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Leishmania Inhibitor of Serine Peptidase 2 Prevents TLR4 Activation by Neutrophil Elastase Promoting Parasite Survival in Murine Macrophages

Marilia S. Faria,* Flavia C. G. Reis,* Ricardo L. Azevedo-Pereira,* Lesley S. Morrison,† Jeremy C. Mottram,§,¶ and Ana Paula C. A. Lima*†

Leishmania major is a protozoan parasite that causes skin ulcerations in cutaneous leishmaniasis. In the mammalian host, the parasite resides in professional phagocytes and has evolved to avoid killing by macrophages. We identified L. major genes encoding inhibitors of serine peptidases (ISPs), which are orthologs of bacterial ecotins, and found that ISP2 inhibits trypsin-fold SIA family peptidases. In this study, we show that L. major mutants deficient in ISP2 and ISP3 (<i>Δisp2/3</i>) trigger higher phagocytosis by macrophages through a combined action of the complement type 3 receptor, TLR4, and unregulated activity of neutrophil elastase (NE), leading to parasite killing. Whereas all three components are required to mediate enhanced parasite uptake, only TLR4 and NE are necessary to promote parasite killing postinfection. We found that the production of superoxide by macrophages in the absence of ISP2 is the main mechanism controlling the intracellular infection. Furthermore, we show that NE modulates macrophage infection in vivo, and that the lack of ISP leads to reduced parasite burdens at later stages of the infection. Our findings support the hypothesis that ISPs function to prevent the activation of TLR4 by NE during the <i>Leishmania</i>–macrophage interaction to promote parasite survival and growth. The Journal of Immunology, 2011, 186: 411–422.

<i>Leishmania</i> is a flagellated protozoan parasite that causes a broad spectrum of diseases ranging from skin ulcerations to visceral damage, depending on the species. In the sandfly, the parasite is found in two major forms, as follows: the logarithmically growing procyclic promastigote and the nondividing metacyclic growing procyclic promastigote, the latter being the virulent disease-inducing form of <i>Leishmania</i>. In the mammalian host, <i>Leishmania</i> transform into the obligate intracellular amastigote stage that has evolved to survive within the phagolysosome of host phagocytes, primarily macrophages (1). Different <i>Leishmania</i> species use several mechanisms to suppress macrophage activation, enabling the formation of a suitable environment for intracellular survival. For example, the binding to and activation of the complement type 3 receptor (CR3) by <i>L. major</i> promastigotes lead to the inactivation of infected cells, contributing to their subsequent intracellular survival (2), and surface lipophosphoglycans (LPG) decrease phagosome fusion with late endocytic organelles and lysosomes, influencing the exposure of internalized promastigotes to hydrolyses (3). <i>L. major</i> lines lacking LPG are highly susceptible to human complement, have lost the ability to inhibit phagolysosomal fusion transiently, and are oxidant sensitive, having drastically reduced capability to establish infections in macrophages (4, 5).

The exposure of infected macrophages to apoptotic neutrophils can also selectively alter the fate of intracellular <i>L. major</i> as neutrophils–macrophage interactions in BALB/c-susceptible mice lead to the production of PGE2 and promote parasite growth, whereas such interactions in C57/B6 mice lead to TNF-α production and parasite killing (6). Recently, it was reported that the ability of neutrophils to induce parasite killing in macrophages of C57/B6 mice is strikingly dependent on the activity of the serine peptidase neutrophil elastase (NE), released by dying neutrophils (7). Although macrophages are the final host cells for <i>Leishmania</i> during the chronic infection, neutrophils have been recognized as the first hosts to metacyclic promastigotes following inoculation by the sandfly bite (8). <i>Leishmania</i> is able to avoid killing by neutrophils, and the phagocytosed parasites reside temporarily as viable metacyclics inside vacuoles, before being released to infect macrophages. Alternatively, parasitized apoptotic neutrophils are taken up by standby macrophages, serving as a “Trojan horse” and promoting a silent transfer of parasites to their final host cell (9). In both settings, the infection of macrophages by metacyclic promastigotes remains the primary route to the establishment of the infection, and the parasite factors acting on the <i>Leishmania</i>–macrophage interaction influence the outcome of the infection.

Recently, we implicated <i>Leishmania</i> ecotin-like inhibitors of serine peptidase (ISPs) as potential virulence factors playing a role in parasite uptake and intracellular survival in macrophages (10). <i>L. major</i> has three ISP genes, but apparently lacks genes encoding serine peptidases (SPs) from the SIA family, favoring the hypothesis that ISPs play a role in controlling host SPs (11). Indeed, ISP2 encodes a functional inhibitor of SIA family members NE (or human leukocyte elastase [HLE]) and cathepsin G (12). These
peptidases are microbicidal components of azurophil granules of neutrophils, a subset of lysosome-like organelles. They are also found in granules or at the cell surface of other cell types such as monocytic or basophilic/mast cell lines, and can be released to the extracellular environment under pathological conditions (13, 14). Considering that *Leishmania* is rapidly engulfed by professional phagocytes, neutrophils, and macrophages, at the site of infection, NE and cathepsin G are candidate targets for ISPs.

*L. major* lacking ISP2 and ISP3 (*isp2/3*) were internalized more efficiently by BALB/c macrophages due to upregulation of phagocytosis, driven by selective engagement of CR3 and unidentified SPs (10). In contrast, ISP deficiency resulted in the partial elimination of intracellular parasites 24 h postinfection, suggesting that ISPs are important for the initiation and persistence of infection of the mammalian host.

In the current study, we addressed the molecular mechanisms by which ISP2 modulates the intracellular development of *L. major*. We present evidence that ISP2 plays a role in oxidant resistance by controlling the activity of NE and preventing the activation of TLR4-mediated responses by *L. major*.

**Materials and Methods**

*Parasites*

*L. major* Friedlin (MHOM/JL/80/Friedlin) were grown as promastigotes in modified Eagle’s medium supplemented with 10% heat-inactivated FCS at 25°C, as described previously (15). Parasite lines deficient in ISP2 and ISP3 (*isp2/3*) and re-expressing ISPs (*isp2/3/isp2/3*) were generated by gene replacement and reintroduction, as described (10).

**Infection assays**

Peritoneal macrophages from C57/B6, C3HEn, C3HeJ (acquired from Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, Brazil), or TLR4- (generated by S. Akira, Osaka University, Osaka, Japan, and donated by M. Bozza, Microbiology Institute, Universidade Federal do Rio de Janeiro, Brazil) mice were elicited with thioglycolate and cultivated overnight in RPMI 1640 medium, 10% FCS before assay, as described (10). Briefly, interactions with stationary-phase promastigotes were performed in RPMI 1640, 0.1% BSA for 3 h, and the cultures were washed before fixation and staining with Giemsa. The number of intracellular parasites was determined by counting at least 100 cells per field under the light microscope. Where indicated in the figures, the parasites were coincubated with 100 ng/ml NE or 5 μM NE inhibitor (NEI) (OmeSuc-AAPF-CMK). For the survival assays, the plated cells were cultivated for 24 h before fixation and staining. No extracellular parasites were observed in the collected peritoneal exudates or in the plates. For lesion progression, 2 × 10³ parasites were injected in the footpads of mice (six mice per group), and the size of the footpads was measured weekly with a dial caliper. The lesion size was estimated by calculating the differences of sizes of infected versus uninfected footpads of each group.

**Parasites**

C3H/HeJ (C3HeJ) and C3H/HeN (C3HeN) mice were elicited with thioglycolate and cultivated overnight in RPMI 1640-FCS and remained in the assay during the 3-h interaction. For the survival assays, the remaining in the assay during the 3-h interaction. Anti-mouse CD11b mAbs (M1/70), was added to macrophages 5 min prior to addition of the parasites and was added to the enzyme immediately before addition of the substrate. The initial velocities were calculated by linear regression of the substrate hydrolysis plots using at least 300 points.

**In vivo infections**

Stationary-phase promastigotes were injected in the peritoneal cavity of C57/B6 mice (2 × 10⁷ parasites/animal, three mice per group) in PBS. After 3 h, the mice were sacrificed and the peritoneal cavity was washed with 5 ml ice-cold RPMI 1640. The collected exudates were centrifuged at 1500 × rpm, and 8 × 10⁶ cells of each animal were plated separately on glass coverslips in 24-well tissue culture plates in 10% RPMI 1640-FCS. The cells were left to adhere for 2 h at 37°C, washed, fixed, and Giemsa stained. Where indicated in the figures, the parasites were coincubated with 100 ng/ml NE or 5 μM NE inhibitor (NEI) (OmeSuc-AAPF-CMK). For the survival assays, the collected peritoneal exudates or in the plates. For lesion progression, 2 × 10³ parasites were injected in the footpads of mice (six mice per group), and the size of the footpads was measured weekly with a dial caliper. The lesion size was estimated by calculating the differences of sizes of infected versus uninfected footpads of each mouse, and the graph represents the mean values of each group. Parasite loads were estimated by limiting dilution; polypeptide lymph nodes were collected and submitted to capture homogeneity with the back of a syringe through a nylon membrane in 6.4-ml promastigotes in modified Eagle’s medium supplemented with 10% FCS. One hundred milliliter of the cell homogenates were submitted to 2-fold serial dilutions in 96-well plates. Plates were cultivated at 27°C for 5–7 d, and then the wells were inspected for parasite growth. The highest dilution well was used to estimate the number of parasites multiplied by the dilution factors. Numbers are total per lymph node.

**Statistical analyses**

Significance of the data was assessed by ANOVA, using the GraphPad Prism 4.0 Program (GraphPad, San Diego, CA). The data were analyzed by one-way ANOVA using the Bonferroni posttest, at a significance level of 5%. In Fig. 2D, the data were analyzed by two-way ANOVA using the Bonferroni posttest, at a significance level of 5%. The scores showing statistical significance are indicated in the figures with asterisks, and the p values are indicated in the legends.

**Results**

Increased infectivity of *L. major* *isp2/3* to C57/B6-derived macrophage is mediated by serine peptidases and CR3

We have previously shown that *L. major* deficient in ISP2 and ISP3 (*isp2/3*) are phagocytosed by macrophages of susceptible BALB/c...
mice more efficiently than wild-type (WT) *L. major*, and that this is mediated by unidentified SPs acting at the initial stages of the parasite–host cell interaction (10). We next verified whether similar characteristics were found with macrophages of resistant C57/B6 mice. Stationary-phase Δisp2/3 promastigotes were internalized more efficiently by elicited peritoneal macrophages as compared with WT or with the line re-expressing ISPs (Δisp2/3: ISP2/ISP3) (Fig. 1A). The increase in macrophage infection was due to higher proportions of infected macrophages as well as increased numbers of parasites per infected cell. The internalization returned to the levels of WT when the interaction was performed in the presence of rISP2 or of the inhibitor of trypsin-like peptidases, APT (Fig. 1A). The higher infection of Δisp2/3 also returned to the levels of WT when macrophages were pretreated with Abs that block CD11b, confirming that increased uptake of Δisp2/3 is mediated by CR3 in macrophages of both BALB/c (10) and C57/B6 mice (Fig. 1B). We next found that Δisp2/3 had reduced capacity to establish infection in macrophages of C57/B6 mice (Fig. 1C). After the initial higher infection, the numbers of intracellular Δisp2/3 were significantly reduced in the subsequent 72 h, as compared with WT or with the lines re-expressing ISP2/ISP3 (Fig. 1C). The addition of APT or rISP2 during the initial 3 h of interaction rescued the mutant parasites from cell death, resulting in numbers of intracellular Δisp2/3 similar to those of WT, 72 h postinfection (Fig. 1C). However, blocking of CD11b at the entry stage had no effect on the subsequent survival and development of intracellular Δisp2/3 (Fig. 1D). These observations indicate that ISP2 and/or ISP3 are required for the establishment of a productive infection in C57/B6 macrophages and that SP activity, but not CR3, compromises the capability of Δisp2/3 to survive in the host cell.

**Macrophage-associated NE modulates the entry and survival of *L. major* in macrophages**

Two S1A family SPs are found in association with CR3 in polymorphonuclear leukocytes: NE (16) and factor Xa (17), so we tested whether NE was involved in up-regulation of *Leishmania* uptake by macrophages using an irreversible NEI (Fig. 2). The infections with Δisp2/3 returned to the levels of those of WT in the presence of APT or of the NEI (Fig. 2A), suggesting that this peptidase is responsible for the increased internalization of Δisp2/3. The analyses of the intracellular growth of parasites over a 3-d period revealed that the high numbers of intracellular Δisp2/3 observed at 3 h infection decreased drastically by 24 h, indicating parasite death (Fig. 2B, triangle). The remaining parasites recovered growth at 48 h, although at much lower rates as compared with WT (Fig. 2B, circle). The addition of the NEI during the initial 3-h interaction led to lower internalization of Δisp2/3, but completely prevented their elimination in 24 h, and the intracellular parasites grew steadily over the 3-d period (Fig. 2B, open triangle). The NEI had no effect on the growth or survival of WT parasites (Fig. 2B, open circles). These results indicate that NE activity present during the early Δisp2/3-macrophage contact leads to subsequent parasite death and to delayed intracellular growth. The selectivity of the synthetic inhibitor used was tested against other S1A family SPs (Fig. 2C). The activity of purified

**FIGURE 1.** Enhanced uptake of Δisp2/3 by macrophages of C57/B6 mice is dependent on CD11b and serine peptidases. Stationary-phase promastigotes were incubated with elicited peritoneal macrophages from C57/B6 mice in RPMI 1640-BSA for 3 h. Extracellular parasites were removed with three washes, and the adhered cells were fixed and subsequently stained with Giemsa. A and C, Macrophages were incubated with aprotinin (APT) or with recombinant ISP2 (rISP2) prior to addition of promastigotes and remained for the duration of the assay. B and D, Macrophages were preincubated with 10 μg/ml anti-CD11b M1/70 (CD11b) or with control rat anti-mouse IgG2b (IgG) for 30 min at 37°C, followed by three washes, before addition of promastigotes. C and D, The extracellular parasites were removed with three washes, and the cells were cultured for an additional 70 h in RPMI 1640-FCS at 37°C before fixation and staining. The number of intracellular parasites was estimated by counting using light microscopy. Where indicated, the interaction was performed in the presence of 4 μg/ml1 purified rISP2 or APT. The experiments were performed in triplicate, three independent times. The graphs show one representative experiment; the data are means of triplicates ± SD. Key: white bars, WT parasites; black bars, Δisp2/3; gray bars, Δisp2/3: ISP2-ISP3. The asterisks indicate statistical significance from all other experimental points at p < 0.001.
NE was readily abolished in the presence of the synthetic inhibitor concentration used in the infection assays (Fig. 2C). This inhibitor did not affect the activities of cathepsin G, trypsin, or chymotrypsin, demonstrating selectivity to NE among the tested peptidases.

Δisp2/3 were more infective to RAW macrophage cell line than WT, and the increased infectivity was fully reversed in the presence of the NE inhibitor, suggesting that NE endogenously produced by monocytes/macrophages is sufficient to upregulate the internalization of the mutant parasites and that infection did not depend on the exposure of macrophages to neutrophils (Fig. 2D).

Although NE present in macrophages of C57/B6 mice is sufficient to promote uptake of Δisp2/3, WT parasites are refractory to this action (Fig. 2A). We hypothesized that ISPs produced by WT L. major were sufficient to control the limited amount of NE activity found in macrophages of C57/B6 mice, preventing up-regulation of phagocytosis and guaranteeing parasite survival. To verify this, we added exogenous NE to macrophages during the infection and absent in the following 24–72 h. Key: filled circle, WT; open circle, WT + NEI; filled triangle, D isp2/3; open triangle, D isp2/3 + NE. A and C. The asterisk indicates statistical significance from all other experimental points at p < 0.001 (*). The graphs show one representative experiment. C. Selectivity of the inhibitor to NE. The peptidase activity of NE, cathepsin G (catG), trypsin, and chymotrypsin was tested in the absence or presence of 10 μM OMeSuc-AAPV-CMK in 50 mM HEPES and 150 mM NaCl (pH 7.5), using the appropriate substrates at 25 μM. The graph shows the initial velocities of substrate hydrolysis. Key: white bars, no inhibitor; black bars, with NEI.

Localization of CR3 and NE in macrophages

The physical association between NE and CD11b has been described in neutrophils, so we asked whether this was also observed in monocytes/macrophages. Immunofluorescence of uninfected peritoneal macrophages showed that CD11b is found throughout the cell periphery (Fig. 4A). The evaluation of 0.9-μm optical sections of the cells revealed that staining with anti-CD11b Abs is observed preferably at the lower sections (Fig. 4A), and very rarely at the upper sections (Fig. 4B). This suggests that CD11b is most likely located at the regions of contact between the cell and the slide surface (Fig. 4A) and is present at very low levels at the apical surface (Fig. 4B). In contrast, NE was clearly observed in the upper sections (Fig. 4B). We did not observe colocalization of CD11b and NE (Fig. 4B, merge) in uninfected macrophages, indicating that these molecules are not constitutively associated in macrophages. In contrast, we observed intense colocalization of NE and CD11b in macrophages infected with Δisp2/3 (arrow) (Fig. 4C, 4D, arrowheads). The colocalization was found exclusively at the apical sections, suggesting that the distribution of CD11b is altered upon infection, relocating to the apical regions of the cell, in close proximity to NE. CD11b was predominantly observed at the basal sections of uninfected macrophages (Fig. 4C, 4D, asterisk) that were adjacent to an infected cell (Fig. 4C, arrow), suggesting that the redistribution of CD11b to the upper region of macrophages requires contact with the parasite.
TLR 4 is required for enhanced uptake and elimination of \( \Delta isp2/3 \) Leishmania

Having determined that CR3 is not required for the elimination of intracellular parasites, we asked whether an alternative cell surface receptor was recruited during the \( \Delta isp2/3 \)-macrophage interaction, possibly contributing to parasite death. Recently, it has been shown that exogenous NE added to macrophages previously infected with \( L. \) major prevents parasite growth, and that this effect is dependent on TLR4 (7).

We therefore investigated whether TLR4 is involved in the phagocytosis and death of \( \Delta isp2/3 \) by blocking TLR4 with the mAb MTS510 (Fig. 5). The neutralization of TLR4 prior to the interaction with \( L. \) major reduced significantly the uptake of \( \Delta isp2/3 \) (Fig. 5A, black bars), whereas it had no effect in the internalization of WT (Fig. 5A, white bars). The neutralization of both CD11b and TLR4 had similar effects (Fig. 5A) to the neutralization of either CD11b (Fig. 1C) or TLR4 alone, suggesting that those receptors act in a common pathway. As seen with the inhibition of NE, neutralization of TLR4 at the entry stage prevented death of intracellular \( \Delta isp2/3 \) 24 h postinfection (Fig. 5B, black bars), as compared with macrophages that were pretreated with control IgG. These results indicate that early mobilization of TLR4 by \( \Delta isp2/3 \) is required for subsequent parasite death.

When TLR4 was blocked before parasite entry, we observed a 2-fold increase in intracellular \( \Delta isp2/3 \) in 24 h, whereas the numbers of intracellular WT were similar during this time period (Fig. 5B). This suggests that blockade of TLR4 promotes rapid intracellular growth of ISP-deficient parasites. To verify this further, we followed the effect of TLR4 neutralization in the kinetics of \( L. \) major intracellular growth (Fig. 5C). Pretreatment of the macrophages with anti-TLR4 had a minimal effect in the initial intracellular growth of WT parasites. In contrast, neutralization of TLR4 not only prevented the death of \( \Delta isp2/3 \) in the 24 h following infection, but also promoted growth in that time period, whereas pretreatment with control IgG had no effect. Intracellular \( \Delta isp2/3 \) were unable to sustain growth in macrophages pretreated with anti-TLR4 Abs in the following 48 and 72 h.

To investigate this further, we infected macrophages derived from C3HeJ mice (Fig. 6C, 6D), which are defective in TLR4 signaling, as compared with macrophages from the background mice strain C3HeN (Fig. 6A, 6B). As seen with macrophages from BALB/c (10) and C57/B6, \( \Delta isp2/3 \) were more infective to macrophages of CH3eN mice, as compared with WT (Fig. 6A), and this was reversed by rISP2 or by the NEI. We observed an effect of NEI in the phagocytosis of WT parasites by those macrophages (Fig. 6A), which might be a result of intrinsic differences in the contents of NE between different strains of mice. \( \Delta isp2/3 \) were poorly infective to TLR4-defective macrophages (Fig. 6C). rISP2 or NEI led to a subtle increase in the uptake of the mutant parasites in TLR4-defective macrophages (Fig. 6C), but with no statistical
significance, indicating that SP activity and TLR4 are acting in conjunction for the enhanced uptake of \( \text{D}_{\text{isp2/3}} \). After 3 d, the numbers of intracellular \( \text{D}_{\text{isp2/3}} \) in CH3eN-derived macrophages were reduced by 50% (Fig. 6B), as compared with the numbers 3 h postinfection (Fig. 6A), indicating parasite death. Noteworthily, the numbers of intracellular \( \text{D}_{\text{isp2/3}} \) at 3 d remained approximately half of that observed at 3 h, indicating that parasite growth was severely compromised in C3HeN macrophages within 24–72 h. This is different from BALB/c macrophages, in which intracellular \( \text{D}_{\text{isp2/3}} \) were able to recover growth at 48 h, albeit slower than WT, resulting in similar numbers of intracellular parasites at 3 h or 3 d postinfection (10). Exposure to rISP2 or to the NEI partially

**FIGURE 6.** Functional TLR4 is required for increased uptake and elimination of \( \text{D}_{\text{isp2/3}} \) in macrophages. Stationary-phase promastigotes were incubated with elicited peritoneal macrophages from C57/B6 mice, in RPMI 1640-BSA, at a 3:1 parasite:macrophage ratio for 3 h. Where indicated, macrophages were preincubated with 10 \( \mu \)g/ml \( \text{anti-CD11b (M1/70)} \), with 5 \( \mu \)g/ml neutralizing anti-TLR4 (MTS510) or with control rat anti-mouse IgG2b for 30 min in RPMI 1640-FCS, followed by three washes, before addition of promastigotes. A, The cultures were fixed and stained after the 3-h interaction. B and C, The extracellular parasites were removed with three washes after 3 h, and the cells were cultured for an additional 20 h (B) or for 20, 48, and 72 h (C), in RPMI 1640-FCS before fixation and staining. The number of parasites was estimated by counting using light microscopy. The experiments were performed in triplicate, two independent times. The graphs show one representative experiment; the data are means of triplicates \( \pm \) SD. Key: white bars, WT; black bars, \( \Delta \text{isp2/3} \); C, Filled symbols, WT; open symbols, \( \text{isp2/3} \); circle, pretreatment of macrophages with anti-TLR4; triangle, pretreatment with rat IgG2b. The asterisks indicate statistical significance from other experimental points at \( p < 0.01 \) (A, B); the double asterisks indicate significance at \( p < 0.001 \) from \( \Delta \text{isp2/3} \); and from WT at \( p < 0.001 \) (C).

**FIGURE 5.** TLR4 is involved in enhanced uptake and elimination of \( \Delta \text{isp2/3} \) in macrophages. Stationary-phase promastigotes were incubated with elicited peritoneal macrophages from C3HeN (A and B) or C3HeJ mice (C and D), in RPMI 1640-BSA, at a 3:1 parasite:macrophage ratio, for 3 h. Where indicated, macrophages were preincubated with 10 \( \mu \)g/ml \( \text{anti-CD11b (M1/70)} \), with 5 \( \mu \)g/ml neutralizing anti-TLR4 (MTS510) or with control rat anti-mouse IgG2b for 30 min in RPMI 1640-FCS, followed by three washes, before addition of promastigotes. A, The cultures were fixed and stained after the 3-h interaction. B and C, The extracellular parasites were removed with three washes after 3 h, and the cells were cultured for an additional 20 h (B) or for 20, 48, and 72 h (C), in RPMI 1640-FCS before fixation and staining. The number of parasites was estimated by counting using light microscopy. The experiments were performed in triplicate, two independent times. The graphs show one representative experiment; the data are means of triplicates \( \pm \) SD. Key: white bars, WT; black bars, \( \Delta \text{isp2/3} \); C, Filled symbols, WT; open symbols, \( \text{isp2/3} \); circle, pretreatment of macrophages with anti-TLR4; triangle, pretreatment with rat IgG2b. The asterisks indicate statistical significance from other experimental points at \( p < 0.01 \) (A, B); the double asterisks indicate significance at \( p < 0.001 \) from \( \Delta \text{isp2/3} \); and from WT at \( p < 0.001 \) (C).
rescued intracellular growth of Δisp2/3 in C3HeN macrophages, demonstrating that NE also modulates the intracellular growth of Δisp2/3 in macrophages of those mice. In constrast, even though Δisp2/3 were poorly internalized by TLR4-defective macrophages at 3 h (Fig. 6C), they grew efficiently in these macrophages, reaching numbers equivalent to those of WT at 3 d (Fig. 6D). The accelerated growth of Δisp2/3 in TLR4-defective macrophages implicates TLR4 in the control of Leishmania growth throughout the intracellular cycle. Importantly, intracellular Δisp2/3 are rescued from death in TLR4-defective macrophages to the same extent as in macrophages of C57/B6 mice that were exposed to NEI (Fig. 2B). Taken together, these results define TLR4 as a receptor responsible for promoting the elimination of Δisp2/3 and restraining their intracellular growth in murine macrophages.

**NE promotes Leishmania uptake and killing through TLR4**

We next evaluated the effects of NE in the infection of macrophages of TLR4-deficient mice, as compared with the infection of macrophages of the genetic background mice, C57/B6 (Fig. 7). As shown previously in Fig. 3, the addition of exogenous NE to macrophages of C57/B6 mice increased the uptake of WT L. major at 3 h (Fig. 7A, white bars), which was abolished upon enzyme pre-inhibition with the NEI. Importantly, pretreatment of macrophages with neutralizing Abs to TLR4 (anti-TLR4), but not with control IgG, abolished the increase in phagocytosis provoked by exogenous NE, indicating that TLR4 is required for the effect of NE in parasite uptake. This supports the hypothesis that the effects of NE and TLR4 are not independent of each other. The engagement of TLR4 with abs to TLR4 (UT12) or by LPS also promoted increased uptake of WT L. major, further confirming that the activation of this receptor leads to upregulation of parasite phagocytosis. The concomitant addition of NE and activating Abs to TLR4 led to a subtle, but significant increase in the upregulation of phagocytosis, as compared with the upregulation caused by either exogenous NE or TLR4-activating Abs alone. Finally, pre-inhibition of exogenous NE was overcome by the activation of TLR4 with TLR4-activating Abs, indicating that NE is acting upstream of TLR4 in the signaling pathway that leads to increased parasite internalization (Fig. 7A). As shown previously, the NEI reversed the enhanced phagocytosis of Δisp2/3 (Fig. 7A, black bars), linking increased uptake of the mutant line with NE activity. The negative effect of NEI on the uptake of Δisp2/3 was abolished by the activation of TLR4 with Abs, corroborating that NE activity promotes parasite internalization through TLR4. Exogenous NE had no effect in the uptake of WT Leishmania by macrophages of TLR4 knockout mice (TLR4−/−) (Fig. 7B, white bars), providing further evidence that TLR4 is required for the upregulation of Leishmania phagocytosis caused by NE. Δisp2/3 was as infective as WT in those macrophages (Fig. 7B, black bars), and this was unaffected by neutralizing Abs to TLR4, providing further support that ISPs control the TLR4–NE pathway.

Next, we asked whether, to promote parasite killing, triggering of the TLR4–NE pathway was required to take place solely at the initial stages of the parasite–macrophage interaction. To address this, we infected macrophages of C57/B6 or TLR4−/− mice with WT Leishmania and triggered this pathway by adding exogenous NE or TLR4-activating Abs after parasite entry (Fig. 7C, 7D, white bars). At 24 h postinfection, the numbers of intracellular WT parasites were equivalent in all tested conditions (Fig. 7C, white bars), indicating that the TLR4–NE pathway has little effect on parasite survival if triggered after parasite phagocytosis, or that this pathway is unavailable for activation immediately after parasite uptake. We found similar results when analyzing the survival of Δisp2/3, which were detected at lower numbers as compared with WT parasites at 24 h postinfection, a phenotype that was unaffected by inhibitors to NE or neutralization of TLR4 if incubated immediately after parasite entry (Fig. 7C, black bars). In contrast to what we observed in macrophages of C57/B6 mice, those parasites were found at equal numbers to WT at 24 h postinfection in TLR4−/− macrophages (Fig. 7D, black bars). When added after parasite uptake, exogenous NE led to an unexpected subtle increase in the numbers of intracellular WT parasites in TLR4−/− macrophages after 24 h, suggesting that NE might influence parasite survival through multiple mechanisms.

**Superoxide mediates the killing of intracellular Δisp2/3 in macrophages**

To determine whether production of superoxide was the mechanism downstream of the TLR4–NE pathway, we verified the effect of the superoxide scavenger EUK134 in infected macrophages (Fig. 8). This compound did not affect the uptake of either WT or Δisp2/3 in macrophages at 3 h (Fig. 8A, 3 h). As expected, in untreated macrophages, the numbers of intracellular Δisp2/3 dropped by 50% 24 h postinfection (Fig. 8A, 24 h), as compared with the number of Δisp2/3 present at 3 h. In contrast, when the infected cultures were treated with EUK134 immediately after parasite entry, the numbers of Δisp2/3 at 24 h postinfection were similar to those at 3 h, indicating that superoxide strongly contributes to the killing of intracellular Δisp2/3. Treatment of macrophages infected with WT parasites with EUK134 had no effect on parasite survival. We followed the intracellular growth of L. major in macrophages treated with EUK134, showing that Δisp2/3 were rescued from death at 24 h, and that the growth was partially recovered in the following days (Fig. 8B, closed triangles). We further verified the involvement of reactive oxygen species (ROS) by treatment of infected macrophages with catalase. As expected, Δisp2/3 were engulfed at higher numbers (Fig. 8C, 3 h) and were partially eliminated in the following 24 h (Fig. 8C, 24 h). As observed with EUK 134, the addition of catalase immediately after the 3-h infection rescued Δisp2/3 from death in the following 24 h, whereas it did not influence the survival of either WT or the re-expressing lines (Fig. 8C, 24-h catalase). Taken together, these results implicate superoxide as the main mechanism driving the intracellular elimination of ISP-deficient parasites by macrophages.

**ISPs and NE modulate the infection in vivo**

To evaluate parasite uptake by phagocytes at the initial stages of infection, we inoculated parasites in the peritoneal cavity of mice and collected the exudate after 3 h. The majority of the collected cells were neutrophils, revealing early recruitment of those cells to the site of infection, and no free parasites were observed in the exudates (data not shown). Δisp2/3 were found at higher numbers than WT parasites, and this was reversed when Δisp2/3 were incubated with the NEI (Fig. 9A). Exogenous NE promoted higher uptake of WT parasites by macrophages in vivo, showing that this peptidase promotes the phagocytosis of L. major by macrophages at early stages of infection in mice. We found that a significant number of macrophages was infected by L. major a few hours after parasite inoculation, and no free parasites were observed, suggesting that they were rapidly engulfed by local cells (i.e., neutrophils, macrophages) or disseminated to other locations. The fate of the parasites internalized by macrophages in vivo was further evaluated by collecting the peritoneal exudates 3 h after parasite infection, followed by cultivation of the cells for 24 h in vitro (Fig. 7B). We found approximately half intracellular Δisp2/3 as compared with WT, which was fully reversed when


\[ \Deltaisp^{2/3} \] were injected together with the NEI. We observed no significant differences in the sizes of lesions in the footpad of C57/B6 mice infected with either WT parasites and two independent clones of \( \Deltaisp^{2/3} \) (Fig. 9C), suggesting that tissue infiltrate and/or inflammation was similar. However, there was a 10-fold reduction in the numbers of \( \Deltaisp^{2/3} \) parasites in the lymph nodes of the infected mice as compared with WT.

**Discussion**

In the current study, we used parasites deficient in ISP2 and ISP3 as a tool to investigate the molecular pathways through which these natural inhibitors exert their role in the Leishmania–macrophage interaction. As the expression of ISP2 is abundant in WT, but ISP3 was never detected in the parasites, the phenotypes characterized in the mutant parasites are most likely related to the loss of ISP2.
7 d later. The discrepancies between the two studies might result after parasite phagocytosis led to reduced growth of parasites 5–6 d later. The utilization of a peptidyl synthetic irreversible inhibitor to NE has been recently proposed (7). The addition of NE to the infected cultures after removal of extracellular parasites and remained in the culture for 24 h. The infections were performed for 3 h in untreated macrophages, the extracellular parasites were removed, and cells were cultured in RPMI 1640-FCS, supplemented or not with 50 mM EUK 134 where indicated, at 37°C for an additional 45 or 70 h. C. Infections were performed in untreated macrophages for 3 h, extracellular parasites were removed, and cells were fixed and stained (left panel, 3 h) or further cultured for 21 h in RPMI 1640-FCS at 37°C (right panel, 24 h). Where indicated, 2000 U/ml catalase was added to the infected cultures after removal of extracellular parasites and remained in the culture for the 21 h. Key: white bars, WT; black bars, Δisp2/3; gray bars, Δisp2/3:ISP2-ISP3. The experiments were performed in triplicate, three independent times. The graphs show one representative experiment; the data are means of triplicates ± SD. The asterisks indicate statistical significance from the remaining experimental points at p < 0.001 (**) and at p < 0.001 (**) (B), and at p < 0.01 (*) and p < 0.0001 (***) (C).

function. We observed that Δisp2/3 were internalized more efficiently than WT parasites by macrophages of C57/B6 mice, in a route dependent on CD11b, a subunit of CR3, and on NE activity.

The utilization of a peptidyl synthetic irreversible inhibitor to NE enabled the identification of NE as the primary target of ISP2 in macrophages. The kinetics of intracellular parasite growth showed that, in addition to preventing death at 24 h postinfection, the NE rescued the capability of Δisp2/3 to grow inside macrophages. Although not reported in macrophages, studies in vivo showed that the phagocytosis of zymosan particles by neutrophils was diminished in NE-deficient animals, whereas the phagocytosis of fluorescein-conjugated Escherichia coli particles was unchanged, suggesting that NE might affect phagocytosis in leukocytes in a selective way (18). Importantly, our data indicate that activation pathways triggered by NE at early stages, that is, during parasite phagocytosis, are detrimental for the survival and intracellular development of amastigotes 3 d later. A deleterious effect of NE derived from apoptotic neutrophils on the survival of intracellular L. major in murine macrophages has been recently proposed (7). However, the role of NE in the uptake of L. major by macrophages was not addressed in that study, because exogenous NE was added after the infection of macrophages (7). In our model, the addition of exogenous NE to macrophages after parasite uptake had no effect in parasite survival within 24 h, whereas in the abovementioned study, the authors observed that exogenous NE added after parasite phagocytosis led to reduced growth of parasites 5–7 d later. The discrepancies between the two studies might result from the different time points used to evaluate parasite load. Importantly, we have shown that NE present at the surface of macrophages is readily available to influence parasite phagocytosis in the absence of neutrophils. Furthermore, our studies support the idea that, even in the absence of neutrophils, NE activity must be controlled during parasite–macrophage contact to avoid future elimination of intracellular Leishmania.

We showed that exogenous NE promotes increased phagocytosis of WT promastigotes, and partial elimination of intracellular amastigotes within 24 h. NE added exogenously did not affect the uptake or survival of Δisp2/isp3, suggesting that, in the absence of ISP2 and ISP3, the amounts of active NE present in macrophages are sufficient to promote parasite elimination. This is, to our knowledge, the first time that the involvement of serine peptidases from macrophages in the uptake and survival of L. major has been described and is important in light of the current view that NE secreted from neutrophils surrounding infected macrophages is the main mechanism of parasite elimination involving neutrophils. We showed that Δisp2/3 were likewise more infective to the murine macrophage cell line RAW, and increased infection was abolished by the inhibitor to NE, demonstrating that macrophages that had not been previously exposed to neutrophils are also capable of killing intracellular Leishmania through NE-dependent mechanisms. In this scenario, we postulate that, in the absence of neutrophils, ISP2 present in Leishmania might be sufficient to control local NE activity at the early stages of interaction with macrophages, preventing activation signals that would result in

FIGURE 8. Superoxide mediates the elimination of intracellular Δisp2/3. Stationary-phase promastigotes were incubated with elicited peritoneal macrophages from C57/B6 mice, in RPMI 1640-BSA, at a 3:1 parasite:macrophage ratio for 3 h. A. Left panel (3 h). Where indicated, the assays were performed in the presence of 50 mM superoxide scavenger EUK134, the extracellular parasites were removed after 3 h, and the cells were fixed and stained with Giemsa. Right panel (24 h). Infections were performed in untreated macrophages for 3 h, extracellular parasites were removed, and the cells were cultured for an additional 21 h in RPMI 1640-FCS at 37°C before fixation and staining. Where indicated, 50 mM EUK 134 was added to the infected cultures after removal of extracellular parasites and remained in the culture for 24 h. B. The infections were performed for 3 h in untreated macrophages, the extracellular parasites were removed, and the cells were cultured in RPMI 1640-FCS, supplemented or not with 50 mM EUK 134 where indicated, at 37°C for an additional 45 or 70 h. C. Infections were performed in untreated macrophages for 3 h, extracellular parasites were removed, and cells were fixed and stained (left panel, 3 h) or further cultured for 21 h in RPMI 1640-FCS at 37°C (right panel, 24 h). Where indicated, 2000 U/ml catalase was added to the infected cultures after removal of extracellular parasites and remained in the culture for the 21 h. Key: white bars, WT; black bars, Δisp2/3; gray bars, Δisp2/3:ISP2-ISP3. The experiments were performed in triplicate, three independent times. The graphs show one representative experiment; the data are means of triplicates ± SD. The asterisks indicate statistical significance from the remaining experimental points at p < 0.001 (**) and at p < 0.001 (**) (B), and at p < 0.01 (*) and p < 0.0001 (**) (C).
subsequent parasite death. We also observed a mild effect of NEI in the uptake of WT parasites by macrophages of C3HeN mice, suggesting that the amount of ISP2 might not be sufficient to inactivate all the NE present in macrophages from that strain of mice. However, we did not observe significant differences in the contents of NE of macrophages from the mouse strains used in this study (data not shown) by Western blotting.

Neutrophils are recruited to the site of tissue damage caused by sandfly probing and phagocytose 80–90% of parasites (8). Whereas the phagocytosis of parasites acquired following release from infected neutrophils is most likely influenced by ISP levels, parasites acquired via parasitized neutrophils, the “Trojan horse” route (9), would not be influenced by ISP2 as proposed. We showed that the lack of ISP2 influences parasite uptake by macrophages in vivo, in the presence of additional cells either resident in the tissue or recruited early to the site of infection. ISP2 might show that the lack of ISP2 influences parasite uptake by macrophages deficient in functional TLR4 or lacking TLR4 upon interaction with the parasite, via an unidentified mechanism.

Our immunofluorescence studies revealed NE at the surface of elicited macrophages. It was shown that subpopulations of the human promonocytic cell line U937 express NE at distinct subcellular sites, that is, granule-like compartments or at the cell surface (20). Cell surface NE is available for binding to the inhibitors α-1-proteinase and α-1-antitrypsin, and behaves as a receptor (14). We seldomly detected colocalization of NE and CD11b in the elicited macrophages, having observed a clear pattern of CD11b distribution at the edge of the cell, at basal regions, whereas NE was also observed at the apical region of the cell. Importantly, there was colocalization between CD11b and NE in macrophages exposed to Δisp2/3, indicating that both molecules are in close proximity at the site of parasite entry. Furthermore, there was an exclusive clear pattern of CD11b located at the upper regions of infected cells, suggesting that the parasite induces the surface redistribution of this receptor in macrophages. Our results suggest that NE present at the surface of elicited macrophages is active because it is subject to modulation by ISP2, APT, or the synthetic NEI. Alternatively, the enzyme could be produced in an inactive form and fully activated upon interaction with the parasite, via an unidentified mechanism. In either case, NE is clearly available for binding to and regulation by ISP2. In monocytes, the association of active cell surface NE to inhibitors induces enzyme clustering with CD4 and CXCR4 in a plasma membrane configuration, demonstrating that ligand binding to cell surface NE is capable of changing its distribution, promoting association with different cell surface receptors (14). At the moment, we do not know how NE and CD11b or TLR4 interact promoting association with different cell surface receptors (14). At the moment, we do not know how NE and CD11b or TLR4 interact.

Our real-time PCR studies revealed that the neutralization of TLR4 by Abs or the infection with Δisp2/3 and prevented parasite elimination in the following 24 h, similarly to pretreatment with NEIs. We also showed that the engagement of TLR4 by exogenous

FIGURE 9. In vivo parasite uptake and survival in phagocytes are modulated by NE. Stationary-phase promastigotes were injected in the peritoneal cavity (A, B) or in the footpads (C, D) of C57/B6 mice. A and B, The peritoneal cavity exudates were collected 3 h after injection of parasites, and the cells were plated in RPMI 1640-FCS for 2 h. B, The numbers of intracellular parasites in WT, Δisp2/3, and Δisp2/3 and Δisp2/3 and prevented parasite elimination in the following 24 h, similarly to pretreatment with NEIs. We also showed that the engagement of TLR4 by exogenous

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agents such as LPS or activating Abs promoted enhanced uptake of WT parasites, showing that the activation of this receptor modulates the phagocytosis of \textit{L. major}. Importantly, the effects of exogenous NE on the increased uptake and fate of internalized WT parasites were completely prevented when TLR4 is previously blocked, establishing that NE and TLR4 act in a common pathway. This hypothesis is further supported by the finding that the activation of TLR4 by Abs can bypass the detrimental effect of NE inhibition in the upregulation of parasite phagocytosis, observed either with \Deltaisp2/3 or with WT in the presence of exogenous NE. It is tempting to speculate that, to act in conjunction, CR3, TLR4, and NE join at a cell surface signaling platform to mediate such events. A link between TLR4-mediated responses and NE activity was previously suggested in RAW cells in which pretreatment with a NEI before exposure to LPS resulted in a significant dose-dependent decrease in MIP-2 production, comparable to that seen following pretreatment with TLR4 Ab (21). We observed that the neutralization of TLR4 before the \textit{Leishmania}–macrophage phagocytosis exerted a protective effect to \Deltaisp2/3 only until 24 h postinfection, but the subsequent intracellular growth was practically arrested after 48–72 h. In contrast, \Deltaisp3/3 grew efficiently macrophages defective in TLR4 signaling, increasing ∼6-fold in 3 d, as compared with ∼2-fold increase in the numbers of WT, in the same period. These results suggest that TLR4 is not only implicated in parasite elimination at short times postinfection (24 h), but has an additional role in controlling amastigote growth at later stages of the parasite intracellular life cycle.

The activation of TLR4 after parasite internalization had no effect on the outcome of infection 24 h later (Fig. 7), suggesting that the TLR4–NE pathway must be activated concomitantly to parasite phagocytosis to promote intracellular killing. It has been reported that phagosome maturation in macrophages is influenced by TLR signaling coupled to phagocytosis (22). Furthermore, it has been proposed that intraphagosomal sensing and modulation of the nascent phagosome by TLR act through inducible phago-

The superoxide scavenger EUK134 or treatment of macrophages with catalase rescued \Deltaisp2/3 from intracellular death after 24 h, establishing that ROS are responsible for the killing of intracellular ISP2-deficient parasites. This is important because it is known that \textit{Leishmania} have evolved multiple ways to avoid ROS production by macrophages, causing superoxide to play a limited role in the control of \textit{Leishmania} infection in mice (25) or in human macrophages (26). Our findings suggest that ISP2 has an important role in the control of ROS production during infection. \textit{L. major} lacking all phosphoglycans (LPG and PPG) reside in fusogenic phagosomes of macrophages, where they are subsequently destroyed, although they can survive in macrophages defective in oxidative burst (27). Although we cannot discount that other pathways that contribute to the downregulation of ROS production are affected in \Deltaisp2/3, this seems unlikely because parasite death is completely reversed by NEIs or in macrophages defective in TLR4 signaling. Furthermore, we found no differences in the production of NO by macrophages infected with WT or with \Deltaisp2/3 (data not shown).

In epithelial cells, stimulation with NE is known to induce ROS (28) as part of an intricate cascade leading to gene expression (29). Although the mechanisms underlying triggering of TLR4 by NE in epithelial cells are not fully understood, a recent study provides strong evidence that this is done indirectly, by promoting the di-

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