Leishmania donovani Lipophosphoglycan Increases Macrophage-Dependent Chemotaxis of CXCR6-Expressing Cells via CXCL16 Induction

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ABSTRACT CXCL16 is a multifunctional chemokine that is highly expressed by macrophages and other immune cells in response to bacterial and viral pathogens; however, little is known regarding the role of CXCL16 during parasitic infections. The protozoan parasite *Leishmania donovani* is the causative agent of visceral leishmaniasis. Even though chemokine production is a host defense mechanism during infection, subversion of the host chemokine system constitutes a survival strategy adopted by the parasite. Here, we report that *L. donovani* promastigotes upregulate CXCL16 synthesis and secretion by bone marrow-derived macrophages (BMDM). In contrast to wild-type parasites, a strain deficient in the virulence factor lipophosphoglycan (LPG) failed to induce CXCL16 production. Consistent with this, cell treatment with purified *L. donovani* LPG augmented CXCL16 expression and secretion. Notably, the ability of BMDM to promote migration of cells expressing CXCR6, the cognate receptor of CXCL16, was augmented upon *L. donovani* infection in a CXCL16- and LPG-dependent manner. Mechanistically, CXCL16 induction by *L. donovani* required the activity of AKT and the mechanistic target of rapamycin (mTOR) but was independent of Toll-like receptor signaling. Collectively, these data provide evidence that CXCL16 is part of the inflammatory response elicited by *L. donovani* LPG *in vitro*. Further investigation using CXCL16 knockout mice is required to determine whether this chemokine contributes to the pathogenesis of visceral leishmaniasis and to elucidate the underlying molecular mechanisms.

KEYWORDS CXCL16, *Leishmania*, cell signaling, chemokine, inflammation, lipophosphoglycan, macrophage

The CXC chemokine ligand 16 (CXCL16) is expressed mainly by macrophages (M ϕ) and dendritic cells but is also produced by B and T cells, fibroblasts, and activated endothelial cells (1–4). Along with CX3CL1/fractalkine, CXCL16 is one of two transmembrane chemokines identified to date (5). The protein structure of CXCL16 comprises a small C-terminal intracellular domain and an extracellular N-terminal chemokine domain bound to the transmembrane region through a heavily glycosylated mucin-like stalk (4). CXCL16 was initially described as a scavenger receptor for phosphatidylserine and oxidized low-density lipoprotein (oxLDL) and therefore was named SR-PSOX (1). In addition to its function as a scavenger receptor, the transmembrane form of CXCL16 mediates adhesion to cells expressing its specific receptor, CXCR6/BONZO (2). In response to proinflammatory stimuli such as interferon gamma (IFN- γ) and tumor necrosis factor (TNF), the chemokine domain of CXCL16 is cleaved by the action of the metalloproteinase ADAM10 (6). Upon release into the extracellular milieu, the soluble

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form of CXCL16 acts as a chemoattractant for CXCR6⁺ cells such as activated NKT cells, NK cells, B cells, monocytes, and T cells (4, 7, 8). Increased CXCL16 production is part of the inflammatory response elicited during bacterial and viral infections and has been associated with either protective or harmful effects (9, 10). For instance, CXCL16 expression in the liver and spleen is required to control the bacterial burden during experimental salmonellosis (9). Conversely, surface-bound CXCL16 acts as an entry receptor for an equine virus and is essential for host cell permissiveness to viral infection (10). In regard to parasitic infections, a recent study suggested that CXCL16 is dispensable for controlling *Leishmania donovani* infection in the liver (11); however, the impact of the CXCL16-CXCR6 axis was not investigated in other organs (e.g., spleen, bone marrow, and lymph nodes) that also contribute to the pathogenesis of the infection.

Visceral leishmaniasis is a neglected tropical disease caused by parasites of the *L. donovani* and *L. infantum* species (12). The World Health Organization estimates that nearly 300,000 new cases and 20,000 deaths occur annually in areas of endemicity inhabited by more than 600 million people at risk of infection (13). The promastigote form of the parasite is transmitted by the sandfly vector to the mammalian host, where it is rapidly internalized by phagocytic cells, including macrophages (14). Promastigotes subsequently differentiate into amastigotes that replicate within phagosomes, also named parasitophorous vacuoles (15). To establish infection within phagocytes, *Leishmania* relies on a set of virulence factors that modulate immune and microbicidal macrophage functions (16, 17). Lipophosphoglycan (LPG) is one of the major surface glycoconjugates of *Leishmania* promastigotes which forms a dense glycocalyx covering the entire surface of the parasite and the flagellum (15). In addition, LPG can be released from promastigotes into the extracellular milieu (18). The structure of LPG consists of a polymer of repeating Gal(β 1,4)Man(α 1)-PO₄ units bound to the membrane via a core glycan structure attached to a glycosyl phosphatidylinositol (GPI) anchor (19). The lipid anchor and the glycan core of LPG are conserved, but the sugar composition and sequence of branching sugars attached to or capping the repeat units vary among *Leishmania* species (15). LPG is a multifaceted molecule that plays a crucial role in the establishment of the infection (15–17). Notably, LPG prevents phagosome maturation (20), inhibits phagosomal acidification and oxidative burst (21, 22), reduces the phagocytic capacity of host macrophages (23), and hampers the lytic action of the complement system (24). In addition to its inhibitory effects, extensive work supports the notion that LPG alters macrophage signaling and contributes to the inflammatory response triggered during *Leishmania* infection (25–29). For example, *L. mexicana* LPG stimulates the synthesis of TNF, interleukin-1 β (IL-1 β), IL-12, and nitric oxide (NO) through the activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) signaling (28). Moreover, *L. major* LPG interacts with Toll-like receptor 2 (TLR2) and induces the secretion of IL-12 and TNF in a MyD88-dependent manner (26). Similarly, *L. amazonensis* LPG and *L. braziliensis* LPG upregulate NO and TNF production via TLR2- and TLR4-mediated mechanisms (27). More recently, it was shown that *L. infantum* LPG interacts with TLR1/2, activates ERK1/2 and Jun N-terminal protein kinase (JNK) signaling, and induces the release of prostaglandin E₂, NO, TNF, IL-6, IL-12, and CCL2 (25). Although less investigated than for other *Leishmania* spp., purified *L. donovani* LPG also modulates proinflammatory mediator production, as evidenced by the upregulation of the nuclear translocation of the transcription factor AP-1 and the synthesis of NO in a macrophage cell line (29). Interestingly, LPG has the opposite effect during infection of primary macrophages with *L. donovani* promastigotes (30). Extending these findings, here we report that the ability of *L. donovani* promastigotes to induce the synthesis of CXCL16 in infected macrophages is largely dependent on the presence of LPG. Accordingly, the ability of macrophages to induce migration of cells expressing CXCR6, the cognate receptor of CXCL16, is enhanced upon treatment with *L. donovani* LPG or infection with wild-type (WT), but not with LPG-deficient, *L. donovani* promastigotes.

FIG 1 *Leishmania donovani* augments CXCL16 production in macrophages. BMDM cultures were inoculated with *L. donovani* (*Ld*) promastigotes (MOI 10:1) and treated with 100 U/ml IFN- γ or 100 ng/ml LPS or left untreated/uninfected (control) for 24 h (A and D) or for the indicated times (B and C). (A) Secreted CXCL16 was measured by sandwich ELISA. (B) *Cxcl16* mRNA amounts were quantified by RT-qPCR (normalized to β -actin); data are expressed as fold change in infected over control samples. (C) Total CXCL16 protein levels were monitored by Western blotting; total amounts of β -actin were used as a loading control. Parasite infection was monitored by probing for *Leishmania* IMPDH. (D) Expression of the transmembrane form of CXCL16 was examined by flow cytometry (left). Total CXCL16 protein levels were monitored by Western blotting as indicated in panel C (right). (A and B) Results are presented as mean $\pm$ standard deviation (SD) (biological replicates, $n = 3$). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (compared to uninfected). (C and D) Data are representative of three independent experiments.

RESULTS

***Leishmania donovani* induces CXCL16 expression in macrophages.** CXCL16 is induced during bacterial and viral infections (9, 10) and is highly expressed by activated macrophages (1). Notably, *L. donovani* promotes the synthesis of a number of chemokines *in vitro* and *in vivo* (31–35). These findings prompted us to investigate whether the expression of CXCL16 was modulated during *L. donovani* infection. To begin addressing this issue, we incubated bone marrow-derived murine macrophages (BMDM) with or without *L. donovani* 15 promastigotes and measured levels of CXCL16 in the culture supernatant by enzyme-linked immunosorbent assay (ELISA). Infected BMDM secreted larger amounts of CXCL16 than uninfected cultures (Fig. 1A). Interestingly, CXCL16 secretion in response to *L. donovani* was similar to that induced by IFN- γ stimulation. The expression of CXCL16 is regulated at the transcriptional level (6). Thus, we next assessed whether enhanced CXCL16 production resulted from changes in mRNA expression. Reverse transcription-quantitative PCR (RT-qPCR) experiments revealed an increasing accumulation of *Cxcl16* mRNA in *L. donovani*-infected BMDM compared to uninfected controls that was detectable as early as 4 h postinfection and was maximal after 24 h (Fig. 1B). CXCL16 is synthesized as an intracellular precursor form that is glycosylated before being translocated to the cell membrane (4). Accordingly, Western blot analyses showed that *L. donovani* rapidly induces the expression of

an ~50-kDa migrating form of CXCL16 that corresponds to the size of the glycosylated protein (36) (Fig. 1C). We next examined whether the increase in total CXCL16 levels correlated with the upregulation of the transmembrane form of the protein. In contrast to lipopolysaccharide (LPS), which served as a positive control, no changes in the cell surface expression of CXCL16 were detected upon *L. donovani* infection (Fig. 1D, left). These data are consistent with a greater induction of CXCL16 in response to LPS than in response to *L. donovani* infection, as monitored by Western blotting (Fig. 1D, right). Thus, *L. donovani* promotes the synthesis and subsequent secretion of CXCL16 by macrophages.

Induction of CXCL16 by *L. donovani* is dependent on the virulence factor LPG.

LPG is one of the major surface glycoconjugates of *Leishmania* promastigotes and plays a central role in the establishment of infection (17). Importantly, macrophages secrete several immune mediators following exposure to purified LPG (25–28). Therefore, we hypothesized that this virulence factor could be implicated in the upregulation of CXCL16 in *L. donovani*-infected macrophages. To test this, we initially treated BMDM with increasing concentrations of LPG purified from promastigote cultures of *L. donovani* 1S. We observed a dose-dependent effect of LPG on the induction of CXCL16, as monitored by Western blotting (Fig. 2A). Of note, LPG concentrations as low as 250 ng/ml augmented CXCL16 protein levels to the same extent as live parasites (Fig. 2A); thus, subsequent cell treatments were carried out using this experimental condition. Additionally, time course experiments revealed that CXCL16 protein expression is augmented in response to LPG following kinetics similar to those observed in *L. donovani*-infected BMDM (Fig. 2B). To further investigate the role of LPG in the modulation of CXCL16 during *Leishmania* infection, we employed an LPG-defective strain of *L. donovani* 1S (Sudan) (i.e., *lpg1* knockout [*lpg1*-KO]) and an *lpg1*-KO add-back strain (i.e., *lpg1*-KO+LPG1) (30). In contrast to WT parasites, *L. donovani lpg1*-KO promastigotes failed to upregulate the expression of CXCL16 in BMDM (Fig. 2C). Remarkably, genetic rescue of the *lpg1* gene restored the ability of the parasite to augment CXCL16 production. Moreover, heat-killed or formalin-fixed *L. donovani* promastigotes retained their capacity to increase CXCL16 levels (Fig. 2C), an indication that the observed effect does not require metabolically active parasites. In addition to *L. donovani* 1S, CXCL16 was also upregulated upon infection with the Ethiopian strain *L. donovani* LV9 (promastigotes and amastigotes) and with a different Old World species, *L. major* Seidman A2 (Senegal) (Fig. 2C). Notably, genetic deletion of GP63, another potent leishmanial virulence factor (16, 37), did not prevent CXCL16 induction by *L. major* promastigotes. Moreover, New World strains *L. infantum* Ba262 (Brazil) and *L. mexicana* M379 (Belize) were also able to enhance CXCL16 expression in BMDM (Fig. 2D). Thus, the effect of *Leishmania* on CXCL16 does not appear to be stage, species, or strain specific and does not depend on GP63. Further supporting the notion that LPG is required for CXCL16 induction by *L. donovani*, BMDM infected with *lpg1*-KO promastigotes did not secrete CXCL16 above basal levels (Fig. 2E). In stark contrast, *L. donovani* WT and *lpg1*-KO+LPG1 strains markedly increased the release of CXCL16 by infected cells compared to uninfected controls (fold changes of 2.65 ± 0.05 and 2.79 ± 0.18 , respectively) (Fig. 2E). Similarly, treatment of BMDM cultures with purified LPG significantly augmented CXCL16 secretion by BMDM (fold change, 5.05 ± 0.23). In agreement with our Western blot and ELISA data, RT-qPCR experiments showed a substantial accumulation of *Cxcl16* mRNA in BMDM infected with either WT or *lpg1*-KO+LPG1 parasites or incubated with purified LPG (fold changes of 2.11 ± 0.05 , 2.08 ± 0.03 , and 2.45 ± 0.07 , respectively) (Fig. 2F). Conversely, infection with the *lpg1*-KO strain led to only a modest (yet significant) increase in *Cxcl16* mRNA levels compared to control cultures (fold change, 1.33 ± 0.04). Collectively, this set of experiments indicates that upregulation of CXCL16 expression following *L. donovani* infection is largely dependent on the virulence factor LPG.

***L. donovani* induces the expression of CXCL16 in macrophages via AKT/mTOR-dependent but TLR-independent mechanisms.** CXCL16 is induced via surface and endosomal TLR stimulation (38), and LPG triggers TLR-mediated signaling (25, 26, 28,

FIG 2 Induction of macrophage CXCL16 by *L. donovani* is dependent on the virulence factor LPG. (A and B) BMDM cultures were treated with increasing concentrations of *L. donovani* LPG for 24 h (A) or with 250 ng/ml LPG for the indicated times (B), and the expression of CXCL16 was monitored by Western blotting. β -Actin was used as a loading control. (C and D) BMDM cultures were infected at an MOI of 10:1 with different *L. donovani* strains and stages (*L. donovani* 1S promastigotes [WT, *lpg1*-KO, *lpg1*-KO+LPG1, WT heat killed {hk}, and WT formalin fixed {ff}] and *L. donovani* LV9 promastigotes and amastigotes) or with promastigotes of other *Leishmania* spp. (*L. major* SA2 WT or *gp63*-KO, *L. infantum* Ba262, and *L. mexicana* M379) for 24 h or left uninfected. CXCL16 protein levels were examined as for panel A. Parasite infection was monitored by probing for *Leishmania* IMPDH. Efficient KO of *gp63* in *L. major* was verified by probing with an anti-*Leishmania* GP63 antibody. (E and F) Cells were infected as for panel C or treated with 250 ng/ml LPG as indicated in panel A. (E) Secreted CXCL16 was measured by sandwich ELISA. (F) The fold change in *Cxcl16* mRNA expression (normalized to β -actin) in *L. donovani*-infected or LPG-treated samples over control samples was determined by RT-qPCR. (A to D) Results are representative of three independent biological replicates. (E and F) Data are presented as mean $\pm$ SD (biological replicates, $n = 3$). ***, $P < 0.001$; ****, $P < 0.0001$; ns, not significant (compared to uninfected/untreated).

39). Therefore, we postulated that the upregulation of CXCL16 in macrophages incubated with *L. donovani* promastigotes could be dependent on TLR activation by LPG. To test this, we employed BMDM isolated from mice deficient in either MyD88 or UNC93B, the two main adaptor proteins required for TLR-mediated cell responses (40). As shown in Fig. 3A, MyD88 KO and UNC93B KO BMDM produced levels of CXCL16 similar to

FIG 3 CXCL16 induction in *L. donovani*-infected macrophages is AKT/mTOR dependent but TLR independent. (A and B) WT, MyD88 KO, and UNC93B KO BMDM cultures were inoculated with *L. donovani*, treated with 250 ng/ml LPG, 100 ng/ml LPS, or 250 ng/ml poly(I:C), or left uninfected/untreated for 24 h. CXCL16 protein expression was assessed by Western blotting. β -Actin was used as a loading control. Parasite infection was monitored by probing for *Leishmania* IMPDH. Induction of COX-2 served as a surrogate for disrupted TLR signaling in MyD88 KO cells. (C) BMDM cultures were inoculated with *L. donovani* WT, *lpg1*-KO, or *lpg1*-KO+LPG1 for 24 h or left uninfected, and the activity of AKT/mTOR signaling was monitored by Western blotting using phospho-specific and total antibodies against AKT and rpS6, respectively. CXCL16 expression and efficacy of infection were examined as for panel A. (D) BMDM cultures were pretreated with 2 μ M MK-2206, 200 nM Torin-1, or an equal volume of dimethyl sulfoxide (DMSO) (vehicle) for 1 h and then infected with *L. donovani* promastigotes for 24 h. The phosphorylation status and total levels of AKT and rpS6 were assessed as for panel C. Results are representative of at least two independent biological replicates.

those produced by WT cells following incubation with either *L. donovani* promastigotes or purified LPG. Similarly, *Escherichia coli* LPS, a TLR4 ligand, augmented the expression of CXCL16 in a MyD88-independent manner (Fig. 3B, left). Unlike *L. donovani* and LPG, upregulation of CXCL16 in response to the TLR3 ligand poly(I:C) was abrogated in the absence of UNC93B (Fig. 3B, right). Thus, the regulatory mechanisms of CXCL16 expression in macrophages appear to be stimulus specific. In addition to TLRs, the induction of CXCL16 involves phosphatidylinositol 3-kinase (PI3K)-AKT signaling (41). Interestingly, it was recently reported that the activity of AKT is sustained during *L. donovani* infection, which appears to be mediated by phosphoinositides present in the parasitophorous vacuole membrane (42). Consistent with this, we observed that AKT remained phosphorylated in cells infected with WT and *lpg1*-KO+LPG1 parasites but was substantially reduced upon infection with *lpg1*-KO promastigotes (Fig. 3C). Signaling through the kinase mechanistic target of rapamycin (mTOR) is a major output of AKT activity (43). Notably, increased mTOR signaling has been observed in *L. donovani*-infected macrophages (44). In line with our data on AKT, sustained phosphorylation of ribosomal protein S6 (rpS6), a downstream target of mTOR, was also LPG dependent (Fig. 3C). Accordingly, upregulation of CXCL16 in response to *L. donovani* infection was affected when the activity of AKT or mTOR was blocked by the chemical inhibitor MK-2206 or Torin-1, respectively (Fig. 3D). Note that these compounds do not exert toxic effects on BMDM up to 24 h, as we previously described (45). Thus, these data provide evidence that *L. donovani* induces the expression of CXCL16 in macrophages via AKT/mTOR-dependent and TLR-independent mechanisms.

CXCL16 secretion by *L. donovani*-infected macrophages promotes the migration of CXCR6-expressing cells. In response to proinflammatory cytokines, the N-terminal chemokine domain of CXCL16 is cleaved by the metalloproteinase ADAM10 (6) and released into the extracellular milieu to promote the migration of CXCR6-expressing cells (e.g., monocytes, neutrophils, and NKT, B, T, and dendritic cells) (4, 7, 8). Thus, we hypothesized that elevated CXCL16 secretion by *L. donovani*-infected macrophages would enhance chemotactic migration of CXCR6⁺ cells. Initially, CXCR6 was overexpressed in RAW 264.7 cells by transient transfection, and increased surface expression of the receptor was confirmed by flow cytometric analysis (see Fig. S1 in the supplemental material). CXCR6⁺ RAW 264.7 cells were then used as effector cells in chemotaxis assays performed in the presence of conditioned medium (CM) from *L. donovani*-infected or uninfected BMDM (Fig. 4A). A greater number of CXCR6⁺ cells migrated toward CM from BMDM infected with WT *L. donovani* promastigotes than from those infected with *lpg1*-KO promastigotes (fold changes, 30.03 ± 7.53 and 9.01 ± 0.74 , respectively, over CM from uninfected BMDM) (Fig. 4B). Consistent with this, CXCR6⁺ cell migration was induced in the presence of CM from LPG-treated BMDM (see Fig. S2 in the supplemental material). Conversely, LPG added directly during migration assays failed to attract CXCR6⁺ cells (Fig. S2). Notably, CXCR6⁺ cell migration in response to recombinant CXCL16 or CM from *L. donovani*-infected BMDM was dramatically reduced in the presence of a neutralizing antibody against CXCL16 (Fig. 4B). Accordingly, CM from *L. donovani*-infected CXCL16 KO BMDM was a much less potent chemoattractant for CXCR6⁺ cells than CM from *L. donovani*-infected WT BMDM (~75% reduction in migration of CXCR6⁺ cells) (Fig. 4C). Note that similar infection rates were observed in WT and CXCL16 KO BMDM up to 24 h (see Fig. S3 in the supplemental material), ruling out the possibility that differences in chemotactic activity between CM from *L. donovani*-infected WT and CXCL16 KO BMDM were caused by changes in the percentage of infected cells. Thus, *L. donovani*-infected BMDM promote migration of CXCR6⁺ cells via LPG-inducible CXCL16 secretion.

DISCUSSION

The multifaceted chemokine CXCL16 displays a number of functions, including lipid scavenging, cell-to-cell adhesion, and chemoattraction (1, 2, 4, 7, 8). Here, we demonstrate that the intracellular protozoan parasite *L. donovani* modulates the expression of CXCL16 in infected macrophages at both the mRNA and soluble protein levels, thereby

FIG 4 *L. donovani* promotes macrophage-mediated chemotaxis of CXCR6⁺ cells via LPG-dependent CXCL16 production. (A) Experimental design to assess chemotactic activity of secreted CXCL16. Conditioned medium from infected or uninfected BMDM cultures was collected after 24 h and added to the lower chamber of Transwell plates. CXCR6-transfected RAW 264.7 cells were added to the upper Transwell insert (8- μ m pore size) and allowed to migrate for 4 h. Migrated cells adhering to the insert underside were counted by microscopy. (B and C) Conditioned medium (CM) from WT or CXCL16 KO BMDM cultures infected with *L. donovani* WT or *lpg1*-KO for 24 h or left uninfected were tested for chemotactic activity. Fresh medium (DMEM, nonconditioned) was included to monitor basal migration activity. As a positive control, murine recombinant CXCL16 (rCXCL16) was added (500 pg/ml) to the lower chamber. A CXCL16-neutralizing antibody was added (250 ng/ml) to specifically block CXCL16-mediated chemotactic activity. Data are presented as mean $\pm$ SD (biological replicates, $n = 3$). **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (for the indicated comparisons).

influencing their ability to mediate chemotaxis of CXCR6⁺ cells. Using an LPG-deficient parasite mutant and purified LPG, we show that the induction of CXCL16 is dependent on this potent virulence factor.

Accumulating evidence supports the notion that different *Leishmania* spp. modulate the host chemokine system to attract cells that represent their natural niche for replication (34, 46). This becomes particularly relevant since we observed that CXCL16 upregulation is not exclusive to *L. donovani* and is not limited to species causing visceral leishmaniasis. Indeed, we detected a similar phenotype in macrophages infected with *Leishmania* spp. of different geographical origins (i.e., Old World and New World species), including two that cause cutaneous leishmaniasis (i.e., *L. major* and *L. mexicana*). Importantly, CXCR6, the cognate receptor of CXCL16, is expressed by subsets of monocytes/macrophages (8, 47) and neutrophils (38); therefore, it is tempting to speculate that CXCL16 induced by *Leishmania* spp. helps attract immune cells that are subsequently infected to promote pathogen dissemination. Of note, we did not detect any changes in the membrane-bound form of CXCL16 in macrophages upon infection with *L. donovani*. Despite this observation, the ability of the parasite to infect

other immune cells (34, 46) and the potential effect of extracellular LPG (18) in uninfected bystander cells could influence the expression of membrane-bound CXCL16 during *L. donovani* infection *in vivo*.

In a recent report, Murray and colleagues (11) used *cxcr6* KO mice as surrogates to explore the role of CXCL16 during experimental visceral leishmaniasis. The authors concluded that the CXCL16-CXCR6 axis was dispensable for the control of *L. donovani* infection, since granuloma formation and parasite clearance in the liver were not affected by the absence of CXCR6. However, the pathology of visceral leishmaniasis spans throughout different tissues and organs (48). For instance, disruption of spleen microarchitecture is associated with local TNF production during visceral leishmaniasis (49). CXCL16 expression is upregulated by proinflammatory cytokines such as TNF and IFN- γ (6). Hence, recruitment of TNF- and IFN- γ -producing cells could contribute to an inflammatory loop in the spleen involving CXCL16.

The role of LPG in host cell inflammatory responses has been extensively described for different *Leishmania* spp. (25–28) but is understudied in *L. donovani* (29), an important consideration given the species-specific chemical structures of LPG molecules (15–17). By genetic and biochemical approaches (i.e., using an *lpg1*-KO mutant and purified LPG, respectively), our work uncovered a novel function for *L. donovani* LPG via the induction of CXCL16. Interestingly, we observed that amastigotes of *L. donovani* are also able to augment the expression of CXCL16 in infected macrophages although to a lesser extent than promastigotes. Given that differentiation of *L. donovani* promastigotes into amastigotes leads to a drastic reduction in LPG levels (50), our data suggest that CXCL16 induction by the amastigote stage is likely to be LPG independent. Despite the drastic reduction in LPG, amastigotes retain a glycocalyx of glycosylinositol phospholipids (51) that could account, at least in part, for the induction of CXCL16. Further *in vitro* and *in vivo* studies are required to elucidate the mechanism of CXCL16 upregulation by *L. donovani* amastigotes and to define its role during visceral leishmaniasis.

LPG molecules from other *Leishmania* spp. were reported to interact with several surface TLRs (i.e., TLR1, -2, and -4) (25–27). Unexpectedly, we observed that *L. donovani* LPG-mediated upregulation of CXCL16 was independent of TLR signaling, as indicated by our data using MyD88 KO and UNC93B KO macrophages. The presence of the lipid anchor in *L. major* LPG was shown to be required for cytokine induction in macrophages (26). In contrast, intact *L. infantum* LPG, but not its glycan and lipid moieties, exhibited proinflammatory activity (25). Structure-function studies of *L. donovani* LPG will shed light on the molecular components necessary to enhance CXCL16 production in macrophages.

Although the exact signaling events linked to *L. donovani*-driven CXCL16 induction remain to be established, our results indicate that this event relies on AKT/mTOR signaling, an important node in the regulation of macrophage immune functions (52). Indeed, within the upstream region of the *cxl16* promoter, binding sites for several transcription factors have been predicted, including sites for CREB, SMAD, GATA, IRF, NF- κ B, and AP-1, the last being the main driver of IL-18-induced CXCL16 expression in aortic smooth muscles cells (41). LPG from *L. donovani* was shown to activate DNA binding of AP-1 in macrophages (29). Moreover, *L. major* LPG was able to induce an NF- κ B activity in 293T cells (26). Thus, it is conceivable that *L. donovani* LPG promotes *Cxl16* mRNA expression via AP-1- and/or NF- κ B-dependent transcriptional activity in macrophages. Our data indicate that the increase in CXCL16 protein expression occurs more rapidly than the accumulation of *Cxl16* mRNA in *L. donovani*-infected macrophages. These results suggest that in addition to transcription, *L. donovani* might enhance the stability and/or the translation efficiency of *Cxl16* mRNA. Of note, we recently demonstrated that another protozoan parasite, *Toxoplasma gondii*, selectively regulates translation of immune-related transcripts in macrophages via the mTOR complex 1 (mTORC1), including chemokines (45). Therefore, sustained mTOR activity during *L. donovani* infection might be required to upregulate CXCL16 expression through both transcriptional and posttranscriptional mechanisms. In addition to a

direct effect on *Cxcl16* mRNA metabolism, we cannot rule out the possibility that increased production of CXCL16 during *L. donovani* infection is at least in part dependent on the secretion of an LPG-inducible factor acting in an autocrine and/or paracrine fashion. Finally, given the importance of LPG in the establishment of infection with *Leishmania* spp. (15–17), the inability of LPG-deficient parasites to enhance CXCL16 synthesis and secretion may also be related to reduced fitness within macrophages.

The immune response to *L. donovani* integrates a complex network of pro- and anti-inflammatory modulators, the balance of which determines the outcome of the infection (48). Our study identifies an additional immune mediator, the chemokine CXCL16, as part of macrophage responses to *L. donovani* promastigotes. Importantly, this phenotype is associated with the virulence factor LPG. Further *in vivo* and *in vitro* studies are required to assess the impact of CXCL16 in the development of visceral leishmaniasis. Ultimately, CXCL16 might emerge as a useful biomarker for disease severity and perhaps as a promising target for therapeutic intervention.

MATERIALS AND METHODS

Reagents. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), 0.05% EDTA-trypsin, penicillin, and streptomycin were acquired from Wisent Inc. Zeocin was purchased from Gibco. Medium 199, HEPES, hemin, hypoxanthine, biotin, 6-biopterin, lipopolysaccharide (LPS) (*Escherichia coli* serotype 0111:B4), 10-phenanthroline monohydrate, and polyinosinic-polycytidylic acid [poly(I:C)] were acquired from Sigma-Aldrich. Recombinant mouse interferon gamma (IFN- γ) was purchased from Cedarlane Laboratories. Torin-1 and MK-2206 were acquired from Cayman Chemical. Complete EDTA-free protease inhibitor and PhosSTOP phosphatase inhibitor tablets were purchased from Roche.

Mice. Animal procedures were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care, and all animal work was approved by the Comité Institutionnel de Protection des Animaux (CIPA) of INRS-Institut Armand-Frappier (CIPA no. 1710-02). Four- to 6-week-old C57BL/6J and DBA/2J mice were purchased from The Jackson Laboratory and maintained in the Centre National de Biologie Expérimentale (CNBE) at INRS-Institut Armand-Frappier. *cxcl16* KO mice were generated as previously described (3) and housed within the Carleton Animal Care Facility at Dalhousie University. Hind legs from *unc93b* KO mice were provided by Simona Stäger (INRS-Institut Armand-Frappier). Hind legs from *myd88* KO mice were a gift from Alain Lamarre (INRS-Institut Armand-Frappier).

Differentiation of BMDM. Bone marrow precursor cells were obtained from femurs and tibias of commercial C57BL/6J (The Jackson Laboratory), *cxcl16* KO, *unc93b* KO, and *myd88* KO mice and differentiated into bone marrow-derived macrophages (BMDM) as previously described (45). Briefly, bone marrow precursor cells were flushed from femurs and tibias maintained in HBSS (100 U/ml penicillin, 100 μ g/ml streptomycin, 4.2 mM sodium bicarbonate) at 4°C. Red blood cells were lysed in ACK lysis buffer (150 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) for 7 min at room temperature. Precursor cells were then resuspended in BMDM culture medium (DMEM, 10% heat-inactivated FBS, 2 mM L-glutamate, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μ g/ml streptomycin) supplemented with 15% L929 fibroblast-conditioned culture medium (LCCM). Cells were seeded in 10-cm-diameter tissue culture-treated dishes and incubated overnight at 37°C with 5% CO₂. The following day, nonadherent cells were collected, resuspended in BMDM culture medium supplemented with 30% LCCM, and plated in 10-cm-diameter nontreated petri dishes (5 $\times$ 10⁶ cells per dish). Medium was replenished 2 days later, and differentiated BMDM were collected 7 days after marrow extraction. Differentiation of precursor cells into macrophages was routinely assessed by monitoring for CD11b and F4/80 coexpression by flow cytometry using allophycocyanin (APC)-anti-mouse/human CD11b antibody no. 101211 and phycoerythrin (PE)-anti-mouse F4/80 antibody no. 123109 (BioLegend), as previously described (53).

Parasites. *Leishmania donovani* (1S and LV9 strains), *L. major* (SA2 strain), *L. mexicana* (M379 strain), and *L. infantum* (Ba262 strain) promastigotes were cultured at 26°C in M199 medium supplemented with 10% heat-inactivated FBS, 100 μ M hypoxanthine, 5 μ M hemin, 3 μ M biopterin, 1 μ M biotin, 50 U/ml penicillin, and 50 μ g/ml streptomycin. The isogenic *L. donovani* LPG-defective *lpg1*-KO mutant and *lpg1*-KO+LPG1 add-back (i.e., rescue) strains were described previously (30). The *lpg1*-KO mutant secretes repeating Gal(β 1,4)Man(α 1)-PO₄-containing molecules but lacks the ability to assemble a functional LPG glycan core (54), precluding synthesis and expression of LPG. The *L. donovani* *lpg1*-KO+LPG1 add-back strain was cultured in the presence of 100 μ g/ml Zeocin. Stationary-phase promastigotes were used for macrophage infections. *L. donovani* amastigotes (LV9 strain) were isolated from the spleens of infected female Golden Syrian hamsters (The Jackson Laboratory, Bar Harbor, ME, USA), as previously described (55).

LPG purification. LPG from *L. donovani* promastigotes (1S strain) was purified by chromatography as previously described (56). Briefly, 10⁹ exponentially growing parasites were centrifuged at 1,900 $\times$ g for 10 min at room temperature and washed with 5 ml of PBS. Cells were delipidated by sequential extraction at 4°C with 25 ml of chloroform-methanol-water (3:2:1) (three times), 25 ml of chloroform-methanol-water (1:1:0.3) (three times), and 25 ml of 4 mM MgCl (three times). LPG was extracted from the resulting delipidated residue fraction by four extractions at 4°C with 25 ml of the solvent water-ethanol-diethyl ether-pyridine-concentrated NH₄OH (15:15:5:1:0.017). The solvent supernatant was taken to

dryness under reduced pressure. The residue was resuspended in 5 ml of 40 mM NH₄OH–1 mM EDTA, and the insoluble material was removed by centrifugation at 15,000 $\times g$ for 10 min. The supernatant was applied to a Sephadex G-150 column (1 by 40 cm) equilibrated with the same buffer. Samples containing LPG were pooled and lyophilized. The sample was resuspended in 40 mM NH₄OH, desalted on a Sephadex G-25 column (1 by 5 cm) equilibrated in 40 mM NH₄OH, and lyophilized. LPG was resuspended in 10 ml of water-ethanol-diethyl ether-pyridine-concentrated NH₄OH (15:15:5:1:0.017) and precipitated at –20°C for 18 h with the addition of 10 ml of methanol. Precipitated LPG was resuspended in sterile phosphate-buffered saline (PBS), and endotoxin levels were measured with the *Limulus* amoebocyte lysate (LAL) chromogenic endotoxin quantitation kit (Pierce), according to the manufacturer's specifications.

BMDM infection. *L. donovani* promastigotes were opsonized with 10% serum from DBA/2J mice, which are naturally deficient in complement component 5 (C5) (57), for 20 min at 37°C, 5% CO₂. Adherent BMDMs (2 $\times 10^5$ cells per cm²) were inoculated with opsonized promastigotes of the different *Leishmania* species and strains at a multiplicity of infection (MOI) of 10:1. Noninternalized parasites were removed after 6 h, and cells were incubated in BMDM culture medium overnight at 37°C with 5% CO₂. The percentage of infected cells was assessed by microscopic examination using the Protocol Hema3 manual staining system (Thermo Fisher Scientific).

RNA extraction and quantitative RT-qPCR. Total BMDM RNA was isolated using QIAzol (Qiagen), according to the manufacturer's protocol. One microgram of RNA was reverse transcribed using the Superscript IV VLO master mix (Invitrogen). Quantitative PCR was performed with PowerU SYBR green master mix (Applied Biosystems) according to the manufacturer's instructions, using a QuantStudio 3 real-time PCR system (Applied Biosystems). Analysis was carried out by relative quantification using the comparative C_t method ($\Delta\Delta C_t$) (58). Experiments were performed in independent biological replicates ($n = 3$), whereby every sample was analyzed in a technical triplicate. Relative mRNA amounts were normalized to the β -actin gene. Primers were designed using NCBI Primer-BLAST (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) for the mouse *cxcl16* gene (forward, 5'-AGACCAGTGGGTCCGTGAAC-3'; reverse, 5'-ACTATGTGCAGGGGTGCTCG-3') and for the mouse β -actin gene (forward, 5'-ACTGTCTGAGTCGCTCCA-3'; reverse, 5'-ATGGCTACGTACATGGCTCG-3').

Western blot analysis. Following infection and other treatments, BMDM cultures were collected in ice-cold PBS (pH 7.4) by scraping, centrifuged, and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (25 mM Tris-HCl [pH 7.6], 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, cOmplete EDTA-free protease inhibitor cocktail, PhosStop phosphatase inhibitor, and 10 mM 10-phenanthroline). Cell debris was removed by centrifugation (15,000 $\times g$ for 15 min at 4°C), and protein content was estimated using the bicinchoninic acid (BCA) protein assay kit (Pierce). Protein extracts were subjected to SDS-PAGE, and the resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad). Membranes were blocked for 1 h in 5% skim milk–Tris-buffered saline (TBS) with 0.1% Tween 20 and probed with the following primary antibodies overnight at 4°C: anti-CXCL16 (no. AF503) from R&D Systems; anti-phospho-AKT (Thr308) (no. 2965), anti- β -actin (no. 3700), anti-COX-2 (no. 4842), and anti-phospho rpS6 (Ser235/236) (no. 2211) from Cell Signaling Technologies; anti-*Leishmania* GP63 (a gift from Robert McMaster, University of British Columbia, Vancouver, Canada); and anti-*Leishmania* IMP dehydrogenase (IMPDH) (59). Membranes were then incubated with the following IgG horseradish peroxidase-linked antibodies: goat anti-rabbit IgG (no. A0545) and goat anti-mouse IgG (no. A4416) from Sigma-Aldrich and donkey anti-goat IgG (no. HAF109) and goat anti-guinea pig IgG (no. CLAS10-653) from R&D Systems. Proteins were then detected by chemiluminescence using Clarity Western ECL substrate (Bio-Rad) and exposure of membranes to autoradiography film (Denville Scientific).

ELISA. Soluble CXCL16 in the supernatants from infected, treated, and control BMDM cultures were assessed using the ELISA capture mouse CXCL16 antibody (no. MAB503) and ELISA detection mouse CXCL16 biotinylated antibody (no. BAF503) from R&D Systems. Concentrations were calculated from standard curves generated using linear regression analysis of data obtained from serial dilutions with the recombinant murine CXCL16 chemokine domain (no. 503-CX/CF).

Macrophage cell line transfection. The RAW 264.7 mouse macrophage cell line was maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM L-glutamine at 37°C with 5% CO₂. Cell transfection with the Bonzo/CXCR6 open reading frame (ORF) pCMV3 expression plasmid (Sino Biological) was carried out using Lipofectamine 2000 (Invitrogen), according to the manufacturer's specifications. Transfection efficiency was monitored by flow cytometry.

Flow cytometry staining. BMDM and CXCR6-overexpressing RAW 264.7 cultures were harvested, washed, and resuspended in fluorescence-activated cell sorter (FACS) buffer (PBS [pH 7.2 to 7.4] with 0.1% bovine serum albumin [BSA]). Fc receptors were blocked with anti-mouse CD16/32 (Fc γ III/II) (clone 93; no. 101302) (BioLegend) and then probed with the following antibodies for 30 min: goat anti-mouse CXCL16 (R&D Systems) or fluorescein isothiocyanate (FITC)–anti-CXCR6 (CD186) (clone SA051D1; no. 151108) (BioLegend). When required, samples were then stained with a chicken anti-goat IgG coupled to Alexa Fluor 488 (Invitrogen). Isotype-matched FITC-coupled rat IgG2b, κ (clone RTK4530; no. 400605) (BioLegend) or AF488-coupled chicken anti-goat IgG only was included to control for nonspecific staining. After staining, cells were fixed in PBS with 1% paraformaldehyde (PFA) for 15 min on ice. The fixative was quenched with PBS with 0.1 M glycine, and cells were washed twice in FACS buffer. Samples were acquired using a BD FACSCalibur, and data were analyzed using Flowing software (Turku, Finland).

Chemotactic migration assays. Chemotactic migration of CXCR6-overexpressing RAW264.7 macrophages was monitored by Transwell assays using 8- μ m-pore-size membrane inserts (Corning). Conditioned medium from untreated, *L. donovani*-infected, and LPG-treated WT and CXCL16 KO BMDM was added to the lower chambers of Transwell plates. CXCR6-overexpressing RAW264.7 macrophages

(5×10^5 /well) were added to Transwell inserts and were incubated at 37°C with 5% CO₂ for 4 h. Cell migration was quantified by manual staining of the insert membranes with the Protocol Hema3 system, and the remaining cells in the upper chamber were removed after the last wash using a cotton swab.

Statistical analysis. Statistical significance was determined using Student's *t* test for simple comparisons and one-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test for multiple comparisons. Calculations were performed using Prism software (GraphPad Software, La Jolla CA). Significant differences are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00064-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.04 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.01 MB.

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V.C., L.-P.L., A.Z., and M.J. conceived and designed the experiments. V.C., L.-P.L., and A.Z. performed the experiments. V.C., L.-P.L., A.Z., and M.J. analyzed the data. A.J., B.J., and A.D. contributed reagents and materials. V.C., L.-P.L., and M.J. wrote the manuscript.

We declare no competing interests.

REFERENCES

- Shimaoka T, Kume N, Minami M, Hayashida K, Kataoka H, Kita T, Yonehara S. 2000. Molecular cloning of a novel scavenger receptor for oxidized low density lipoprotein, SR-PSOX, on macrophages. *J Biol Chem* 275:40663–40666. <https://doi.org/10.1074/jbc.C000761200>.
- Shimaoka T, Nakayama T, Fukumoto N, Kume N, Takahashi S, Yamaguchi J, Minami M, Hayashida K, Kita T, Ohsumi J, Yoshie O, Yonehara S. 2004. Cell surface-anchored SR-PSOX/CXC chemokine ligand 16 mediates firm adhesion of CXC chemokine receptor 6-expressing cells. *J Leukoc Biol* 75:267–274. <https://doi.org/10.1189/jlb.1003465>.
- Veinotte L, Gebremeskel S, Johnston B. 2016. CXCL16-positive dendritic cells enhance invariant natural killer T cell-dependent IFN γ production, and tumor control. *Oncoimmunology* 5:e1160979. <https://doi.org/10.1080/2162402X.2016.1160979>.
- Izquierdo MC, Martin-Cleary C, Fernandez-Fernandez B, Elewa U, Sanchez-Niño MD, Carrero JJ, Ortiz A. 2014. CXCL16 in kidney and cardiovascular injury. *Cytokine Growth Factor Rev* 25:317–325. <https://doi.org/10.1016/j.cytogfr.2014.04.002>.
- Shimaoka T, Nakayama T, Kume N, Takahashi S, Yamaguchi J, Minami M, Hayashida K, Kita T, Ohsumi J, Yoshie O, Yonehara S. 2003. Cutting edge: SR-PSOX/CXC chemokine ligand 16 mediates bacterial phagocytosis by APCs through its chemokine domain. *J Immunol* 171:1647–1651. <https://doi.org/10.4049/jimmunol.171.4.1647>.
- Abel S, Hundhausen C, Mentlein R, Schulte A, Berkhout TA, Broadway N, Hartmann D, Sedlacek R, Dietrich S, Muetze B, Schuster B, Kallen KJ, Saftig P, Rose-John S, Ludwig A. 2004. The transmembrane CXC-chemokine ligand 16 is induced by IFN- γ and TNF- α and shed by the activity of the disintegrin-like metalloproteinase ADAM10. *J Immunol* 172:6362–6372. <https://doi.org/10.4049/jimmunol.172.10.6362>.
- Johnston B, Kim CH, Soler D, Emoto M, Butcher EC. 2003. Differential chemokine responses and homing patterns of murine TCR $\alpha\beta$ NKT cell subsets. *J Immunol* 171:2960–2969. <https://doi.org/10.4049/jimmunol.171.6.2960>.
- Huang Y, Zhu XY, Du MR, Li DJ. 2008. Human trophoblasts recruited T lymphocytes and monocytes into decidua by secretion of chemokine CXCL16 and interaction with CXCR6 in the first-trimester pregnancy. *J Immunol* 180:2367–2375. <https://doi.org/10.4049/jimmunol.180.4.2367>.
- Fahy OL, Townley SL, McColl SR. 2006. CXCL16 regulates cell-mediated immunity to *Salmonella enterica* serovar Enteritidis via promotion of gamma interferon production. *Infect Immun* 74:6885–6894. <https://doi.org/10.1128/IAI.01065-06>.
- Sarkar S, Chelvarajan L, Go YY, Cook F, Artiushin S, Mondal S, Anderson K, Eberth J, Timoney PJ, Kalbfleisch TS, Bailey E, Balasuriya UB. 2016. Equine arteritis virus uses equine CXCL16 as an entry receptor. *J Virol* 90:3366–3384. <https://doi.org/10.1128/JVI.02455-15>.
- Murray HW, Luster AD, Zheng H, Ma X. 2017. Gamma Interferon-Regulated Chemokines in *Leishmania donovani* infection in the liver. *Infect Immun* 85:e00824-16. <https://doi.org/10.1128/IAI.00824-16>.
- Wyllie S, Patterson S, Stojanovski L, Simeons FR, Norval S, Kime R, Read KD, Fairlamb AH. 2012. The anti-trypansome drug fexinidazole shows potential for treating visceral leishmaniasis. *Sci Transl Med* 4:119re1. <https://doi.org/10.1126/scitranslmed.3003326>.
- WHO. 2017. Global leishmaniasis update, 2006–2015: a turning point in leishmaniasis surveillance. *Wkly Epidemiol Rec* 92:557–565.
- Kaye P, Scott P. 2011. Leishmaniasis: complexity at the host-pathogen interface. *Nat Rev Microbiol* 9:604–615. <https://doi.org/10.1038/nrmicro2608>.
- de Assis RR, Ibraim IC, Nogueira PM, Soares RP, Turco SJ. 2012. Glycoconjugates in New World species of *Leishmania*: polymorphisms in lipophosphoglycan and glycoinositolphospholipids and interaction with

- hosts. *Biochim Biophys Acta* 1820:1354–1365. <https://doi.org/10.1016/j.bbagen.2011.11.001>.
16. Olivier M, Atayde VD, Isnard A, Hassani K, Shio MT. 2012. *Leishmania* virulence factors: focus on the metalloprotease GP63. *Microbes Infect* 14:1377–1389. <https://doi.org/10.1016/j.micinf.2012.05.014>.
 17. Podinovskaia M, Descoteaux A. 2015. *Leishmania* and the macrophage: a multifaceted interaction. *Future Microbiol* 10:111–129. <https://doi.org/10.2217/fmb.14.103>.
 18. King DL, Chang YD, Turco SJ. 1987. Cell surface lipophosphoglycan of *Leishmania donovani*. *Mol Biochem Parasitol* 24:47–53. [https://doi.org/10.1016/0166-6851\(87\)90114-9](https://doi.org/10.1016/0166-6851(87)90114-9).
 19. Descoteaux A, Turco SJ. 1999. Glycoconjugates in *Leishmania* infectivity. *Biochim Biophys Acta* 1455:341–352. [https://doi.org/10.1016/S0925-4439\(99\)00065-4](https://doi.org/10.1016/S0925-4439(99)00065-4).
 20. Desjardins M, Descoteaux A. 1997. Inhibition of phagolysosomal biogenesis by the *Leishmania* lipophosphoglycan. *J Exp Med* 185:2061–2068. <https://doi.org/10.1084/jem.185.12.2061>.
 21. Lodge R, Diallo TO, Descoteaux A. 2006. *Leishmania donovani* lipophosphoglycan blocks NADPH oxidase assembly at the phagosome membrane. *Cell Microbiol* 8:1922–1931. <https://doi.org/10.1111/j.1462-5822.2006.00758.x>.
 22. Vinet AF, Fukuda M, Turco SJ, Descoteaux A. 2009. The *Leishmania donovani* lipophosphoglycan excludes the vesicular proton-ATPase from phagosomes by impairing the recruitment of synaptotagmin V. *PLoS Pathog* 5:e1000628. <https://doi.org/10.1371/journal.ppat.1000628>.
 23. Vinet AF, Jananji S, Turco SJ, Fukuda M, Descoteaux A. 2011. Exclusion of synaptotagmin V at the phagocytic cup by *Leishmania donovani* lipophosphoglycan results in decreased promastigote internalization. *Microbiology* 157:2619–2628. <https://doi.org/10.1099/mic.0.050252-0>.
 24. Puentes SM, Da Silva RP, Sacks DL, Hammer CH, Joiner KA. 1990. Serum resistance of metacyclic stage *Leishmania major* promastigotes is due to release of C5b-9. *J Immunol* 145:4311–4316.
 25. Lima JB, Araujo-Santos T, Lazaro-Souza M, Carneiro AB, Ibraim IC, Jesus-Santos FH, Luz NF, Pontes SM, Entringer PF, Descoteaux A, Bozza PT, Soares RP, Borges VM. 2017. *Leishmania infantum* lipophosphoglycan induced-prostaglandin E2 production in association with PPAR-gamma expression via activation of Toll like receptors-1 and 2. *Sci Rep* 7:14321. <https://doi.org/10.1038/s41598-017-14229-8>.
 26. de Veer MJ, Curtis JM, Baldwin TM, DiDonato JA, Sexton A, McConville MJ, Handman E, Schofield L. 2003. MyD88 is essential for clearance of *Leishmania major*: possible role for lipophosphoglycan and Toll-like receptor 2 signaling. *Eur J Immunol* 33:2822–2831. <https://doi.org/10.1002/eji.200324128>.
 27. Ibraim IC, de Assis RR, Pessoa NL, Campos MA, Melo MN, Turco SJ, Soares RP. 2013. Two biochemically distinct lipophosphoglycans from *Leishmania braziliensis* and *Leishmania infantum* trigger different innate immune responses in murine macrophages. *Parasit Vectors* 6:54. <https://doi.org/10.1186/1756-3305-6-54>.
 28. Rojas-Bernabé A, García-Hernández O, Maldonado-Bernal C, Delegado-Domínguez J, Ortega E, Gutiérrez-Kobeh L, Becker I, Aguirre-García M. 2014. *Leishmania mexicana* lipophosphoglycan activates ERK and p38 MAP kinase and induces production of proinflammatory cytokines in human macrophages through TLR2 and TLR4. *Parasitology* 141:788–800. <https://doi.org/10.1017/S0031182013002187>.
 29. Balaraman S, Singh VK, Tewary P, Madhubala R. 2005. *Leishmania* lipophosphoglycan activates the transcription factor activating protein 1 in J774A.1 macrophages through the extracellular signal-related kinase (ERK) and p38 mitogen-activated protein kinase. *Mol Biochem Parasitol* 139:117–127. <https://doi.org/10.1016/j.molbiopara.2004.10.006>.
 30. Prive C, Descoteaux A. 2000. *Leishmania donovani* promastigotes evade the activation of mitogen-activated protein kinases p38, c-Jun N-terminal kinase, and extracellular signal-regulated kinase-1/2 during infection of naive macrophages. *Eur J Immunol* 30:2235–2244. [https://doi.org/10.1002/1521-4141\(2000\)30:8<2235::AID-IMMU2235>3.0.CO;2-9](https://doi.org/10.1002/1521-4141(2000)30:8<2235::AID-IMMU2235>3.0.CO;2-9).
 31. Dasgupta B, Roychoudhury K, Ganguly S, Akbar MA, Das P, Roy S. 2003. Infection of human mononuclear phagocytes and macrophage-like THP1 cells with *Leishmania donovani* results in modulation of expression of a subset of chemokines and a chemokine receptor. *Scand J Immunol* 57:366–374. <https://doi.org/10.1046/j.1365-3083.2003.01227.x>.
 32. Gregory DJ, Godbout M, Contreras I, Forget G, Olivier M. 2008. A novel form of NF-kappaB is induced by *Leishmania* infection: involvement in macrophage gene expression. *Eur J Immunol* 38:1071–1081. <https://doi.org/10.1002/eji.200737586>.
 33. Matte C, Olivier M. 2002. *Leishmania*-induced cellular recruitment during the early inflammatory response: modulation of proinflammatory mediators. *J Infect Dis* 185:673–681. <https://doi.org/10.1086/339260>.
 34. Oghumu S, Lezama-Dávila CM, Isaac-Márquez AP, Satoskar AR. 2010. Role of chemokines in regulation of immunity against leishmaniasis. *Exp Parasitol* 126:389–396. <https://doi.org/10.1016/j.exppara.2010.02.010>.
 35. Cotterell SE, Engwerda CR, Kaye PM. 1999. *Leishmania donovani* infection initiates T cell-independent chemokine responses, which are subsequently amplified in a T cell-dependent manner. *Eur J Immunol* 29:203–214. [https://doi.org/10.1002/\(SICI\)1521-4141\(199901\)29:01<203::AID-IMMU203>3.0.CO;2-B](https://doi.org/10.1002/(SICI)1521-4141(199901)29:01<203::AID-IMMU203>3.0.CO;2-B).
 36. Gough PJ, Garton KJ, Wille PT, Rychlewski M, Dempsey PJ, Raines EW. 2004. A disintegrin and metalloproteinase 10-mediated cleavage and shedding regulates the cell surface expression of CXCL chemokine ligand 16. *J Immunol* 172:3678–3685. <https://doi.org/10.4049/jimmunol.172.6.3678>.
 37. Arango Duque G, Descoteaux A. 2015. *Leishmania* survival in the macrophage: where the ends justify the means. *Curr Opin Microbiol* 26:32–40. <https://doi.org/10.1016/j.mib.2015.04.007>.
 38. Steffen S, Abraham S, Herbig M, Schmidt F, Blau K, Meisterfeld S, Beissert S, Guck J, Gunther C. 2018. Toll-like receptor-mediated upregulation of CXCL16 in psoriasis orchestrates neutrophil activation. *J Invest Dermatol* 138:344–354. <https://doi.org/10.1016/j.jid.2017.08.041>.
 39. Srivastava S, Pandey SP, Jha MK, Chandel HS, Saha B. 2013. *Leishmania* expressed lipophosphoglycan interacts with Toll-like receptor (TLR)-2 to decrease TLR-9 expression and reduce anti-leishmanial responses. *Clin Exp Immunol* 172:403–409. <https://doi.org/10.1111/cei.12074>.
 40. Kawai T, Akira S. 2011. Toll-like receptors and their crosstalk with other innate receptors in infection and immunity. *Immunity* 34:637–650. <https://doi.org/10.1016/j.immuni.2011.05.006>.
 41. Chandrasekar B, Mummidi S, Valente AJ, Patel DN, Bailey SR, Freeman GL, Hatanoto M, Tokuhisa T, Jensen LE. 2005. The pro-atherogenic cytokine interleukin-18 induces CXCL16 expression in rat aortic smooth muscle cells via MyD88, interleukin-1 receptor-associated kinase, tumor necrosis factor receptor-associated factor 6, c-Src, phosphatidylinositol 3-kinase, Akt, c-Jun N-terminal kinase, and activator protein-1 signaling. *J Biol Chem* 280:26263–26277. <https://doi.org/10.1074/jbc.M502586200>.
 42. Zhang N, Prasad S, Huyghe Despointes CE, Young J, Kima PE. 2018. *Leishmania* parasitophorous vacuole membranes display phosphoinositides that create conditions for continuous Akt activation and a target for miltefosine in *Leishmania* infections. *Cell Microbiol* 11:e12889. <https://doi.org/10.1111/cmi.12889>.
 43. Roux PP, Topisirovic I. 2018. Signaling pathways involved in the regulation of mRNA translation. *Mol Cell Biol* 38:e00070-18. <https://doi.org/10.1128/MCB.00070-18>.
 44. Cheekatla SS, Aggarwal A, Naik S. 2012. mTOR signaling pathway regulates the IL-12/IL-10 axis in *Leishmania donovani* infection. *Med Microbiol Immunol* 201:37–46. <https://doi.org/10.1007/s00430-011-0202-5>.
 45. Leroux LP, Lorent J, Graber TE, Chaparro V, Masvidal L, Aguirre M, Fonseca BD, van Kempen LC, Alain T, Larsson O, Jaramillo M. 2018. The protozoan parasite *Toxoplasma gondii* selectively reprograms the host cell transcriptome. *Infect Immun* 86:e00244-18. <https://doi.org/10.1128/IAI.00244-18>.
 46. Hurrell BP, Beaumann M, Heyde S, Regli IB, Muller AJ, Tacchini-Cottier F. 2017. *Leishmania mexicana* amastigotes can replicate within neutrophils. *J Leukoc Biol* 102:1187–1198. <https://doi.org/10.1189/jlb.4HI0417-158R>.
 47. Linke B, Meyer Dos Santos S, Picard-Willems B, Keese M, Harder S, Geisslinger G, Scholich K. 2017. CXCL16/CXCR6-mediated adhesion of human peripheral blood mononuclear cells to inflamed endothelium. *Cytokine* <https://doi.org/10.1016/j.cyto.2017.06.008>.
 48. Kumar R, Nylen S. 2012. Immunobiology of visceral leishmaniasis. *Front Immunol* 3:251. <https://doi.org/10.3389/fimmu.2012.00251>.
 49. Engwerda CR, Ato M, Cotterell SE, Mynott TL, Tschannerl A, Gorak-Stolinska PM, Kaye PM. 2002. A role for tumor necrosis factor-alpha in remodeling the splenic marginal zone during *Leishmania donovani* infection. *Am J Pathol* 161:429–437. [https://doi.org/10.1016/S0002-9440\(10\)64199-5](https://doi.org/10.1016/S0002-9440(10)64199-5).
 50. Bahr V, Stierhof Y-D, Ilg T, Demar M, Quinten M, Overath P. 1993. Expression of lipophosphoglycan, high-molecular weight phosphoglycan and glycoprotein 63 in promastigotes and amastigotes of *Leishmania mexicana*. *Mol Biochem Parasitol* 58:107–121. [https://doi.org/10.1016/0166-6851\(93\)90095-F](https://doi.org/10.1016/0166-6851(93)90095-F).
 51. Naderer T, McConville MJ. 2008. The *Leishmania*-macrophage

- interaction: a metabolic perspective. *Cell Microbiol* 10:301–308. <https://doi.org/10.1111/j.1462-5822.2007.01096.x>.
52. Katholnig K, Linke M, Pham H, Hengstschlager M, Weichhart T. 2013. Immune responses of macrophages and dendritic cells regulated by mTOR signalling. *Biochem Soc Trans* 41:927–933. <https://doi.org/10.1042/BST20130032>.
 53. Leroux LP, Dasanayake D, Rommereim LM, Fox BA, Bzik DJ, Jardim A, Dzierszinski FS. 2015. Secreted *Toxoplasma gondii* molecules interfere with expression of MHC-II in interferon gamma-activated macrophages. *Int J Parasitol* 45:319–332. <https://doi.org/10.1016/j.ijpara.2015.01.003>.
 54. Huang C, Turco SJ. 1993. Defective galactofuranose addition in lipophosphoglycan biosynthesis in a mutant of *Leishmania donovani*. *J Biol Chem* 268:24060–24066.
 55. Matte C, Descoteaux A. 2010. *Leishmania donovani* amastigotes impair gamma interferon-induced STAT1alpha nuclear translocation by blocking the interaction between STAT1alpha and importin-alpha5. *Infect Immun* 78:3736–3743. <https://doi.org/10.1128/IAI.00046-10>.
 56. Turco SJ, Wilkerson MA, Clawson DR. 1984. Expression of an unusual acidic glycoconjugate in *Leishmania donovani*. *J Biol Chem* 259:3883–3889.
 57. Howell GR, Soto I, Ryan M, Graham LC, Smith RS, John SW. 2013. Deficiency of complement component 5 ameliorates glaucoma in DBA/2J mice. *J Neuroinflammation* 10:76. <https://doi.org/10.1186/1742-2094-10-76>.
 58. Taylor S, Wakem M, Dijkman G, Alsarraj M, Nguyen M. 2010. A practical approach to RT-qPCR—publishing data that conform to the MIQE guidelines. *Methods* 50:S1–S5. <https://doi.org/10.1016/j.ymeth.2010.01.005>.
 59. Fulwiler AL, Soysa DR, Ullman B, Yates PA. 2011. A rapid, efficient and economical method for generating leishmanial gene targeting constructs. *Mol Biochem Parasitol* 175:209–212. <https://doi.org/10.1016/j.molbiopara.2010.10.008>.