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Comparative Study of the Ability of *Leishmania mexicana* Promastigotes and Amastigotes To Alter Macrophage Signaling and Functions[▽]

Issa Abu-Dayyeh,[1,2] Kasra Hassani,[1,2] Edze R. Westra,[1,3] Jeremy C. Mottram,[4] and Martin Olivier[1,2]*

Department of Microbiology and Immunology, McGill University, Montréal, QC, Canada[1]; Centre for the Study of Host Resistance, Research Institute of the McGill University Health Centre, Montréal, QC H3A 2B4, Canada[2]; Laboratory of Microbiology, Department of Agrotechnology and Food Sciences, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, Netherlands[3]; and Wellcome Centre for Molecular Parasitology and Division of Infection & Immunity, Faculty of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8TA, United Kingdom[4]

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Leishmania **alternates between two morphologically different stages, promastigotes and amastigotes. While the majority of reports focused on how the promastigote form can alter macrophage (Mϕ) signaling and function, fewer reports investigated signaling alterations mediated by amastigotes, and there is a lack of comparative studies. In this study, we performed a comparison between the ability of both forms of the parasite to alter Mϕ signaling and functions. Here, we show that both promastigotes and amastigotes were able to rapidly activate host protein tyrosine phosphatases (PTPs), importantly the Src homology 2 domain-containing PTP (SHP-1). However, we found that PTP-1B is specifically activated by promastigote but not amastigote infection and that *lmcpb*$^{-/-}$ promastigotes were no longer able to activate PTP-1B. We also show a similarity in the way promastigotes and amastigotes inactivate the transcription factors (TFs) STAT-1α and AP-1, but we show differences in the modulation of NF-κB, with promastigotes cleaving the p65 subunit, generating a smaller p35 subunit, and amastigotes fully degrading the p65 subunit with no p35 production. Importantly, we show that the cysteine proteinase LmCPb plays a key role in the alteration of NF-κB, STAT-1α, and AP-1 by promastigote and amastigote infections, ultimately leading to the inability of these TFs to translocate to the nucleus in response to gamma interferon (IFN-γ) stimulation and thus contributing to the ability of both parasite forms to effectively block IFN-γ-mediated nitric oxide (NO) production in Mϕs.**

Leishmania parasites are prevalent in more than 80 countries in the world. It is estimated that there are 12 million cases of leishmaniasis worldwide, with 2 million new cases emerging every year (8). The most common manifestations of the disease are as follows: (i) visceral leishmaniasis caused by *Leishmania donovani* and *Leishmania chagasi* and responsible for the majority of mortality cases, (ii) cutaneous leishmaniasis caused principally by *Leishmania major* and *Leishmania mexicana* and is the most common manifestation of the disease, and (iii) the disfiguring mucocutaneous leishmaniasis caused by *Leishmania braziliensis* (23).

Leishmania is a dimorphic protozoan alternating between the promastigote and amastigote stages. Promastigotes have an elongated shape with long flagella and live inside the sand fly vector. Once promastigotes are deposited into mammalian skin while the sand fly is having a blood meal, they are phagocytosed by cells of the monocyte/macrophage (Mϕ) lineage and rapidly transform into round amastigotes that are smaller than the promastigotes and lack external flagella (23). In addition to being morphologically different, the two life stages of the parasite have different surface molecule compositions. While infectious metacyclic promastigotes have a thick glycocalyx, this cover is almost completely absent in amastigotes (33). The glycocalyx is made of glycoproteins and other glycosylated species anchored to the surface membrane by a glycosylphosphatidylinositol (GPI) linkage (10). The promastigote surface is predominantly covered by lipophosphoglycan (LPG), a GPI-anchored molecule made of repeating units of a disaccharide and a phosphate. Buried in a sea of LPG, promastigotes have another important GPI-anchored molecule, the surface protease gp63. Interestingly, amastigotes have been shown to produce very little LPG compared to promastigotes (21) and have less gp63 (38) that, in the case of *L. mexicana*, has been shown to lack a GPI anchor and to be confined to the flagellar pocket (22).

Although LPG and, to a lesser extent, gp63 are the most studied virulence factors in *Leishmania*, other important virulence factors found in several *Leishmania* species include clan CA, family C1 cysteine proteinases (CPs) (26). *L. mexicana* has been shown to have cathepsin L-like CP genes (*lmcpb*) that are present in multiple copies and occur in a tandem array (39) and two single-copy CP genes, the cathepsin L-like gene (*lmcpa*) and the other cathepsin B-like gene (*lmcpc*). Unlike LPG and gp63, which are plentiful in promastigotes and are downregulated in amastigotes, LmCPb (*L. mexicana* cysteine proteinase b) is expressed at low levels in metacyclic promastigotes and is strongly upregulated in amastigotes (39), indicating that the protein may play a crucial role in the intracellular survival of the parasite.

Given that promastigotes have to avoid Mϕ microbicidal

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Duff Medical Building, Room 610, McGill University, 3775 University Street, Montréal, QC, Canada H3A 2B4. Phone: (514) 398-5592. Fax: (514) 398-7052. E-mail: martin.olivier@mcgill.ca.

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action in order to establish themselves in the host and that amastigotes have to suppress Mϕ killing abilities when they try to invade new Mϕs in the course of a persistent *Leishmania* infection, it is not surprising that both forms of the parasite can alter key Mϕ signaling pathways (31). Indeed, several studies have previously shown that promastigotes (1, 12, 35, 36) and amastigotes (24, 36, 41), or molecules derived from them, are able to block nitric oxide (NO) production by host Mϕs in response to activating stimuli, such as gamma interferon (IFN-γ) or bacterial lipopolysaccharide (LPS). The promastigote surface contains several glycoconjugates allowing interaction with Mϕs and internalization via several types of receptors, such as complement receptors 1, 3, and 4 (18, 40), the mannose fucose receptor (3), the C-reactive protein receptor (9), and the fibronectin receptor (6). On the other hand, amastigotes lack many of those glycoconjugates and seem to interact with Mϕs mainly through glycosylinositol phospholipids (GIPLs) and to be phagocytosed via the Fc receptor following opsonization by antibodies or via complement receptors. Although redundancies might exist in the way promastigotes and amastigotes interact with Mϕs and modulate their signaling in order to block their killing functions, the differences between both forms, whether at the gene expression, metabolic, or surface molecule level suggest to us that some differences ought to exist in the way those two forms can modulate Mϕ signaling to their own favor. This work is an effort to compare the similarities and differences between promastigotes and amastigotes of *L. mexicana* in terms of their ability to alter key signaling molecules, namely, protein tyrosine phosphatases (PTPs) and the transcription factors (TFs) nuclear factor kappa B (NF-κB), signal transducer and activator of transcription 1alpha (STAT-1α), and activating protein 1 (AP-1), known to play a pivotal role in NO production (19, 42) as well as other Mϕ functions detrimental to the survival of the *Leishmania* parasite (31). In addition, given the established role of LmCPb as a virulence factor and an immunomodulator (2, 27), this work explores the role of this proteinase in the *Leishmania*-induced alterations of signaling molecules that we observed.

MATERIALS AND METHODS

Cell culture and reagents. The immortalized B10R bone marrow-derived Mϕs (BMDMs) were derived from B10A.Bcg^r mice (37). The immortalized me-3 (SHP-1$^{-/-}$) and LM-1 (wild-type [WT]) BMDMs were generated from moth-eaten mice (*Ptpn6$^{me/me}$*; C3HeBFeJ *me*/*me*) and their respective wild-type littermates (C3HeBFeJ *me*/+) as previously described (14). Recombinant murine IFN-γ was purchased from Cedarlane, NC. Antibodies used to immunoprecipitate SHP-1 and PTP-1B were purchased from Upstate, NY.

***In vitro* infection.** Promastigotes of *L. mexicana* (MNYC/BZ/62/M379), and *L. mexicana* deficient for LmCPb generated by targeted gene deletion as previously described (27) were kept in SDM medium (10% fetal bovine serum [FBS]) (32), and stationary-phase parasites were used to infect cells in a parasite-to-Mϕ ratio ranging from 5:1 to 20:1. Axenic amastigotes of WT and *lmcpb*$^{-/-}$ *L. mexicana* were transformed from promastigote cultures, kept in MAA medium (medium for axenically grown amastigotes, pH 5.6, 20% FBS) (27), and incubated at a temperature of 32°C. When parasites were used for infections, noninternalized ones were removed by washing the plates with phosphate-buffered saline (PBS), after which Mϕs were collected for subsequent experiments.

Western blot analysis. Western blotting was performed as previously described (32). Proteins were detected using antibodies directed against gp63 (provided by Robert McMaster, University of British Columbia, Canada), LACK (provided by Eric Prina, Institut Pasteur, France), A2 (provided by Greg Matlashewski, McGill University, Canada), SHP-1 (Chemicon, CA), and PTP-1B (Upstate). Proteins were detected using a horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibody (Amersham, QC, Canada) and visualized using ECL Western blotting detection system (Amersham).

NO assay. NO production was evaluated by measuring the accumulation of nitrite in the culture medium by the Griess reaction, as previously described (13).

pNPP phosphatase assay. Mϕs were collected, lysed in PTP lysis buffer (50 mM Tris [pH 7.0], 0.1 mM EDTA, 0.1 mM EGTA, 0.1% β-mercaptoethanol, 1% Igepal, 25 μg/ml aprotinin, and 25 μg/ml leupeptin), and kept on ice for 45 min. Lysates were cleared by centrifugation, and protein content was determined by using the Bradford reagent. For experiments measuring phosphatase activity in total cell lysates, the lysates (30 μg) were incubated in a phosphatase reaction mix (50 mM HEPES [pH 7.5], 0.1% β-mercaptoethanol, 10 mM *p*-nitrophenylphosphate [pNPP]) for 30 min at 37°C, and the optical density (OD) at 405 nm was read. For experiments measuring phosphatase activity in immunoprecipitates (IPs), cell lysates were subjected to immunoprecipitation using protein A/G agarose beads (Santa Cruz, CA) and 3 μg of the SHP-1, PTP-1B, or the anti-rat (Sigma-Aldrich, ON, Canada) antibody for nonspecific binding. The beads were spun down and washed three times with the PTP lysis buffer and then incubated with the phosphatase reaction mix for 4 to 6 h at 37°C, and the OD at 405 nm was read.

In-gel PTP assay. Cell lysates (30 μg) were obtained using the PTP lysis buffer previously described. Samples were loaded on a gel containing a γ-^{32}P-labeled poly(Glu4Tyr) peptide (Sigma-Aldrich), and PTP bands were observed by in-gel PTP assay as previously described (20).

EMSA. Nuclear extracts were prepared by a standard protocol, and electrophoretic mobility shift assays (EMSAs) were performed as previously described (17). Briefly, nuclear extracts were incubated with binding buffer (11) containing 1.0 ng of [γ-^{32}P]dATP radiolabeled double-stranded DNA oligonucleotide for 20 min at room temperature. The DNA binding consensus sequences used for transcription factors are as follows: 5′-AGTTGAGGGG ACTTTCCCAGGC-3′ for NF-κB, 5′-AAGTACTTTCAGTTTCATATTACT CTA-3′ for STAT-1 (gamma interferon activation site [GAS]/interferon-stimulated response element [ISRE] consensus sequence), and 5′-AGCTCG CGTGACTCAGCTG-3′ for AP-1. Sp1 consensus oligonucleotide was used as a nonspecific control (5′-ATTCGATCGGGGCGGGGCGAGC-3′) (all DNA oligonucleotides were purchased from Santa Cruz). DNA-protein complexes were resolved by electrophoresis in native 4% (wt/vol) polyacrylamide gels. The gels were then dried and autoradiographed.

RESULTS

Promastigotes and amastigotes of *L. mexicana* efficiently block IFN-γ-mediated NO production in Mϕs. To investigate whether both promastigotes and amastigotes were able to inhibit IFN-γ-mediated NO production in our experimental system, NO levels were measured in B10R Mϕs infected with one of the two forms, followed by IFN-γ stimulation. Results showed that both promastigotes and axenic amastigotes of *L. mexicana* were able to significantly block IFN-γ-mediated NO production in Mϕs (Fig. 1A). Successful differentiation of *L. mexicana* promastigotes to amastigotes was confirmed by several methods: morphological changes seen by phase-contrast microscopy (data not shown), the remarkably decreased level of production of gp63 in amastigotes compared to promastigotes (Fig. 1B, top panel), and the detection of the amastigote-specific A2 protein in amastigote but not promastigote lysates (Fig. 1B, middle panel). The LACK D protein was used as a loading control (Fig. 1B, bottom panel).

Promastigotes and amastigotes of *L. mexicana* activate PTPs in Mϕs. We have previously reported that *Leishmania donovani* promastigotes can rapidly increase total PTP activity in Mϕs to alter their signaling pathways (5). Here, we performed pNPP phosphatase assays to compare the ability of promastigotes and amastigotes of *L. mexicana* to rapidly activate PTPs in Mϕs. Results indicated that both promastigotes and amastigotes were able to rapidly increase total PTP activity in infected Mϕs, reaching a peak activation value at 6 h postinfec-

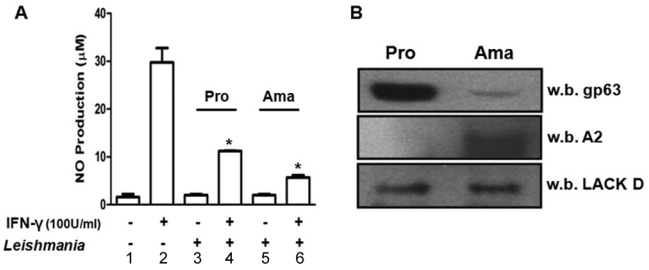

FIG. 1. Inhibition of IFN-γ-mediated NO production by *L. mexicana* promastigotes (Pro) and amastigotes (Ama). (A) NO assay of B10R Mϕs left untreated (−) (bar 1), stimulated with IFN-γ (100 U/ml) for 24 h (+) (bar 2), infected with *Leishmania* (overnight [O/N], 20:1 ratio) (+) (bars 3 and 5), or infected with *Leishmania* (O/N, 20:1 ratio) and then stimulated with IFN-γ (100 U/ml) for 24 h (bars 4 and 6). The values are means plus standard errors of the means (SEMs) (error bars). Values that were significantly different ($P < 0.05$, analysis of variance [ANOVA] test) are indicated by an asterisk. (B) Western blot analysis of *L. mexicana* promastigote and amastigote cell lysates (40 μg). The membrane was cut and blotted for gp63 (top panel) and A2 (middle panel). The membrane was then stripped and reblotted for LACK D (bottom panel) to demonstrate equal loading. All results are representative of at least three independent experiments. w.b., Western blotted.

tion and decreasing thereafter (Fig. 2A). To obtain a better understanding of the specific PTPs involved in this activation, we performed in-gel PTP assays screening for different Mϕ PTPs and monitoring their alterations with promastigote or amastigote infections over a 24-h infection period. In-gel PTP assays showed that both promastigotes and amastigotes were able to rapidly activate the Src homology 2 domain-containing protein tyrosine phosphatase (SHP-1) seen by the appearance of a cleavage product associated with the activation of SHP-1 (Fig. 2B, top two arrows). Interestingly, while promastigote infection clearly activated PTP-1B, resulting in a cleavage fragment, the generation of this active cleaved form by amastigotes was minimal (Fig. 2B, bottom two arrows).

***L. mexicana*'s cysteine proteinase LmCPb plays a role in PTP-1B but not SHP-1 activation in Mϕs.** One important virulence factor expressed in metacyclic promastigotes and in amastigotes of *L. mexicana* is the cysteine proteinase LmCPb. To evaluate a possible role for LmCPb in the activation of SHP-1 and PTP-1B, we performed in-gel PTP assays, Western blotting, and pNPP phosphatase assays on lysates and immunoprecipitates (IPs) of Mϕs infected with WT or *lmcpb*$^{-/-}$ promastigotes and amastigotes and evaluated the effects of these parasites on SHP-1 and PTP-1B cleavage and activation. Although there was some reduced cleavage of SHP-1's top band in Mϕs infected with *lmcpb*$^{-/-}$ promastigotes and amastigotes (Fig. 3A, top arrow, and Fig. 3B, top panel), its lower cleavage band was still prominent (Fig. 3A, second arrow from the top, and Fig. 3B, top panel), and SHP-1's IP from Mϕs infected by *lmcpb*$^{-/-}$ parasites exhibited elevated phosphatase activity, similar to that caused by WT parasites (Fig. 3C, top graph), suggesting that LmCPb is not required in the *Leishmania*-induced SHP-1 activation.

Conversely, infection with *lmcpb*$^{-/-}$ parasites did not lead to the cleavage of the top PTP-1B band seen with WT promastigotes (Fig. 3A, third arrow from the top, and Fig. 3B, bottom panel), resulting in a strong reduction in the cleavage band that we observe in WT promastigote infection (Fig. 3A, bottom arrow, and Fig. 3B, bottom panel). This decrease in PTP-1B cleavage was associated with the inability of *lmcpb*$^{-/-}$ parasites to cause PTP-1B activation compared to WT promastigote infection (Fig. 3C, bottom graph). This set of data suggests that LmCPb seems to play a role in the activation of PTP-1B in *L. mexicana* promastigotes.

Ability of *L. mexicana* promastigotes and amastigotes to alter Mϕ TFs. Because TFs NF-κB, STAT-1α, and AP-1 are strong modulators of Mϕ signaling, we decided to study the ability of promastigotes and amastigotes of *L. mexicana* to alter key Mϕ TFs, namely, NF-κB, STAT-1α, and AP-1. We also evaluated the lowest parasite-to-cell ratio required to cause these alterations. EMSAs revealed that both promastigotes and amastigotes were able to cause the disappearance of the p65-containing subunit of NF-κB but with different results. Promastigotes cleaved p65 into a p35-containing subunit, while amastigotes caused a total degrada-

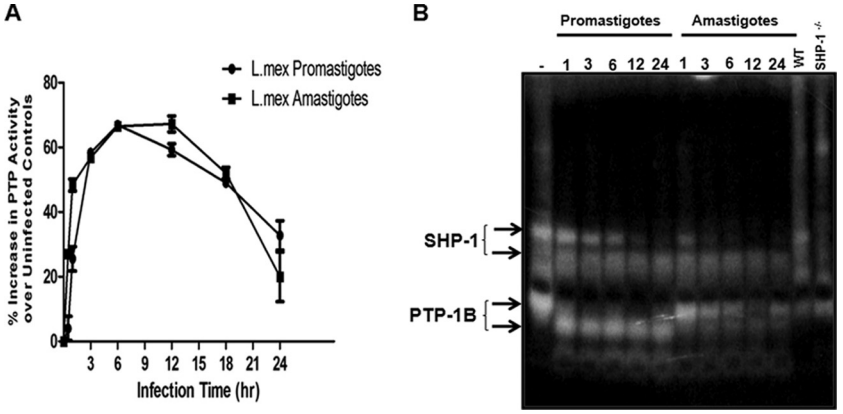

FIG. 2. Activation of host PTPs by *L. mexicana* promastigotes and amastigotes. (A) B10R Mϕs were infected with *L. mexicana* promastigotes or amastigotes for the specified time course (0 to 24 h, 20:1 ratio), and total PTP activity of Mϕ lysates (20 μg) was measured using the pNPP phosphatase assay. (B) Cell lysates (30 μg) of Mϕs infected with promastigotes or amastigotes of *L. mexicana* for the specified time course (0 to 24 h, 20:1 ratio) subjected to an in-gel PTP assay. The rightmost two lanes contain lysates (30 μg) from WT and SHP-1$^{-/-}$ Mϕs to confirm the band corresponding to SHP-1. All results are representative of at least three independent experiments.

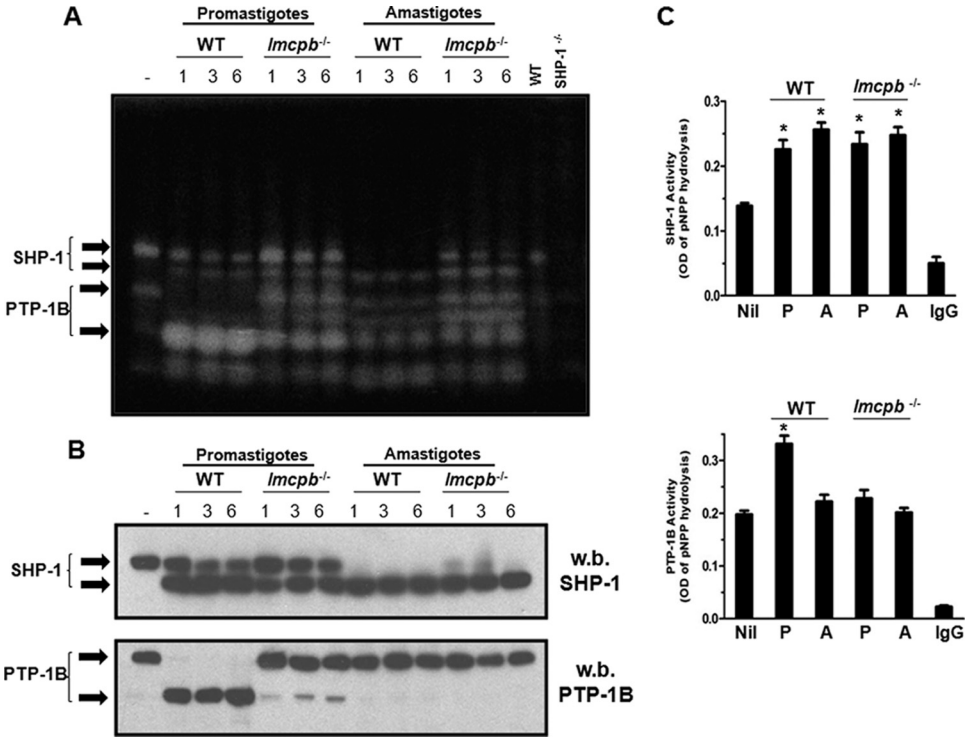

FIG. 3. Role of LmCPb in PTP-1B activation. (A) B10R Mϕs were infected with *L. mexicana* promastigotes and amastigotes of WT and *lmcpb*$^{-/-}$ parasites for the specified time course (0 to 6 h, 20:1 ratio), and cell lysates (30 μg) were subjected to an in-gel PTP assay. The rightmost two lanes contain lysates (30 μg) from WT and SHP-1$^{-/-}$ Mϕs to confirm the band corresponding to SHP-1. (B) Samples in panel A were run on gels by SDS-PAGE and blotted for SHP-1 (top panel) and PTP-1B (bottom panel). w.b., Western blotted. (C) B10R Mϕs were infected with *L. mexicana* promastigotes (P) and amastigotes (A) of WT and *lmcpb*$^{-/-}$ parasites (3 h, 20:1 ratio) followed by lysis and immunoprecipitation of SHP-1 or PTP-1B. The SHP-1 and PTP-1B IPs were subjected to a pNPP phosphatase assay (top and bottom graphs, respectively). The values are means plus standard errors of the means (SEMs) (error bars). Values that were significantly different ($P < 0.05$, ANOVA test) are indicated by an asterisk. Rabbit anti-rat IgG was used as a negative control for the IPs. All results are representative of at least three independent experiments.

tion of this subunit with no observable production of the p35 subunit (Fig. 4A). Interestingly, both promastigotes and amastigotes inhibited STAT-1α and AP-1 activity, but generally, a stronger effect was seen with amastigote infection (Fig. 4B and C). Additionally, a parasite-to-cell ratio of 5:1 was sufficient to observe the previously mentioned alterations (Fig. 4A, B, and C) and was thus selected for the rest of the EMSA experiments conducted.

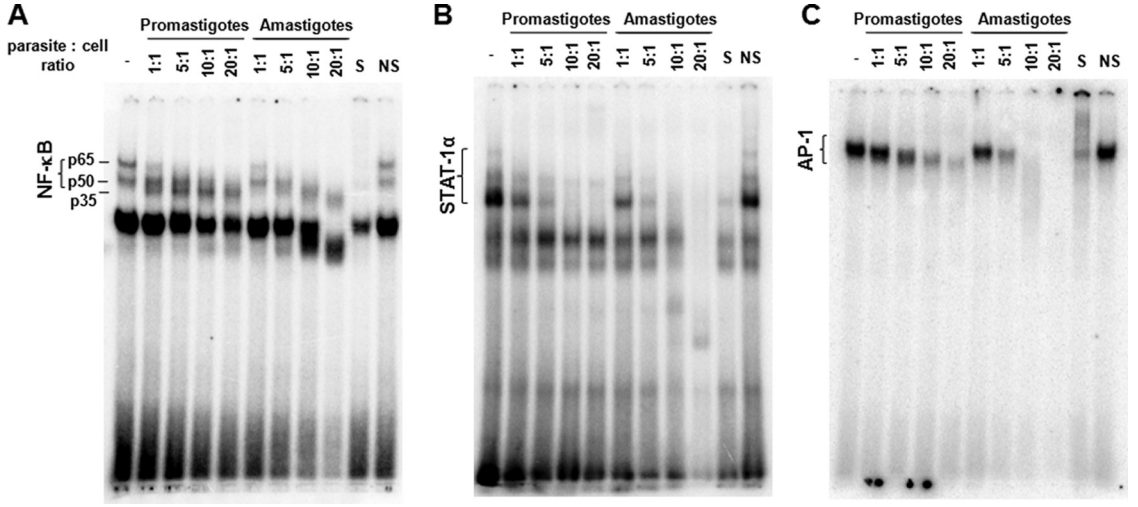

FIG. 4. Modulation of Mϕ TFs by *L. mexicana* promastigotes and amastigotes. B10R Mϕs were infected with *L. mexicana* promastigotes and amastigotes at the specified parasite-to-cell ratios (3-h infection). (A to C) Nuclear proteins were extracted and subjected to EMSA to evaluate DNA-binding activity of NF-κB (A), STAT-1α (B), and AP-1 (C). S, specific competition (100-fold excess of specific nonradioactive oligonucleotide); NS, nonspecific competition (100-fold excess of nonspecific, nonradioactive Sp-1 oligonucleotide).

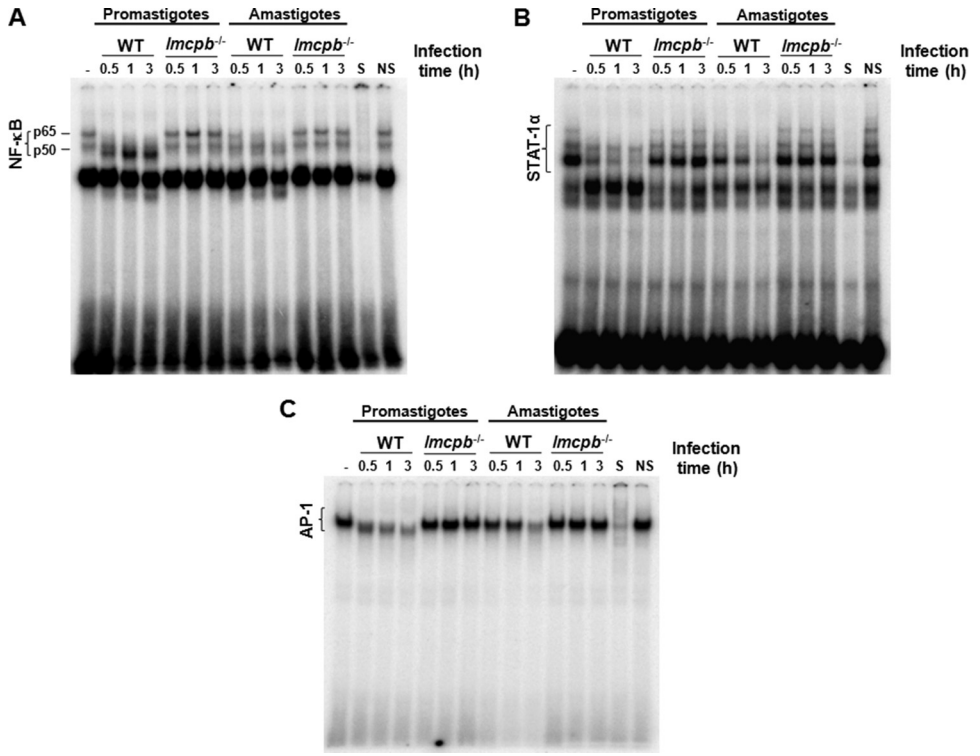

FIG. 5. Role of LmCPb in the modulation of Mϕ TFs. B10R Mϕs infected for the specified time course (0.5 to 3 h) with WT or *lmcpb*$^{-/-}$ promastigotes and amastigotes of *L. mexicana* in a 5:1 parasite-to-cell ratio. Nuclear proteins were extracted and subjected to EMSA to evaluate DNA-binding activity of NF-κB (A), STAT-1α (B), and AP-1 (C). S, specific competition (100-fold excess of specific nonradioactive oligonucleotide); NS, nonspecific competition (100-fold excess of nonspecific, nonradioactive Sp-1 oligonucleotide).

LmCPb plays a crucial role in the ability of promastigotes and amastigotes of *L. mexicana* to modulate NF-κB, STAT-1α, and AP-1. To study the role of LmCPb in the modulation of the different transcription factors, we performed EMSAs to evaluate the ability of *lmcpb*$^{-/-}$ parasites to alter NF-κB, STAT-1α, and AP-1. Results revealed that both promastigotes and amastigotes of *lmcpb*$^{-/-}$ parasites were not able to alter the p65 subunit of NF-κB (Fig. 5A) or to inhibit STAT-1α (Fig. 5B) and AP-1 (Fig. 5C) activity. The previously mentioned TFs of Mϕs infected with *lmcpb*$^{-/-}$ parasites remained intact and similar to that of uninfected Mϕs, suggesting a crucial role for LmCPb in the cleavage/degradation of NF-κB and in the blockage of STAT-1α and AP-1 activity in host Mϕs.

***lmcpb*-rescued parasites alter Mϕ signaling in a similar manner to WT parasites.** To rule out the possibility that the protection of transcription factor alterations that we observe when Mϕs are infected with *lmcpb*$^{-/-}$ parasites is due to the attenuation of those parasites as they are multiply passaged in the knocking-out process, we investigated whether *lmcpb* add-back parasites were also able to inhibit NF-κB the way WT parasites did. Results showed that indeed this was the case (Fig. 6), suggesting that the lack of effect observed in *lmcpb*$^{-/-}$ parasite-infected Mϕs is indeed due to the absence of this protease and not an effect of parasite attenuation.

Unlike infection by WT parasites, TFs of Mϕs infected with *lmcpb*$^{-/-}$ parasites retain their ability to be activated by IFN-γ. To evaluate whether the alteration of NF-κB, STAT-1α, and AP-1 by WT promastigotes and amastigotes interferes with the ability of those TFs to translocate to the nucleus and to evaluate the role of LmCPb in this context, we performed EMSAs. Results indicated that infection with WT promastigotes and amastigotes of *L. mexicana* rendered p65-containing subunits of NF-κB, STAT-1α, and AP-1 unresponsive to IFN-γ stimulation (Fig. 7A, B, and C, respectively). Interestingly, when Mϕs were infected with *lmcpb*$^{-/-}$ parasites (Fig. 7A, B, and C), these TFs were able to translocate to the nucleus and

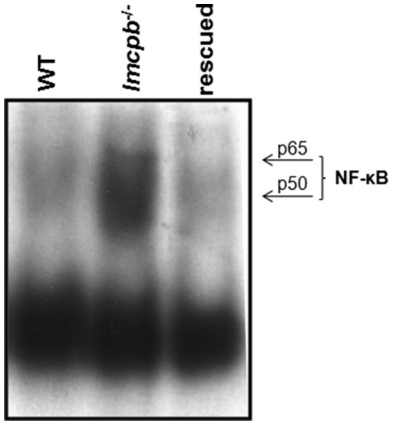

FIG. 6. Effect of *lmcpb*-rescued parasites on Mϕ NF-κB. B10R Mϕs were infected for 1 h with *L. mexicana* amastigotes of WT, *lmcpb*$^{-/-}$, or *lmcpb*-rescued parasites. Nuclear proteins were extracted and subjected to EMSA to evaluate DNA-binding activity of NF-κB.

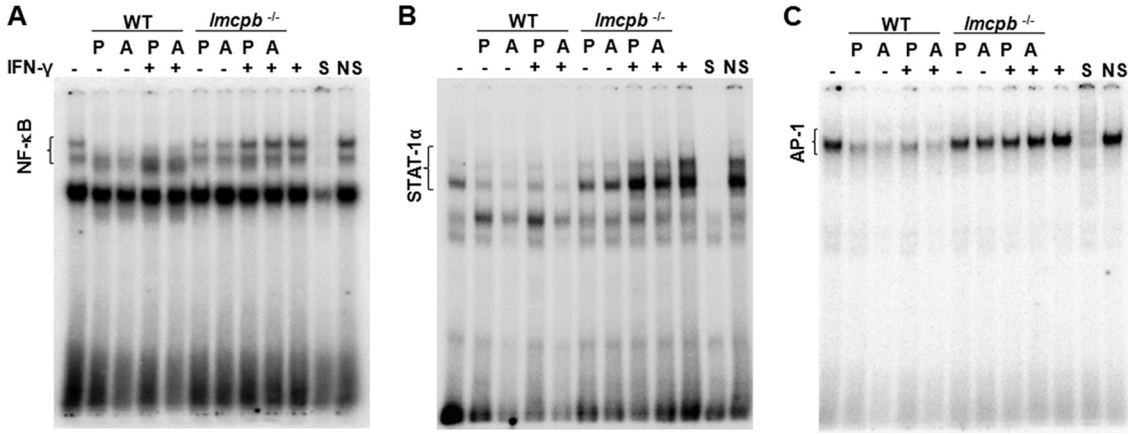

FIG. 7. Nuclear translocation of TFs in response to IFN-γ in Mϕs infected with WT or *lmcpb*$^{-/-}$ *L. mexicana* promastigotes (P) and amastigotes (A). B10R Mϕs were left uninfected, stimulated with IFN-γ (100 U/ml) for 6 h (+), and infected (overnight [O/N]) with WT or *lmcpb*$^{-/-}$ promastigotes and amastigotes of *L. mexicana* in a 5:1 parasite-to-cell ratio, or they were infected (O/N) and then stimulated with IFN-γ (100 U/ml) for 6 h. (A to C) Nuclear proteins were extracted and subjected to EMSA to evaluate the DNA-binding activity of NF-κB (A), STAT-1α (B), and AP-1 (C). S, specific competition (100-fold excess of specific nonradioactive oligonucleotide); NS, nonspecific competition (100-fold excess of nonspecific, nonradioactive Sp-1 oligonucleotide).

bind their consensus sequences in a manner similar to that of the IFN-γ-only positive control.

Promastigotes and amastigotes of WT *L. mexicana* but not *lmcpb*$^{-/-}$ parasites are able to inhibit Mϕ NO production in response to IFN-γ stimulation. To evaluate the impact of LmCPb on Mϕ function, we performed NO assays to test whether *lmcpb*$^{-/-}$ parasites are able to block IFN-γ-mediated NO production by Mϕs in a fashion similar to that observed by WT parasites. Results showed that while WT promastigotes and amastigotes successfully inhibited IFN-γ-mediated NO production as illustrated in Fig. 1A, *lmcpb*$^{-/-}$ parasites were not able to do so. The levels of NO produced in response to *lmcpb*$^{-/-}$ parasites were comparable to those produced by uninfected cells stimulated with IFN-γ (Fig. 8).

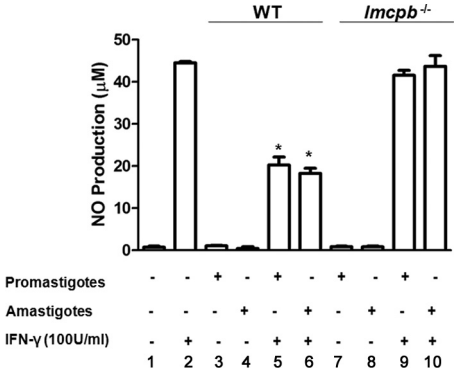

FIG. 8. Inability of *lmcpb*$^{-/-}$ parasites to inhibit IFN-γ-mediated Mϕ NO production. NO assay of B10R Mϕs left untreated (−) (bar 1), stimulated with IFN-γ (100 U/ml) for 24 h (+) (bar 2), infected with WT (bars 3 and 4) or *lmcpb*$^{-/-}$ (bars 7 and 8) *L. mexicana* (O/N, 20:1 ratio) (+), or infected (O/N, 20:1 ratio) and then stimulated with IFN-γ (100 U/ml) for 24 h (bars 5 and 6 and 9 and 10). The values are means plus standard errors of the means (SEMs) (error bars). Values that were significantly different ($P < 0.05$, ANOVA test) are indicated by an asterisk. The results are representative of at least three independent experiments.

DISCUSSION

NO production by Mϕs plays a key role in the resolution of *Leishmania* infections. Indeed, there are several publications reporting the ability of promastigotes, amastigotes, or various parasite molecules, such as lipophosphoglycan (LPG) (35) and glycosylinositol phospholipids (GIPLs) (36), to inhibit Mϕ NO production in response to activating stimuli. Of utmost interest, none of those previous studies compared the ability of both forms of the parasite to inhibit Mϕ NO production and the negative regulatory mechanisms they employ to cause this inhibition.

We have previously reported the ability of *Leishmania* promastigotes to rapidly activate host PTPs (5). Furthermore, we and others have shown that host SHP-1 is one of the key PTPs activated by *Leishmania* (5, 12–14, 30). However, very little work has been done concerning the ability of amastigotes to activate host PTPs and SHP-1 in particular. The key role that SHP-1 plays in amastigote infection can be deduced from *in vivo* studies where amastigotes are exclusively found and where the absence of SHP-1 was associated with increased resistance to *Leishmania* infection (14). Additionally, it has been previously reported that *Leishmania donovani* amastigotes are able to activate host SHP-1, yet the effect was claimed to be observable after 17 h of infection (29). In this work, we confirm previous observations regarding the ability of promastigotes to activate host PTPs (e.g., SHP-1). Importantly, we demonstrate that amastigotes can rapidly activate host PTP activity (as early as 0.5 h postinfection) (Fig. 2A) and that SHP-1 is a key phosphatase rapidly activated by amastigote infection (Fig. 2B and Fig. 3A, B, and C). This suggests that SHP-1 is a critical phosphatase utilized by both forms of the parasite to inactivate key Mϕ signaling pathways.

Interestingly, PTP-1B was activated by promastigote but not amastigote infection (Fig. 2B and Fig. 3A, B, and C). This phosphatase has been reported to bind to and negatively regulate JAK2 (28), while knockdown and overexpression studies

suggested its role in the regulation of MyD88-dependent proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), and the inhibition of NF-κB and STAT-1 activation in Toll-like receptor (TLR)-triggered Mϕs (43). Given that SHP-1 has also been associated with the ability to negatively regulate JAK2 (5), block LPS-mediated TNF production (1), and control NF-κB and AP-1 activity (4), this finding suggests that PTP-1B activated by promastigotes might have an additive effect to SHP-1 action to help establish the parasite in host cells. Indeed, data from our laboratory demonstrated that *Leishmania* promastigote gp63 activated PTP-1B in a cleavage-dependent manner and that PTP-1B$^{-/-}$ mice infected with *L. major* promastigotes showed a significant delay in the onset and progression of footpad inflammation as well as lower parasite burden (15). Therefore, the selective activation of PTP-1B by promastigotes is plausible, given that they are less adapted to the host environment compared to amastigotes that might find SHP-1 action sufficient to ensure their safe entry to new uninfected phagocytes, especially since they seem to affect some signaling molecules more drastically upon initial contact with Mϕs, such as the strong inhibition they cause to NF-κB, STAT-1, and AP-1 compared to promastigotes (Fig. 4). It is worth mentioning, however, that the utilization of host PTPs other than SHP-1 by amastigotes is a possibility that cannot be disregarded and deserves further investigation.

Consistent with a previous finding from our group, we have shown that promastigotes cause cleavage of NF-κB's p65 subunit, generating a smaller p35 subunit (16). On the other hand, amastigotes caused complete NF-κB degradation (Fig. 4A) as previously shown (7). Also, consistent with previous findings, promastigotes were able to cause degradation of STAT-1α (11) and inhibition of AP-1 (12) in Mϕs, but whether LmCPb degrades STAT-1α on its own or in conjunction with the proteasome, as we previously described for *L. donovani* (11), remains to be determined. Importantly, we were able to demonstrate for the first time that amastigotes of *L. mexicana* can also cause rapid STAT-1α and AP-1 inhibition (Fig. 4). Very interestingly, all the alterations caused by promastigotes and amastigotes to NF-κB, STAT-1α, and AP-1 did not take place when Mϕs were infected with *lmcpb*$^{-/-}$ parasites. LmCPb has been previously reported to play a role in the degradation of IκB and NF-κB (7), interleukin 2 receptor (IL-2R), and the immunoglobulin E receptor(IgER) (34). Our data confirm the role of LmCPb in NF-κB degradation (Fig. 5A) and demonstrate for the first time that LmCPb leads to the inhibition of STAT-1α and AP-1 nuclear translocation and DNA-binding activity, possibly by causing their degradation (Fig. 5B and C) or by interfering with key phosphorylation steps (25, 44) required for their translocation to the nucleus. The protection of these transcription factors seen when Mϕs were infected with *lmcpb*$^{-/-}$ parasites is reflected by the ability of these transcription factors to translocate to the nucleus and bind their target sequences in response to IFN-γ stimulation (Fig. 7), which correlated with the ability of Mϕs to produce NO when stimulated with IFN-γ following infection with *lmcpb*$^{-/-}$ parasites (Fig. 8). Importantly, the protection observed with *lmcpb*$^{-/-}$ parasites cannot be due to their lower ability to enter Mϕs, since staining experiments that we performed showed that WT and *lmcpb*$^{-/-}$ parasites could infect Mϕs at similar rates (around 35% of total Mϕs become infected in a 1-h period [data not shown]), nor can it be due to their attenuation while they were multiply passaged in the knocking-out process, as signaling alterations could again be seen when the Mϕs were infected with *lmcpb*-rescued parasites (Fig. 6). Collectively, the LmCPb-related findings presented here could help partially explain the previously observed low infectivity of *cpb* mutants (27), as well as further confirm the role of this cysteine proteinase as a *Leishmania* virulence factor.

In conclusion, our comparison of promastigotes and amastigotes of *L. mexicana* in terms of their ability to alter Mϕ signaling yielded several interesting similarities as well as differences. While both forms were able to activate total PTP activity and to rapidly activate SHP-1, PTP-1B was activated only by promastigotes. Additionally, while both forms caused full inhibition of STAT-1α and AP-1, NF-κB seemed to be altered differently. Promastigotes cleaved NF-κB's p65 subunit to p35 while amastigotes caused complete p65 inhibition with no significant production of the smaller subunit. Importantly, this study also revealed new roles for LmCPb as a virulence factor by demonstrating its ability to interfere with STAT-1α and AP-1 transcriptional activity, therefore contributing to the parasite's ability to block IFN-γ-induced NO production in Mϕs, securing its survival and propagation within its mammalian host.

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