Exclusion of synaptotagmin V at the phagocytic cup by *Leishmania donovani* lipophosphoglycan results in decreased promastigote internalization

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Regulators of membrane fusion play an important role in phagocytosis, as they regulate the focal delivery of endomembrane that is required for optimal internalization of large particles. During internalization of *Leishmania* promastigotes, the surface glycolipid lipophosphoglycan (LPG) is transferred to the macrophage membrane and modifies its fusogenic properties. In this study, we investigated the impact of LPG on the recruitment of the exocytosis regulator synaptotagmin V (Syt V) at the area of internalization and on the early steps of phagocytosis. Using *Leishmania donovani* LPG-defective mutants and LPG-coated particles, we established that LPG reduces the phagocytic capacity of macrophages and showed that it causes exclusion of Syt V from the nascent phagosome. Silencing of Syt V inhibited phagocytosis to the same extent as LPG, and these effects were not cumulative, consistent with a Syt V-dependent mechanism for the inhibition of phagocytosis by LPG. Previous work has revealed that LPG-mediated exclusion of Syt V from phagosomes prevents the recruitment of the vacuolar ATPase and acidification. Thus, whereas exclusion of Syt V from phagosomes in the process of formation may be beneficial for the creation of a hospitable intracellular niche, it reduces the phagocytic capacity of macrophages. We propose that the cost associated with a reduced internalization rate may be compensated by increased survival, and could lead to a greater overall parasite fitness.

Received 6 April 2011
Revised 30 May 2011
Accepted 14 June 2011

INTRODUCTION

The various species of the protozoan parasite *Leishmania* are responsible for a spectrum of human diseases ranging from a relatively confined cutaneous lesion to a progressive visceral infection that can be fatal. Transmission of the parasite is mediated by blood-sucking sandflies, either of the genus *Phlebotomus* or of the genus *Lutzomyia*. Upon the bloodmeal of an infected sandfly, infectious promastigotes are inoculated into the mammalian host, where they are phagocytosed by macrophages. Promastigotes subsequently differentiate into amastigotes and replicate within phagolysosomal vacuoles (Alexander & Russell, 1992).

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Abbreviations: BMM, bone marrow-derived macrophages; LPG, lipophosphoglycan; LPG-Zym, zymosan coated with purified LPG; M β CD, methyl- β -cyclodextrin; siRNA, small interfering RNA; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; Syt, synaptotagmin; WT, wild-type; Zym, zymosan.

Internalization of *Leishmania* promastigotes is a classical receptor-mediated endocytic event involving multiple *Leishmania* and macrophage surface molecules. On macrophages, the complement receptors 1 and 3, the mannose receptor p150,95, DC-SIGN, and the scavenger receptors have been implicated in promastigote binding, although their relative roles in internalization remain to be firmly established (Akilov *et al.*, 2007; Colmenares *et al.*, 2004; Gomes *et al.*, 2009; Mosser, 1994). Two major *Leishmania* promastigote surface components have been shown to participate in the attachment process. One is the glycosylphosphatidylinositol (GPI)-anchored metalloprotease gp63, which acts as a primary acceptor for C3 deposition on the promastigote surface (Brittingham *et al.*, 1995; Russell, 1987). The second is lipophosphoglycan (LPG), an abundant virulence-related surface glycolipid consisting of a polymer of Gal β (1,4(-Man(α 1)-PO₄ units anchored into the promastigote membrane via an unusual GPI (Descoteaux & Turco, 1999). LPG has been reported to be the parasite receptor for macrophages, and has been proposed to be involved in the initiation of infection

through interactions with CR3 and p150,95, members of the CD18 family of integrins (Handman & Goding, 1985; Talamás-Rohana *et al.*, 1990). However, subsequent studies have revealed that internalization of LPG-defective *Leishmania* promastigotes is superior to that of wild-type (WT) parasites (Descoteaux *et al.*, 1992; Holm *et al.*, 2003; McNeely & Turco, 1990; Späth *et al.*, 2003), suggesting that LPG is unlikely to play a major role in promastigote adhesion to macrophages and that it may possibly interfere with the phagocytic process.

To accommodate the extension of the plasma membrane that takes place during the phagocytosis of large particles such as *Leishmania* promastigotes, focalized exocytosis of endomembrane occurs at the phagocytic cup (Booth *et al.*, 2001; Hackam *et al.*, 1998; Huynh *et al.*, 2007). Various intracellular compartments, including recycling endosomes, late endosomes and the endoplasmic reticulum, contribute membrane for phagosome formation through fusion events regulated by soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs), such as VAMP3, VAMP7 and syntaxin 18 (Bajno *et al.*, 2000; Braun & Niedergang, 2006; Cox *et al.*, 2000; Desjardins, 2003; Hatsuzawa *et al.*, 2006; Niedergang *et al.*, 2003). The activity of SNARE complexes is regulated by synaptotagmins (Syts), a family of transmembrane proteins containing two tandem C2 domains that act as Ca^{2+} sensors (Chapman, 2008; Jahn *et al.*, 2003). The best-characterized Syt in phagocytosis is the lysosomal Syt VII, which regulates Ca^{2+} -dependent exocytosis of lysosomes (Martinez *et al.*, 2000) and the delivery of lysosomal membrane to the phagosome (Czibener *et al.*, 2006). More recently, we identified Syt V as a recycling endosome-associated protein recruited to the forming phagosome, and we showed that this exocytosis regulator controls the phagocytic process (Vinet *et al.*, 2008).

Upon contact between promastigotes and macrophages, LPG transfers from the parasite surface to the nascent phagosome membrane (Tolson *et al.*, 1990), where it disrupts existing lipid microdomains and alters the formation of these structures after promastigote internalization (Dermine *et al.*, 2005; Winberg *et al.*, 2009). One consequence of LPG membrane insertion is the exclusion of Syt V (Vinet *et al.*, 2009). Given the role of Syt V in the regulation of phagocytosis, we investigated the impact of LPG on the association of Syt V to the area of internalization and on the initial steps of phagocytosis. We provide evidence that LPG impairs the recruitment of Syt V to the nascent phagosome, resulting in a reduction in the phagocytic capacity of host macrophages.

METHODS

Macrophages. All animals were handled in strict accordance with good animal practice as defined by the Canadian Council on Animal Care, and all animal work was approved by the Comité institutionnel de protection des animaux of INRS-Institut Armand-Frappier (protocol 0811-08). Bone marrow-derived macrophages (BMM) were

obtained by growing bone marrow cells from female BALB/c mice at 37 °C in 5 % CO_2 for 7 days in Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine (Life Technologies) supplemented with 10 % heat-inactivated fetal bovine serum (Hyclone), 10 mM HEPES (pH 7.4) and antibiotics (complete medium) in the presence of 15 % (v/v) L929 cell-conditioned medium as a source of colony-stimulating factor-1 (Descoteaux & Matlashewski, 1989). BMM were made quiescent by culturing them in the absence of colony-stimulating factor-1 for 18 h prior to being used. The murine macrophage cell line RAW 264.7 was grown in complete medium in a 37 °C incubator with 5 % CO_2 . Stably transfected RAW 264.7 cells expressing Syt V–GFP (Syt V–GFP RAW 264.7 cells) have been described previously (Vinet *et al.*, 2008).

Parasites. *Leishmania donovani* promastigotes (Sudanese strain 1S) derived from splenic amastigotes were grown at 26 °C in RPMI 1640 medium supplemented with 20 % heat-inactivated fetal bovine serum, 100 μM adenine, 20 mM MES (pH 5.5), 5 μM haemin, 3 μM bioppterin, 1 μM biotin and antibiotics. The isogenic mutants *lpg1*-KO and *lpg2*-KO have been described previously (Privé & Descoteaux, 2000). The *lpg1*-KO mutant secretes repeating $\text{Gal}(\beta 1,4)\text{Man}(\alpha 1)\text{-PO}_4$ -containing molecules, but lacks the ability to assemble a functional LPG glycan core (Huang & Turco, 1993), precluding synthesis and expression of LPG. The *lpg2*-KO mutant expresses the truncated LPG $\text{Gal}(\alpha 1,6)\text{Gal}(\alpha 1,3)\text{Gal}(\beta 1,3)[\text{Glc}(\alpha 1\text{-P})]\text{Man}(\alpha 1,3)\text{Man}(\alpha 1,4)\text{GlcNAc}(\alpha 1,6)\text{-phosphatidyl inositol}$, because it is unable to add repeating $\text{Gal}(\beta 1,4)\text{Man}(\alpha 1)\text{-PO}_4$ units to LPG (Descoteaux *et al.*, 1995). The *lpg2*-KO + LPG2 add-back was grown in the presence of 50 μg G418 ml^{-1} . For infections, promastigotes were used in the late stationary phase of growth.

Reagents and antibodies. The affinity-purified rabbit anti-Syt V antibody has been described previously (Saegusa *et al.*, 2002). The mouse monoclonal antibody against LPG (CA7AE) was from Cedarlane Laboratories (Tolson *et al.*, 1989). Methyl- β -cyclodextrin (M β CD) was from Sigma. LPG was isolated from exponential phase cultures of *L. donovani* promastigotes as previously described (Orlandi & Turco, 1987; Russo *et al.*, 1992).

RNA interference. Syt V silencing by RNA interference was performed as previously described (Vinet *et al.*, 2008) by using a small interfering RNA (siRNA) corresponding to nucleotides 94–112 of Syt V cDNA (Iezzi *et al.*, 2005), whereas an siRNA specific to GFP was used as a negative control (Flandin *et al.*, 2006). Adherent RAW 264.7 cells were transfected with siRNA duplexes at a final concentration of 240 nM using Oligofectamine reagent (Invitrogen) as described by Flandin *et al.* (2006). A BLAST search against the mouse genome sequence database was performed to ensure that the chosen siRNA sequences targeted only the mRNA of interest.

Cholesterol depletion. Cholesterol depletion was achieved by incubating macrophages with 10 mM M β CD (Sigma) in serum-free medium at 37 °C for 1 h, followed by washing with PBS before particle internalization, as described previously (Vinet *et al.*, 2009). The effect of this treatment on lipid raft integrity was monitored by confocal microscopy following labelling of macrophages with Alexa Fluor 594-coupled cholera toxin β (Molecular Probes).

Coating and opsonization of particles. Purified LPG was sonicated and added to the zymosan (Zym) particles at a final concentration of 25 μM in PBS, pH 7.3, incubated at 37 °C for 1 h, washed and resuspended in complete medium prior to phagocytosis experiments. The efficiency of LPG coating was assessed by immunofluorescence using the anti-LPG antibody CA7AE. Serum opsonization of *L. donovani* promastigotes was done as described previously (Lodge & Descoteaux, 2005), and serum opsonization of Zym was carried out by incubating the particles in DMEM

supplemented with 10 % mouse serum for 30 min at 37 °C prior to phagocytosis.

Phagocytosis assays. For synchronized phagocytosis assays, macrophages were incubated with particles (Zym or *L. donovani* promastigotes) at a particle-to-cell ratio of 15:1 (unless otherwise specified) for 15 min at 4 °C. Excess particles were removed by several thorough washes with DMEM, and phagocytosis was triggered by transferring the cells to 37 °C for the indicated time points before processing for microscopy.

Immunofluorescence. Macrophages were fixed for 10 min using 2 % paraformaldehyde in PBS, permeabilized using 0.1 % Triton X-100, and non-specific surface Fc γ receptor binding was blocked using 1 % BSA, 2 % goat serum, 6 % milk and 50 % fetal bovine serum. For immunostaining, cells were labelled with the appropriate combinations of primary antibodies or antisera (anti-Syt V, anti-LPG), and secondary antibodies (anti-rabbit or anti-mouse Alexa Fluor 488 or 568 conjugates, Molecular Probes). Syt V-GFP cells were fixed and directly incubated with the DNA-detecting far-red-fluorescing dye DRAQ5 before being mounted or subjected to immunofluorescence. The use of Syt V-GFP RAW 264.7 cells to localize Syt V following infection with *L. donovani* promastigotes was necessary because our antiserum against Syt V cross-reacts with *Leishmania* epitopes. All coverslips were mounted on slides with Fluoromount-G (Southern Biotechnology Associates). Detailed analysis of protein presence and localization on the phagosome was performed using an oil immersion Nikon Plan Apo $\times$ 100 (numerical aperture 1.4) objective mounted on a Nikon Eclipse E800 microscope equipped with a Bio-Rad Radiance 2000 confocal imaging system (Bio-Rad Laboratories, Zeiss). Images were obtained using appropriate filters, through the sequential scanning mode of the LaserSharp software (Bio-Rad Laboratories, Zeiss) with a Kalman filter of at least 6.

Statistical analyses. Statistical analyses were performed using Student's two-tailed two-sample unequal variance test.

RESULTS

L. donovani LPG impairs promastigote internalization

To investigate the mechanism by which LPG interferes with the uptake of *L. donovani* promastigotes (Descoteaux *et al.*, 1992; Holm *et al.*, 2003; McNeely & Turco, 1990), we first compared the internalization of WT, LPG-defective *lpg1*-KO mutants, Gal(β 1,4)Man(α 1)-PO $_4$ -defective *lpg2*-KO mutants or *lpg2*-KO add-back (*lpg2*-KO + LPG2) serum-opsonized promastigotes in BMM. Under these experimental conditions, internalization of WT and mutant *L. donovani* promastigotes occurs predominantly via complement receptor 3 (Lodge *et al.*, 2006). Consistent with previous reports, internalization of WT promastigotes was significantly lower with respect to that of either *lpg1*-KO or *lpg2*-KO promastigotes (Fig. 1a) (200 WT parasites per 100 BMM, compared with 360 *lpg1*-KO parasites per 100 BMM and 350 *lpg2*-KO parasites per 100 BMM). Internalization of the *lpg2*-KO add-back (*lpg2*-KO + pLPG2) promastigotes was similar to that of WT promastigotes (Fig. 1a, 230 parasites per 100 BMM). These results confirmed that promastigotes lacking LPG were internalized more efficiently than WT promastigotes.

To directly address the effect of LPG on phagocytosis, we compared the internalization of Zym and Zym coated with purified LPG (LPG-Zym). Fig. 1(b) shows that phagocytosis of unopsonized LPG-Zym was reduced by 2.6-fold with respect to that of unopsonized Zym. A reduction in phagocytosis was also observed for LPG-Zym compared with Zym when particles were opsonized with serum (Fig. 1c). To determine whether impaired phagocytosis was accentuated with an increased number of particles, we performed a phagocytosis assay of serum-opsonized Zym or LPG-Zym at various particle-to-cell ratios. Our results indicated that the inhibitory effect of LPG on phagocytosis increased with the number of particles, ranging from 1.3 for a particle-to-cell ratio of 10:1 to 1.6 for a particle-to-cell ratio of 40:1 (Fig. 1c). Moreover, the percentage of cells containing a high particle load was greater for Zym than for LPG-Zym. Indeed, quantification of the distribution of the particle number per macrophage showed that most macrophages contained five Zym but only one LPG-Zym (Fig. 1d). These results indicated that LPG interfered with the internalization process and that this effect was more important at a high particle load.

Internalization of *L. donovani* promastigotes requires intact lipid microdomains

Previous studies have revealed that LPG is transferred from the promastigote surface into lipid microdomains present in the nascent phagosome membrane, causing a disorganization of these membrane microdomains (Dermine *et al.*, 2005; Tolson *et al.*, 1990; Vinet *et al.*, 2009; Winberg *et al.*, 2009). The well-documented importance of host cell cholesterol-enriched lipid microdomains for microbial invasion (Lafont & van der Goot, 2005; Zaas *et al.*, 2005) suggests that disorganization of those structures by LPG may contribute to the reduced internalization of WT promastigotes and LPG-Zym. To verify this possibility, we examined the role of lipid microdomains in the internalization of serum-opsonized WT and *lpg2*-KO *L. donovani* promastigotes. To this end, we extracted cholesterol from the membranes by treating macrophages with 10 mM M β CD for 1 h before phagocytosis, and we confirmed the efficacy of this treatment by confocal microscopy following labelling of macrophages with Alexa Fluor 594-cholera toxin β . As shown in Fig. 2(a), the distribution of ganglioside GM1 was punctate in control macrophages. In contrast, ganglioside GM1 was evenly distributed in the cellular membrane after cholesterol extraction, indicating disruption of lipid rafts (Fig. 2a) (Orlandi & Fishman, 1998). Infection of M β CD-treated macrophages revealed that cholesterol extraction inhibited the uptake of WT *L. donovani* promastigotes by 72 % (Fig. 2b). Similar to WT promastigotes, uptake of the *lpg2*-KO promastigotes was inhibited by 64 % in macrophages treated with M β CD. Cholesterol extraction had no effect on promastigote attachment to macrophages (data not shown). As a control, we also showed that internalization of Zym was inhibited to a similar extent under these

Fig. 1. LPG decreases the phagocytic capacity of macrophages. (a) BMM cells were infected with WT, *lpg1*-KO, *lpg2*-KO or *lpg2*-KO + LPG2 *L. donovani* promastigotes for 10 min and fixed, and the number of parasites per 100 macrophages was determined. (b) RAW 264.7 cells were allowed to internalize non-opsonized Zym or LPG-Zym for 10 min, and the number of particles per 100 macrophages was determined. (c, d) RAW 264.7 cells were allowed to internalize serum-opsonized Zym or LPG-Zym for 10 min at different particle-to-cell ratios. The number of particles per 100 macrophages (c) and the particle distribution in macrophages for the ratio 40 : 1 were determined (d). For each condition, the phagocytic index was calculated on at least 100 cells in triplicate, and at least three independent experiments were performed. (a–c) Error bars, SD of one representative triplicate determination. * $P \leq 0.005$, ** $P \leq 0.0005$; in (a), P values compare the phagocytic index of macrophages infected with WT and *lpg2*-KO + LPG2 versus *lpg1*-KO and *lpg2*-KO parasites.

conditions (Fig. 2c). These observations are consistent with a role for lipid microdomains in the phagocytic process in general and in the internalization of *L. donovani* promastigotes (Pucadyil *et al.*, 2004; Rodríguez *et al.*, 2006). Moreover, these results are consistent with the hypothesis that insertion into and disorganization of lipid microdomains by LPG (Dermine *et al.*, 2005; Vinet *et al.*, 2009; Winberg *et al.*, 2009) may reduce the phagocytic capacity of macrophages.

LPG-mediated inhibition of phagocytosis is associated with the exclusion of Syt V at the phagocytic cup

Syt V is recruited to the nascent phagosome and participates in phagocytosis, possibly by regulating focal exocytosis of recycling endosomes (Vinet *et al.*, 2008). We recently reported that LPG excludes Syt V from lipid microdomains present in the membrane of maturing

Fig. 2. Cholesterol depletion inhibits the phagocytosis of serum-opsonized *L. donovani* promastigotes. (a) RAW 264.7 macrophages were treated with 10 mM MβCD for 1 h and washed several times prior to phagocytosis. To evaluate the integrity of lipid rafts, cells were treated with 2 μg ml⁻¹ cholera toxin β-Alexa Fluor 594, which binds to the lipid raft marker ganglioside GM1. Left panel, control cells; right panel, cells treated with MβCD. Bars, 5 μm. (b) Macrophages were incubated with either WT or *lpg2*-KO serum-opsonized promastigotes for 30 min. Phagocytosis was determined as the number of internalized parasites per 100 cells. (c) Macrophages were treated as in (a), and were allowed to internalize Zym for 30 min. Phagocytosis was determined as the number of internalized Zym particles per 100 cells. Experiments were done in triplicate and similar results were obtained in three independent experiments. (b, c) **P* < 0.05, ***P* < 0.01, as compared with cells treated with DMSO.

phagosomes (Vinet *et al.*, 2009). This finding raised the possibility that LPG interferes with phagocytosis by inhibiting the recruitment of Syt V to the phagocytic cup. To test this hypothesis, we first incubated Syt V-GFP RAW 264.7 cells (Vinet *et al.*, 2008) with WT, *lpg1*-KO, *lpg2*-KO or *lpg2*-KO + LPG2 serum-opsonized *L. donovani* promastigotes and allowed phagocytosis to proceed for 10 min, before assessing the presence of Syt V-GFP at the phagocytic cup. Whereas Syt V-GFP was detected on over 65 % of phagocytic cups induced by *lpg1*-KO and *lpg2*-KO promastigotes, it was detected on 35 % of phagocytic cups induced by WT and *lpg2*-KO add-back promastigotes (Fig. 3a). These results suggested that LPG interfered with the recruitment of Syt V to the phagocytic cups. We next directly addressed the effect of LPG on the association of Syt V with phagocytic cups, by feeding Syt V-GFP RAW 264.7 cells and BMM with either Zym or LPG-Zym. Recruitment of Syt V-GFP to the phagocytic cup was impaired in the presence of LPG-Zym (50 % of phagocytic cups positive for Syt V-GFP) compared with Zym (over 85 % of phagocytic cups positive for Syt V-GFP) (Fig. 3b, upper panel). We made similar observations for endogenous Syt V in BMM cells, with 20 % of phagocytic cups positive for Syt V in the presence of LPG-Zym, compared with 65 % of phagocytic cups positive for Syt V in the presence of Zym (Fig. 3b, lower panels). Interestingly, Fig. 3(c) shows that in the same cell, Syt V-GFP was excluded from a phagocytic cup containing an LPG-Zym particle, while being recruited to a phagocytic cup containing a Zym particle not coated with LPG. These observations are consistent with the notion that LPG locally impairs recruitment of Syt V to the phagocytic cup.

To further demonstrate that the inhibitory effect of LPG on promastigote internalization was dependent on its action on Syt V, we assessed the effect of Syt V silencing on the phagocytosis of Zym and LPG-Zym in RAW 264.7 cells. As shown in Fig. 4(a), confocal immunofluorescence analysis confirmed the knockdown of Syt V in RAW 264.7 macrophages transfected with siRNA Syt V. Control and siRNA Syt V-treated cells were exposed to various particle-to-cell ratios to mimic an increased membrane demand. Consistent with our previous observations (Vinet *et al.*, 2008), we found that regardless of the particle-to-cell ratio, Syt V silencing caused a significant decrease in the ability of macrophages to internalize Zym (Fig. 4b). In contrast, Syt V silencing did not have a significant effect on the internalization of LPG-Zym for all the tested particle-to-cell ratios (Fig. 4b). Importantly, internalization of LPG-Zym by either control or siRNA Syt V-treated macrophages was similar to the internalization of Zym by cells treated with siRNA Syt V (Fig. 4b). These results indicated that Syt V silencing and LPG impaired phagocytosis to a similar extent, and that these effects were not cumulative, consistent with a Syt V-dependent mechanism for the inhibition of phagocytosis by LPG.

In agreement with its role under phagocytic conditions, where substantial quantities of membrane are required

Fig. 3. Recruitment of Syt V to the phagocytic cup is impaired by LPG. (a) Syt V-GFP RAW 264.7 cells were incubated with WT, *lpg1*-KO, *lpg2*-KO or *lpg2*-KO + LPG2 *L. donovani* promastigotes for 10 min, fixed and stained for DNA. Representative confocal image(s) illustrate the presence of Syt V-GFP on phagocytic cups containing parasites (shown by arrowheads). (b, c) Syt V-GFP

RAW 264.7 cells (b, upper panels; c) or BMM cells (b, lower panel) were allowed to internalize Zym and/or LPG-Zym for 10 min and fixed. Cells were stained for Syt V and LPG (b, lower panel) or for LPG alone (b, upper panel; c). Endogenous Syt V or Syt V-GFP recruitment is indicated by white arrowheads. (a–c) Bars, 3 μ m.

(Vinet *et al.*, 2008), silencing of Syt V had a significant impact on the ability of macrophages to internalize three or more Zyms at a particle-to-cell ratio of 40 : 1 (Fig. 5a). We next compared the distribution of Zym and LPG-Zym in control and siRNA Syt V-treated macrophages to further investigate the effect of LPG on Syt V during internalization. In control macrophages (siRNA GFP- and mock-transfected RAW 264.7 cells), the number of cells containing three or more LPG-Zyms was significantly lower than the number containing three or more Zym particles (Fig. 5b). Interestingly, for siRNA Syt V-treated macrophages, the number of cells containing three or more Zyms was significantly reduced, to levels comparable with the number of cells containing three or more LPG-Zyms. These results indicate that LPG had no additional inhibitory effect on phagocytosis in siRNA Syt V-treated macrophages, supporting the conclusion that exclusion of Syt V from the phagocytic cup is an important factor associated with the inhibitory effect of LPG on phagocytosis.

DISCUSSION

The present study was aimed at investigating the mechanism by which the *L. donovani* promastigote surface glycolipid LPG interferes with the phagocytic process. We report that LPG impairs the recruitment of Syt V to the nascent phagosome, resulting in a critical reduction in the host macrophage phagocytic capacity.

Upon contact between *L. donovani* promastigotes and the macrophage, LPG is incorporated from the parasite surface into the macrophage membrane, where it associates with lipid microdomains. This transfer is facilitated by the detergent-like 1-alkyl-2-lyso-phosphatidylinositol anchor of LPG and causes disorganization of these lipid microdomains (Dermine *et al.*, 2005; Tolson *et al.*, 1990; Winberg *et al.*, 2009). Host cell plasma membrane lipid microdomains are regarded as important regulators in the internalization of various micro-organisms, including *Leishmania* promastigotes (Gatfield & Pieters, 2000; Grassmé *et al.*, 2003; Lafont & van der Goot, 2005; Peyron *et al.*, 2000; Rodríguez *et al.*, 2006; Schneider *et al.*, 2007; Zaas *et al.*, 2005). In this context, lipid microdomains are believed to serve as platforms for actin organization and for clustering of signalling molecules involved in phagocytosis. An additional mechanism by which lipid microdomains may contribute to the phagocytic process may be related to the fact that these structures are also central to regulated exocytosis (Lang, 2007; Salaün *et al.*, 2004), a process essential to the recruitment of endomembrane

Fig. 4. LPG reduces phagocytosis by a Syt V-dependent mechanism. RAW 264.7 cells were transfected with either an siRNA to Syt V or an siRNA to GFP, or only mock-transfected (control), and incubated for 24 h. (a) siRNA to Syt V reduces Syt V levels as assessed by confocal immunofluorescence microscopy using an anti-Syt V antibody. (b) Cells were then allowed to internalize Zym or LPG-Zym for 10 min and fixed. The number of particles per 100 macrophages was determined for the particle-to-cell ratios 10 : 1, 20 : 1 and 40 : 1. For each condition, the phagocytic index was calculated for at least 100 cells in triplicate, and at least three independent experiments were performed. Error bars, SD of one representative triplicate; * $P \leq 0.005$, ** $P \leq 0.0005$.

during phagocytosis (Bajno *et al.*, 2000; Hackam *et al.*, 1998; Huynh *et al.*, 2007). In macrophages, the exocytosis regulator Syt V is a recycling endosome-associated protein that is recruited to the phagocytic cup. Syt V plays a role during phagocytosis possibly through the mobilization of recycling endosomes as a source of endomembrane (Vinet *et al.*, 2008). Our finding that LPG prevented the recruitment to or association with the nascent phagosome of Syt V provides insight into the mechanism by which LPG interferes with the phagocytic process. Indeed, consistent with its role in the regulation of phagocytosis (Vinet *et al.*, 2008), our data show that silencing of Syt V had a significant impact on the phagocytic capacity of macrophages, as evidenced by their greatly reduced ability, with respect to control macrophages, to internalize three or more Zym particles under conditions of high membrane demand. Strikingly, the phagocytic capacity of siRNA Syt V-treated cells towards Zym particles was similar to that of control macrophages towards LPG-Zym particles. In addition, the phagocytic capacity of siRNA-treated macrophages was not further reduced by LPG. These results are consistent with a model in which insertion of LPG into the macrophage membrane at the phagocytic cup modifies the biophysical properties of this membrane. This precludes the recruitment of Syt V-containing endosomes and impairs the endomembrane delivery necessary for efficient internalization.

Work by Hackam and colleagues (Hackam *et al.*, 1998) has revealed that vesicle-SNARE-mediated exocytosis of endomembranes is essential for optimal completion of

particle internalization during phagocytosis. Consistently, several SNAREs, including VAMP3, syntaxin 4 and syntaxin 13, are recruited to the nascent phagocytic cup to mediate the membrane fusion events involved in exocytosis (Bajno *et al.*, 2000; Collins *et al.*, 2002; Fratti *et al.*, 2002; Murray *et al.*, 2005). Similar to Syt V (Vinet *et al.*, 2009), intact lipid microdomains are required for the recruitment of syntaxin 4 and VAMP3 to phagosomes (Kay *et al.*, 2006). Therefore, the finding that LPG locally impairs recruitment of Syt V at the phagocytic cup suggests that LPG may also affect the association of those lipid microdomain-associated SNAREs to the phagocytic cup. Inasmuch as syntaxin 4 and VAMP3 are also necessary for the secretion of TNF- α from recycling endosomes recruited to the phagocytic cup (Kay *et al.*, 2006; Murray *et al.*, 2005), it is reasonable to infer that LPG could interfere with the release of proinflammatory cytokines during phagocytosis. This scenario remains to be experimentally addressed.

Lipid microdomains play an important role in phagolysosome biogenesis (Dermine *et al.*, 2001, 2005), since various processes take place within these domains, including assembly of the NADPH oxidase and the recruitment of the v-ATPase (Dermine *et al.*, 2001; Galli *et al.*, 1996; Lafourcade *et al.*, 2008; Vilhardt & van Deurs, 2004). Disruption of lipid microdomains may therefore represent a very efficient way to create a hospitable intracellular niche (Arellano-Reynoso *et al.*, 2005). In this regard, we have recently shown that one

Fig. 5. Phagocytosis inhibition mediated by LPG and Syt V silencing affects cells with a high particle load. (a, b) RAW 264.7 cells were transfected with either an siRNA to Syt V or an siRNA to GFP, or were only mock-transfected, and were incubated for 24 h. Cells were then allowed to internalize Zym or LPG-Zym for 10 min and fixed. The particle distribution per macrophage was determined for a particle-to-cell ratio of 40 : 1 and expressed as a percentage of macrophages containing 1–2, 3–4 or 5+ particles. For each condition, the particle distribution was calculated for at least 100 cells in triplicate, and at least three independent experiments were performed. Error bars, SD of one representative triplicate; * $P \leq 0.005$, ** $P \leq 0.0005$.

direct consequence of LPG insertion into phagosome lipid microdomains is the exclusion or dissociation of Syt V, which participates in phagolysosomal acidification (Vinet *et al.*, 2009). Whereas LPG-mediated exclusion of Syt V from phagosomes in the process of formation may be beneficial for the establishment of infection, it paradoxically reduces the phagocytic capacity of macrophages. This finding may appear surprising, as intracellular pathogens should promote their internalization to reach their replication niche within host cells. In evolutionary terms, although an obligatory fitness cost for the parasite to reach its intracellular niche, LPG-mediated exclusion of Syt V from membranes is a predominant process in the impairment of macrophage microbicidal activities. Thus, the imposed cost associated with a reduced parasite internalization rate may be compensated by an increased survival rate and could lead to greater overall fitness for the parasite.

ACKNOWLEDGEMENTS

This work was supported by Canadian Institutes of Health Research grant MOP-12933. A. D. is the holder of a Canada Research Chair and

was a chercheur-boursier from the Fonds de la recherche en santé du Québec. A. F. V. was partly supported by a doctoral fellowship from the Fondation Armand-Frappier. The Center for Host–Parasite Interactions is supported by the Fonds Québécois de la Recherche sur la Nature et les Technologies. We are grateful to Marcel Desrosiers for his expert assistance with confocal immunofluorescence microscopy, and to Guillermo Arango Duque for critical comments on the manuscript.

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Edited by: L. Knoll