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# *Leishmania* Promastigotes Induce Cytokine Secretion in Macrophages through the Degradation of Synaptotagmin XI

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## Abstract

Synaptotagmins (Syts) are type-I membrane proteins that regulate vesicle docking and fusion in processes such as exocytosis and phagocytosis. We recently discovered that Syt XI is a recycling endosome- and lysosome-associated protein that negatively regulates the secretion of TNF and IL-6. In this study, we show that Syt XI is directly degraded by the zinc metalloprotease GP63 and excluded from *Leishmania parasitophorous* vacuoles by the promastigotes surface glycolipid lipophosphoglycan. Infected macrophages were found to release TNF and IL-6 in a GP63-dependent manner. To demonstrate that cytokine release was dependent on GP63-mediated degradation of Syt XI, small interfering RNA-mediated knockdown of Syt XI before infection revealed that the effects of small interfering RNA knockdown and GP63 degradation were not cumulative. In mice, i.p. injection of GP63-expressing parasites led to an increase in TNF and IL-6 secretion and to an augmented influx of neutrophils and inflammatory monocytes to the inoculation site. Both of these cell types have been shown to be infection targets and aid in the establishment of infection. In sum, our data revealed that GP63 induces proinflammatory cytokine release and increases infiltration of inflammatory phagocytes. This study provides new insight on how *Leishmania* exploits the immune response to establish infection.

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## Introduction

Parasites of the *Leishmania* genus are the causative agents of the leishmaniasis, a group of human neglected parasitic diseases endemic in many regions of the world (1). The life cycle of *Leishmania* is digenetic. Promastigote forms are inoculated into the mammalian host by infected sand flies and are ingested by phagocytes, where they form parasitophorous vacuoles (PV). Phagocytes are crucial for both immunity and development; they ingest apoptotic bodies, foreign particles, destroy microbes, and present Ags (2, 3). However, many pathogens have evolved the ability of using phagocytosis to hide from the immune system and replicate intracellularly (4, 5). Macrophages and other phagocytes also secrete a panoply of pleiotropic cytokines—such as TNF and IL-6—that are responsible for establishing an effective immune response, and for linking innate and adaptive immunity (3, 6, 7). Within phagocytic cells, promastigotes differentiate into amastigotes that replicate in phagolysosomes. Infected phagocytes are characterized by a deactivation of their effector functions, which may contribute to the suppression of cell-mediated immunity observed during leishmaniasis. *Leishmania* parasites have the capacity of subverting phagocytosis (8) and of modulating cytokine secretion (9), allowing the parasite to thrive within phagocytic cells and within the organism as a whole.

A salient feature of *Leishmania* parasites is their capacity to alter phagocyte biology through pathogenicity factors such as lipophosphoglycan (LPG) and the zinc metalloprotease GP63, both of which are predominant at the promastigote stage of the parasite (8, 10–12). GP63 cleaves proteins involved in the regulation of phagocyte functions (13), leading to altered cell signaling, to subversion of transcription and translation (14–17), Ag cross-

presentation (18), lipid metabolism (19, 20), and likely to other unknown effects. LPG is a complex glycosphospholipid that supports parasite survival both in the sand fly gut and in the phagosome (8, 21). In the macrophage phagosome, LPG inhibits phagolysosomal biogenesis through alteration of membrane fusogenic properties (22). In this regard, we recently reported that *Leishmania* targets Synaptotagmin (Syt) V—a regulator of particle uptake and phagosome maturation—by impeding the recruitment of this Syt to phagocytic cups via LPG (23, 24). Syts constitute a large family of membrane-associated proteins that regulate vesicle-associated processes ranging from exocytosis (25, 26) to phagocytosis (27, 28). Because of the roles of Syts in vesicle trafficking, they are attractive targets for pathogens. For instance, the parasite *Trypanosoma cruzi* uses Syt VII, a Ca<sup>2+</sup>-dependent regulator of lysosome exocytosis, to invade target cells (29). In the case of Syt V, abrogation of Syt V recruitment by LPG results in the exclusion of the V-ATPase and in reduced phagosomal acidification (23).

During phagocytosis, both IL-6 and TNF are shepherded to the macrophage surface and to the site of phagocytic cup formation via a highly orchestrated pathway that is directed by members of the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptors (SNARE) family. From the Golgi apparatus, SNARE proteins Syntaxin-6, -7, and Vti1b control trafficking to recycling endosomes, and thence, the vesicle-associated membrane protein 3 guides trafficking of TNF- and IL-6-containing vesicles from recycling endosomes to the cell surface (7, 30). A pivotal function of these two proinflammatory cytokines is to help recruit phagocytes to the inflammation site through modulation of chemokine release and expression of adhesion molecules (31–34). Inoculation of *L. major* promastigotes has been shown to be followed by the infiltration of neutrophils and inflammatory monocytes. Both of these cell types are infection targets and important for the establishment of infection (35–37). Syts are regulators of SNARE complex activity and play a role in cytokine release. Indeed, we recently reported that Syt XI, which inhibits vesicle fusion (38), dampens the secretion of both TNF and IL-6 (26). Interestingly, it has been demonstrated that *L. major* promastigotes induce the secretion of TNF and IL-6 following their internalization by macrophages (9, 39–42). This raises the possibility that these parasites could disrupt membrane fusion regulators to induce proinflammatory cytokine release. Given the inhibitory role of Syt XI in cytokine secretion (26), we hypothesized that *Leishmania* could target Syt XI to facilitate release of TNF and IL-6 postinfection. This may then regulate the infiltration of inflammatory phagocytes to the infection site.

In this research, we demonstrate that in macrophages, Syt XI was excluded from PVs via LPG and degraded by *Leishmania* promastigotes in a GP63-dependent fashion, leading to increased secretion of TNF and IL-6. Furthermore, we show that in early infection, GP63 augmented *in vivo* TNF and IL-6 release, as well as the accrual of neutrophils and inflammatory monocytes. These data indicate that GP63 enables *Leishmania* promastigotes to elicit cytokine release and inflammatory phagocyte recruitment to the site of infection, both of which may contribute to the establishment of infection.

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## Materials and Methods

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### Ethics statement

Experiments involving mice and hamsters were done as prescribed by protocols 1312-03 and 1302-02, respectively, which were approved by the Comité Institutionnel de Protection des Animaux of the INRS-Institut Armand-Frappier. These protocols respect procedures on good animal practice provided by the Canadian Council on animal care.

### Abs and plasmids

The mouse monoclonal anti-LPG (CA7AE) Ab (43) was from Cedarlane and the mouse mAb anti-GP63 was a gift from Dr. W. R. McMaster (University of British Columbia, Vancouver, BC, Canada) (44, 45). The rabbit polyclonal Ab targeting the C2A domain of Syt XI was purified by affinity chromatography (46). Rabbit polyclonal Abs anti-p38 and -GFP were obtained from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. For flow cytometrical analysis of cellular infiltrates, the following anti-mouse Abs recognizing various Ags were used: CD11c-allophycocyanin (clone HL3), CD11b-biotin (clone MI/70), MHCII-FITC (clone 2G9), and Gr1-PE (clone RB6-8C5) (all from BD Biosciences). Streptavidin V450 was from eBioscience. The anti-mouse CCR2-AlexaFluor 700 and anti-CD-23-PerCp-Cy5 (clone B3B4) Abs were obtained from R&D Systems and BioLegend, respectively.

To express GST-Syt XI-cyto in *Escherichia coli*, the Syt XI-cyto construct was cloned into the BamHI/NotI site of the pGEX-4T3 vector (Amersham Biosciences) as described previously (47).

### Cell culture

The mouse macrophage cell line RAW264.7 and RAW264.7 cells expressing the FLAG-Syt XI-GFP construct (26) were cultured in complete DMEM with L-glutamine (Life Technologies) and complemented with 10% heat-

inactivated FBS (PAA Laboratories), 10 mM HEPES (Bioshop) at pH 7.4, and antibiotics (Life Technologies) in a 37°C incubator with 5% CO<sub>2</sub>. Bone marrow-derived macrophages (BMM) were extracted from the bone marrow (48) of 6- to 8-wk-old female BALB/c mice (Charles River Laboratories), and differentiated with complete DMEM supplemented with 15% v/v L929 cell-conditioned medium as a source of CSF 1. To render BMM quiescent prior to experiments, cells were transferred to tissue-culture treated petris and kept for 16 h in complete DMEM without L929 cell-conditioned medium.

Promastigotes were cultured in *Leishmania* medium (medium 199 [Sigma-Aldrich] with 10% heat-inactivated FBS, 40 mM HEPES at pH 7.4, 100 µM hypoxanthine, 5 µM hemin, 3 µM bipterin, 1 µM biotin, and antibiotics), in a 26°C incubator with 5% CO<sub>2</sub>. *Leishmania major* NIH S clone A2 promastigotes (wild-type [WT],  $\Delta gp63$ , and  $\Delta gp63+gp63$ ) were provided by Dr. W. R. McMaster (University of British Columbia), and *Leishmania tarentolae* Parrot-TarII promastigotes were a gift from Dr. B. Papadopoulou (Université Laval, Quebec, QC, Canada). Amastigotes of the LV9 strain of *Leishmania donovani* were extracted from the spleens of infected female HsdHan:AURA hamsters (Harlan Sprague Dawley) as described previously (49). Incubation in *Leishmania* medium at pH 7.4, 26°C, for 10–15 d was used to differentiate LV9 amastigotes into promastigotes. The  $\Delta gp63+gp63$  *L. major* and  $\Delta lpg2$  *L. donovani* LV9 promastigotes were grown in *Leishmania* medium supplemented with 50 µg/ml G418 (Life Technologies) or 100 mg/ml hygromycin (Roche), respectively. Prior to infections, promastigotes in late stationary phase were opsonized with BALB/c mouse serum (23).

### Transfections, infections, and cytokine quantification

For small interfering RNA (siRNA) transfections, RAW264.7 macrophages were plated in the absence of antibiotics for 16 h on glass coverslips (Fisher), or on 24-well plates. Then, macrophages were subjected to two rounds of transfection with Oligofectamine (Life Technologies), with each transfection being 24 h apart (30). Macrophages were mock transfected, transfected with siRNA to GFP (50), or with the ON-TARGETplus SMARTpool siRNA to Syt XI (Thermo Scientific), which contains four siRNA with the following sequences: sequence 1, 5'-CGAUCGACUACUAAGAAU-3'; sequence 2, 5'-GAGAGAGGUCUGCGAGAGU-3'; sequence 3, 5'-AUGUCAAGGUGAACGUCUA-3'; and sequence 4, 5'-GCACAGUCUGAGCGAGUAC-3'. Basic local alignment search tool searches were performed to confirm that these sequences targeted only the Syt XI mRNA. After the second transfection, macrophages were cultured for 24 h prior to stimulation or infection.

Using 24-well plates and a final volume of 300 µl, 3 × 10<sup>6</sup> adherent BMM or RAW264.7 macrophages were stimulated with 0.2 ng/ml LPS from *E. coli* Serotype O127:B8 (Sigma-Aldrich), or infected with opsonized *L. major*, *L. tarentolae*, or *L. donovani* promastigotes or amastigotes. After stimulation or infection, cell culture supernatants were collected and centrifuged to remove non-internalized parasites and debris. ELISA kits were used as per the manufacturers' protocols to quantify murine IL-6 (BD OptiEIA; BD Biosciences) and TNF (Ready-SET-Go! Mouse TNF-α Kit; eBioscience) secretion.

### Synchronized phagocytosis assays

For synchronized phagocytosis using zymosan or parasites, macrophages were incubated at 4°C for 15 min (26). Macrophages were washed with cold complete DMEM to remove excess particles, and internalization was triggered by transferring cells to 37°C for the required times (27). Cells were then washed with PBS and prepared for confocal immunofluorescence microscopy. Zymosan was either opsonized with mouse serum (51), or coated (23, 24) with purified *L. donovani* LPG (52, 53).

### Confocal immunofluorescence microscopy

Macrophages on coverslips were fixed with 2% paraformaldehyde (Canemco and Mirvac) for 7 min and blocked and permeabilized for 17 min with a solution of 0.1% Triton X-100, 1% BSA, 6% nonfat milk, 2% goat serum, and 50% FBS. This was followed by a 2-h incubation with primary Abs diluted in PBS. Then, macrophages were incubated with a suitable combination of secondary Abs (anti-rabbit Alexa Fluor 488, anti-rat 568, and anti-mouse 568; Molecular Probes) and DRAQ5 (Biostatus). Coverslips were washed three times with PBS after every step. After the final washes, Fluoromount-G (Southern Biotechnology Associates) was used to mount coverslips on glass slides (Fisher), and coverslips were sealed with nail polish (Sally Hansen). Macrophages were imaged with the ×63 objective of an LSM780 microscope (Carl Zeiss Microimaging), and images were taken in sequential scanning mode. Image analysis was performed with the ZEN 2011 software. For fluorescence intensity profiles, a 1-pixel line was traced around phagocytic cups using the DIC (TPMT) channel as guide; the same microscope settings were used for all conditions.

### Lyses, SDS-PAGE, and Western blotting

Prior to lysis, adherent macrophages or parasites were placed on ice and washed with PBS containing 1 mM sodium orthovanadate and 2 mM 1,10-phenanthroline. Cells were scraped in the presence of lysis buffer containing 1% Nonidet P-40, 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8), 2 mM 1,10-phenanthroline, and phosphatase and protease inhibitors (Roche). Alternatively, macrophages were lysed in 8 M

Urea supplemented with 400 U/ml benzonase nuclease (Qiagen) (54). After incubation at  $-70^{\circ}\text{C}$ , lysates were centrifuged for 10 min to remove insoluble matter. After protein quantification, 30  $\mu\text{g}$  sample was boiled ( $100^{\circ}\text{C}$ ) for 6 min in SDS sample buffer and migrated in 10% SDS-PAGE gels. Proteins were transferred onto Hybond-ECL membranes (Amersham Biosciences), blocked for 2 h in TBS1X-0.1% Tween containing 5% skim milk, incubated with primary Abs (diluted in TBS1X-0.1% Tween containing 5% BSA) overnight at  $4^{\circ}\text{C}$ , and thence with appropriate HRP-conjugated secondary Abs for 1 h at room temperature. Then, membranes were incubated in ECL (GE Healthcare), and immunodetection was achieved via chemiluminescence.

#### Protein purification and in vitro degradation assays

The pGEX-4T3-Syt XI-cyto plasmid was transformed into *E. coli* BL21 ( $\lambda\text{DE3}$ ), and protein production was induced in 200 ml Luria-Bertani (+100  $\mu\text{g}/\text{ml}$  ampicillin) containing 10 mM isopropyl  $\beta$ -D-thiogalactoside (Roche) for 24 h. Bacteria were centrifuged and resuspended in GST binding buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 1 mM EDTA) containing 0.5% Triton X-100 and complete protease inhibitors. This mixture was frozen for 30 min at  $-70^{\circ}\text{C}$ , thawed on ice, and sonicated with a Branson Sonifier 150 (three 20-s pulses in continuous mode at intensity 5; 20-s pause among pulses). After centrifugation, 500  $\mu\text{l}$  glutathione beads (Amersham Biosciences) was added to supernatant and incubated at  $4^{\circ}\text{C}$  with agitation for 2 h. Beads were then spun and washed three times with 20 ml GST binding buffer. GST-Syt XI-cyto was eluted by resuspending beads—twice—in a solution containing 10 mM reduced glutathione, 50 mM Tris-HCl (pH 8), and 5% glycerol for 15 min at room temperature.

For in vitro GST-Syt XI-cyto cleavage assays, 1  $\mu\text{g}$  eluted GST-Syt XI-cyto was incubated with  $30 \times 10^6$  *L. major* promastigotes in 200  $\mu\text{l}$  *Leishmania* medium, or with the equivalent amount of promastigote lysate in 200  $\mu\text{l}$  binding buffer (PBS1X, 1 mM  $\text{ZnCl}_2$ ). Incubations were carried out in 0.6-ml tubes at  $37^{\circ}\text{C}$  with agitation. For GP63 inhibition experiments, *Leishmania* lysates were preincubated with 2 mM 1,10-phenanthroline for 1 h prior to addition of GST-Syt XI-cyto. After incubations, parasites were discarded, SDS loading buffer containing 2 mM 1,10-phenanthroline was added, and samples were boiled at  $100^{\circ}\text{C}$  for 6 min. Ten microliters of each sample was loaded on SDS-PAGE gels, and degradation was analyzed by Western blot.

#### Intraperitoneal infections and FACS analysis

Female BALB/c mice were inoculated i.p. with  $2 \times 10^6$  *L. major* promastigotes (WT,  $\Delta\text{gp63}$ , and  $\Delta\text{gp63}+\text{gp63}$ ) in late stationary phase (35). Promastigotes were gently resuspended in 0.5 ml filtered HBSS (Life Technologies) and injected into the lower left abdominal quadrant using a 1 ml syringe mounted with a  $26\text{g} \times 1/2$  needle. Four hours postinoculation, mice were euthanized, and i.p. cavities were flushed with 5 ml cold HBSS containing 10 mM EDTA (pH 7.4). Lavages were centrifuged, supernatants were probed for cytokine content via ELISA, and cell populations were characterized via FACS. Cells were resuspended in cold PBS containing 1% horse serum (Sigma-Aldrich), 0.1%  $\text{NaN}_3$ , and 5 mM EDTA (pH 8) and stained as previously described (55) using the aforementioned FACS Abs. Flow cytometric analysis was carried out using the LSRFortessa cytometer (Special Order Research Product; BD Biosciences), and the BD FACSDiva Software (version 6.2) was used for data acquisition and analysis.

#### Data analysis

Statistical significance was assessed via the two-tailed, unequal variance *t* test, or the two-tailed Mann–Whitney *U* test. Error bars denote the SEM. Graphs were plotted with SigmaPlot or GraphPad Prism 5.

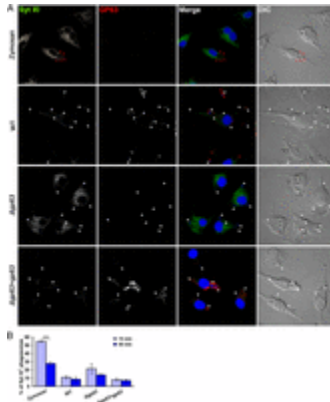
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## Results

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### *Leishmania* degrades Syt XI in a GP63-dependent manner

We previously discovered that Syt XI negatively regulates cytokine secretion and modulates phagosome maturation (26). Because *Leishmania* affects these processes, we postulated that this parasite may alter Syt XI integrity and function. For these reasons, we sought to investigate the fate of Syt XI in macrophages infected with *Leishmania* promastigotes. To this end, we infected BMM with WT *L. major* promastigotes for 1 h (Supplemental Fig. 1) or 6 h (Fig. 1A) and assessed Syt XI distribution by confocal microscopy. Because we recently discovered that *Leishmania* promastigotes cleave various SNAREs through GP63 (18), we included the isogenic GP63 null mutant ( $\Delta\text{gp63}$ ) and its complemented counterpart ( $\Delta\text{gp63}+\text{gp63}$ ) in our study. As shown in Fig. 1A (and Supplemental Fig. 1), we observed an absence of Syt XI staining in macrophages infected with WT or  $\Delta\text{gp63}+\text{gp63}$  promastigotes. This indicates that Syt XI levels were diminished in a GP63-dependent fashion. Syt XI recruitment to PVs also appeared greatly reduced in contrast to phagosomes containing zymosan. Quantification of PVs positive for Syt XI revealed that recruitment to PVs was hampered even in those containing the  $\Delta\text{gp63}$  mutant (Fig. 1B), suggesting that other factors may also aid in excluding Syt XI from PVs. Altogether, these data suggested that Syt XI was downmodulated in response to infection with GP63-expressing parasites and that Syt XI was excluded from PVs.



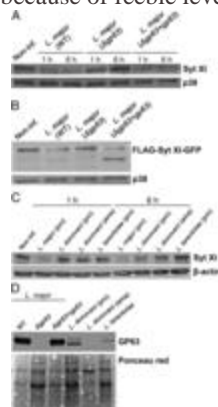
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### FIGURE 1.

GP63 lowers Syt XI levels in infected macrophages. (A) BMM were infected with opsonised *L. major* WT,  $\Delta gp63$ , or  $\Delta gp63+gp63$  promastigotes or with opsonized zymosan for 6 h. Syt XI (green) and GP63 (red) levels were then visualized by confocal microscopy. DNA is shown in blue (DRAQ5); white and red arrowheads denote internalized parasites and sites of Syt XI recruitment around zymosan phagosomes, respectively. (B) PVs hindered Syt XI recruitment independently of GP63. Syt XI recruitment to PVs was quantified after 15 min or 1 h of infection for 100 phagosomes. Experiments were repeated twice in triplicate, and error bars represent the SEM. \*\*\* $p < 0.001$ . Original magnification  $\times 63$ .

Given that GP63 cleaves a variety of macrophage proteins (14–19) and that we observed decreased Syt XI levels upon infection by GP63-expressing parasites, we set out to determine whether GP63 was degrading Syt XI. Indeed, Western blot analyses on lysates from BMM infected with WT,  $\Delta gp63$ , and  $\Delta gp63+gp63$  *L. major* promastigotes revealed a degradation of Syt XI that was dependent on the presence of GP63 (Fig. 2A). We also infected RAW264.7 macrophages expressing a FLAG-Syt XI-GFP fusion protein, and 6 h postinfection, we observed that this protein also was cleaved by parasites expressing GP63 (Fig. 2B). We then assayed whether promastigotes and amastigotes from other *Leishmania* species degraded Syt XI (Fig. 2C). Infection with the viscerotropic species *L. donovani* also resulted in Syt XI degradation 6 h postinfection, albeit to a lesser degree than observed for *L. major*. Interestingly, this reduced degradation of Syt XI observed in BMM infected with *L. donovani* promastigotes correlates with lesser GP63 levels in these parasites (Fig. 2D). Consistent with the absence of detectable GP63 (Fig. 2D), we observed that *L. donovani* amastigotes did not induce Syt XI degradation (Fig. 2C) (18,56). Similarly, infection with promastigotes of the nonpathogenic *L. tarentolae* species did not lead to Syt XI degradation (Fig. 2C), because of feeble levels (Fig. 2D) of inactive GP63 (57).



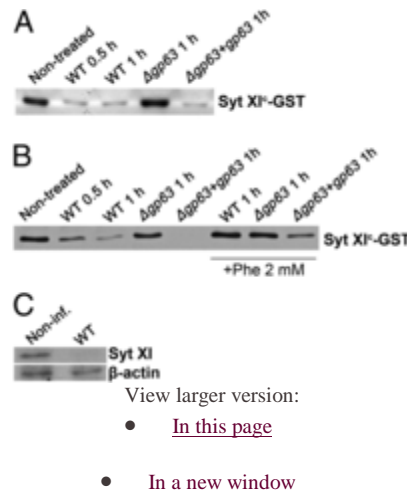
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**FIGURE 2.**

*Leishmania* promastigotes use GP63 to cleave Syt XI. (A) Syt XI degradation by *Leishmani*promastigotes is dependent on the GP63 metalloprotease. BMM were infected with opsonised *L. major* WT,  $\Delta gp63$ , or  $\Delta gp63+gp63$  promastigotes for 1 or 6 h, and Syt XI levels were assessed via Western blot analysis with an anti-Syt XI Ab. (B) RAW264.7 macrophages expressing FLAG-Syt XI-GFP also were infected for 6 h, and cleavage was evaluated with an anti-GFP Ab. (C) Syt XI degradation was also assayed in BMM infected with *L. donovani* promastigotes and amastigotes and with *L. tarentolae* promastigotes. (D) GP63 levels in stationary-phase promastigotes and amastigotes were assessed via Western blot analysis with an anti-GP63 Ab; a ponceau red staining of the gel was included as loading control. The lysis buffer used contained 1,10-phenanthroline to inhibit GP63 activity during lysate processing. All experiments were repeated at least three times. ama, amastigote; pro, promastigote.

Having established that Syt XI was degraded by GP63, we investigated whether this degradation happened directly or was because of an intermediate molecule. To this end, we incubated purified recombinant GST-Syt XI with either live parasites or with parasite lysates. In both cases, we observed that this recombinant Syt XI was degraded by parasites expressing GP63 and that this degradation increased with time (Fig. 3A, 3B). Moreover, we showed that the catalytic site of GP63 is important for the degradation of Syt XI because phenanthroline-mediated chelation of  $Zn^{2+}$  ions, which are critical for GP63 function, effectively inhibited cleavage by GP63-expressing promastigotes (Fig. 3B). Degradation by proteases also can be artifactual and may happen only after they are exposed to potential substrates after cell lysis (54). To exclude this possibility, we lysed control and WT *L. major*-infected macrophages in 8 M urea, a chaotropic agent. After Western blot analysis, we observed that Syt XI was still degraded in infected macrophages (Fig. 3C), similar to results obtained with the Nonidet P-40-based lysis buffer containing 1,10-phenanthroline (Fig. 2). Having established that *Leishmania* promastigotes degraded Syt XI through GP63 proteolysis, we explored the potential role of LPG as a contributor to Syt XI exclusion from PVs.

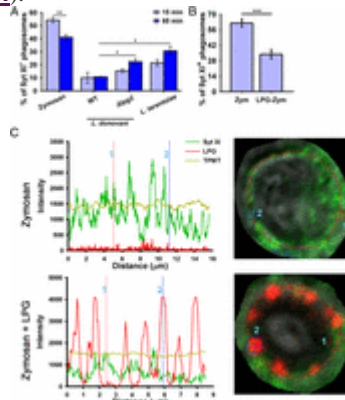
**FIGURE 3.**

Syt XI degradation is direct and does not occur during lysate processing. (A and B) GP63 degrades Syt XI directly. A GST-Syt XI<sup>c</sup> fusion protein was incubated with live *L. major* WT,  $\Delta gp63$ , or  $\Delta gp63+gp63$  promastigotes (A) or with parasite lysates (B), and levels were assessed via Western blot analysis with an anti-Syt XI Ab. Coincubation of parasite lysates with 2 mM 1,10-phenanthroline effectively blocks GP63 degradation of GST-Syt XI. (C) Lysates of *L. major* WT-infected BMM were prepared using 8 M urea to denature proteins during lysate processing; Syt XI degradation was assessed by Western blot analysis. Experiments were repeated at least three times. <sup>c</sup>, cytoplasmic domain of Syt XI.

**LPG mediates exclusion of Syt XI from PVs**

LPG inhibits phagolysosome maturation by impairing recruitment of antimicrobial effectors (23, 58). Because cleavage by GP63 does not account for the exclusion of Syt XI from PVs, we investigated whether LPG is mediating this phenotype. To address this question, we infected BMM with either WT or phosphoglycan-defective  $\Delta lpg2$  *L. donovani* promastigotes or with *L. tarentolae* promastigotes. Using confocal microscopy, we observed that after 1 h of phagocytosis, recruitment of Syt XI was higher in PVs containing LPG-defective  $\Delta lpg2$  *L. donovani* and the nonpathogenic *L. tarentolae* promastigotes, compared with PVs containing WT *L.*

*donovani* promastigotes (**Fig. 4A**). To directly show that LPG contributes to impaired Syt XI recruitment, we coated zymosan particles with LPG (**23, 24**) and analyzed Syt XI recruitment to phagosomes by confocal immunofluorescence microscopy. In LPG-coated zymosan phagosomes, we observed a ~2-fold decrease in Syt XI recruitment with respect to uncoated zymosan (**Fig. 4B**). Fluorescence intensity profile analysis of LPG-coated zymosan phagosomes revealed that LPG staining was not concomitant with Syt XI staining, implying that LPG-containing microdomains created in the membrane of phagosomes prevent Syt XI from associating to phagosomes (**Fig. 4C**). These data show that LPG also targeted Syt XI by excluding this protein from PVs, similar to what was found for Syt V (**24**).



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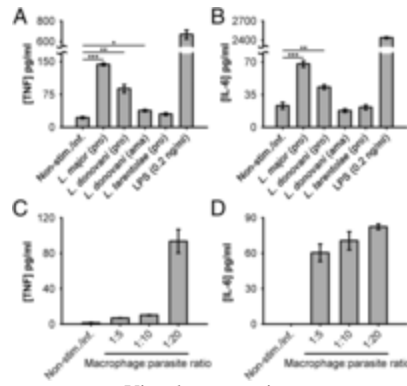
#### FIGURE 4.

LPG mediates exclusion of Syt XI from the phagosome. (A) LPG modulates the recruitment of Syt XI to the phagosome. BMM were given zymosan or infected with *L. donovani*LV9 (WT or  $\Delta lpg2$ ) or *L. tarentolae* promastigotes. (B) To directly assess the effect of LPG on Syt XI recruitment, BMM were fed with LPG-coated zymosan for 15 min. Syt XI recruitment on LPG-coated or naked zymosan was then assessed via confocal microscopy. (C) LPG excludes Syt XI from the phagocytic cup. Fluorescence profiles on images of representative phagosomes revealed that LPG (red) excluded Syt XI (green) from phagocytic cups (DIC, olive line). Recruitment was assessed for 100 phagosomes, and results are the average of two experiments done in triplicate. Error bars denote the SEM. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . Original magnification  $\times 63$ .

#### *Leishmania* induces TNF and IL-6 secretion through degradation of Syt XI

Previous studies showed that *L. major* promastigotes induce TNF and IL-6 secretion in macrophages (**9, 39, 40**). Because GP63 degrades a negative regulator of cytokine secretion (Syt XI), we set out to investigate whether GP63 had an impact on cytokine release induced by *L. major* promastigotes. We first compared promastigotes and amastigotes for their capacity to modulate TNF and IL-6 secretion in BMM. Six hours postinfection, both TNF and IL-6 were induced by *L. major* and *L. donovani* promastigotes, although *L. major* was a stronger inducer (**Fig. 5A, 5B**). In contrast, *L. tarentolae* promastigotes induced neither TNF nor IL-6, whereas *L. donovani* amastigotes failed to induce IL-6 but were able to elicit a slight increase in TNF secretion. Next, we infected BMM with *L. major* promastigotes at various doses, and secretion of TNF and IL-6 was found to be dose dependent (**Fig. 5C, 5D**).





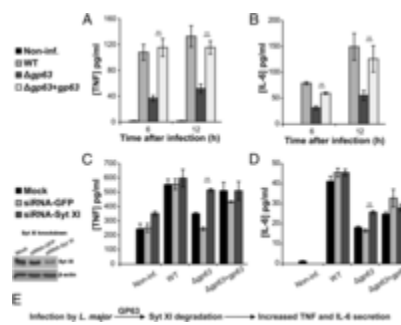
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### FIGURE 5.

*Leishmania* promastigotes induce TNF and IL-6 release. Infection with *L. major* and *L. donovani* promastigotes triggers the secretion of TNF (A) and IL-6 (B). BMM were either stimulated with LPS (0.2 ng/ml) or infected with promastigotes or amastigotes as indicated. Cytokine release in culture supernatants was then measured by ELISA. Cytokine secretion from infected cells is dependent on parasite dose. BMM were infected with the indicated *L. major* promastigote dose for 6 h, and TNF (C) and IL-6 (D) secretion were then quantified. Results are the mean of three independent experiments done in triplicate. Error bars denote the SEM. \* $p < 10^{-3}$ , \*\* $p < 10^{-5}$ , \*\*\* $p < 10^{-7}$ . ama, amastigote; pro, promastigote.

We next sought to establish whether GP63 contributes to the release of TNF and IL-6, because it had never been studied whether this protease plays a role in *Leishmania*-induced cytokine release. Wild-type and  $\Delta gp63+gp63$  *L. major* promastigotes induced significantly more TNF and IL-6 in BMM compared with  $\Delta gp63$  parasites (Fig. 6A, 6B). Because decreased levels of Syt XI lead to increased TNF and IL-6 secretion (26), it is possible that degradation of Syt XI is responsible for the increase in cytokine release induced by GP63. To directly test this possibility, we knocked down Syt XI in RAW264.7 macrophages (blot in Fig. 6C) and measured cytokine release following infection with WT,  $\Delta gp63$ , or  $\Delta gp63+gp63$  *L. major* promastigotes. Our data show that promastigotes expressing GP63 did not induce more cytokine secretion in macrophages treated with siRNA to Syt XI, with respect to macrophages transfected with siRNA to GFP, or mock transfected (Fig. 6C, 6D). In contrast,  $\Delta gp63$  promastigotes elicited significantly more cytokine secretion from macrophages treated with siRNA to Syt XI than from control-transfected macrophages. In addition, the cytokine levels induced by  $\Delta gp63$  parasites in macrophages treated with siRNA to Syt XI resembled the levels elicited by parasites expressing GP63. Hence, the effects of siRNA and GP63 degradation did not have a cumulative impact on parasite-induced TNF and IL-6 secretion. Altogether, our data are consistent with a model where *Leishmania* promastigotes elicit TNF and IL-6 secretion through a mechanism that implicates the degradation of Syt XI by GP63 (Fig. 6E). These data also support the notion that Syt XI is a negative regulator of cytokine secretion.



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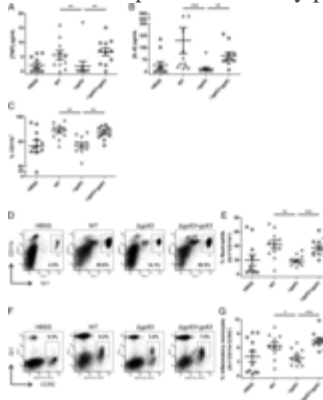
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**FIGURE 6.**

*L. major* induces TNF and IL-6 secretion via degradation of Syt XI. (A and B) The release of TNF and IL-6 is regulated by GP63. BMM were infected with WT,  $\Delta gp63$ , or  $\Delta gp63+gp63$  *L. major* promastigotes for 6 or 12 h. The results are the mean of three independent experiments done in triplicate. The effects of siRNA and GP63 on TNF (C) and IL-6 (D) secretion are not cumulative. After mock or siRNA transfections (Syt XI knockdown was assessed via Western blot analysis as shown), RAW264.7 macrophages were infected with *L. major* WT,  $\Delta gp63$ , or  $\Delta gp63+gp63$  promastigotes for 6 h. Culture supernatants were then subjected to ELISA. The results represent one representative experiment—done in triplicate—of three. Statistical significance was assessed with the Mann–Whitney *U* test. (E) Model for *L. major*-triggered secretion of TNF and IL-6 via GP63-mediated degradation of Syt XI. Error bars denote the SEM.  $**p < 0.01$ .

**GP63-expressing promastigotes elicit increased TNF and IL-6 release in vivo as well as increased neutrophil and inflammatory monocyte recruitment**

Having shown that infection with GP63-containing promastigotes caused an increase in TNF and IL-6 secretion from cultured macrophages, we sought to determine whether this phenomenon occurred in vivo. To test this hypothesis, we injected  $2 \times 10^6$  stationary-phase *L. major* WT,  $\Delta gp63$ , or  $\Delta gp63+gp63$  promastigotes in the peritoneal cavities of BALB/c mice, and 4 h later, we assessed cytokine levels and cell content in peritoneal lavages. As demonstrated with BMM (Figs. 5, 6), mice inoculated i.p. with GP63-expressing parasites displayed augmented TNF and IL-6 (Fig. 7A, 7B) levels in comparison with mice inoculated with  $\Delta gp63$  parasites or to control mice. TNF and IL-6 are cytokines that act on a variety of cells, including those of the endothelium (31), to induce chemokine release and adhesion molecules and thence inflammatory phagocyte accrual to the inflammation site (31, 33, 34). Taken together with the fact that *L. major* promastigotes induce inflammatory phagocyte recruitment to the infection site (35, 36), we investigated whether GP63 played a role in this phenomenon. Four hours postinoculation, we observed a GP63-dependent increase in CD11b<sup>+</sup> myeloid cell recruitment (Fig. 7C). Further characterization of this population revealed that GP63-expressing parasites induced a 2-fold increase in the percentages of neutrophils (Gr1<sup>hi</sup>CD11b<sup>hi</sup>) and inflammatory monocytes (Gr1<sup>+</sup>CD11b<sup>+</sup>CCR2<sup>+</sup>) recruited to the infection site (Fig. 7D–G). These data support a role for GP63 in proinflammatory cytokine release and recruitment of proinflammatory phagocytes to the infection site.



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**FIGURE 7.**

In vivo modulation of cytokine secretion and phagocyte infiltration by GP63. Mice were inoculated i.p. with WT,  $\Delta gp63$ , or  $\Delta gp63+gp63$  *L. major* promastigotes for 4 h, and TNF (A) and IL-6 (B) levels in i.p. lavages were measured. The abundance of CD11b<sup>+</sup> cells was assessed by flow cytometry (C). Neutrophils were analyzed by gating on live cells and then on CD11b<sup>hi</sup> and Gr1<sup>hi</sup> and inflammatory monocytes by gating on CD11b<sup>+</sup> and subsequently on Gr1<sup>+</sup> and CCR2<sup>+</sup> cells. Representative dot plots and relative percentages of neutrophils (D and E) and inflammatory monocytes (F and G) are shown. Graphs show the mean of three independent experiments—of five—performed using at least three mice per group; error bars denote the SEM.  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ .

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## Discussion

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We previously characterized Syt XI as a negative regulator of cytokine secretion and of phagosome maturation (26). In this work, we demonstrated that Syt XI is both degraded by the zinc metalloprotease GP63, and excluded from the phagosome by LPG. Given that *L. major* promastigotes trigger the release of TNF and IL-6, we found that in macrophages, degradation of Syt XI by GP63 modulated this proinflammatory cytokine release. Importantly, GP63 augmented cytokine release in vivo and promoted infiltration of neutrophils and inflammatory monocytes to the inoculation site. Taken together, our results support the notion that GP63 contributes to the inflammatory response induced early during infection.

*Leishmania* parasites have evolved to thwart the immune response by alteration of signaling pathways that would otherwise coalesce to mount a strong microbicidal response by macrophages (59). Albeit known as anti-inflammatory, some *Leishmania* species such as *L. major* can upregulate the secretion of proinflammatory cytokines (9, 39, 40). Other studies have shown that *L. major* promastigotes trigger the release of TNF and IL-6, at least during the initial stages of infection (39, 41, 42). Furthermore, *L. major* promastigotes triggers the infiltration of inflammatory phagocytes shortly after inoculation (35–37). We expanded upon these findings by demonstrating that infected BMM secrete both of these cytokines and that release of TNF and IL-6 was dependent on the presence of GP63. Knowing that *L. major* promastigotes induce the secretion of proinflammatory cytokines, we hypothesized that Syt XI could also be targeted by *Leishmania*. Because Syt XI is a negative regulator of cytokine secretion (26), we inferred that its degradation could be a strategy used by the parasite to modulate cytokine release. Indeed, decreased levels of Syt XI in *L. major*-infected macrophages were caused by GP63-dependent cleavage. Interestingly, reduced or absence of Syt XI degradation by either *L. donovani* or *L. tarentolae* promastigotes, respectively, correlated with a smaller or no induction of TNF and IL-6 by these parasites. When we treated macrophages with siRNA to Syt XI prior to infection with GP63-expressing promastigotes, we did not observe increased cytokine secretion in comparison with control-transfected macrophages. On the contrary, GP63-lacking promastigotes triggered significantly more cytokine release from macrophages treated with siRNA to Syt XI. These findings show that *Leishmania* uses GP63 to degrade a negative regulator of cytokine secretion to augment secretion of proinflammatory cytokines from macrophages. Our data confirmed that GP63 boosts TNF and IL-6 secretion in vivo. Inoculation of GP63-expressing parasites also resulted in augmented infiltration of inflammatory monocytes and neutrophils early in infection. In vivo induction of TNF and IL-6 secretion by *L. major* promastigotes may help recruit phagocytes (9), because both of these inflammatory cytokines mediate monocyte and neutrophil recruitment via stimulation of chemokine production and release (31, 33). TNF and IL-6 can be produced by infected resident macrophages, and these cytokines may then act on surrounding PBMCs (33) and endothelium (31) to trigger release of chemokines such as CXCL2 and CCL2. These can in turn recruit neutrophils and inflammatory monocytes to the infection site. Such an early effect of GP63 on cytokine secretion and phagocyte recruitment can have important consequences in the infection process. Infection of neutrophils can transfer infection to macrophages once infected apoptotic neutrophils are phagocytosed (60). In addition, neutrophil depletion can severely hamper infection (36). Inflammatory monocytes are also infected, and although they have been shown to kill *Leishmania* in vitro (35), infected cells in peritoneal infiltrates are present after 4 h of infection (36). Infected inflammatory monocytes and resident macrophages can also produce IL-10, which promotes the establishment of chronic infection (61, 62). Hence, the finding that GP63 modulates phagocyte recruitment to the infection site may contribute to the development of pathologies associated with *L. major* infection. Whether phagocyte recruitment and release of other cytokines and chemokines are altered through GP63-mediated degradation of Syt XI in vivo is an issue that will deserve further investigation.

There is clear evidence that *Leishmania* promastigotes use at least two distinct mechanisms to impair phagolysosome composition and function through subversion of the membrane fusion machinery. The first mechanism consists in disrupting lipid microdomains of the phagosome membrane through the insertion of LPG (63, 64). We showed that this prevents association of Syt V with the phagosome membrane, impairing phagosomal acidification (23). The second mechanism consists in cleaving SNAREs through the action of GP63, causing a modification of phagosomal properties required for optimal Ag processing and cross-presentation (18). The finding that Syt XI is both degraded by GP63 and excluded from the phagosome membrane by LPG indicates that these two major promastigote surface molecules can act in concert to prevent the action of this membrane fusion regulator. To our knowledge, this is the first example of a macrophage molecule targeted by those two *Leishmania* virulence factors. We recently showed that Syt XI regulates the recruitment of gp91<sup>phox</sup> and of the lysosomal-associated membrane protein-1 to phagosomes, suggesting that the LPG-mediated exclusion of Syt XI from phagosomes may contribute to the phagosome remodelling induced by *Leishmania* promastigotes (18). Identification of Syt XI binding partners will be necessary to understand how Syt XI regulates cytokine secretion and phagolysosome biogenesis. In addition, it will be of interest to investigate whether those Syt XI interactors are also targeted by GP63 and LPG.

How GP63 accesses and cleaves Syt XI is not known. In macrophages, Syt XI is found on recycling endosomes and lysosomes (26), and GP63 is known to redistribute throughout the macrophage in vesicular structures throughout the cytoplasm (Fig. 1, Supplemental Fig. 1) (18), in lipid rafts (14, 17), and in the perinuclear area (17). Because GP63 is GPI anchored, it is likely to come into close proximity with Syt XI in membranes. Addressing this issue and characterizing the trafficking of GP63 in infected cells will result in a better understanding of how this protease accesses its multiple targets.

In summary, our data reveal that GP63 induces early TNF and IL-6 release both in vitro and in vivo and contributes to the increase in inflammatory phagocyte infiltration to the inoculation site. Improved knowledge of *Leishmania*-induced inflammation will further our understanding of how the parasite establishes infection, modulates the immune response, metastasizes, and causes pathology.

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## Disclosures

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The authors have no financial conflicts of interest.

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## Footnotes

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