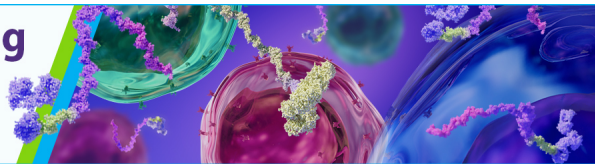


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Inhibition of Lipopolysaccharide-Induced Macrophage IL-12 Production by *Leishmania mexicana* Amastigotes: The Role of Cysteine Peptidases and the NF- κ B Signaling Pathway¹

Pamela Cameron,^{2,3†} Adrienne McGachy,^{3*} Mary Anderson,[†] Andrew Paul,[†] Graham H. Coombs,[‡] Jeremy C. Mottram,[§] James Alexander,^{4*} and Robin Plevin[†]

Infection with lesion-derived *Leishmania mexicana* amastigotes inhibited LPS-induced IL-12 production by mouse bone marrow-derived macrophages. This effect was associated with expression of cysteine peptidase B (CPB) because amastigotes of CPB deletion mutants had limited ability to inhibit IL-12 production, whereas preincubation of cells with a CPB inhibitor, cathepsin inhibitor IV, was able to suppress the effect of wild-type amastigotes. Infection with wild-type amastigotes resulted in a time-dependent proteolytic degradation of I κ B α and I κ B β and the related protein NF- κ B. This effect did not occur with amastigotes of CPB deletion mutants or wild-type promastigotes, which do not express detectable CPB. NF- κ B DNA binding was also inhibited by amastigote infection, although nuclear translocation of cleaved fragments of p65 NF- κ B was still observed. Cysteine peptidase inhibitors prevented I κ B α , I κ B β , and NF- κ B degradation induced by amastigotes, and recombinant CPB2.8, an amastigote-specific isoenzyme of CPB, was shown to degrade GST-I κ B α in vitro. LPS-mediated I κ B α and I κ B β degradation was not affected by these inhibitors, confirming that the site of degradation of I κ B α , I κ B β , and NF- κ B by the amastigotes was not receptor-driven, proteosomal-mediated cleavage. Infection of bone marrow macrophages with amastigotes resulted in cleavage of JNK and ERK, but not p38 MAPK, whereas preincubation with a cysteine peptidase inhibitor prevented degradation of these proteins, but did not result in enhanced protein kinase activation. Collectively, our results suggest that the amastigote-specific cysteine peptidases of *L. mexicana* are central to the ability of the parasite to modulate signaling via NF- κ B and consequently inhibit IL-12 production. *The Journal of Immunology*, 2004, 173: 3297–3304.

Leishmania are obligate intracellular parasites that live as nonmotile amastigotes within cells of the mononuclear phagocyte lineage of their mammalian hosts (1). Enormous progress has been made in recent years in elucidating the mechanisms by which successful intracellular infection is achieved (1), with the majority of studies demonstrating that acquired protective immunity against murine leishmaniasis is dependent upon an IL-12-driven type 1 response and IFN- γ production (2–5). However, the immunological pathways resulting in nonhealing disease are less well characterized. This is particularly true for the role of IL-4 and the Th2 response. For example, studies of *Leishmania major* suggest that disease susceptibility is a result of *Leishmania* homolog of receptors for activated C kinase Ag-specific V β 4⁺ V α 8⁺ CD4⁺ T cells producing an early IL-4-driven Th2 response that down-regulates IL-12 and IFN- γ production and IL-12R expression (6, 7). However, further studies using *L. major* as

well as other *Leishmania* species suggest that IL-4 has little influence on disease outcome, and the absence of a Th1 response, which may be a direct result of the failure to produce or respond to IL-12, is the primary default mechanism leading to nonhealing disease (8–11). Indeed, it is well established that all *Leishmania* species studied to date are not only capable of entering macrophages silently as metacyclic promastigotes (when transmitted from the sandfly vector of the disease), but can also significantly down-regulate the IL-12 production associated with macrophage activation. Although IL-10, TGF- β , and PGE₂ are capable of mediating such activity and are up-regulated during *Leishmania* infection, the parasites have been demonstrated to inhibit host cell IL-12 production independently of these pathways (12).

The activation and nuclear translocation of NF- κ B, which consists of hetero- or homodimers of RelA, RelB, c-Rel, p50, and p52, lead to increased transcription of a number of genes, including those encoding IL-12 due to the IL-12p40 promoter having two NF- κ B binding sites (13). However, studies using *L. major* metacyclic promastigotes and synthetic lipophosphoglycan (LPG)⁵ indicated that LPG differentially regulates IL-12 as well as NO production independently of NF- κ B activation by targeting ERK and p38 MAPK, respectively (14). It remains controversial whether *L. major* amastigotes, which are largely deficient in LPG, can directly down-regulate IL-12, but recent evidence suggest they do this indirectly via Fc γ R-mediated uptake into macrophages, which results in IL-10 production (15). *L. mexicana* amastigotes, in contrast, were demonstrated to directly down-regulate macrophage IL-12 production by an at that time unknown mechanism (12). Our

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⁵ Abbreviations used in this paper: LPG, lipophosphoglycan; BMM ϕ , bone marrow-derived macrophage; CPB, cysteine peptidase B.

present study clearly demonstrates that *L. mexicana* disrupts NF- κ B activation, and the data strongly suggest that this leads to the down-regulation of macrophage IL-12 production. Furthermore, we have established that this activity is mediated by amastigote-specific cysteine peptidases that we have previously identified as virulence factors (16–18).

Materials and Methods

Materials

All chemicals and reagents were obtained from appropriate commercial sources. The *Escherichia coli* expression plasmid for GST-I κ B α was a gift from R. Hay (University of St. Andrews, St. Andrews, U.K.). Morpholinecarboxyl-phenylalanine-homophenylalanine-vinyl sulfone phenyl (K11002) was a gift from J. H. McKerrow (University of California, San Francisco, CA).

Parasites and infection protocols

Leishmania mexicana (MYNC/BZ/62/M379) was maintained by serial passage of amastigotes inoculated s.c. into the shaven rumps or footpads of BALB/c mice. Amastigotes for use in experimental studies were isolated and purified from lesions and enumerated as previously described (19). The cysteine peptidase B (CPB)-deficient mutants (Δ *cpb*) used in this study have been described previously (16). Promastigotes were grown in Schneider's insect medium (Sigma-Aldrich, Poole, U.K.) with 20% (v/v) heat-inactivated FCS and were used when in stationary phase; axenic amastigotes were grown as described by Bates et al. (20). All parasites were washed three times in RPMI 1640 before use.

Purification of recombinant CPB2.8

The recombinant CPB, without the C-terminal domain, was expressed in *E. coli* and activated as described previously (21). The enzyme, designated CPB2.8 for this study, was stored frozen at -20°C until used.

Cell culture

Bone marrow-derived macrophages (BMM ϕ) were grown in DMEM, containing 20% (v/v) heat-inactivated FCS and 20% (v/v) L cell-conditioned medium. Adherent cells were harvested using ice-cold buffer, washed three times in RPMI 1640, and incubated at 33°C for 24 h. Macrophages were then infected (at a ratio of five parasites per macrophage) with different life cycle stages of *L. mexicana*: stationary phase metacyclic promastigotes harvested from in vitro cultures or lesion-derived amastigotes purified from infected mice (19).

Western blotting

Detection of I κ B α , I κ B β , and NF- κ B (p65 isoform) using SDS-PAGE was conducted as outlined previously (22). All Abs (Santa Cruz Biotechnology, Santa Cruz, CA) were titrated for optimum blotting conditions.

EMSA

After termination by washing in ice-cold PBS, agonist-stimulated cells were scraped, pelleted, and resuspended in buffer. After scraping, cellular material was recovered by centrifugation (13,000 rpm for 1 min), the supernatant was aspirated, and the pellet was resuspended in 400 μ l of buffer 1 (10 mM HEPES (pH 7.9) containing 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, and 20 μ g/ml E64) and allowed to swell on ice for 15 min. After incubation, 25 μ l of 10% (w/v) Nonidet P-40 (prepared in buffer 1) was added, and samples were vortexed at full speed for 10 s before centrifugation at 13,000 rpm for 1 min. The pellet was resuspended in 50 μ l of buffer 2 (20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 10 μ g/ml pepstatin, and 20 μ g/ml E64). The extracts from the cells were agitated at 4°C for 15 min and then sonicated on ice in a bath-type sonicator twice for 30 s each time. Extracted nuclear material was recovered as the supernatant after centrifugation (13,000 rpm for 15 min) at 4°C . The nuclear extracts were extracted and incubated with an NF- κ B consensus nucleotide (Promega, Madison, WI) that was labeled with ^{32}P as previously described (23). Samples were run on 5% nondenaturing polyacrylamide gels, and DNA binding was identified by autoradiography.

Epifluorescent microscopy

Cells were grown on coverslips and stimulated in the usual manner. Briefly, reactions were stopped by washing twice with ice-cold PBS, fol-

lowed by the addition of ice-cold methanol for 15 min. Coverslips were incubated with 1% (w/v) BSA solution for 30 min before addition of primary Ab (1/200) for 60 min. After washing, the coverslips were incubated with a secondary FITC-conjugated Ab (1/400) for 60 min, after which the coverslips were washed, dried, and mounted in Mowiol. Slides were examined using a Zeiss 4 laser scanning epifluorescent microscope (Zeiss, Oberkochen, Germany) with a $\times 40$ lens.

Macrophage IL-12 production

Bone marrow-derived macrophages (BMM ϕ) were resuspended in complete RPMI 1640 and plated onto 96-well plates (100 μ l/well at 2×10^6 BMM ϕ /ml). Medium alone or 1 μ g/ml LPS was added to cultures with or without *L. mexicana* amastigotes and with or without cathepsin L inhibitor IV (10 μ M; Calbiochem, Nottingham, U.K.) to a final volume of 200 μ l/well. Supernatants were collected after 48 h, and IL-12 levels were measured. IL-12 (p70 p40) levels were measured by two-site ELISA. The capture Ab was anti-mouse IL-12 (c15.6; BD Pharmingen, San Diego, CA) at 2 μ g/ml. Murine rIL-12 (R&D Systems, Abingdon, U.K.) was used as standard. Biotinylated anti-mouse IL-12 (c17.8; BD Pharmingen) was used for detection after binding of 1/1000 streptavidin/alkaline phosphatase conjugate with *p*-nitrophenol phosphate (1 mg/ml; Sigma-Aldrich) as substrate.

Results

Inhibition by *L. mexicana* amastigotes of LPS-induced IL-12 production is modulated by active cysteine peptidases

L. mexicana amastigotes have previously been shown to be able to inhibit IL-12 production after activation (12). This effect was confirmed in this study (Fig. 1). However, two additional studies implicated the involvement of cysteine peptidase B (CPB) in this effect. Firstly, pretreatment of cells with cathepsin L inhibitor IV, an inhibitor of CPB in vitro (see Fig. 5), significantly reversed the ability ($p < 0.05$) of wild-type *L. mexicana* to down-regulate LPS-induced macrophage IL-12 production (Fig. 1A). Secondly, amastigotes of the CPB gene deletion mutant Δ *cpb* had a reduced ability to down-regulate LPS-induced macrophage IL-12 production, such that the addition of LPS to macrophage cultures infected with CPB-deficient parasites resulted in significantly increased ($p < 0.01$) IL-12 production (Fig. 1B). Furthermore, contrary to the ability of the cysteine peptidase inhibitor to suppress the ability of wild-type amastigotes to inhibit LPS-induced macrophage IL-12 production (Fig. 1A), the same inhibitor had no effect when used with CPB-deficient parasites (Fig. 1B). Nevertheless, although previous studies indicate that parasites enter macrophages silently, in six separate experiments we noted that infection with both wild-type and Δ *cpb* mutant parasites increased basal IL-12 production by BMM ϕ .

L. mexicana degrades NF- κ B, I κ B α , and I κ B β

To investigate the mechanisms by which amastigote infection results in reduction in IL-12 production, a number of relevant cell signaling pathways were examined, including NF- κ B. After infection of BMM ϕ with *L. mexicana* amastigotes, harvested from lesions, a time-dependent degradation of the p65 Rel A form of NF- κ B was observed (Fig. 2A). When the blot was overexposed, a C-terminal fragment of ~ 37 kDa (the Ab was raised against the C terminus of I κ B) and another smaller fragment were found to be generated over 4 h. Only p65 and c-Rel were activated in BMM ϕ after stimulation with LPS (results not shown), and it was found that c-Rel was similarly degraded after *L. mexicana* amastigote infection (Fig. 2B). The cellular content of α -actin remained constant after *L. mexicana* stimulation (Fig. 2C), suggesting a degree of selectivity of the parasite's action. Both isoforms of I κ B were also degraded almost fully by 60 min (Fig. 2, D and E). This profile of I κ B degradation contrasts greatly with the well-recognized profiles of LPS-induced I κ B degradation (Fig. 2, D and E, right panels). The loss of cellular I κ B α in response to LPS was transient,

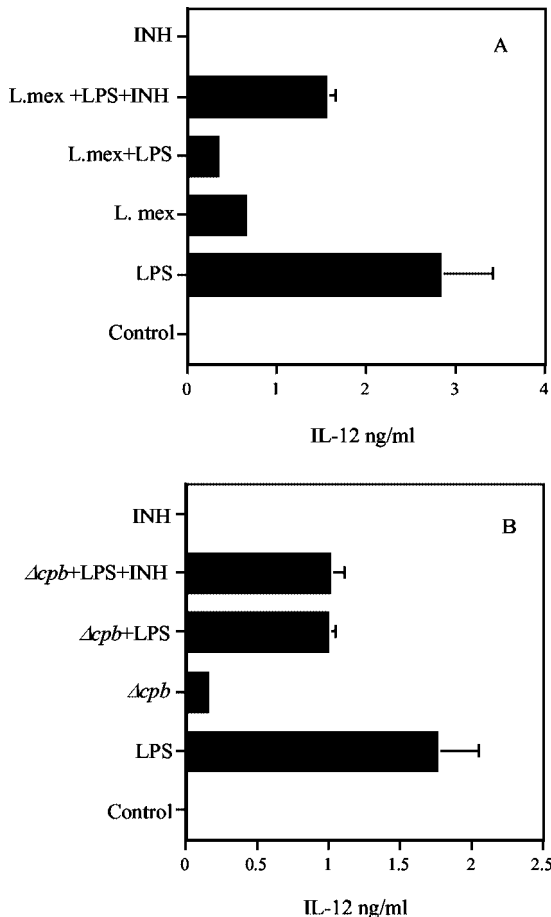


FIGURE 1. The effect of amastigotes on LPS-induced IL-12 synthesis. BMM ϕ produced significant quantities of IL-12 when stimulated with LPS for 48 h (A). Infection of macrophages with *L. mexicana* amastigotes for 48 h resulted in minimal IL-12 induction and greatly inhibited LPS-induced IL-12 production ($p < 0.01$; A). The addition of cathepsin L inhibitor IV (INH; 10 μ M) significantly reversed the ability of the parasite to inhibit LPS-induced IL-12 production ($p < 0.05$; A). The Δcpb amastigotes had limited ability to inhibit LPS-induced macrophage IL-12 production (B), and the cathepsin L inhibitor did not further promote LPS-induced IL-12 production (B). The data are representative from six independent experiments.

with the protein level returning toward control (unstimulated) values by 90 min, whereas $I\kappa B\beta$ degradation was substantially delayed, only being apparent by 90 min.

Further characterization of the *L. mexicana*-induced response found that the effect was specific for the amastigote form of the parasite harvested from lesions. Infection with promastigotes for up to 4 h did not initiate $I\kappa B\alpha$ degradation (Fig. 2F). As Kane and Mosser (15) have demonstrated that entry of lesion-derived amastigotes into macrophages, unlike other life cycle stages, is primarily via Fc γ Rs, we postulated that this could influence activation of signal transduction pathways. Thus, in vitro cultured parasites were also incubated with either antiserum from infected mice or in parasite-free supernatants from amastigote lesions for 30 min before use. However, such Ab-coated parasites failed to degrade $I\kappa B\alpha$ (results not shown)

L. mexicana amastigotes do not affect NF- κ B nuclear localization, but do affect DNA binding

Having demonstrated that *L. mexicana* amastigotes cause cleavage of NF- κ B isoforms, we studied the effect of this on NF- κ B acti-

vation. In Fig. 3, BMM ϕ were stimulated with LPS in the presence or the absence of amastigotes, and the cellular localization of p65 was visualized by epifluorescence microscopy (Fig. 3). In unstimulated cells, p65 was retained within the cytoplasm (Fig. 3A), which was translocated to the nucleus after stimulation with LPS for 60 min (Fig. 3B). Translocation was almost 100% under these conditions. Infection with amastigotes alone had no significant effect on the subcellular localization of p65, although cytoplasmic staining was more diffuse in these cells (Fig. 3C). Nevertheless, after infection with amastigotes and stimulation with LPS, strong nuclear staining was still observed (Fig. 3D).

Having established that NF- κ B could still be translocated to the nucleus despite being partially degraded, we examined the potential for the protein to bind to an appropriate DNA sequence, as assessed by EMSA. Fig. 3E demonstrates that LPS stimulated an increase in basal NF- κ B-DNA binding in nuclear extracts. Infection with amastigotes resulted in the abolition of both basal and LPS-stimulated NF- κ B-DNA binding. However, by immunoblotting nuclear extracts from each condition (Fig. 3F), it was found that whereas in LPS-stimulated cells the level of NF- κ B was enhanced relative to that in controls, no full-length NF- κ B was observed in amastigote-infected cells. However, once again a smaller protein fragment was found to be present at greater levels than in the control or LPS-stimulated samples. This suggests that the apparent nuclear translocation of p65 NF- κ B, as observed by immunofluorescence (Fig. 3D), is, in fact, of a truncated form of the protein.

The ability of *L. mexicana* amastigotes to cleave $I\kappa B$ is cysteine peptidase dependent

Having established the effect of amastigotes on LPS-induced NF- κ B signaling, we assessed more closely the enzymatic dependence of the effect. Cleavage of $I\kappa B$ and associated proteins was found to be dependent upon the enzymatic activity of the amastigote-specific cysteine peptidases known as CPB. Incubation of recombinant CPB2.8 with GST- $I\kappa B\alpha$ in vitro caused a rapid and complete degradation (Fig. 4A). Furthermore, infection of mouse macrophages with lesion-derived amastigotes of a mutant lacking the CPB gene (Δcpb) failed to initiate the degradation of $I\kappa B\alpha$, $I\kappa B\beta$, or NF- κ B observed with the wild-type organism (Fig. 4, B–D).

Cysteine peptidase inhibitors prevent $I\kappa B\alpha$ degradation

Cysteine peptidase inhibitors were examined for their ability to abolish the *L. mexicana* amastigote-induced degradations. Preincubation with the cell-permeable cathepsin L inhibitor IV prevented CPB2.8-induced cleavage of GST- $I\kappa B\alpha$ in vitro over the concentration range 1–10 μ M (Fig. 5A). Similar results were observed in *L. mexicana*-infected macrophages for both $I\kappa B\alpha$ (Fig. 5B) and $I\kappa B\beta$ (Fig. 5C). In contrast, cathepsin L inhibitor IV, at a concentration that fully reversed the effect of *L. mexicana*, was found to be without effect on LPS-induced $I\kappa B\alpha$ or $I\kappa B\beta$ degradation (Fig. 5, B and C). *L. mexicana*-induced cleavage of NF- κ B was also reversed by cathepsin L inhibitor IV over the same concentration range (Fig. 5D).

Similar results were obtained with another cell-permeable inhibitor, K11002. K11002 was found to prevent cleavage of $I\kappa B\alpha$ mediated by CPB2.8 in vitro (Fig. 6A). K11002 at a concentration of 1 μ M or greater fully reversed *L. mexicana*-induced $I\kappa B\alpha$ degradation (Fig. 6B). However, K11002 did not affect LPS-induced $I\kappa B\alpha$ degradation. In contrast, the nonpermeable inhibitor E64, although effectively preventing CPB2.8-mediated degradation of GST- $I\kappa B\alpha$ in vitro (Fig. 6C), was without effect on *L. mexicana*- or LPS-induced $I\kappa B\alpha$ degradation (Fig. 6D). This strongly indicates that the degradation of $I\kappa B\alpha$ and related proteins due to *L. mexicana* amastigotes takes place intracellularly.

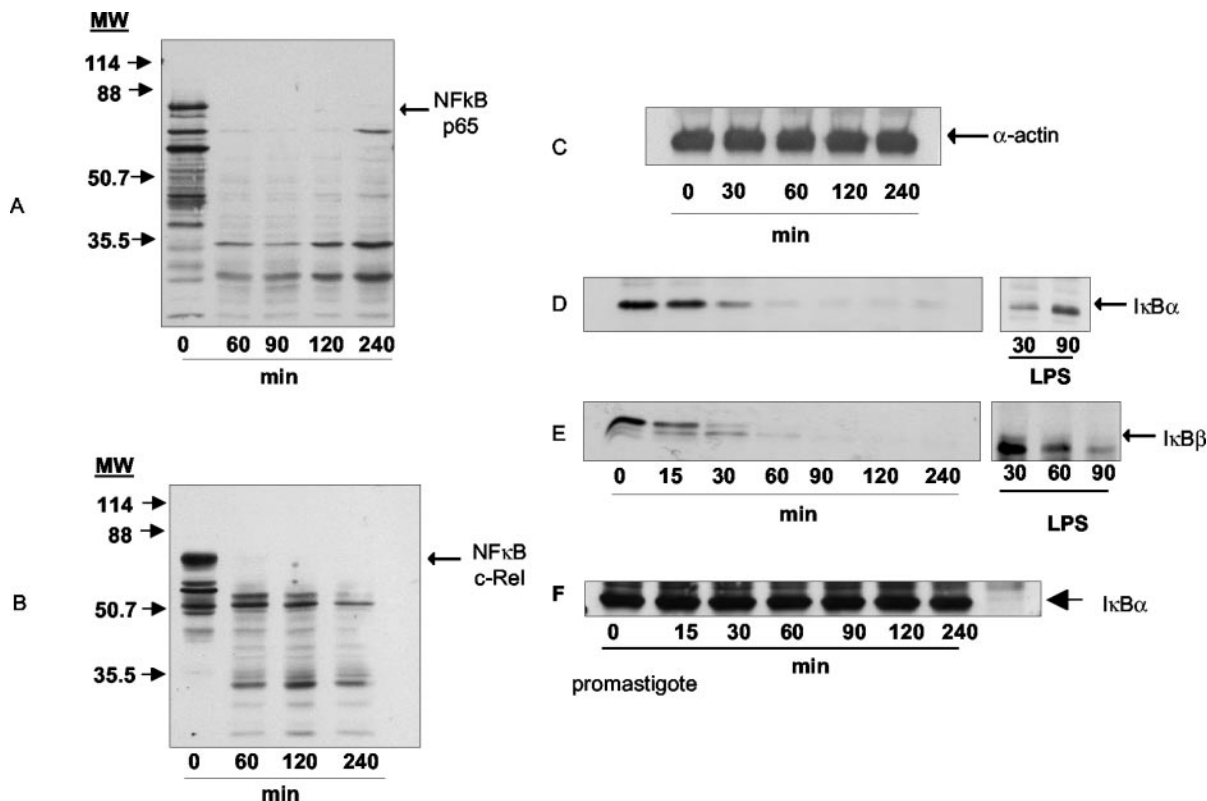


FIGURE 2. Effect of amastigotes of *L. mexicana* on degradation of NF- κ B, I κ B α , and I κ B β in mouse BMM ϕ . BMM ϕ were infected with *L. mexicana* amastigotes (five per cell) or incubated with 1 μ g/ml LPS for the times indicated. Whole-cell extracts were assessed for p65 (A), c-Rel (B), α -actin (C), I κ B α (D), and I κ B β (E) by Western blotting as outlined in *Materials and Methods*. BMM ϕ infected with promastigotes under similar conditions were assessed for I κ B α expression (F). Each blot is representative of at least three independent experiments.

L. mexicana cleaves MAPKs

Although our studies have revealed that the NF- κ B pathway is strongly affected by amastigote infection, we also examined effects on the MAPK signaling cascades, because these pathways may also be involved in the effects of LPS. Initially we examined phosphorylation of JNK by Western blotting, because this pathway has been shown to be strongly activated by LPS, and, indeed, in mouse monocytes both 46- and 54-kDa isoforms of JNK were strongly activated (Fig. 7A). However, after pretreatment of cells with amastigotes, LPS-stimulated phosphorylation of JNK was lost. Western blotting revealed that this was due to degradation of JNK after amastigote infection. As early as 30 min after infection, full-length JNK had been completely degraded, and proteins of lower mass had appeared (Fig. 7B). Of the two other MAPK family members assessed, ERK was also found to be rapidly degraded (Fig. 7C), whereas, over a similar time course, p38 MAPK was reduced by only a minor extent (Fig. 7D). Furthermore, similar to I κ B α , degradation of ERK and JNK could be prevented by preincubation with cathepsin inhibitor IV (not shown). Infection with *L. mexicana* alone failed to stimulate an increase in phospho-p38 levels (Fig. 7E). Moreover, preincubation with cathepsin inhibitor IV did not enable amastigotes to further activate p38 MAPK (Fig. 7E), JNK, or ERK (not shown), suggesting that infection alone is insufficient to activate these kinases.

Discussion

The factors that determine susceptibility to cutaneous leishmaniasis have been a matter of some controversy. Many studies indicate that nonhealing disease is associated with the parasite's ability to generate a type 2 response and IL-4/IL-13 production, which

counter-regulates the development of a type 1 response via the inhibition of IL-12 and IFN- γ production (6, 7, 24–28). Conversely, several studies in various murine models indicate that the inability to develop a type 1 response could occur independently of type 2 cytokine involvement, and that the parasites themselves are inherently capable of regulating the ability of macrophages to produce IL-12 (8–12). Indeed, *L. major* promastigotes have previously been shown to inhibit IL-12 production, this effect being mediated by cell surface LPG targeting ERK MAPK (14). However, little or no LPG is synthesized by amastigotes (29), and other mechanisms of regulating IL-12 production must operate after infection with this life cycle stage. Although *L. major* promastigotes inhibit IL-12 production independently of NF- κ B (14, 30), the involvement of the NF- κ B signaling pathway is well established as a requirement for IL-12 production in a number of other experimental models. In the present study we have demonstrated that *Leishmania* amastigotes, but not promastigotes, are able to proteolytically cleave NF- κ B and I κ B such that transcriptional activation is impaired. Consequently, the ability of the macrophage to mount a proinflammatory IL-12-driven response is also impaired. We also have identified the amastigote's stage-specific CPB as being most likely responsible for this cleavage. Although a recent study suggests that immunity to *L. mexicana* may be independent of IL-12 (31), two additional studies indicate that IL-12 is important in controlling chronic disease (27, 32). Furthermore, the growth of *L. mexicana* CPB-deficient mutants is controlled in C57BL/6 mice, and unlike their wild-type counterparts, these mutants are unable to suppress a type 1 response (33). Conversely, CPB-deficient parasites do induce nonhealing lesions in IL-12^{-/-} mice on this background (33).

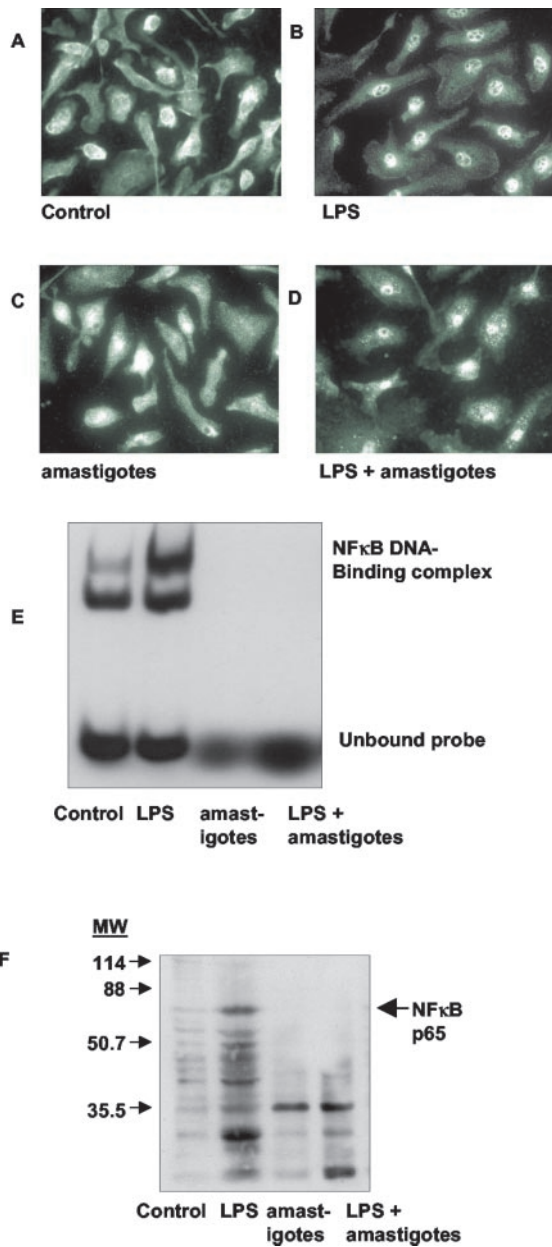


FIGURE 3. The effect of amastigotes on LPS-induced NF-κB nuclear localization and DNA binding. BMMφ were infected with *L. mexicana* amastigotes (five per cell) or incubated with 1 μg/ml LPS for 60 min. Cells were grown on glass coverslips and stained with anti-p65, and localization of p65 was determined by microscopy as described in *Materials and Methods*. Cells were untreated (A), stimulated with LPS (B), infected with amastigotes (C), or infected with amastigotes, then stimulated with LPS (D). E, An EMSA in which nuclei were extracted and incubated with an NF-κB consensus oligonucleotide before electrophoresis on native gels, as described in *Materials and Methods*. The gel was then dried and subjected to autoradiography for a minimum of 12 h. F, Western blotting of nuclear extracts for p65 NF-κB. Each blot is representative of at least three separate experiments.

Members of the NF-κB family of transcription factors exist as homodimers or heterodimers in the cytoplasm, complexed to inhibitory proteins of the IκB family (34). Appropriate cell stimulation activates the NF-κB signaling pathway and results in the phosphorylation, ubiquitination, and degradation of IκB, which facilitates the translocation of NF-κB to the nucleus (35). These transcription factors regulate the expression of numerous proinflammatory cytokines, chemokines, and adhesion molecules and

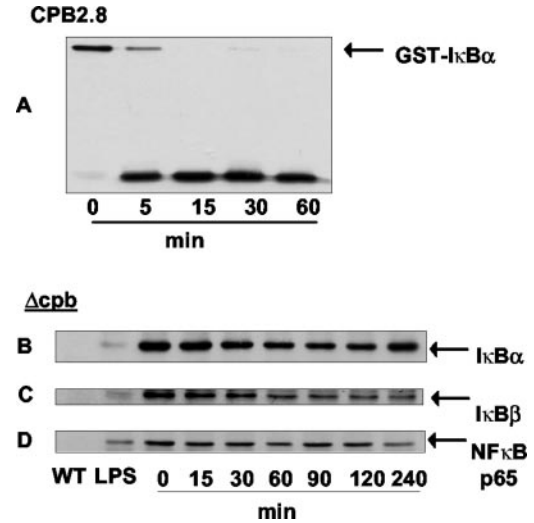


FIGURE 4. Recombinant amastigote-specific CPB2.8 cleaves IκBα, whereas Δ*cpb* amastigotes fail to degrade IκBα, IκBβ, and p65 NF-κB in mouse BMMφ. GST-IκBα (1 μg) was incubated with 10 ng of recombinant CPB2.8 for the times indicated in a final volume of 100 μl (A). BMMφ were infected with wild-type *L. mexicana* or Δ*cpb* lesion amastigotes (five per cell) or were incubated with 1 μg/ml LPS for the times indicated. Whole-cell extracts were assessed for IκBα (B), IκBβ (C), and NF-κB p65 (D) by Western blotting as outlined in *Materials and Methods*. Each blot is representative of at least three independent experiments.

thus play a major regulatory role in the development of immune responses (36). It is not surprising, therefore, that infectious agents have evolved mechanisms to circumvent or subvert the NF-κB

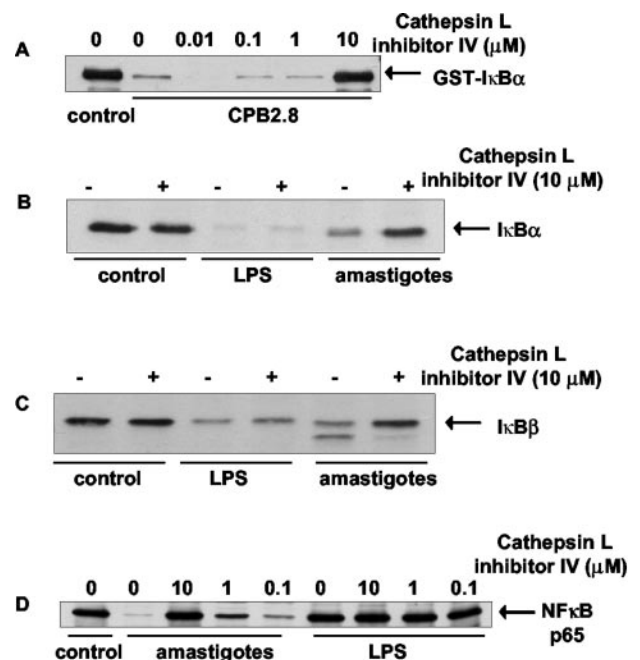


FIGURE 5. Reversal of amastigote cleavage of IκBα, IκBβ, and p65 NF-κB by cathepsin inhibitor IV. Recombinant CPB2.8 (10 ng) was incubated with cathepsin L inhibitor IV (1–10 μM) for 30 min on ice before incubation with 1 μg of recombinant GST-IκBα for 10 min at 37°C (A). BMMφ were preincubated with 10 μM cathepsin inhibitor IV for 30 min before infection with *L. mexicana* amastigotes (five per cell) for an additional 30 min. Cells were then stimulated with vehicle or 1 μg/ml LPS for 30 min (B and D) or 2 h (C). Lysates were assessed for IκBα (A and B), IκBβ (C), and NF-κB p65 (D). Each blot represents at least three independent experiments.

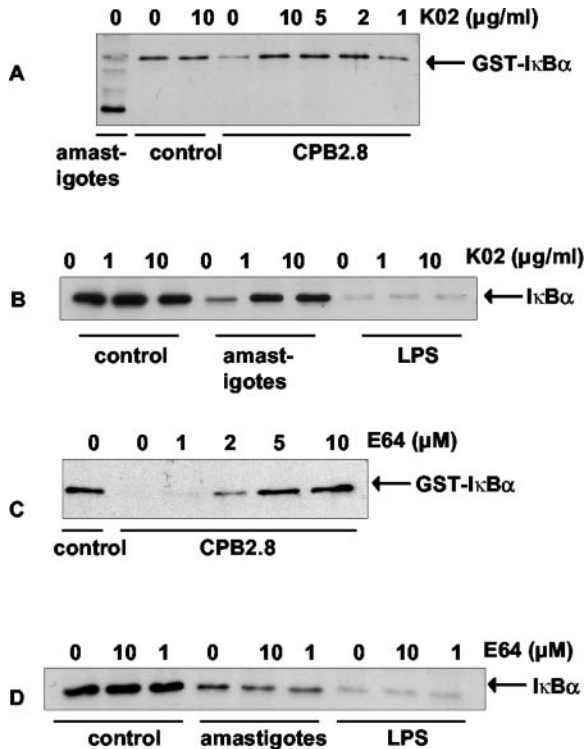


FIGURE 6. Reversal of amastigote-induced cleavage of I κ B α by K11002, but not E64. Increasing concentrations of K11002 (A) or E64 (C) were incubated with 10 ng of recombinant CPB2.8 for 30 min at room temperature before incubation with 1 μ g of recombinant GST-I κ B α for 10 min at 37°C. BMM ϕ were incubated with 1 or 10 μ M K11002 (B) or E64 (D) for 30 min before infection with *L. mexicana* amastigotes (five per cell) for an additional 30 min. Cells were then stimulated with vehicle or 1 μ g/ml LPS for 30 min more. Lysates were assessed for I κ B α by Western blotting as outlined in *Materials and Methods*. Each blot is representative of at least three independent experiments.

signaling pathway to facilitate their successful invasion of the host (37). Many organisms target the regulatory I κ B protein to inhibit its phosphorylation, ubiquitination, or degradation (38–43) or even produce molecules that mimic its activity (44), all of which prevent the nuclear translocation of NF- κ B. Other pathogens have been demonstrated to subvert NF- κ B activity downstream of the degradation of I κ B by preventing nuclear translocation of unbound NF- κ B or its subsequent binding to DNA after translocation (45–49). We have now shown that *L. mexicana* amastigotes are similar to the apicomplexan parasite *Toxoplasma gondii* in inducing rapid degradation of I κ B (46, 47). However, the mechanisms of immune evasion differ between the parasites. After infection of macrophages by *T. gondii*, NF- κ B fails to translocate to the nucleus by as yet uncharacterized mechanisms (46, 47). We have shown in this study that *L. mexicana* amastigotes, in addition to degrading I κ B, degrade NF- κ B. Nevertheless, the epifluorescent microscopy studies presented here show that *L. mexicana* amastigotes, unlike *T. gondii*, are unable to completely prevent LPS-induced nuclear translocation of NF- κ B, yet NF- κ B DNA binding is totally inhibited. Because infection with *L. mexicana* amastigotes almost totally inhibits the ability of LPS to induce macrophage IL-12 production, it seems that those NF- κ B fragments reaching the nucleus fail to induce transcription, perhaps because they lack the DNA-binding sequence located in the N terminus (50), but retain a nuclear location sequence that is located in the C terminus (51). This idea is consistent with results shown in Fig. 3F. Nuclear extracts

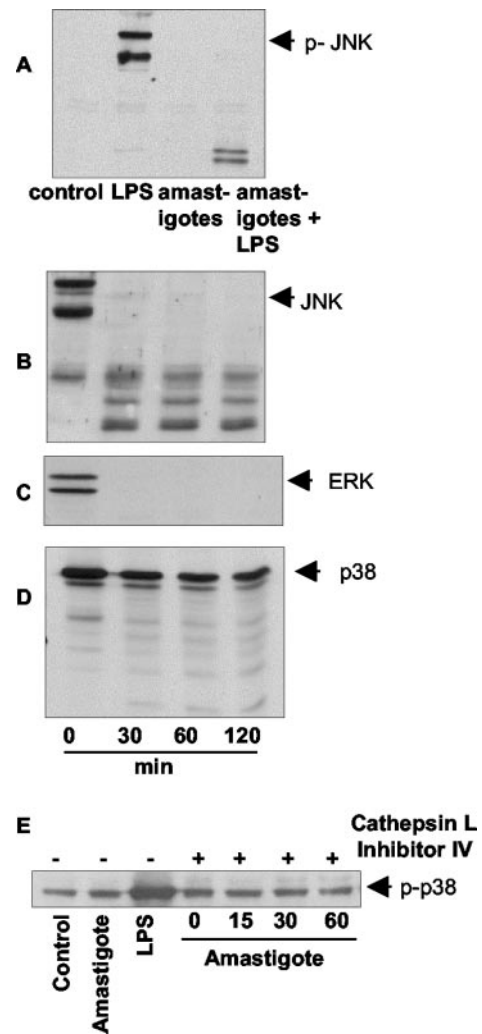


FIGURE 7. The effect of amastigotes on MAPK family members. BMM ϕ were infected with *L. mexicana* amastigotes (five per cell) or were incubated with 1 μ g/ml LPS for 30 min. E, Cells were treated with 10 μ M cathepsin inhibitor IV for 30 min before stimulation with *L. mexicana* amastigotes or LPS (where indicated). Whole-cell extracts were assessed for phospho (p)-JNK (A), JNK (B), ERK (C), p38 MAPK (D), and p-p38 MAPK (E) by Western blotting as outlined in *Materials and Methods*. Each blot is representative of at least three independent experiments.

from cells infected with *L. mexicana* amastigotes contained a 35-kDa truncated form of NF- κ B.

We have previously demonstrated that *L. mexicana* amastigote cysteine peptidases are virulence factors (16) that are instrumental in the parasite generating a type 2 response (17, 18). Consequently, CPB-deficient mutants have reduced infectivity for mice and promote a type 1 response. During the course of the present study we observed that CPB-deficient mutants failed to cleave NF- κ B and I κ B, and that an enzymatically active recombinant cysteine peptidase (CPB2.8) had such activity against GST-I κ B. This suggests that CPB can act as a virulence factor by disrupting the NF- κ B signaling pathway. This hypothesis was further strengthened by the use of cell-permeable cysteine peptidase inhibitors. These were able to inhibit NF- κ B and I κ B degradation by amastigotes and limited the ability of *L. mexicana* wild-type amastigotes to inhibit LPS-induced IL-12 production. Similar to previous studies (12), we found that *L. mexicana* amastigote infection did not modulate CPB-induced NO production, although we did measure a small CPB-dependent modification of TNF- α production (results not

shown). Thus, although several signaling pathways as well as NF- κ B were disrupted, global defects are not identified, suggesting a degree of redundancy. Studies of *L. major* amastigotes and *L. donovani* indicate that the induction of IL-10, probably as a result of Fc γ -mediated parasite uptake, is the major regulator of IL-12 production and susceptibility. However, our studies and those of others (12) suggest that the inhibition of macrophage IL-12 production by *L. mexicana* amastigotes is independent of IL-10 induction. Furthermore, IL-10^{-/-} mice develop nonhealing lesions similar to their wild-type counterparts when infected with *L. mexicana* (52). Nevertheless, as CPB-deficient amastigotes maintain some ability to inhibit LPS-induced IL-12 production by BMM ϕ , other CPB-independent mechanisms of down-regulating this response remain to be identified.

The fact that the amastigotes do not prevent NF- κ B nuclear localization, yet inhibit DNA binding, lends weight to the premise that there is some degree of specificity in the actions of cysteine peptidases. Moreover, in additional experiments it was found that the amastigotes could cause the cleavage of both JNK and ERK, but not p38, within the time period examined. Interestingly, previous studies have demonstrated that p38 induces IL-12 transcription (53), but that both ERK and JNK negatively regulate IL-12 production (14, 54). Thus, cleavage of ERK and JNK should actually increase IL-12 transcription. Furthermore, we observed that amastigotes alone or in the presence of inhibitor are unable to phosphorylate p38 MAPK. This suggests that an increase in basal IL-12 production, observed in response to entry of the amastigotes, is unlikely to be mediated by any activation of p38 MAPK. Thus, some other mechanisms must be responsible for this effect and for the observation that inhibition of CPB activity does not fully reverse the inhibitory effects of the amastigotes on LPS induced IL-12 production. Among those currently being examined include effects on JAK/STAT signaling. Nevertheless, as discussed previously, it is NF- κ B that plays a significant role in IL-12 transcription. Consequently, the weight of evidence provided strongly suggests that *L. mexicana* amastigotes subvert macrophage LPS-induced IL-12 production by disrupting the NF- κ B signaling pathway. It seems likely that the effect on this pathway is at least partly responsible for the survival of *L. mexicana* in its mammalian host.

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