (**A**) Promastigotes with FITC- surface proteins were added to RAW cells and infected cells were processed for confocal microscopy at 1, 3 and 6 hours after infection (6 hour insert is another representative cell from the same slide). White asterisks indicate *Leishmania*, arrowheads indicate exported proteins. Scale bars: 50 μm. (**B-D**) High-pressure frozen *L. donovani*-infected THP-1 cells. (**B**) Exosome-like microvesicles associated with the PLM, arrow. Scale bar: 500 nm. (**B′**) ×10,000 magnification of the microvesicles in (B). Scale bar: 100 nm. (**C**) MVB (black arrowhead) associated with the PLM. Scale bar: 100 nm. (**D**) MVB (white arrowhead) associated with the PLM of a *Leishmania* in the processes of being degraded. Scale bar: 500 nm. Ld, *L. donovani*; n, *Leishmania* nucleus; cyt, macrophage cytoplasm; mit, macrophage mitochondrion; v, macrophage vacuole. (**E**) The cytosol of infected and uninfected RAW cells was isolated after 6 hours of infection. The blot was probed with α-mature cathepsin D to control for disruption of the phagosome. The data represent three identical experiments with similar results.

in a time-dependent manner (Fig. 6A, concave arrowheads), These punctate structures were of an appropriate size for MVBs containing multiple FITC-labeled exosomes (Fig. 6A) and ultrastructural studies provided additional support for this.

Examination of *Leishmania*-infected cells by TEM after high-pressure cryopreservation provided direct ultrastructral evidence that *Leishmania* mediate protein release into infected cells using exosomes. We observed vesicles with the morphology of exosomes apparently budding from the phagolysosomal membrane (PLM) of internalized *Leishmania*, both as lone vesicles 30-70 nm in diameter with the classic cup shape of exosomes (Fig. 6B,B′), and also within MVBs (Fig. 6C). Note that the intraluminal vesicles of the MVB in Fig. 6C are uniform in size, ranging from 30-50 nm, as well as in electron density; similarly to the purified exosomes described earlier (Fig. 1F-G). By contrast, Fig. 6D shows a dead *Leishmania* undergoing early degeneration where no exosomal budding can be seen. This implies, as would be expected, that exosome release from the phagosome requires intact, viable amastigotes. Moreover, Fig. 6D shows an apparent MVB (white arrowhead) associated with the PLM. However, its morphology is highly disorganized and strikingly different from the MVB in Fig. 6C, consistent with the degenerative state of the organism in the phagolysosome.

To examine whether *Leishmania* exosomal cargo is delivered to the cytoplasm of infected or exosome-treated cells, we used *Leishmania*-specific antibodies against HSP70 and HSP90. Both proteins were detected in the cyotosolic compartment of

Leishmania-infected macrophages (Fig. 6E). It is important to note that this finding was not an artifact due to disruption of the phagosome during subcellular fractionation, because the phagolysosomal marker mature cathepsin D was not detected in these cytosolic fractions (Fig. 6E). By contrast, none of the *Leishmania* exosomal markers (HSP70, HSP90) found in the cytosol of infected cells [including EF-1α and aldolase (Nandan et al., 2002; Nandan et al., 2007)] were detected by western blotting in the cytosol or whole-cell lysates of exosome-treated cells (data not shown). This might seem contradictory to our findings that *Leishmania* GFP$^+$ exosomes bound to and appeared to be internalized by treated macrophages (Fig. 5E-F). However, we believe these results indicate that once *Leishmania* exosomes are bound to naive macrophages, they are rapidly internalized and subsequently undergo processing and degradation. Whatever these events involve, it appears that the fate of these exosomes is somewhat different from exosomes that arise from within an infected cell.

In summary, we have observed: (1) the release of *Leishmania* GFP$^+$ vesicles into infected cells (Fig. 5A-D); (2) isolated vesicles with exosome morphology and MVBs containing similar vesicles budding from the phagolysosome of live internalized *Leishmania* (Fig. 6B-C); (3) the uptake of GFP$^+$ exosomes by treated, uninfected cells (Fig. 5E-F); and (4) *Leishmania* exosomal proteins in the cytosol of infected macrophages (Fig. 6E). Together, these data suggest that *Leishmania* use exosomes to deliver molecular messages to infected as well as neighboring uninfected macrophages. In addition, our data suggests that *Leishmania* might use these vesicles to deliver cargo to the cytoplasm of infected cells.

Leishmania exosomes selectively induce macrophage secretion of interleukin-8

Having shown internalization of *Leishmania* exosomes by treated macrophages, we then examined whether exosome treatment altered macrophage cytokine production. Incubation of differentiated THP-1 cells with endotoxin-free *Leishmania* exosomes did not result in induction of TNF-α secretion, although both LPS and IFNγ brought about such a response (data not shown). By contrast, exosome treatment did induce macrophage secretion of IL-8 in a dose-dependent manner (Fig. 7) and this was qualitatively similar to what was observed with infection per se. IL-8 was induced by vesicles harvested at both neutral and acidic pH (Fig. 7). Moreover, this was true for both *L. donovani* (Fig. 7) and *L. major* exosomes (data not shown). It is clear from these data that *Leishmania* exosomes modify the specific cytokine profile of treated macrophages.

Discussion

The findings reported above indicate that *L. donovani*, *L. mexicana* and *L. major* release microvesicles possessing the known biochemical and morphological characteristics of mammalian

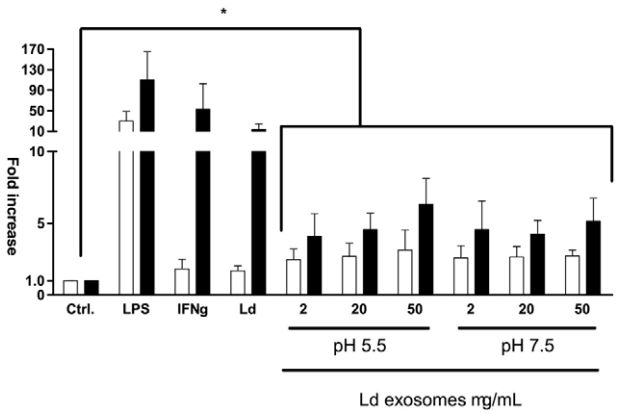

Fig. 7. *Leishmania* exosomes induce IL-8 secretion. Macrophages were treated with endotoxin-free *Leishmania* exosomes for up to 24 hours. Supernatant cytokines were quantified by ELISA. Fold change was determined by dividing the amount of cytokine induced under each condition by that for the untreated control. Fold changes from four separate experiments are shown. White bars, 2 hours; black bars, 24 hours. A significant difference was observed between untreated control cells and all exosome treatments at 24 hours; *$P \leq 0.05$.

exosomes. These *Leishmania* organelles contain exosomal markers, display exosome morphology, and migrate through a linear sucrose gradient in exactly the same manner as classical exosomes (Fig. 1). Moreover, the protein cargo of these exosomes accounts for over half – and very likely more – of total protein secretion by *L. donovani* (Fig. 4). This provides strong evidence for a model in which exosome release is the major mechanism of protein secretion from *Leishmania* spp.

During the past decade, a large number of proteomic studies of mammalian exosomes have been performed (for a review, see Simpson et al., 2008). A common pattern has emerged from these, with approximately 60 proteins or protein families (e.g. histone, annexin) having been identified as classical exosomal markers, whereas other exosomal proteins are recognized as cell-type specific. Our results show that the *Leishmania* exosome proteome overlaps with the common mammalian exosome proteome by >50% (Fig. 4D and supplementary material Table S1), firmly establishing the *Leishmania* secreted microvesicles as exosomes.

Leishmania exosome phenotype is modulated in response to infection-like conditions

It was of interest to examine whether heat shock, or both heat shock and acidic pH would bring about changes in exosome cargo and release, perhaps reflecting a role for exosomes in the initial infection of a mammalian host. Exosome release increased significantly in response to temperature elevation (Fig. 2). Additionally, the cargo

Table 1. *Leishmania* exosomes carry candidate virulence factors

Functional class	Gene DB accession no.	Protein identity	Enriched in acidic pH	Reference
Immune evasion/suppression	LmjF10.0460	GP63, leishmanolysin	–	(Joshi et al., 2002; Kulkarni et al., 2006)
	LmjF26.0620	Heat shock protein 10	+	(Galli et al., 1996; Johnson et al., 2005b)
	LmjF26.1240	Heat shock protein 70	–	(Cho et al., 2008; 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)

of *Leishmania* exosomes was found to be both temperature and pH sensitive. Not only was there specific packaging of individual proteins, we also detected specific functional enrichments based on changes in both temperature and pH (supplementary material Fig. S2 and Table S5). This probably reflects a sophisticated packaging of virulence factors by *Leishmania* in response to specific environments. Table 1 lists a few 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.

Leishmania exosomes are delivered to host cells and facilitate pathogen-host communication

In the present study, using GFP-*Leishmania* we observed the release of *Leishmania*-derived vesicles into infected cells (Fig. 5A-D). Furthermore, after infecting cells with FITC-surface-labeled organisms, we detected the time-dependent accumulation of $FITC^+$ vesicles in the cell cytosol with a size range consistent with MVBs (Fig. 6A). In addition, we observed the uptake of *Leishmania* exosomes from the extracellular environment by naive cells (Fig. 5D-F and Fig. 6A). Taken together, these results suggest a model in which *Leishmania* exosomes deliver cargo to host cells. This model is supported by the detection, both in this report and two previous reports, of the exosomal markers HSP70 and HSP90 (Fig. 6E) as well as EF-1α and aldolase in the cytosol of infected cells (Nandan et al., 2002; Nandan et al., 2007). Moreover, our results clearly show that these exosomes selectively induced IL-8 secretion (Fig. 7). In summary, these results strongly support the conclusion that *Leishmania* use exosomes to deliver effector molecules to host cells as well as to communicate with the wider host cellular environment.

Induction by *Leishmania* exosomes of macrophage IL-8 might be important in disease pathogenesis. A model has emerged recently to suggest that *Leishmania* use neutrophils as Trojan horses to deliver themselves to macrophages via a 'silent phagocytosis' pathway to avoid cell activation (van Zandbergen G. et al., 2004). Clearly, our results show that exosomes from *L. donovani*, as was true for infection per se, induced IL-8 secretion (Fig. 7), a response that is likely to bring about the early recruitment of neutrophils to the site of infection (Peters and Sacks, 2009). Understanding the full impact of the interaction of *Leishmania* exosomes with host cells and the molecules responsible for these interactions is the focus of ongoing work.

Our results suggest three possible mechanisms for delivery of exosomes or exosomal cargo to infected cells. First, as shown in Fig. 6B,B′, *Leishmania* exosomes might bud off the PLM as de novo microvesicles. In this case, the exosomal cargo could be transported to the host cytosol either by membrane transporters embedded in the exosomal membrane (see Fig. 8, mechanism 1 and Table 2) or via retrograde trafficking to the Golgi. In this regard, *Leishmania* exosomes contain many proteins involved in direct membrane transport (Table 2 and supplementary material Tables S1, S5 and S6) and these transporters could be used to transport exosomal cargo into the macrophage cytosol. Conversely, depending upon their orientation, the transporters could move host anti-*Leishmania* effectors into exosomes, effectively sequestering them within these vesicles, thereby abrogating their effects. Second, the ultrastructural findings of Fig. 6C, depicting an MVB associated with the phagosome, in particular support a mechanism whereby exosomes could be reverse endocytosed out of the phagosome as

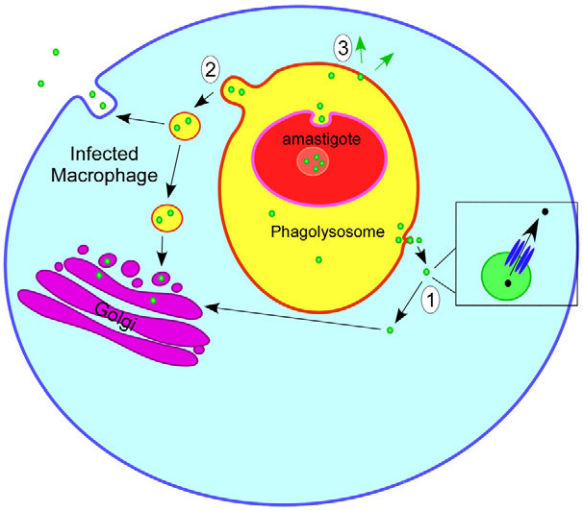

Fig. 8. Three potential mechanisms by which *Leishmania* exosomes might deliver cargo to the host cell cytoplasm. (1) Direct membrane fusion with the PLM, releasing exosome cargo into cytoplasm. (2) Retrograde trafficking of *Leishmania* exosomes through the host vesicle trafficking system. (3) Transport of cargo across the exosomal membrane by a transport protein complex.

MVBs into the cell, similarly to what was observed with trichosanthin-loaded exosomes (Zhang et al., 2009). From this location, they could hijack the host retrograde trafficking pathway to deliver *Leishmania* exosomes to the host trans-Golgi (Fig. 8, mechanism 2). Here, the cargo would have access to the entire host secretion system, including the cytosol (Vago et al., 2005). Third, as demonstrated by Fig. 5D-F, *Leishmania* exosomes released by promastigotes or amastigotes could bind to and perhaps fuse with the plasma or phagolysosome membrane, respectively, directly dumping exosomal cargo into the host cell (see Fig. 8, mechanism 3).

Conclusions

For many years, a mechanism accounting for protein secretion from *Leishmania* has remained elusive. The findings presented in this report identify a novel exosome-based pathway as a general mechanism of protein secretion used by *Leishmania* spp. Moreover, our data suggest that this pathway participates in pathogen-to-host communication and in the delivery of exosomal cargo into

Table 2. Transmembrane-transport-related proteins in *Leishmania* exosomes

Transporter category	Transported compounds	GeneDB accession no.
ATP-binding-cassette transporters	Lipids and sterols Metabolic products Drugs	LmjF29.0620 LmjF27.0980 LmjF15.0890 LmjF06.0080
ATPase subunits	Cations	LmjF25.1170 LmjF35.2080 LmjF05.0500 LmjF31.1220
Nucleoside transport	Nucleobases and nucleosides	LmjF10.0380 LmjF36.1940
Glucose transport	Glucose	LmjF36.6290

macrophages. The results also show that *Leishmania* upregulate exosome production and modify exosomal cargo in response to environmental factors that mimic infection. The specific identities of the cargo proteins within *Leishmania* exosomes suggest that they have the potential to modulate macrophage cell regulation and functional properties. Indeed, the finding that these vesicles selectively induced IL-8 secretion provided direct evidence to support this. Although this study, by design, focused on the protein cargo found in *Leishmania* exosomes, it is also highly likely that other classes of bioactive molecules contribute to the biological properties of these microvesicles. It has recently been shown that exosomes from human mast cells are a source of bioactive shuttle RNAs – both mRNAs and microRNAs – that can be transferred between cell types (Valadi et al., 2007). Our initial unpublished results show that *Leishmania* exosomes indeed contain RNAs and these vesicles could serve as an excellent delivery mechanism into host cytosol.

The implications for the discovery of exosomes as the major mechanism of secretion from *Leishmania* are significant. For example, our preliminary data show that *Leishmania* isolates of the same species, but causing divergent disease phenotypes clinically, displayed distinct exosome proteomic profiles. This suggests that exosome-based secretion contributes to different disease phenotypes. In addition, investigation of *Leishmania* exosomes has the potential to highlight important drug targets that are active in the host cytoplasm and are released extracellularly, where they are relatively accessible locations for drug targeting. Finally, the biological similarity between *Leishmania* and other trypanosomatids is very high, and insights from *Leishmania* exosome biology might inform typanosome biology more generally and, for that matter, could have relevance to other eukaryotic intracellular pathogens such as *Toxoplasma* and *Plasmodia spp*.

Materials and Methods

Reagents, materials and antibodies

Except where otherwise noted, reagents were obtained from the Sigma. FBS and RPMI-1640 were purchased from Invitrogen. All ultracentrifugation hardware including tubes, rotors and centrifuges were purchased from Beckman Coulter. *Leishmania*-specific anti-HSP70 and anti-HSP90 were described previously (Hubel et al., 1995). *Leishmania*-specific anti-histidine secreted acid phosphatase (SAcP) was from Dennis Dwyer. Anti-cathepsin-D was purchased from CalBiochem; anti-EF-1α from Upstate Biotechnologies, antibodies against mouse, rabbit and goat HRP-conjugated IgG were from CedarLane; HRP-conjugated and 4-nm-gold-conjugated anti-chicken IgY was from Jackson ImmunoResearch.

Cell culture

L. donovani Sudan S2, *L. mexicana* MNYC/BZ/62/M379 and *L. major* Fredlin (MHOM/IL/80/Friedlin) promastigotes were cultured as described (Hubel et al., 1995; Silverman et al., 2008). The murine macrophage cell lines RAW264.7 and J774, and the human pro-monocytic cell line THP-1 were cultured in RPMI-1640 + 10% FBS, as described (Bhatnagar et al., 2007; Nandan et al., 2002). *Leishmania* were transfected with pXG-eGFP (a gift from Stephen Beverley, Washington University, St Louis, MO) as described and selected for by semi-solid plating (Ha et al., 1996). GFP expression was maintained with 0.5-1 μg/ml G418.

Isolation of exosomes

Stationary-phase promastigotes, ~50.0×10^6 cells/ml, were washed three times with HBSS to remove FBS and resuspended in buffered exosome collection medium (ECM). To collect exosomes under conditions of neutral pH, RPMI-1640 was buffered with 20 mM HEPES and supplemented with 2 mM L-glutamine, 1% penicillin-streptomycin, and 1% D-glucose. For collection under acidic conditions, RPMI-1640 was buffered with 25 mM MES (Debrabant et al., 2004) and supplemented as above. The pH of MES-buffered ECM was lowered to pH 5.5 using HCl.

Exosomes were isolated as described (Raposo et al., 1996) with minor modifications. After 24 hours, promastigotes were removed from ECM by centrifugation at 300 ***g*** for 10 minutes. Cell pellets were washed three times in PBS + 1 mM EDTA and 10 μg/ml PMSF and stored at –70°C. The cell-free ECM was centrifuged at 700 ***g*** for 30 minutes and 15,000 ***g*** for 45 minutes to remove residual cells and debris. Exosomes were pelleted from the cleared ECM at 110,000 ***g*** for 1 hour in a Type 70ti rotor followed by resuspension in either 1 ml cold PBS, or 1 ml of 2.5 M sucrose (20 mM HEPES, pH 7.4). For immediate analysis, exosomes in PBS were concentrated further by ultracentrifugation at 110,000 ***g*** for 1.5-2 hours in a TLA-100.3 rotor and resuspended in 50-100 μl PBS.

Purification of exosomes

Exosomes were purified by flotation in a linear sucrose gradient as previously described (Raposo et al., 1996). Briefly, pelleted exosomes were resuspended in 2.5 M sucrose, which was overlaid with a step-wise gradient (2.5-0.25 M sucrose, 20 mM HEPES, pH 7.4) followed by ultracentrifugation at 200,000 ***g*** for 15-20 hours at 4°C in a SW40 rotor. Gradient fractions (1 ml) were harvested from the bottom of the tube with a 23-gauge needle. Fraction densities were read with a hand-held refractometer followed by dilution in 23 ml PBS and ultracentrifugation at 110,000 ***g*** for 2 hours in a Type 70 Ti rotor to pellet vesicles. Pellets were immediately solubilized by boiling for 10 minutes in 100 μl reducing Laemmli sample buffer. Samples were either stored at –20°C or immediately resolved by SDS-PAGE followed by western blotting using standard procedures, as previously described (Silverman et al., 2008).

Exosome isolation for proteomic analysis

To increase yield and purity of the exosomes, we substituted the 110,000 ***g*** spins with filter concentration (Lamparski et al., 2002). The cleared conditioned medium (see above) was concentrated in a 100 kDa MWCO Centricon Plus-70 (Millipore), and purified in a sucrose cushion consisting of 4 ml PBS layered on top of 0.5 ml of 2.5 M sucrose (20 mM Tris-HCl, pH 7.4 in D_2O) underlying 0.75 ml of 1M sucrose. The cushions were ultracentrifuged (SW55 rotor) at 200,000 ***g*** for 3 hours. Cushion fractions were collected from the bottom of the tube and the 1 M sucrose, the exosome-containing, fraction was washed and concentrated with a 100 kDa MWCO Ultrafree-15 filter (Millipore), followed by pelleting at 110,000 ***g*** in a TLA-100.3 rotor for 1.5 hours. The exosome pellet was solubilized in digestion buffer (50 mM NH_4OH, 1% sodium deoxycholate, pH 8.0), denatured by boiling for 10 minutes, and adjusted to a protein concentration of 1 g/ml using the Pierce Micro-BCA Protein Assay. 25 μg exosome protein was trypsinized, as previously described (Silverman et al., 2008).

Peptide labeling and mass spectrometry

Tryptic peptide digests were desalted and concentrated as described previously (Chan et al., 2006) and reductive dimethylation using formaldehyde isotopologues was performed to differentially label peptides from the different growth conditions. Peptides from pH 5.5 cultures and from 26°C cultures were labeled with light formaldehyde (CH_2O) whereas peptides from pH 7 cultures and from 37°C cultures were labeled with heavy formaldehyde (C^2H_2O). The labeling reactions were performed as described (Chan and Foster, 2008) and combined peptide samples were analyzed by LC-MS/MS with a linear-trapping quadrupole-OrbitrapXL (ThermoFisher Scientific).

LC-MS/MS data analysis

Fragment spectra were searched against the *L. major* (May 2006 compilation, 17392 sequences) protein database using Mascot (v2.2, Matrix Science) with the following parameters; trypsin specificity allowing up to one missed cleavage, cysteine carbamidomethylation as a fixed modification and heavy and light dimethylated lysine side chains and peptide amino termini as variable modifications, ESI-trap fragmentation characteristics, 3 p.p.m. mass tolerance for precursor ion masses, 0.8 Da tolerance for fragment ion masses. As we have published previously (Silverman et al., 2008), proteins were considered identified when at least two unique peptides of eight or more amino acids and with Mascot IonsScores >25 resulting in an estimated false discovery rate of less than 0.5% based on reversed database searching. All peptide and protein identification information acquired in this study can be found in supplementary material Table S1 and all quantitative information can be found in supplementary material Table S2.

Gene ontology annotation enrichment analysis

The AmiGO term enrichment tool (Boyle et al., 2004; Carbon et al., 2009) performed the GO enrichment analysis. Test sets examined were proteins with peptide ratios falling above the 75th percentile: >3.789 in the 37°C/26°C analysis and >1.258 in the pH comparison, and below the 25th percentile: <1.884 in the temperature analysis and <0.861 in the pH comparison. The exosome proteomes (n=233 for 37°C/26°C; n=175 for pH 7.5/pH 5.5) were the reference data sets. Only 159 of 175 proteins from the pH experiment and 216 of 233 proteins from the temperature experiment had assigned annotations (supplementary material Table S5). The remaining proteins were hypothetical and have no assigned GO terms.

Exosome isolation for macrophage treatment

Endotoxin-free exosomes were isolated following the procedure for proteomic analysis substituting all reagents with culture-grade solutions. In addition, concentration of vesicles prior to sucrose purification was performed using 100,000 kDa MWCO VivaCell 100 filtration devices (Sartorius AG). The 1 M sucrose was filtered through Mustange E endotoxin removal filters (Pall Corporation). The Ultraclear 5 ml tubes were incubated with 30% H_2O_2 for 4 hours to remove endotoxin, followed by extensive washing with water. 2 ml PBS was underlaid with 0.75 ml of 1 M sucrose and concentrated samples were then overlaid. After ultracentrifugation, the exosome fractions were collected from

the top. After resuspension in 15 ml PBS, sucrose was removed by concentration to ~200 µl with 100,000 kDa MWCO Vivaspin 20 filtration devices (Sartorius AG). 20 µl of 1 M sucrose was added to exosomes before storage at –80°C.

Leishmania viability after exosome collection

After removal of supernatant for exosome isolation, 50 million *Leishmania* were washed in PBS and resuspended in 100 µl PBS + 1 ng/ml fluorescein diacetate. After 15 minutes in the dark, the cells were washed and resuspended in 200 µl PBS, 0.4% paraformaldehyde (PFA) + 2 ng/ml propidium iodide. The populations were analyzed on a FACSCanto Flow Cytometer (BD Biosciences) using FL1 and FL3. Dead cell controls were generated by incubation with 10% PFA for 30 minutes followed by washing and staining.

Macrophage infection and exosome treatment

Infection of macrophages, confocal microscopy and isolation of cytosolic fractions were performed as described (Nandan et al., 2002). For confocal microscopy, surface proteins of stationary phase promastigotes were covalently labeled (or not) with a FITC-succinimidyl ester (Invitrogen) following the manufacturer's instructions. Washed stationary phase *L. donovani* were incubated in dye solution in the dark for 1 hour at room temperature. *Leishmania* (+GFP or +FITC) were resuspended in 1 ml RPMI-1640 and added to macrophages (RAW or J774) grown on sterile glass coverslips at a MOI of 20:1. The infection proceeded at 37°C. Infected cells were washed, fixed with 4% PFA, permeabilized with 0.01% Triton X-100, incubated with either 5 U/ml Alexa Fluor 546 Phalliodin (Invitrogen) or 10 ng/ml Hoechst 33342 and mounted in Prolong Gold with DAPI (Invitrogen) or SlowFade (CalBiochem), respectively. GFP-Ld infections were imaged at ×100 with a Leica TCS SP2 inverted microscope at the iCapture Institute (Vancouver, BC, Canada). Time-course images were captured at ×63, with a BioRad Radiance 2000 scanning confocal system coupled to a Nikon Eclipse TE300 inverted epi-fluorescence microscope. Brightest-point projections of serial *z*-stacks were generated with ImageJ software

Endotoxin-free exosomes collected from *L. donovani* at 37°C ± pH 5.5 were incubated with differentiated THP-1 cells in 96-well plates. In parallel, cells were treated with 1 µg/ml *E. coli* 0111:B4 LPS, 1 ng/ml human IFN-γ (BioSource International) and *L. donovani* at an MOI of 10:1, as positive controls. Cell-free supernatants were collected at indicated times and stored at –80°C. Cytokines were quantified by ELISA: IL-8 ELISA Kit (BD Biosciences Pharmingen), TNF-α ELISA reagents (EBiosciences).

Electron microscopy

For analysis by electron microscopy, purified exosomes were fixed in 2% glutaraldehyde, 4% PFA in cacodylate buffer for 2 hours at room temperature. Samples were washed and absorbed onto formvar/carbon-coated copper grids for 10 minutes. Grids were washed and stained with 1% aqueous uranyl acetate (Ted Pella). Samples were viewed on a JEOL 1200EX TEM (Jeol, Peabody, MA). Immunolabeling was done following a published procedure (Brandau et al., 1995), and primary antibodies were detected with 4 nm gold anti-chicken secondary antibody. Grids were imaged with a 1K AMT side mount camera on a Hitachi H7600 TEM operated at 80 kV.

Mid-log *L. mexicana* promastigotes were washed twice in Dulbecco's PBS and then fixed for 24 hours (4°C, 1% paraformaldehyde, 0.025% glutaraldehyde, 0.2 M sodium cacodylate-HCl, pH 7.2). Sections were stained with 2% uranyl acetate and Reynold's lead citrate (Giddings, 2003), followed by examination with a Philips CM 10 TEM at room temperature. Negative images were digitized using a Nikon Super Coolscan 4000 film scanner and processed using Adobe Photoshop CS3.

THP-1 cells were infected with stationary phase *L. donovani* promastigotes, as described (Nandan et al., 1999). After 24 hours, cells were dislodged by up and down pipetting in cold PBS without Mg^{2+} and Ca^{2+}, pelleted by centrifugation at 300 ***g*** and, and resuspended in 25 µl 20% BSA in PBS. 1-2 µl of this material was high-pressure frozen, freeze substituted in 1% osmium tetroxide and 0.1% uranyl acetate, embedded and polymerized in Spurr-Epon resin, as described (Giddings, 2003). Grids with 60 nm sections were imaged as for immunolabeled exosomes.

Graphics and statistical analysis

Except where noted, all micrographs were processed with Adobe Photoshop Elements 7. All graphs and statistical analyses were generated in GraphPad Prism 4.00. Proportional Venn diagrams were created with the DrawVenn Application (Chow and Rogers, 2005).

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/6/842/DC1

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