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Neutrophils Activate Macrophages for Intracellular Killing of *Leishmania major* through Recruitment of TLR4 by Neutrophil Elastase[1]

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We investigated the role of neutrophil elastase (NE) in interactions between murine inflammatory neutrophils and macrophages infected with the parasite *Leishmania major*. A blocker peptide specific for NE prevented the neutrophils from inducing microbicidal activity in macrophages. Inflammatory neutrophils from mutant *pallid* mice were defective in the spontaneous release of NE, failed to induce microbicidal activity in wild-type macrophages, and failed to reduce parasite loads upon transfer in vivo. Conversely, purified NE activated macrophages and induced microbicidal activity dependent on secretion of TNF-α. Induction of macrophage microbicidal activity by either neutrophils or purified NE required TLR4 expression by macrophages. Injection of purified NE shortly after infection in vivo reduced the burden of *L. major* in draining lymph nodes of TLR4-sufficient, but not TLR4-deficient mice. These results indicate that NE plays a previously unrecognized protective role in host responses to *L. major* infection. *The Journal of Immunology,* 2007, 179: 3988–3994.

Neutrophils are among the first leukocytes to reach the site of infection (1, 2). Besides microbicidal function, neutrophils secrete cytokines, proteases, and chemokines that alter the connective tissue and recruit inflammatory and immune cells (1–4). Neutrophils play regulatory roles on immune responses to infection (4–8), but the molecular mechanisms involved are poorly understood. Previous studies demonstrate that interactions of live or dead neutrophils with infected macrophages regulate *Leishmania major* infection (9, 10). In resistant mice, neutrophils protect against infection and induce the killing of *L. major* in macrophages (9). Protection induced by neutrophils requires TNF-α production and generation of reactive oxygen species by macrophages (9). Neutrophil elastase (NE)[3] is a serine protease released from neutrophil azurophilic granules in inflammatory exudates (11). NE plays a multifaceted role in tissue injury and in-

flammation, due to its ability to cleave extracellular matrix components and cell surface molecules (12). A specific peptide inhibitor of NE reverts the leishmanicidal activity induced by neutrophils, and exacerbates *L. major* infection in vivo (9), suggesting a role for NE in macrophage activation.

In mammals, TLRs transduce proinflammatory signals delivered by pathogen molecules to the cells of the innate immune system (13). Recent studies indicate that TLRs are involved in immune responses against protozoan parasites (reviewed in Ref. 14). Protective immunity against *L. major* is impaired in mice deficient of the TLR family adaptor protein MyD88 (15, 16), and signaling through TLR4 contributes to early host resistance against infection (17, 18). In addition, endogenous stimuli initiate inflammatory responses through the engagement of TLRs (19, 20). Specifically, pancreatic elastase induces systemic inflammation and myeloid cell activation through TLR4 (21, 22), and NE induces IL-8 secretion by epithelial cells through activation of TLR4 (23). In this study, we sought to determine the role of NE in the proinflammatory interaction of neutrophils with macrophages infected with *L. major*. We found that inflammatory neutrophils activated macrophages to kill *L. major* by a mechanism that required NE and TLR4 signaling. These findings provided new insight into innate protective mechanisms mounted against *Leishmania* infection.

Materials and Methods

Mice and parasite

BALB/c, C3H/HeN, C3H/HeJ, and C57BL/6J (B6) mice were from Oswaldo Cruz Institute, Fiocruz, Rio de Janeiro. Mutant pallid B6 mice (C57BL/6J-pa) were obtained from University of Siena, Italy, and bred in our facilities. All animal work was approved by an institutional review committee. *L. major* strain LV39 (MRHO/Sv/59/P) was isolated from the footpads of BALB/c mice, and maintained in vitro (24). Promastigotes were kept for up to 4 wk.

Abs and reagents

Neutralizing anti-TNF-α (clone MP6-XT3) mAb, rat IgG1, rat IgG2a/κ, anti-CD16/CD32 mAb, and allophycocyanin-labeled anti-Gr-1 (clone RB6-8C5) mAb were obtained from BD Pharmingen. FITC-labeled

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[3] Abbreviations used in this paper: NE, neutrophil elastase; DFO, deferoxamine; SLPI, secretory leukocyte protease inhibitor; AAT, α_1 anti-trypsin; MeOSuc-AAPV-CMK, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone; WT, wild type.

Annexin V and propidium iodide were obtained from R&D Systems. Neutralizing anti-TLR4 (clone MTS510), anti-TLR2 (clone T2.5) mAbs, and mouse IgG1/κ isotype control were obtained from eBioscience. Abs were used at 10 μg/ml. Purified human NE (Calbiochem; 100–200 ng/ml), Deferoxamine (DFO, 100 μM; Sigma-Aldrich), human recombinant secretory leukocyte protease inhibitor (SLPI, 10–200 ng/ml; R&D Systems), NE inhibitor methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (MeOSuc-AAPV-cmk), and collagenase inhibitor Z-Pro-D-Leu-D-Ala-NHOH (both from Calbiochem, 10 μg/ml) were used. Endotoxin content of purified NE was verified by a quantitative chromogenic *Limulus* amebocyte lysate kit (QCL-1000 kit, BioWhittaker). In addition, purified NE and LPS (Sigma-Aldrich) were incubated with polymyxin B-agarose affinity chromatography columns with the capacity to retain 10,000 endotoxin units (Detoxi-Gel columns, Pierce).

Exudate neutrophils

Neutrophils were obtained 7 h after i.p. injection of 1 ml of 3% thioglycollate. Exudate cells were washed and incubated in DMEM at 37°C for 1 h in 250 ml flasks (Corning). Recovered nonadherent cells contained 80–90% neutrophils. Apoptotic neutrophils were obtained by overnight incubation ("aging"), as described (25). Aged Gr-1+ cells contained >90% Annexin-V+, propidium iodide-negative cells. Aged cells were extensively washed in cold DMEM before use.

Inflammatory macrophages, infection, and coculture

Macrophages were obtained 4 days after i.p. injection of 1 ml thioglycollate. Exudate cells were plated in 48-well vessels (Nunc). Adherent cells (1×10^5) received 1×10^6 stationary phase *L. major* promastigotes in complete medium and 10% FCS at 37°C. After 4 h, monolayers were extensively washed. All cultures were done in DMEM (Invitrogen Life Technologies), supplemented with glutamine, 2-ME, gentamicin, sodium pyruvate, MEM nonessential amino acids, HEPES buffer, and 1% v/v Nutridoma-SP (Boehringer Mannheim), instead of FCS. Neutrophils were added at a 10:1 ratio (1×10^6). Cultures were kept for 3 days at 37°C, 7% CO_2. Extracellular parasites were absent throughout this period.

Assessment of intracellular load of L. major

After 3 days, monolayers were washed and fed with Schneider medium (Invitrogen Life Technologies) supplemented with 20% FCS and 2% human urine. Monolayers were cultured at 26°C for an additional 3 days. The relative intracellular load of *L. major* amastigotes was measured by assessing the number of extracellular motile promastigotes produced (26). Profiles of relative parasite loads closely followed the microscopical assessment of the number of amastigotes per 100 macrophages and percentage of infected macrophages (9).

### Treatment with elastase and transfer of neutrophils in vivo

Mice were infected with 3×10^6 *L. major* promastigotes in the hind footpads. After 30 min, mice received 400 ng purified human NE (Calbiochem) in the right hind footpad. Purified NE was passed through polymyxin B columns before injection. In other experiments, mice received 6×10^6 live neutrophils in the right hind footpad instead of NE. After 14 days, parasite loads in right and left draining lymph nodes were separately determined in individual mice by promastigote production in Schneider medium (9).

Cytokine production

Leishmania-infected macrophages (3×10^5) were treated with NE (200 ng/ml), and supernatants were collected after 1–2 days in culture. Supernatants were cleared by centrifugation and assayed for MIP-1 (1-day supernatant) and TNF-α (2-day supernatant) content by sandwich ELISA according to the manufacturer's instructions (R&D Systems). Results are the mean and SE of triplicate cultures.

Elastase activity

Elastase enzymatic activity was measured as described (27). Neutrophils (10^7/ml in serum-free DMEM) were incubated for 4 h at 37°C. Cells were centrifuged to separate into pellet and supernatants. Cell pellets were solubilized in lysis buffer (50 mN Tris (pH 7.4), 1% Triton X-100, 0.25% deoxycholate, 150 mM NaCl, and 1 mM EGTA). For elastase analysis, 20 μl of either supernatant or solubilized pellet were added in triplicate to ELISA plates, followed by 55 μl elastase reaction buffer (0.1 M HEPES, 0.5 M NaCl, 10% dimethylsulfoxide (pH 7.5)). Then, 150 μl of 0.2 mM Elastase substrate I (MeOSuc-AAPV-pna; Calbiochem) was added in reaction buffer, and samples were incubated at 37°C for 1 h. Elastase activity was measured by reading absorbance at 410 nm, using serial dilutions of human elastase (Calbiochem) as standards.
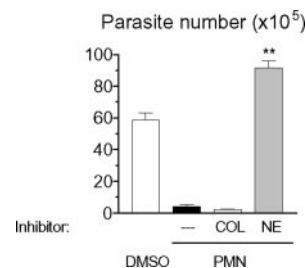
FIGURE 1. Induction of intramacrophagic killing of *Leishmania* by neutrophils requires NE activity. Parasite loads in *Leishmania* infected inflammatory B6 macrophages cultured without (dimethylsulfoxide (DMSO) □) or with apoptotic B6 neutrophils (PMN), in the presence of solvent alone (–, ■), Collagenase inhibitor Z-Pro-D-Leu-D-Ala-NHOH (COL, ▨), or NE inhibitor MeOSuc-AAPV-cmk (NE, ▦), both at 10 μg/ml. All cultures received dimethylsulfoxide, the solvent for protease inhibitors. Intracellular parasite load was evaluated after 3 days in culture, by extracellular promastigote production in Schneider medium. ∗∗, $p < 0.01$, compared with PMN alone. Representative of three experiments with comparable results.

SDS-PAGE and immunoblot analysis

Neutrophils (4×10^7/20 ml in serum-free DMEM) were immediately centrifuged or incubated for 4 h at 37°C, separated into pellet and supernatant and frozen at −70°C. Cell pellets were resuspended in lysis buffer (Invitrogen Life Technologies) with 1% Protease Inhibitor Cocktail set III (Calbiochem) on ice for 30 min. Insoluble material was removed by centrifugation. Supernatants were concentrated by filtration through Centriprep membrane, 3 kDa pore size (Millipore). Protein extracts or concentrated supernatants were boiled and added to each lane. The volume applied per lane was corrected according to the actual proportion of neutrophils, as determined in Giemsa-stained cytospins, to yield the equivalent to 6×10^6 neutrophils. The amount of protein applied to each lane was similar, as determined by Coomassie Blue staining before blotting. Proteins were resolved by SDS-PAGE, transfered to nitrocellulose (Bio-Rad), and subjected to immunoblot analysis. Blocked membranes were incubated with polyclonal Abs to mouse NE (1/200), mouse SLPI (1/200), or mouse α1 antitrypsin (AAT, 1/200; Santa Cruz Biotechnology). Secondary Ab was bovine anti-goat IgG conjugated with HRP (1/1,000), and ECL-Plus detection reagents (Amersham Biosciences) were used to visualize immunoreactive proteins. Gels were calibrated using standard proteins (Santa Cruz Biotechnology) with MW in the range of 23,000 to 132,000 kDa.

Statistical analysis

Data were analyzed by Student' t test for independent samples, using SigmaPlot for Windows. Differences with a p value <0.05 were considered significant. For parasite loads in vivo, counts for left and right draining lymph nodes were first normalized by log transformation, and paired t tests were used instead.

Results

Induction of intramacrophagic microbicidal activity required NE activity

We have previously demonstrated that the interaction of *Leishmania*-infected B6 macrophages with live or apoptotic B6 neutrophils is proinflammatory, leading to TNF-α production and TNF-α-dependent killing of intracellular parasites (9). In agreement with previous results (9), we observed that the microbicidal activity induced by apoptotic neutrophils could be prevented by the addition of MeOSuc-AAPV-cmk, a blocker peptide specific for NE (28), but not by a control peptide inhibitory for collagenase (Fig. 1). These results suggested that NE activity is involved in macrophage activation to a leishmanicidal state.

A protease-antiprotease imbalance in B6 inflammatory neutrophils

The enzymatic activity of NE is regulated by the serpins SLPI and AAT (29, 30). We sought to determine whether unchecked NE
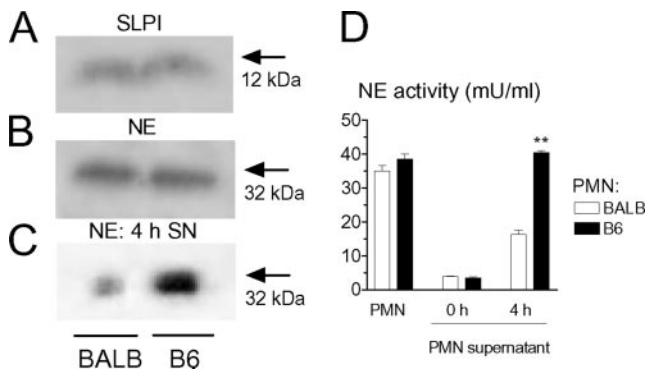
FIGURE 2. A protease-antiprotease imbalance in inflammatory B6 neutrophils. Immunoblots of SLPI (*A*) and NE (*B*) polypeptides in whole cell extracts from 6×10^6 freshly harvested BALB (*left*) and B6 (*right*) inflammatory neutrophils. *C*, Immunoblots of NE polypeptide detected on 4-h supernatants from 6×10^6 BALB and B6 inflammatory neutrophils. Arrows at right, MW markers. The amounts of loaded protein were similar in each lane, as assessed by Coomassie blue staining before blotting. *D*, NE enzymatic activity (mU/ml) measured in whole cell extracts (*left*; PMN), or in 0- and 4-h supernatants (*right*) from 6×10^6 BALB (□) and B6 (■) neutrophils. **, $p < 0.01$. Representative of three experiments with identical results.

proteolytic activity could be responsible for the proinflammatory activity of B6 neutrophils. Analysis by immunoblot did not show any difference between the amounts of expressed SLPI (Fig. 2*A*) or AAT (data not shown) proteins in BALB/c and B6 inflammatory neutrophils. Furthermore, we found similar amounts of NE in freshly explanted BALB/c and B6 neutrophils (Fig. 2*B*). However, after 4 h in culture, we found 2–3-fold more NE released into the supernatant of B6, as compared with BALB/c neutrophils (Fig. 2*C*). This result was reproduced in an additional experiment. To verify whether increased NE secretion resulted in a protease-antiprotease imbalance, we measured the net NE enzymatic activity in the 4-h supernatants. In repeat experiments, NE activity was 2–3-fold higher in B6, compared with BALB/c supernatants (Fig. 2*D*). These results indicated that, compared with BALB/c neutrophils, B6 neutrophils released increased amounts of net NE enzymatic activity into supernatants.

Neutrophils from mutant pallid B6 mice failed to induce microbicidal activity

Pallid is a mutation in the protein Pallidin, affecting the biogenesis of lysosome-related organelles by destabilizing the assembly of the biogenesis of lysosome-related organelles complex-1 (31). Mutant *pallid* B6 mice have reduced coat pigmentation and reduced secretion of lysosomal enzymes in urine (31). In addition, neutrophils from *pallid* mice release reduced amounts of NE in response to chemotactic stimulus (32). We confirmed that inflammatory neutrophils from *pallid* mice released reduced amounts of NE into the supernatant, compared with constitutive release from wild-type (WT) B6 neutrophils (Fig. 3*A*). We sought to determine the outcome of interactions of *pallid* neutrophils with WT macrophages infected with *L. major*. Coculture of infected WT macrophages with live WT B6 neutrophils resulted in a dose-dependent reduction of parasite loads in macrophages (Fig. 3*B*). However, coculture with live *pallid* neutrophils failed to induce parasite killing, and led to an increase of *L. major* replication in macrophages at high doses of added neutrophils (Fig. 3*C*). Culture with dead *pallid* neutrophils induced parasite killing by macrophages (data not shown), presumably because of leakage of NE into the medium (27). We also tested the effect of adoptive transfer of live neutro-
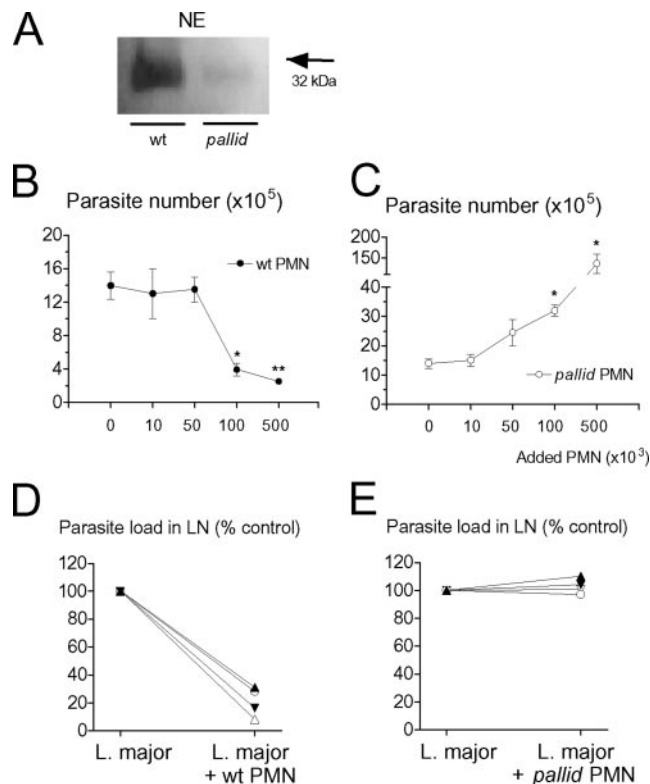

FIGURE 3. Defective induction of intramacrophagic killing of *Leishmania* by mutant *pallid* B6 neutrophils. *A*, Mutant *pallid* neutrophils release reduced amounts of NE into supernatants. Immunoblots of NE polypeptide detected on 4-h supernatants collected from live *pallid* and WT B6 inflammatory neutrophils. Arrow at right, MW marker. The amount of loaded proteins was similar in each lane, as assessed by Coomassie blue staining before blotting. *B* and *C*, Macrophage leishmanicidal activity induced by WT and *pallid* B6 neutrophils. Parasite loads in *Leishmania*-infected WT B6 macrophages (10^5) cultured in the presence of increasing numbers of live WT (*B*) or mutant *pallid* B6 neutrophils (*C*). Intracellular parasite load was evaluated after 3 days in culture, by extracellular promastigote production in Schneider medium. *, $p < 0.05$; **, $p < 0.01$. Representative of two experiments with identical results. *D* and *E*, Transfer of WT and *pallid* B6 neutrophils in vivo. B6 mice were infected with *L. major* in both footpads, and were injected with WT (*D*) or *pallid* (*E*) B6 neutrophils in the right footpad 30 min later. After 14 days, parasite loads were determined in the left (*L. major*) and right (*L. major* + PMN) draining lymph nodes. The figures show the percentages of parasite load in the right lymph nodes of four individual mice per group, taking the left lymph node loads as 100%. Differences between right and left lymph nodes were significant for WT ($p < 0.05$), but not for *pallid* neutrophils (N.S.).

phils in vivo on the extent of infection. Transfer of WT B6 neutrophils to the footpads markedly reduced the parasite load in draining lymph nodes of infected B6 mice (Fig. 3*D*). However, transfer of *pallid* neutrophils failed to reduce the parasite load of infected B6 mice (Fig. 3*E*). Taken together, these results suggested a link between NE release, induction of microbicidal activity in macrophages, and a protective effect in vivo.

NE-induced macrophage activation and killing of L. major

Given the above results, we investigated the effects of purified NE on induction of a microbicidal state in inflammatory macrophages infected with *L. major*. Initially, we found that the addition of NE at doses of 1 μg/ml or higher induced morphological changes in macrophages, which progressed to cell detachment and death in a proportion of cells. However, at doses of 100–200 ng/ml (3.3–6.6 nM), addition of NE induced dramatic membrane spreading in
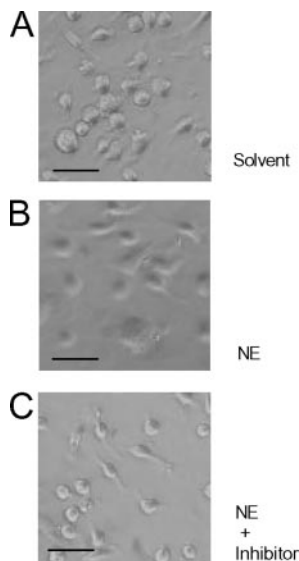
FIGURE 4. Purified NE induces membrane spreading in macrophages. B6 inflammatory macrophage monolayers were cultured in solvent alone (*A*), NE at 100 ng/ml (*B*), or NE plus NE inhibitor MeOSuc-AAPV-cmk, at 10 μg/ml (*C*). After 18 h, phase contrast micrographs were taken. Note the marked membrane spreading in *B*, which is prevented in *C*. Calibration bar, 30 μm. Representative of two experiments with identical results.

macrophages, without any sign of cell death (Fig. 4*B*, compared with Fig. 4*A*). Macrophage membrane spreading could be prevented by adding the specific NE inhibitor MeOSuc-AAPV-cmk (Fig. 4*C*). These results indicated that NE protease activity was required for induction of membrane spreading. In addition, they argued against endotoxin contamination as the cause of cell spreading. Addition of purified NE increased the constitutive secretion of the chemokine MIP-2 by infected macrophages (Fig. 5*A*). Furthermore, addition of purified NE induced intramacrophagic killing of *L. major* at low dosages, and in a dose-dependent fashion, in B6 macrophages (Fig. 5*B*). DFO is an iron chelator that prevents oxidative damage (33). The microbicidal activity induced by NE could be reverted by adding DFO (Fig. 5*C*), or by adding exogenous rSLPI (Fig. 5*D*). These results suggested that activation of a microbicidal state required production of oxidant species and NE enzymatic activity. The results also argued against any effect of endotoxin contamination.

Intramacrophagic microbicidal activity induced by NE required TNF-α production

We investigated whether purified NE induced the secretion of TNF-α by *Leishmania*-infected macrophages. Similar to apoptotic neutrophils (9), NE induced TNF-α secretion after 48 h (Fig. 6*A*). Moreover, addition of a neutralizing anti-TNF-α mAb, but not an isotype control, prevented the leishmanicidal activity of purified NE on macrophages (Fig. 6*B*). These results demonstrated that the leishmanicidal activity of NE depended on TNF-α production by macrophages.
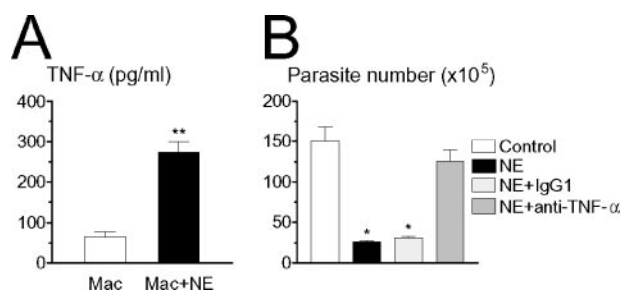
Intramacrophagic microbicidal activity induced by neutrophils or purified NE required TLR4 signaling

Using a chromogenic *Limulus* assay kit, the purified NE preparations gave undetectable endotoxin levels at a concentration 10–20 times higher than the dosages used in the present study. We further investigated whether removal of any residual endotoxin contamination affected microbicidal activity induced by purified NE. Diluted samples of purified NE or LPS were passed through poly-
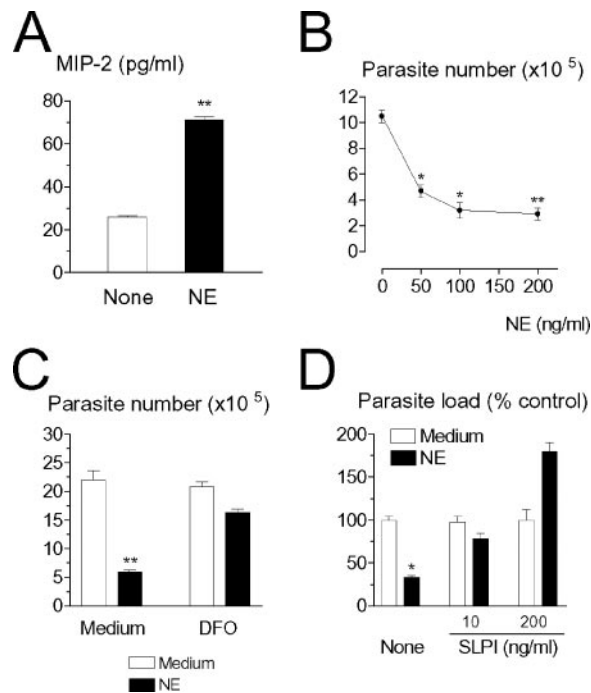

FIGURE 5. Purified NE activates macrophages and induces microbicidal activity. *A*, MIP-2 activity in supernatants from *Leishmania* infected macrophages treated (■) or not (□) with NE, 200 ng/ml. *B*, Intracellular load of *Leishmania* in macrophages treated with the indicated doses of NE. *C*, Parasite loads in infected macrophages treated or not with NE (100 ng/ml) in the absence (Medium), or in the presence of the iron chelator DFO, at 100 μM. *D*, Parasite loads in macrophages treated or not with NE (100 ng/ml) in the absence (None), or in the presence of the indicated doses of rSLPI (10 or 200 ng/ml). All experiments were done with inflammatory B6 macrophages. Intracellular parasite load was evaluated after 3 days in culture by extracellular promastigote production in Schneider medium. *, $p < 0.05$; **, $p < 0.01$, compared with controls. Data are representative of two experiments with identical results.

myxin B-agarose columns with the capacity to retain 10,000 endotoxin units. The leishmanicidal activity induced by LPS was lost following passage through polymyxin B-coupled agarose. However, the activity induced by NE was not affected (Fig. 7*A*). These results confirmed that macrophage activation induced by NE was not due to endotoxin contamination. Secretion of IL-8 induced

FIGURE 6. Microbicidal activity induced by NE depends on TNF-α production by macrophages. *A*, Amounts of TNF-α released into the supernatant by *Leishmania* infected macrophages after 48 h incubation in medium alone (open bar) or in the presence of NE, at 200 ng/ml (■). *B*, Parasite loads in *Leishmania* infected macrophages treated as indicated with NE in the presence of neutralizing anti-TNF-α mAb or an isotype control, both at 10 μg/ml. Intracellular parasite load was evaluated after 3 days in culture, by extracellular promastigote production in Schneider medium. *, $p < 0.05$; **, $p < 0.01$. Representative of two experiments with identical results.
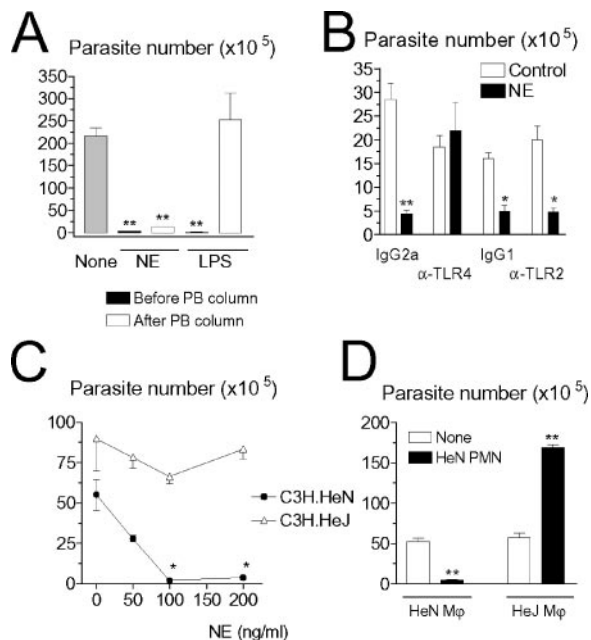
FIGURE 7. Microbicidal activity induced by NE or neutrophils requires TLR4 expression. *A*, Microbicidal activity of NE is independent on endotoxin contamination. Parasite loads in *Leishmania* infected macrophages (untreated: ▨) treated with NE, 100 ng/ml, or with LPS, 10 ng/ml, either before (■) or after (□) passage through polymyxin B agarose columns (PB columns; □). **, $p < 0.01$. *B*, Parasite loads in *Leishmania* infected macrophages, either untreated (□) or treated or not with NE (100 ng/ml) in the presence of neutralizing anti-TLR4 and anti-TLR2 mAbs or their isotype controls (■), all at 10 μg/ml. Treatment with NE induced significant killing of *L. major* in all cases, except in the presence of anti-TLR4. *C*, Parasite loads in *Leishmania* infected C3H/HeN (closed symbols) or C3H/HeJ (open symbols) macrophages treated with the indicated doses of purified NE. *, $p < 0.05$, compared with untreated macrophages. *D*, Parasite loads in *Leishmania* infected inflammatory C3H/HeN (*left*) or C3H/HeJ (*right*) macrophages cultured in the absence (None; □) or in the presence of C3H/HeN neutrophils (■). **, $p < 0.01$. In all experiments, intracellular parasite load was evaluated after 3 days in culture, by extracellular promastigote production in Schneider medium. Data are representative of two experiments with identical results.

by NE on epithelial cells requires TLR4 expression (23). We therefore investigated the role of TLR4 in macrophage activation induced by NE. Infected B6 macrophages were treated with purified NE in the presence of neutralizing anti-TLR2 or anti-TLR4 mAbs, or their isotype controls. The neutralizing mAb specific for TLR4, but not for TLR2, prevented the intramacrophagic killing of *L. major* (Fig. 7*B*).

C3H/HeJ mice express a loss-of-function mutation of the *Tlr4* gene (34, 35). Macrophages from C3H/HeJ or control C3H/HeN mice were infected with *L. major* and treated with purified NE. Addition of purified NE potently inhibited parasite replication in control C3H/HeN macrophages, at the same low dosages which were effective for B6 macrophages (Fig. 7*C*). However, NE failed to inhibit replication of *L. major* in TLR4 mutant C3H/HeJ macrophages at all dosages tested (Fig. 7*C*). These results indicated that TLR4 was required for macrophage activation induced by NE.

We investigated the role of TLR4 expression in macrophage interaction with neutrophils. Coculture of inflammatory C3H/HeN neutrophils with C3H/HeN macrophages induced potent leishmanicidal activity (Fig. 7*D*). However, coculture of the same C3H/HeN neutrophils with TLR4 mutant C3H/HeJ macrophages failed to induce killing, and actually exacerbated the replication of *L. major* in macrophages (Fig. 7*D*). These results indicated that
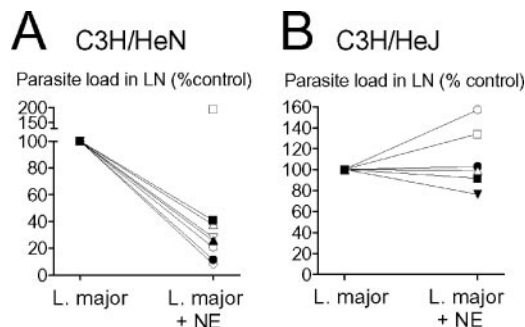
FIGURE 8. Injection of NE reduces *L. major* infection in vivo. C3H/HeN (*A*) and TLR4-mutant C3H/HeJ (*B*) mice were infected with *L. major* in both footpads, and were injected with 0.4 μg NE in the right footpad 30 min later. After 14 days, parasite loads were determined in the left (*L. major*) and right (*L. major* + NE) draining lymph nodes. The figures show the percentages of parasite load in the right lymph nodes of individual mice, taking the left lymph node loads as 100%. Differences between right and left lymph nodes were significant in HeN ($p = 0.006$) mice, but not in HeJ mice ($p = 0.564$). All eight C3H/HeN and 6 C3H/HeJ animals were included in the analysis.

TLR4 expression in macrophages was required for the microbicidal activity induced by inflammatory neutrophils. Together, these results indicated that neutrophils and purified NE induced leishmanicidal activity in macrophages through TLR4 signaling.

NE reduced early parasite replication in vivo

We investigated whether injection of purified NE affected in vivo infection by *L. major* at an early stage. C3H/HeN and C3H/HeJ mice were infected with *L. major* in both footpads, and NE was injected 30 min later in the right footpad. After 14 days, parasite loads were determined separately in the draining left and right lymph nodes. Except for one of eight animals, NE markedly reduced parasite loads in draining lymph nodes of C3H/HeN mice, but failed to alter the course of early infection in C3H/HeJ mice (Fig. 8). In addition, injection of NE reduced by 3-fold the load of *L. major* in both infected B6 and BALB/c mice (data not shown). These results indicated that NE restricted *L. major* infection in vivo, and that this protective effect required TLR4 expression.

Discussion

Regulation of immune responses by neutrophils is under polymorphic genetic control (6, 7, 36, 37). However, the cellular mechanisms involved are poorly understood. Cooperation between macrophages and neutrophils in defense against *L. major* is under genetic control. In susceptible hosts, neutrophils play a deleterious role in vivo and exacerbate the replication of *L. major* in macrophages (9). This interaction requires cell contact (9) and expression of the Fas ligand by live neutrophils to allow rapid cell death and phagocytic removal (10). Increased parasite growth results from the secretion of prostaglandin and TGF-β by macrophages (9), similar to the replication of *Trypanosoma cruzi* driven by engulfment of apoptotic lymphocytes (38). In resistant hosts, however, neutrophils protect against early infection in vivo and induce the killing of *L. major* by macrophages (9). In this study, our results have demonstrated that, in resistant hosts, macrophage activation by inflammatory neutrophils induced the killing of *L. major* through recruitment of TLR4 by NE.

NE is a serine protease constitutively released by inflammatory neutrophils (11, 12). NE is also released from injured resting blood neutrophils and induces production of TNF-α by human macrophages (26). In agreement with previous studies (9), addition of the

NE blocker peptide MeOSuc-AAPV-cmk prevented the ability of B6 neutrophils to induce microbicidal activity in macrophages. Furthermore, administration of MeOSuc-AAPV-cmk to B6 mice at the time of infection with *L. major*, induces an 8-fold increase of the parasite burden in draining lymph nodes (9). These results suggest a nonredundant role for NE in early defense against *L. major* infection. Our data now demonstrated a strain-specific polymorphism in the rate of NE released by inflammatory neutrophils. Neutrophils from B6 mice released 2–3 times more NE protein and enzymatic activity into the supernatant, compared with neutrophils from BALB/c mice. The release of myeloperoxidase was also 2–3 times larger in B6 than in BALB/c neutrophils (data not shown). These results suggest that inflammatory B6 neutrophils undergo more extensive degranulation of azurophilic granules compared with neutrophils from BALB/c mice. The increased amount of NE activity released by B6 neutrophils could explain the proinflammatory response induced in macrophages. In agreement with this possibility, inflammatory neutrophils from mutant *pallid* B6 mice released much less NE than WT neutrophils, failed to activate WT macrophages for killing of *L. major*, and failed to protect against infection upon transfer in vivo. Similar to neutrophils from BALB/c mice (9), neutrophils from *pallid* B6 mice exacerbated replication of *L. major* in vitro. Exacerbated parasite replication could have resulted from the anti-inflammatory clearance of apoptotic neutrophils by macrophages in the absence of simultaneous engagement of a proinflammatory pathway (39, 40). Different from BALB/c neutrophils (9), transfer of *pallid* neutrophils did not exacerbate the parasite load in vivo. Lack of the deleterious effect could have resulted from either the dose of transfered neutrophils used or from release of serine proteases upon cell transfer.

We investigated the effects of NE on macrophage activation. Addition of purified NE to infected macrophages induced membrane ruffling and cell spreading, up-regulated production of the chemokine MIP-2, and induced intramacrophagic killing of *L. major*. NE induces oxidant generation in macrophages (41). Our previous studies indicate that the generation of oxygen intermediates, but not NO, is required for microbicidal activity induced by neutrophils (9). In this study, we found that the microbicidal activity of purified NE could be reverted by DFO, suggesting that oxidant generation was required. Microbicidal activity of NE could also be reverted by the serpin SLPI, suggesting that enzymatic activity is required. Previous studies indicate that microbicidal activity of neutrophils requires TNF-α (9), and that purified NE induces TNF-α secretion from human macrophages (27). In agreement, our data indicated that purified NE induced TNF-α secretion in mouse macrophages infected with *L. major*, and that TNF-α was required for microbicidal activity induced by purified NE.

Signaling through TLRs plays an important role in proinflammatory responses against protozoan parasites (14). In addition, during infection and tissue injury, degradation products of the extracellular matrix function as endogenous ligands of TLRs (19–21). Injection of pancreatic elastase induces systemic proinflammatory responses through TLR4 (21, 22), and NE induces IL-8 secretion in epithelial cells through engagement of TLR4 (23). In smooth muscle cells, NE induces rapid association of MyD88 with IRAK, followed by NFκB activation (42). These studies suggest that NE activates cell responses through TLRs. We demonstrated that macrophage leishmanicidal activity induced by NE required TLR4. Our results ruled out contamination of NE with LPS, because NE effects on macrophage spreading and microbicidal activity could be reverted by NE blocker peptide, by DFO, and by rSLPI. Using a *Limulus* lysate assay, endotoxin levels of purified NE were undetectable at doses 10–20 times higher than those used in the present study. Furthermore, the activity of purified NE could

not be removed by passage through polymyxin B-coupled agarose. Finally, macrophage activation induced by inflammatory neutrophils also required functional TLR4 expression.

Neutrophils play a protective role against early *L. major* infection in vivo (9). Furthermore, mice deficient of the TLR family adaptor protein MyD88 develop a nonhealing phenotype upon infection with *L. major* (15, 16), and protective immunity against *L. major* is partially impaired in mice deficient of TLR4 (17, 18). Our results have demonstrated that early in vivo administration of NE reduced *L. major* infection, and that expression of TLR4 was required for this protective effect.

Early engagement of TLR4 mediated by NE triggered a proinflammatory response and induced microbicidal activity. These results contrasted with the notion that phagocytic removal of apoptotic neutrophils is anti-inflammatory (40). However, a recent study demonstrates that B6 macrophages infected with *L. major* induce neutrophil apoptosis more efficiently than BALB/c macrophages, and that B6 neutrophils are cleared from the site of infection more rapidly than BALB/c neutrophils (43). These findings suggest a mechanism by which proinflammatory neutrophils are rapidly removed from the tissues, helping to resolve inflammation.

Macrophages acquire neutrophil granules from apoptotic neutrophils for antimicrobial activity against *Mycobacteria* (4, 5, 44). We cannot discard that a similar mechanism induces leishmanicidal activity in the presence of NE. However, if this is the case, both TLR4 and production of TNF-α are critically involved. The molecular mechanism by which NE triggers cellular responses through TLR4 was not determined. NE could cleave extracellular matrix molecules, such as glycosaminoglycans, to generate endogenous ligands for TLRs (20, 21, 45, 46). Alternatively, a direct effect of NE on TLR4 molecules cannot be discarded. Our results could help in the design of new therapeutic approaches against *Leishmania* infection. In addition, our studies suggested that genetic polymorphisms affecting interactions between macrophages and neutrophils could play a role in innate resistance to *Leishmania* infection in humans.

Disclosures

The authors have no financial conflict of interest.

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