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

Pamela Cameron, Adrienne McGachy, Mary Anderson, Andrew Paul, Graham H. Coombs, Jeremy C. Mottram, James Alexander, 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 IκBα and IκBβ and the related protein NF-κB. This effect did not occur with amastigotes of CPB deletion mutants or wild-type promastigotes, which do not express detectable CPB. NF-κB DNA binding was also inhibited by amastigote infection, although nuclear translocation of cleaved fragments of p65 NF-κB was still observed. Cysteine peptidase inhibitors prevented IκBα, IκBβ, and NF-κB degradation induced by amastigotes, and recombinant CPB2,8, an amastigote-specific isoenzyme of CPB, was shown to degrade GST-IκBα in vitro. LPS-mediated IκBα and IκBβ degradation was not affected by these inhibitors, confirming that the site of degradation of IκBα, IκBβ, and NF-κB 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-κB 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). Eno-

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present study clearly demonstrates that L. mexicana disrupts NF-κB 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-IκBα was a gift from R. Hay (University of St. Andrews, St. Andrews, U.K.). Morspholinocarbonyl-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 (Δcpb) used in this study have been described previously (16). Promastigotes were grown in Schneiders 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 (BMMφ) 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 IκBα, IκBβ, and NF-κB (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 μl 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 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 20 μg/ml E64) and allowed to swell on ice for 15 min. After incubation, 25 μl of 10% (v/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 μl 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 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 20 μg/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 activated and incubated with an NF-κB consensus nucleotide (Promega, Madison, WI) that was labeled with 32P 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, followed 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 Axioliminar epifluorescent microscope (Zeiss, Oberkochen, Germany) with a ×40 lens.

Macrophage IL-12 production

Bone marrow-derived macrophages (BMMφ) were resuspended in complete RPMI 1640 and plated onto 96-well plates (100 μl/well at 2 × 106 BMMφ/ml). Medium alone or 1 μg/ml LPS was added to cultures with or without L. mexicana amastigotes and with or without cathepsin L inhibitor IV (10 μM; Calbiochem, Nottingham, U.K.) to a final volume of 200 μl/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 μg/ml. Murine rL-L (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 Δcpb 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 Δcpb mutant parasites increased basal IL-12 production by BMMφ.

L. mexicana degrades NF-κB, IκBα, and IκBβ

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-κB. After infection of BMMφ with L. mexicana amastigotes, harvested from lesions, a time-dependent degradation of the p65 Rel A form of NF-κB 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 IκB) and another smaller fragment were found to be generated over 4 h. Only p65 and c-Rel were activated in BMMφ 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 IκB were also degraded almost fully by 60 min (Fig. 2, D and E). This profile of IκB degradation contrasts greatly with the well-recognized profiles of LPS-induced IκBα degradation (Fig. 2, D and E, right panels). The loss of cellular IκBα in response to LPS was transient,
L. mexicana amastigotes do not affect NF-κB nuclear localization, but do affect DNA binding

Having demonstrated that L. mexicana amastigotes cause cleavage of NF-κB isoforms, we studied the effect of this on NF-κB activation. In Fig. 3, BMMφ 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-κB 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-κB-DNA binding in nuclear extracts. Infection with amastigotes resulted in the abolition of both basal and LPS-stimulated NF-κB-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-κB was enhanced relative to that in controls, no full-length NF-κB 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-κB, as observed by immunofluorescence (Fig. 3D), is, in fact, of a truncated form of the protein.

The ability of L. mexicana amastigotes to cleave IκB is cysteine peptidase dependent

Having established the effect of amastigotes on LPS-induced NF-κB signaling, we assessed more closely the enzymatic dependence of the effect. Cleavage of IκB 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-IκBα 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 IκBα, IκBβ, or NF-κB observed with the wild-type organism (Fig. 4, B–D).

Cysteine peptidase inhibitors prevent IκBα 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-IκBα in vitro over the concentration range 1–10 μM (Fig. 5A). Similar results were observed in L. mexicana-infected macrophages for both IκBα (Fig. 5B) and IκBβ (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 IκBα or IκBβ degradation (Fig. 5, B and C). L. mexicana-induced cleavage of NF-κB 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 IκBα mediated by CPB2.8 in vitro (Fig. 6A). K11002 at a concentration of 1 μM or greater fully reversed L. mexicana-induced IκBα degradation (Fig. 6B). However, K11002 did not affect LPS-induced IκBα degradation. In contrast, the nonpermeable inhibitor E64, although effectively preventing CPB2.8-mediated degradation of GST-IκBα in vitro (Fig. 6C), was without effect on L. mexicana or LPS-induced IκBα degradation (Fig. 6D). This strongly indicates that the degradation of IκBα and related proteins due to L. mexicana amastigotes takes place intracellularly.

![Graph](image-url)
L. mexicana cleaves MAPKs

Although our studies have revealed that the NF-κB 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 IκBα, 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-κB (14, 30), the involvement of the NF-κB 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-κB and IκB 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).
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 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

![Diagram A](image1)  
Control  
LPS  
LPS + amastigotes

![Diagram B](image2)  
amastigotes  
LPS + amastigotes

![Diagram C](image3)  
NF-κB DNA-binding complex  
Unbound probe

![Diagram D](image4)  
Control  
LPS  
LPS + amastigotes

![Diagram E](image5)  
MW

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<tr>
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<td>NFκB</td>
<td>p65</td>
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![Diagram F](image6)  
Control  
LPS  
LPS + amastigotes

![Diagram G](image7)  
A  
B  
C  
D

![Diagram H](image8)  
NFκB  
p65

![Diagram I](image9)  
control  
CPB2.8

![Diagram J](image10)  
control  
LPS  
amastigotes

![Diagram K](image11)  
control  
LPS  
amastigotes

![Diagram L](image12)  
control  
LPS  
amastigotes

![Diagram M](image13)  
control  
amastigotes  
LPS

![Diagram N](image14)  
control  
amastigotes  
LPS

![Diagram O](image15)  
control  
amastigotes  
LPS

![Diagram P](image16)  
control  
amastigotes  
LPS

![Diagram Q](image17)  
control  
amastigotes  
LPS

![Diagram R](image18)  
control  
amastigotes  
LPS
signaling pathway to facilitate their successful invasion of the host (37). Many organisms target the regulatory IκB 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-κB. Other pathogens have been demonstrated to subvert NF-κB activity downstream of the degradation of IκB by preventing nuclear translocation of unbound NF-κB 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 IκB (46, 47). However, the mechanisms of immune evasion differ between the parasites. After infection of macrophages by T. gondii, NF-κB 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 IκB, degrade NF-κB. 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-κB, yet NF-κB 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-κB 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 from cells infected with L. mexicana amastigotes contained a 35-kDa truncated form of NF-κB.

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, CBP-deficient mutants have reduced infectivity for mice and promote a type 1 response. During the course of the present study we observed that CBP-deficient mutants failed to cleave NF-κB and IκB, and that an enzymatically active recombinant cysteine peptidase (CBP2.8) had such activity against GST-IκB. This suggests that CBP can act as a virulence factor by disrupting the NF-κB signaling pathway. This hypothesis was further strengthened by the use of cell-permeable cysteine peptidase inhibitors. These were able to inhibit NF-κB and IκB 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 CBP-induced NO production, although we did measure a small CBP-dependent modification of TNF-α production (results not
shown). Thus, although several signaling pathways as well as NF-kB 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 Fcγ-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 CBP-deficient amastigotes maintain some ability to inhibit LPS-induced IL-12 production by BMs, other CBP-independent mechanisms of down-regulating this response remain to be identified.

The fact that the amastigotes do not prevent NF-kB 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 CBP 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-kB 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-kB 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.

References


