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Macrophage Interactions with Neutrophils Regulate *Leishmania major* Infection¹

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Macrophages are host cells for the pathogenic parasite *Leishmania major*. Neutrophils die and are ingested by macrophages in the tissues. We investigated the role of macrophage interactions with inflammatory neutrophils in control of *L. major* infection. Coculture of dead exudate neutrophils exacerbated parasite growth in infected macrophages from susceptible BALB, but killed intracellular *L. major* in resistant B6 mice. Coinjection of dead neutrophils amplified *L. major* replication in vivo in BALB, but prevented parasite growth in B6 mice. Neutrophil depletion reduced parasite load in infected BALB, but exacerbated infection in B6 mice. Exacerbated growth of *L. major* required PGE₂ and TGF- β production by macrophages, while parasite killing depended on neutrophil elastase and TNF- α production. These results indicate that macrophage interactions with dead neutrophils play a previously unrecognized role in host responses to *L. major* infection. *The Journal of Immunology*, 2004, 172: 4454–4462.

Neutrophils provide the first line of defense against infection, and release mediators that alter extracellular environment and recruit inflammatory cells. Neutrophils constitutively die by apoptosis at inflammatory sites, and are ingested by macrophages (1, 2). Neutrophil clearance interrupts the release of inflammatory mediators (2, 3), and administration of apoptotic cells accelerates resolution of inflammation (4). Cells undergoing apoptosis expose ligands for a set of conserved receptors on macrophages (5). These scavenger receptors trigger production of anti-inflammatory mediators such as PGE₂ and TGF- β (6). TGF- β is important for tissue repair, but inactivates macrophages and promotes the growth of intracellular pathogens (7–9). Macrophages phagocytosing apoptotic cells secrete TGF- β , down-regulate NO production, and up-regulate synthesis of the polyamine putrescine, leading to increased replication of the pathogenic parasite *Trypanosoma cruzi* (10). This biochemical pathway starts with PGE₂ production; cyclooxygenase inhibitors block *T. cruzi* growth in vitro, and markedly reduce parasitemia in vivo (10). The pathway triggered by apoptotic cell clearance could play a role in other infections, as polyamine synthesis is essential for replication of many pathogenic parasites (11).

Cutaneous leishmaniasis is an infectious disease that can be approached in murine models. Host genetic factors play an important role in resistance or susceptibility to infection with *Leishmania* (12). Infection with *Leishmania major* spontaneously resolves in resistant B6 mice, but progresses and kills susceptible BALB/c mice (13). Neutrophils are major components of innate immunity to *L. major* infection (14, 15). Neutrophils arrive at the site of infection before inflammatory macrophages, the primary targets for parasite replication (16). Early influx of neutrophils modifies subsequent Th cell responses and susceptibility to *L. major* infection (17). Moreover, growth of *Leishmania* inside macrophages is controlled by arginase, ornithine decarboxylase (ODC),⁴ and polyamine levels (18).

We investigated the roles of inflammatory neutrophils on macrophage activation and *L. major* replication in susceptible and resistant hosts. We found that, in susceptible BALB mice, interaction with dead neutrophils exacerbates *L. major* growth through PGE₂ and TGF- β production by macrophages. However, neutrophils promote parasite killing in resistant B6 mice. Neutrophil elastase (NE) and TNF- α disable the default pathway of dead cell removal, and induce leishmanicidal activity in macrophages. These results indicate that interactions of dead inflammatory neutrophils with macrophages play a previously unrecognized role in host responses to a pathogenic parasite.

Materials and Methods

Mice and parasite

BALB/c, C3H/HeN, and C57BL.6 (B6) mice of both sexes, aging 6–8 wk, were from the Oswaldo Cruz Institute Animal Care Facility (Fiocruz, Rio de Janeiro, Brazil). All animal work was approved and conducted according to humane institutional guidelines. *L. major* strain LV39 (MRHO/Sv/59/P) was isolated monthly from footpad lesions of infected BALB mice (19), and maintained in vitro as proliferating promastigotes (20). Promastigotes were kept for up to 4 wk without losing infectivity.

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⁴ Abbreviations used in this paper: ODC, ornithine decarboxylase; L-NMMA, N^G-monomethyl-L-arginine-monoacetate; MeOSuc-AAPV-CMK, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone; NE, neutrophil elastase; NOS, NO synthase; PI, propidium iodide.

Exudate neutrophils

Neutrophils were obtained 7 h after i.p. injection of 1 ml of 3% thioglycolate broth (Difco, Detroit, MI). Exudate cells were washed and incubated in DMEM at 37°C for 1 h in 250-ml flasks (Corning Glass, Corning, NY), to remove adherent macrophages (1). In some experiments, neutrophils were obtained 16 h after i.p. injection of 2×10^6 *L. major* promastigotes. Nonadherent cells (80–90% neutrophils) were either used directly, or were further purified by density gradient centrifugation on Percoll, as described (21). By flow cytometry, the resulting population contained >97% Gr-1⁺ neutrophils. Neutrophils were killed by two methods. First, neutrophils and Jurkat cells were heated at 56°C for 30 min, in serum-free DMEM, as described (22). Heated cells were extensively washed in cold DMEM before use. Heated neutrophils reproduced late stages of apoptosis, because they had condensed chromatin and nuclear coalescence typical of apoptosis, but were permeable to trypan blue. Heated Jurkat cells were typically early apoptotic, as they had pyknotic nuclei, but excluded trypan blue. Neutrophils were also killed by UV irradiation. Neutrophils (5×10^6 /ml) were suspended in cold DMEM-10% FCS (Life Technologies, Grand Island, NY) in 25-ml flasks (Corning), and placed over an UV transilluminator box, as described (23). UV-treated neutrophils had coalescent and pyknotic nuclei, but excluded trypan blue. Cells were washed and resuspended in complete culture medium. All cultures were done in DMEM (Life Technologies), supplemented with 2 mM glutamine, 5×10^{-5} M 2-ME, 10 µg/ml gentamicin, sodium pyruvate, MEM nonessential amino acids, 10 mM HEPES buffer, and 1% v/v Nutridoma-SP (Boehringer Mannheim, Indianapolis, IN). Except when noted, all experiments were done with heat-killed neutrophils.

Inflammatory macrophages, infection, and coculture

Inflammatory macrophages were obtained 4 days after i.p. injection of 1 ml of thioglycolate. Peritoneal exudate cells were plated in 48-well vessels (Nunc, Roskilde, Denmark) at 1.5×10^5 cells/well, in complete culture medium. Cells immediately received 1×10^6 stationary phase *L. major* promastigotes, and were incubated in complete medium-10% FCS at 37°C. After 4 h, monolayers were extensively washed with warm HBSS, to remove extracellular parasites and nonadherent cells, leaving $\sim 1 \times 10^5$ adherent macrophages. All cultures were done in complete medium-1% Nutridoma-SP, instead of FCS. Neutrophils were added at a 10:1 ratio (1×10^6), in the presence or absence of Abs, solvents, and reagents. In some experiments, macrophage monolayers (2×10^5 adherent cells) were prepared in 24-well vessels (Corning), infected with 2×10^6 promastigotes, and washed. Dead neutrophils (10:1) were either added in the same compartment, or separated by a cell-impermeable culture insert (MilliCell inserts, 0.4 µm; Sigma-Aldrich, St. Louis, MO). Cultures were kept for 3 days at 37°C, 7% CO₂. Extracellular parasites were absent throughout this period.

Assessment of intracellular load of *L. major*

After 3 days, infected macrophage monolayers were extensively washed, and medium was replaced by 0.5 ml of Schneider medium (Life Technologies), supplemented with 20% FCS and 2% human urine (9). Monolayers were cultured at 26°C for additional 3 days. Intracellular load of *L. major* amastigotes was estimated by production of proliferating extracellular motile promastigotes in Schneider medium (9). Alternatively, infected macrophage monolayers (1×10^5) were cocultured or not with dead neutrophils (1×10^6) in glass coverslips placed inside 1-ml culture vessels (Corning). After 7 days, coverslips were washed and stained with May-Grunwald Giemsa (Sigma-Aldrich), and intracellular amastigotes were counted in 200 macrophages. Results are shown as amastigote number per 100 macrophages, and as percentage of infected macrophages. All results are mean and SE of triplicate cultures.

Adhesion and uptake of neutrophils

BALB and B6 macrophage monolayers were prepared in glass coverslips and washed. Dead BALB and B6 neutrophils were added at a 10:1 ratio, and coverslips were incubated for 1 h at 37°C in serum-free DMEM. Coverslips were extensively washed and stained with May-Grunwald Giemsa. Phagocytosis of apoptotic neutrophils induces concurrent ingestion of extracellular fluid into spacious phagosomes (24). Ingested neutrophils were identified as surrounded by a large vacuole. The remaining associated neutrophils were scored as adhered. Percentages of macrophages with adhered and ingested neutrophils, and numbers of ingested and adhered neutrophils per 100 macrophages were scored. Results are mean and SE of triplicate cultures.

Flow cytometry

Peritoneal exudate cells containing neutrophils were obtained 7 h after i.p. injection of 1 ml of 3% thioglycolate broth. Cells were washed, treated with anti-CD16/CD32 (Fc block), and stained with propidium iodide (PI) plus FITC-annexin V, plus PE anti-Gr-1 mAb. Cells (10^4 cells per acquisition) were analyzed on a BD X-Calibur flow cytometer. Gr-1-positive cells were electronically gated, and the percentages of cells positive for annexin V or PI (collected at the FL3 channel) were determined.

Abs and reagents

Anti-CD16/CD32 (Fc block) and PE-labeled anti-Gr-1 mAb RB6-8C5 were from BD Biosciences (San Diego, CA). Annexin V and PI were from R&D Systems (apoptosis detection kit; Minneapolis, MN). Neutralizing anti-TNF-α mAb (clone MP6-XT3), rat IgG1 isotype control (both from BioSource Europe, Nivelles, Belgium), neutralizing anti-TGF-β Ig, and normal chicken Ig (R&D Systems) were used at 10 µg/ml. Recombinant mouse thrombopoietin receptor/Fc chimera and mouse rTGF-β receptor II/Fc chimera (R&D Systems) were used at 50 ng/ml. Neutrophil-depleting anti-Gr-1 mAb RB6-8C5 (25) and control rat IgG (BioSource Europe) were used *in vivo*. Macrophage monolayers were treated with 10 µM PGE₂, 10 µg/ml aspirin, 1 µg/ml indomethacin (all from Sigma-Aldrich), or equivalent dosage of solvent (ethanol for PGE₂ and aspirin, DMSO for indomethacin) for 3 days. Monolayers were also treated with the specific NE inhibitor methoxysuccinyl-Ala-Ala-Pro-Val-chloromethylketone (26) (MeOSuc-AAPV-CMK; Calbiochem-Novabiochem, La Jolla, CA), control collagenase inhibitor Z-Pro-D-Leu-D-Ala-NHOH (Calbiochem-Novabiochem), both at 10 µg/ml, or equivalent dosage of solvent (DMSO) alone. NO synthase (NOS) inhibitor N^G-monomethyl-L-arginine-monoacetate (L-NMMA; Calbiochem-Novabiochem) was used at 1 mM, final concentration. Catalase (from bovine liver, 20,000 U/mg; Sigma-Aldrich) was used at 1,000 U/ml.

Cytokine production

Supernatants from control and infected macrophages, dead neutrophils alone, or dead neutrophil/infected macrophage cocultures were collected after 2 days, cleared by centrifugation, and immediately assayed for IL-10, TGF-β, and TNF-α content, by sandwich ELISA, according to the manufacturer instructions (BD Biosciences).

Neutrophil depletion

BALB and B6 mice received a total of 500 µg of neutrophil-depleting anti-Gr-1 mAb RB6-8C5 (25) or control rat IgG, divided into five i.p. doses of 100 µg each, given at days -1, 2, 5, 8, and 11 after *L. major* infection in the footpads. We confirmed that this dosage ensures peripheral blood neutropenia for at least 3 days after each mAb injection (25). At day 0, control and depleted animals were infected with *L. major* in the footpads (1×10^6 stationary phase promastigotes). After 13 days, animals were sacrificed and parasite loads in draining lymph nodes were determined by promastigote production in Schneider medium. Results were normalized as the ratio between parasite number and lymph node cellularity.

Treatment with killed neutrophils and NE inhibitor *in vivo*

BALB and B6 mice were injected in the footpads with 1×10^6 *L. major* promastigotes, in the absence or in the presence of 4×10^6 dead syngeneic neutrophils. Parasite load in draining lymph nodes was determined after 10 days by promastigote production in Schneider medium. MeOSuc-AAPV-CMK (NE inhibitor) and Z-Pro-D-Leu-D-Ala-NHOH (collagenase inhibitor used as control) were dissolved in DMSO. The doses for the inhibitors were 100 µg/g body weight, as described (26). Because promastigotes are killed when suspended in solvent (DMSO) alone, each inhibitor (in 50 µl of solvent), as well as solvent alone, was injected into the footpads of B6 mice 1 h after *L. major* injection (1×10^6 promastigotes/footpad). After 13 days, parasite load in draining lymph nodes was determined by promastigote production in Schneider medium. Results were normalized as the ratio between parasite number and lymph node cellularity.

Statistical analysis

Data were analyzed by Student's *t* test for independent samples, using SigmaPlot for Windows. Differences with a *p* value <0.05 or lower were considered significant.

Results

Macrophage interactions with dead neutrophils induce growth or killing of *L. major*, depending on host genotype

To mimick the early stage of infection, we cultured live or killed neutrophils with *L. major*-infected inflammatory macrophages from both susceptible (BALB) and resistant (B6) genotypes. By flow cytometry, live Gr-1⁺ exudate neutrophils from BALB mice contained 36%, and Gr-1⁺ exudate neutrophils from B6 mice contained 40% of cells already staining positive for annexin V. BALB neutrophils had 3.3%, and B6 neutrophils had 4.4% of cells taking up PI. Whether dead or alive, syngeneic neutrophils markedly exacerbated *L. major* replication in BALB macrophages, but almost completely eliminated *L. major* from B6 macrophages (Fig. 1A). Apoptotic neutrophils obtained by UV irradiation gave similar results (Fig. 1B). Furthermore, highly purified dead neutrophils (>97% Gr-1⁺ cells) from BALB and B6 mice exacerbated growth and killed *L. major*, respectively (Fig. 1C). Dead neutrophils elicited by i.p. injection of *L. major* promastigotes induced microbicidal activity similar to neutrophils elicited by thioglycolate (Fig. 1D). Microscopic assessment of macrophage infection demonstrated that feeding with dead BALB neutrophils increased intracellular amastigote number and percentage of infected macrophages (Fig. 1E). However, feeding with dead B6 neutrophils

induced a marked reduction of intracellular amastigote number and percentage of infected macrophages (Fig. 1F). Dead neutrophils from resistant C3H mice also induced marked killing of *L. major* in C3H macrophages (data not shown).

To rule out a differential macrophage reaction to different dead cells, we treated infected BALB and B6 macrophages with a common apoptotic stimulus. Xenogeneic apoptotic Jurkat T cells amplified *L. major* growth in both BALB and B6 macrophages (Fig. 2A). We then mixed macrophages and dead neutrophils from different strains. BALB macrophages either nourished or killed intracellular *L. major*, following coculture with killed BALB or B6 neutrophils, respectively (Fig. 2B, left). B6 macrophages treated with B6 neutrophils killed *L. major* with marked efficiency. Dead BALB neutrophils induced some degree of parasite killing, although with much less efficiency (Fig. 2B, right). Together, the results suggest that differences both in macrophage responses and in neutrophils control *L. major* growth or killing. We compared the screening and ingestion of dead B6 and BALB neutrophils by syngeneic macrophages, following 1-h incubation. No significant differences were found in percentages of macrophages ingesting or adhering to neutrophils in the two strains (Fig. 2C, left). Furthermore, the number of neutrophils attached or ingested by macrophages was also similar in the two strains (Fig. 2C, right). Similar

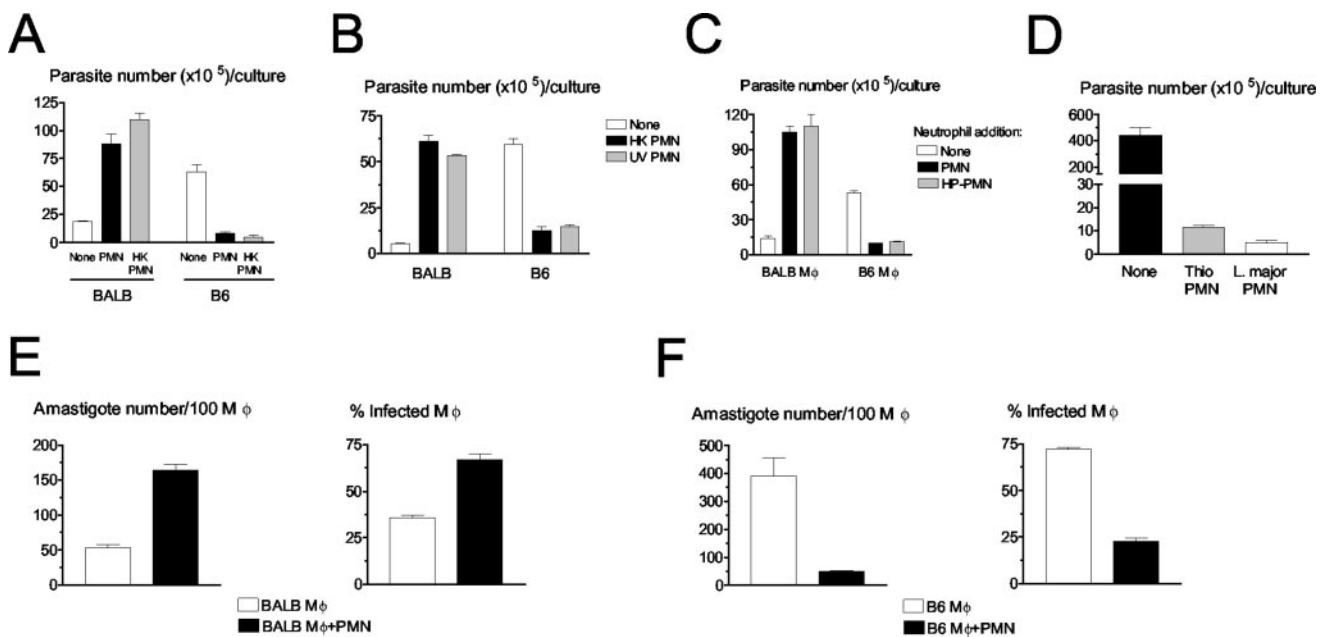


FIGURE 1. Dead neutrophils have opposing effects on *L. major* replication in macrophages from resistant and susceptible mice. **A**, Strain-specific effects of live or dead neutrophils. Inflammatory BALB or B6 macrophages were infected with *L. major* and cultured alone (□), or with 10-fold excess live (■) or heat-killed (HK; gray bars) syngeneic exudate neutrophils (PMN). Parasite load in macrophages was determined after 3 days. Results are the mean and SE of triplicate cultures. Results were significant ($p < 0.01$) for differences induced by PMN. **B**, Apoptotic neutrophil removal. Infected macrophage monolayers were treated with either heat-killed (HK; ■) or UV-treated apoptotic (UV; gray bars) neutrophils (PMN). *L. major* replication in macrophages was measured after 3 days. Results are mean and SE of triplicate cultures. Differences induced by PMN were significant ($p < 0.01$). **C**, Effect of highly purified neutrophils. Infected BALB and B6 macrophage monolayers were treated with heat-killed syngeneic neutrophils (PMN), or highly purified, heat-killed syngeneic neutrophils (HP-PMN). Replication of *L. major* in macrophages was measured after 3 days. Differences induced by PMN and HP-PMN were significant ($p < 0.01$). **D**, Effect of dead neutrophils elicited by parasites. Infected inflammatory B6 macrophages were cultured alone (■), or with dead neutrophils (PMN) elicited by thioglycolate (gray bars), or by *L. major* (□). Intracellular load of *L. major* in macrophages was measured after 3 days. Addition of both PMN induced killing ($p < 0.01$). **E**, Microscopic assessment of exacerbated parasite replication. Infected inflammatory BALB macrophages were cultured either alone (□), or with 10-fold excess killed BALB neutrophils (PMN; ■) for 7 days in glass coverslips. Monolayers were stained, and assessed for amastigote number/100 macrophages (left), and for percentage of infected macrophages (right). Results are mean and SE of triplicate cultures. Addition of PMN exacerbated both parasite replication and percentage of infected macrophages ($p < 0.01$). **F**, Microscopic assessment of leishmanicidal activity. Infected inflammatory B6 macrophages were cultured either alone (□), or with 10-fold excess killed B6 neutrophils (PMN; ■) for 7 days in glass coverslips. Monolayers were stained, and assessed for amastigote number/100 macrophages (left), and for percentage of infected macrophages (right). Results are mean and SE of triplicate cultures. Addition of PMN both induced parasite killing and reduced percentage of infected macrophages ($p < 0.01$).

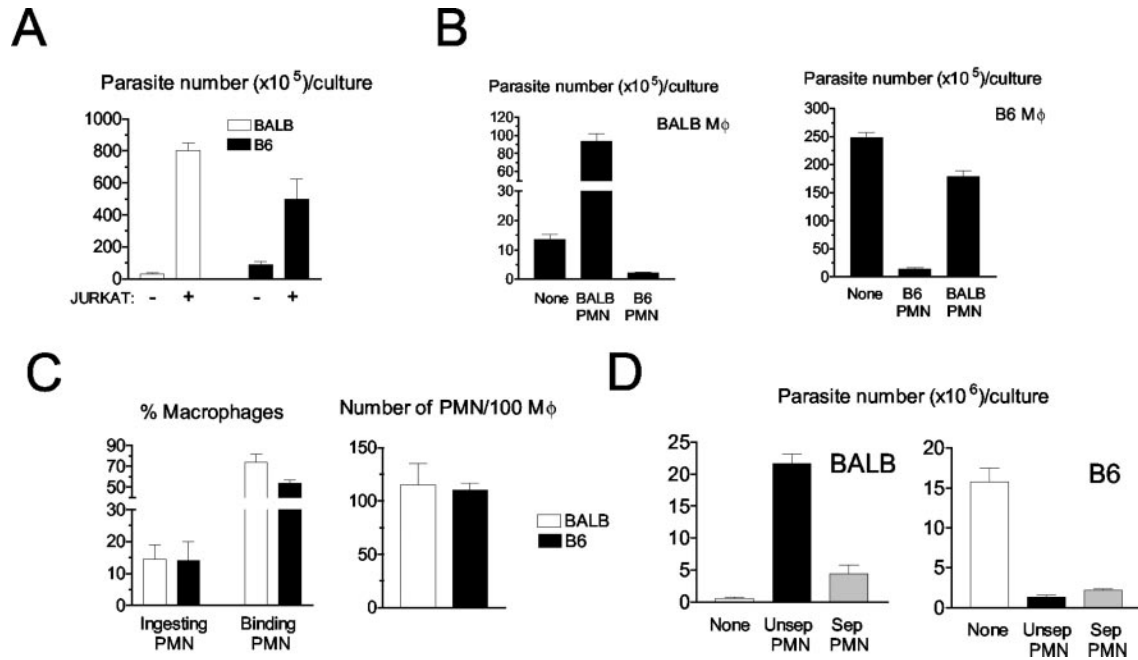


FIGURE 2. Macrophage interactions with dead cells regulate *Leishmania* growth or killing. *A*, Apoptotic lymphocytes drive parasite growth in both BALB and B6 macrophages. Infected macrophage monolayers were cultured either alone (–), or with 5-fold excess apoptotic Jurkat cells (+). Intracellular load of *L. major* was measured after 3 days. Addition of dead Jurkat cells exacerbated parasite growth ($p < 0.01$). *B*, Dead neutrophils regulate macrophage leishmanicidal activity. Infected BALB (left) or B6 (right) macrophages were cultured alone, or with dead neutrophils (PMN) from BALB or B6 mice, as indicated. Intracellular load of *L. major* was measured after 3 days. Changes induced by both PMN were significant ($p < 0.01$ for B6 PMN; $p < 0.05$ for BALB PMN) in BALB macrophages. Changes induced by both PMN were significant ($p < 0.01$ for B6 PMN; $p < 0.05$ for BALB PMN) in B6 macrophages. *C*, Adhesion and ingestion of neutrophils. BALB (□) and B6 (■) macrophage monolayers were cultured with 10-fold excess apoptotic syngeneic neutrophils (PMN) for 60 min, 37°C, in glass coverslips. Coverslips were washed, stained, and counted for number of adherent and ingested PMN. Results show percentage of macrophages ingesting and binding PMN (left), and the combined number of adherent and ingested PMN (right). Differences between strains were NS for all parameters. *D*, Contact dependence of macrophage-neutrophil interactions. Inflammatory BALB (left) or B6 (right) macrophages were infected with *L. major* and cultured, either alone, or in the presence of excess heat-killed syngeneic neutrophils, either in the same compartment (unseparated), or separated by culture inserts. Parasite loads in macrophages were determined after 3 days. Separation decreased parasite replication in BALB macrophages ($p < 0.01$), but had no effect on parasite killing by B6 macrophages (NS).

results were obtained when infected macrophages were treated with dead neutrophils (data not shown). Increased replication of *T. cruzi* in macrophages requires physical contact with apoptotic cells, due to the

need of adhesion and phagocytosis (27). Fueling of *L. major* replication also required cell contact between BALB macrophages and dead neutrophils (Fig. 2*D*, left), but parasite killing in B6 macrophages was

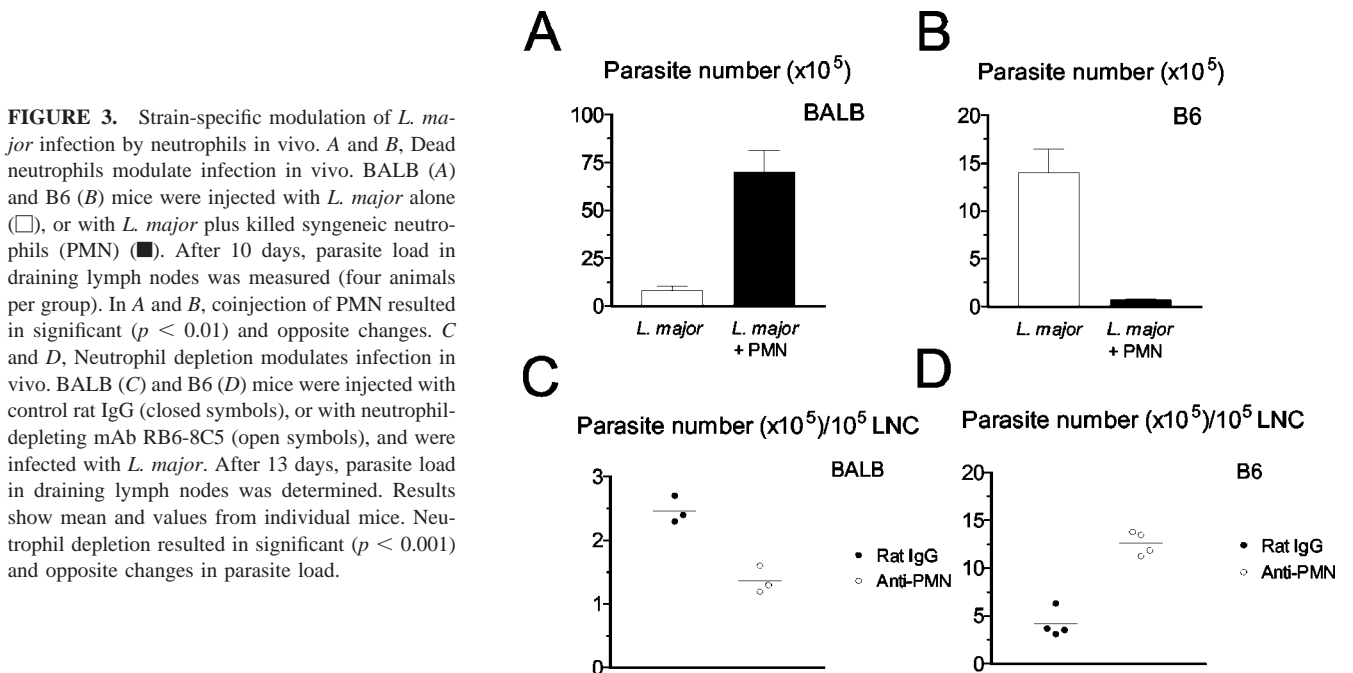


FIGURE 3. Strain-specific modulation of *L. major* infection by neutrophils in vivo. *A* and *B*, Dead neutrophils modulate infection in vivo. BALB (*A*) and B6 (*B*) mice were injected with *L. major* alone (□), or with *L. major* plus killed syngeneic neutrophils (PMN) (■). After 10 days, parasite load in draining lymph nodes was measured (four animals per group). In *A* and *B*, coinjection of PMN resulted in significant ($p < 0.01$) and opposite changes. *C* and *D*, Neutrophil depletion modulates infection in vivo. BALB (*C*) and B6 (*D*) mice were injected with control rat IgG (closed symbols), or with neutrophil-depleting mAb RB6-8C5 (open symbols), and were infected with *L. major*. After 13 days, parasite load in draining lymph nodes was determined. Results show mean and values from individual mice. Neutrophil depletion resulted in significant ($p < 0.001$) and opposite changes in parasite load.

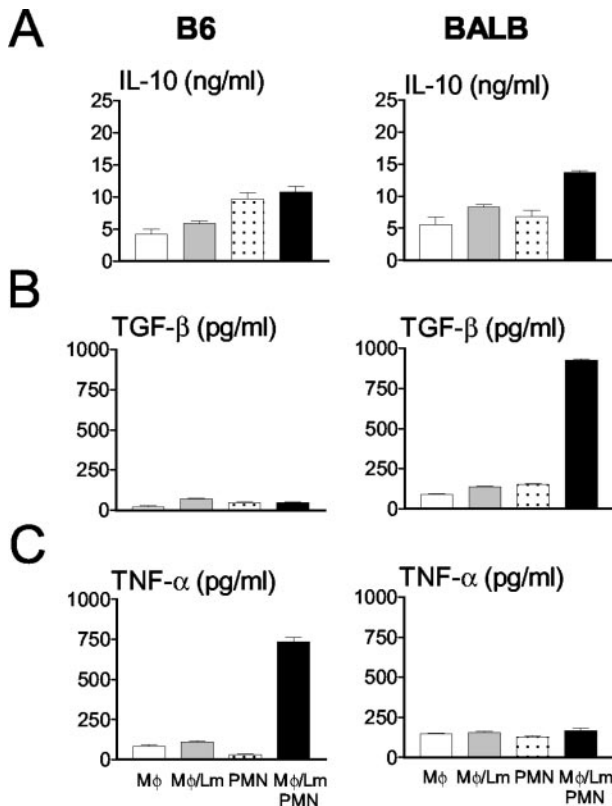


FIGURE 4. Macrophage interactions with dead neutrophils induce strain-specific cytokine production. B6 (left column) and BALB (right column) inflammatory macrophages (□), *L. major*-infected macrophages (gray bars), dead exudate syngeneic neutrophils (PMN; ▤), and infected macrophage/PMN cocultures (■) were cultured for 2 days. Supernatants were collected, cleared by centrifugation, and assayed for IL-10 (A), TGF-β (B), and TNF-α (C) content by sandwich ELISA. Results are mean and SE of triplicates. Dead neutrophils induced significant TGF-β production in BALB macrophages ($p < 0.01$), and significant TNF-α production in B6 macrophages ($p < 0.01$).

equally effective across a cell-impermeable membrane (Fig. 2D, right). In agreement, leishmanicidal activity could be reproduced with supernatants from killed or live B6 neutrophils (data not shown). Together, the data suggest that intracellular growth of *L. major* is amplified by phagocytic removal of susceptible-type neutrophils. However, parasite killing is induced by a soluble mediator (mediators) released by resistant-type neutrophils.

Dead neutrophils modulate *L. major* infection in vivo

BALB or B6 mice were injected in the footpads with *L. major* alone, or with *L. major* plus syngeneic killed neutrophils. Resulting parasite load in draining lymph nodes was measured after 10 days. Injection of BALB neutrophils markedly exacerbated *L. major* replication in draining lymph nodes of BALB hosts (Fig. 3A), while injection of B6 neutrophils almost eliminated *L. major* replication from lymph nodes of the B6 strain (Fig. 3B). Injection of *L. major* induces an early influx of neutrophils (14), associated with susceptibility to infection (17). To determine whether neutrophils are involved in the control of infection in vivo, we depleted BALB and B6 mice from neutrophils with anti-Gr-1 mAb. Control and neutrophil-depleted mice were infected, and parasite loads were determined after 13 days. Neutrophil depletion reduced *L. major* load in lymph nodes of BALB hosts (Fig. 3C), but exacerbated infection in B6 hosts (Fig. 3D). These results suggest an immunopathogenic role for neutrophil clearance in vivo.

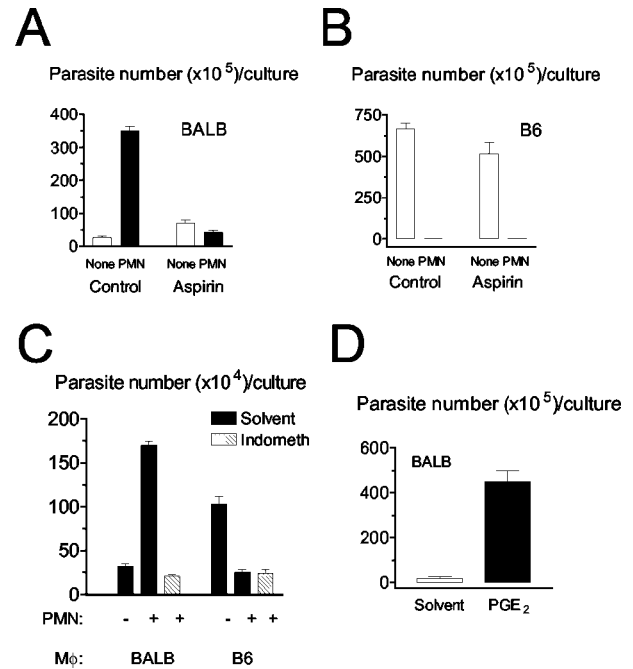


FIGURE 5. PGE₂ as mediator of *Leishmania* growth. A--C, Effects of aspirin (A and B) and indomethacin (C) on *Leishmania* replication. Infected BALB (A) and B6 (B) inflammatory macrophages were cultured alone (□) or with dead syngeneic neutrophils (PMN; ▤), in the presence of aspirin or solvent (A and B). C, Infected BALB and B6 macrophages were also cultured with PMN in the presence of indomethacin (hatched bars) or solvent (■). After 3 days, intracellular load of *L. major* was measured. Results are mean and SE of triplicate cultures. Aspirin and indomethacin blocked *L. major* growth induced by BALB PMN ($p < 0.01$), but had no effect on *Leishmania* killing induced by B6 PMN (not significant). D, Exogenous PGE₂ drives *L. major* growth in BALB macrophages. Macrophages were infected with *L. major*, washed, and recultured with solvent alone (□), or PGE₂ (■) for 3 days, and intracellular load of *L. major* was measured. PGE₂ exacerbated *L. major* growth ($p < 0.01$).

Differential cytokine production controls *L. major* growth

Phagocytic removal of dead cells triggers secretion of regulatory cytokines (6, 10). We measured production of TGF-β, TNF-α, and IL-10 induced by dead neutrophils. BALB and B6 exudate neutrophils released IL-10, and levels of IL-10 were also elevated in BALB and B6 inflammatory macrophages, whether infected or not (Fig. 4A). Exposure to dead neutrophils did not induce additional IL-10 production by macrophages (Fig. 4A). Strikingly, dead BALB neutrophils selectively induced TGF-β (Fig. 4B), but not TNF-α (Fig. 4C) release by infected BALB macrophages. Conversely, dead B6 neutrophils induced TNF-α (Fig. 4C), but not TGF-β (Fig. 4B) release by infected B6 macrophages.

Growth of *T. cruzi* driven by uptake of apoptotic cells depends on PGE₂ and TGF-β production (10). We tested the role of PGE₂ in *L. major* replication induced by dead inflammatory neutrophils. Cyclooxygenase inhibitors aspirin (Fig. 5A) and indomethacin (Fig. 5C) blocked the growth of *L. major* fueled by dead BALB neutrophils, but had little or no effect on the leishmanicidal activity elicited by dead B6 neutrophils (Fig. 5, B and C). In agreement, exogenous PGE₂ markedly exacerbated *L. major* growth in BALB macrophages in the absence of neutrophils (Fig. 5D). PGE₂ is required for TGF-β production by macrophages (6, 10). To investigate whether TGF-β is required for exacerbated *L. major* growth, we neutralized TGF-β activity induced by killed BALB neutrophils. A neutralizing anti-TGF-β Ab completely abolished *L. major* replication, compared with a control chicken Ig (Fig. 6A). To

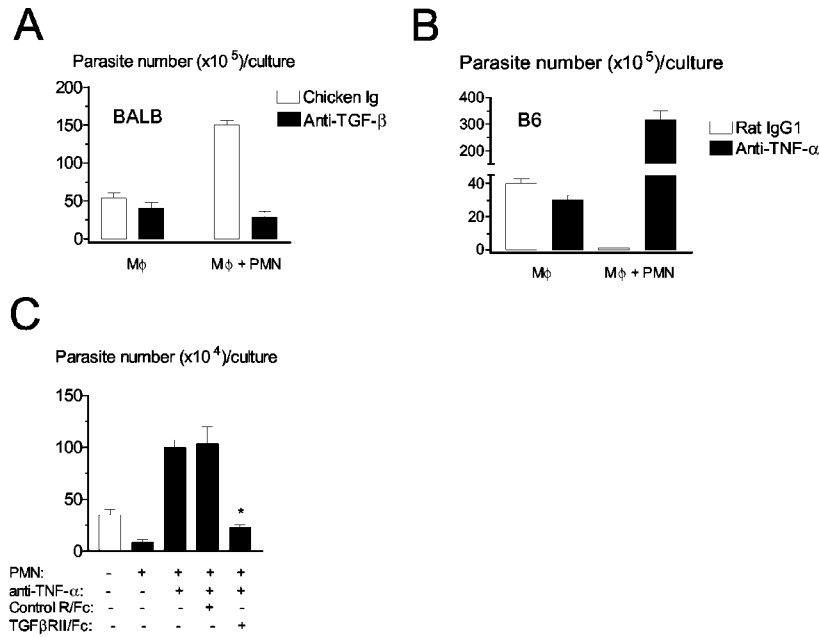


FIGURE 6. Cytokines involved in *Leishmania* growth or killing. *A*, *Leishmania* growth is driven by TGF- β . Infected BALB macrophages were cultured alone or with dead syngeneic neutrophils (PMN), in the presence of control chicken Ig (\square), or a neutralizing anti-TGF- β Ig (\blacksquare) for 3 days, and intracellular load of *L. major* was measured. Results are mean and SE of triplicate cultures. Addition of PMN induced significant differences in the absence ($p < 0.01$), but not in the presence (NS) of anti-TGF- β . *B*, *Leishmania* killing depends on TNF- α . Infected B6 macrophages were cultured alone or with dead syngeneic neutrophils (PMN), in the presence of isotype control (\square) or a neutralizing anti-TNF- α mAb (\blacksquare), and intracellular load of *L. major* was measured after 3 days. Results are mean and SE of triplicate cultures. Addition of PMN induced significant ($p < 0.01$) and opposite changes in the absence and in the presence of anti-TNF- α . *C*, Exacerbated growth induced by anti-TNF is mediated by TGF- β . Infected B6 macrophages were cultured alone (\square), or with dead syngeneic neutrophils (PMN; \blacksquare), in the absence or presence of neutralizing anti-TNF- α mAb, and in the presence of control mouse thrombopoietin receptor/Fc or neutralizing mouse TGF- β receptor II/Fc protein, as indicated. Intracellular load of *L. major* was measured after 3 days. Results are mean and SE of triplicate cultures. Addition of TGF- β receptor II/Fc induced significant reduction of parasite load ($p < 0.01$).

investigate whether TNF- α is required for *L. major* killing, we neutralized TNF- α activity induced by killed B6 neutrophils. A neutralizing anti-TNF- α Ab completely abolished leishmanicidal activity (Fig. 6*B*). Following TNF- α neutralization, the resulting *L. major* load in neutrophil-treated macrophages climbed to a level 10-fold higher than the control (Fig. 6*B*). These results suggest that TNF- α is required for microbicidal activity, and that, in the absence of TNF- α activity, phagocytic removal of B6 neutrophils shifts toward promotion of *L. major* growth. In agreement, increased parasite growth promoted by anti-TNF- α Ab was abolished following TGF- β neutralization with a mouse TGF- β receptor II/Fc chimeric protein, but not by a control mouse thrombopoietin receptor/Fc protein (Fig. 6*C*). These results indi-

cate that increased parasite growth induced by anti-TNF is mediated by TGF- β .

Mechanism of parasite killing: role of NE

We investigated whether NO was involved in parasite killing. Addition of excess NOS inhibitor L-NMMA markedly increased *L. major* growth in B6 macrophages in the absence of neutrophils (Fig. 7*A*). However, L-NMMA had no effect on parasite killing induced by dead B6 neutrophils (Fig. 7*A*). In contrast, exogenous catalase partially abolished leishmanicidal activity (Fig. 7*B*). These results suggest that parasite killing is mediated by reactive oxygen species, and not by NO.

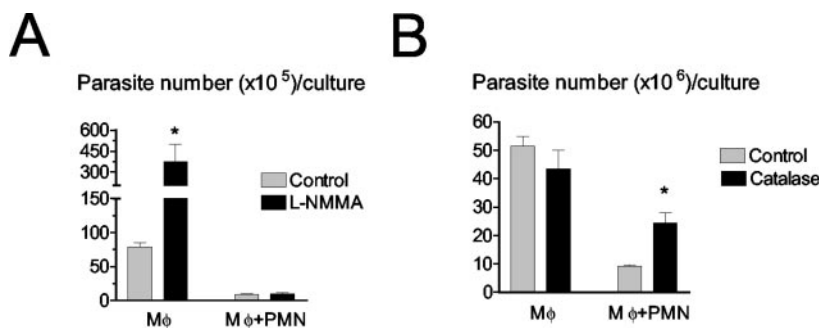


FIGURE 7. Mechanism of *Leishmania* killing. *A*, Leishmanicidal activity does not require NO production. Infected B6 inflammatory macrophages were cultured alone or with dead B6 neutrophils (PMN), in the absence (gray bars) or presence of L-NMMA (\blacksquare). After 3 days, intracellular load of *L. major* was measured. Results are mean and SE of triplicate cultures. *, Indicates $p < 0.01$. *B*, Leishmanicidal activity requires oxidant generation. B6 macrophages were cultured as in *B*, but in the absence (gray bars) or presence (\blacksquare) of exogenous catalase. Parasite load was measured after 3 days. Results are mean and SE of triplicate cultures. *, Indicates $p < 0.05$.

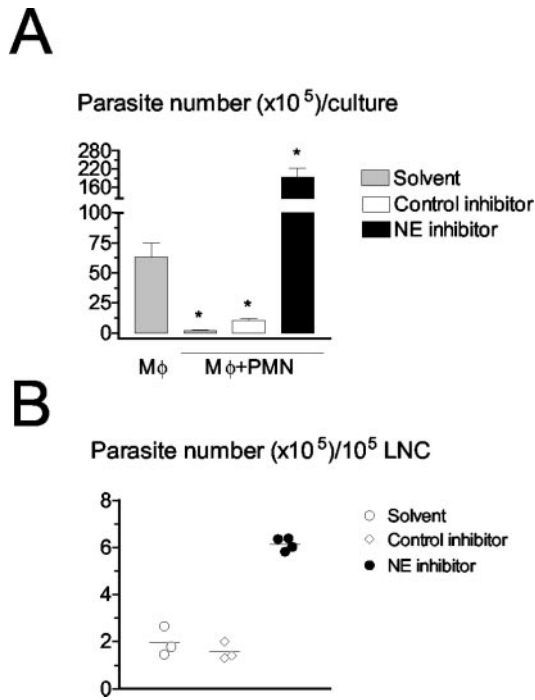


FIGURE 8. Leishmanicidal activity depends on NE. **A**, NE inhibitor abolishes leishmanicidal activity in vitro. Infected B6 inflammatory macrophages were cultured alone (gray bars) or with dead B6 neutrophils (PMN), in the presence of solvent alone (gray bars), collagenase inhibitor (\square), or NE inhibitor (\blacksquare). Intracellular load of *L. major* was measured after 3 days. Results are mean and SE of triplicate cultures. *, Indicates $p < 0.01$. **B**, NE inhibitor exacerbates *L. major* infection in vivo. B6 mice were injected with *L. major* in the footpads, followed by injection of solvent alone (\circ), collagenase inhibitor (\diamond), or NE inhibitor (\bullet) 1 h later. After 13 days, parasite load in draining lymph nodes was determined. Results show mean and values from individual mice. Treatment with NE inhibitor exacerbated infection ($p < 0.001$).

Fadok et al. (28) identified the serine protease NE as an inducer of TNF- α production by human macrophages exposed to blood neutrophil lysates. Because we used inflammatory neutrophils, which are actively degranulating (29), we investigated whether serine proteases are involved in parasite killing induced by B6 exudate neutrophils. Supernatants from dead B6 neutrophils induced marked killing of *L. major* by B6 macrophages, and the microbicidal activity was abrogated by treatment with the serine protease inhibitor PMSF (data not shown). Moreover, addition of the specific NE inhibitor MeOSuc-AAPV-CMK completely abolished the leishmanicidal activity of dead B6 neutrophils (Fig. 8A). Addition of Z-Pro-D-Leu-D-Ala-NHOH, a control collagenase inhibitor, had no effect on leishmanicidal activity (Fig. 8A). Similar to TNF- α neutralization, neutralization of NE activity also resulted in the opposite exacerbation of *L. major* growth (Fig. 8A). To investigate whether NE plays a protective role in vivo, B6 mice were injected in the footpads with *L. major* and with NE inhibitor. Resulting parasite load was measured in draining lymph nodes after 13 days. Injection of NE inhibitor, but not solvent or control collagenase inhibitor, markedly exacerbated *L. major* replication, increasing parasite load in draining lymph nodes (Fig. 8B). These results indicate a protective role for NE in resistant B6 hosts.

Discussion

Neutrophils are constitutively deleted by apoptosis, leading to phagocytic clearance of the intact dying cell (1, 2). Phagocytosis of apoptotic neutrophils has been implicated in resolution of inflam-

mation (3, 4). Furthermore, phagocytic clearance of dead cells plays a regulatory role in immune recognition (30, 31), antitumor activity (32), and intracellular parasitic infection (7, 10). Apoptotic cell removal triggers macrophage deactivation (10) and secretion of anti-inflammatory mediators (6, 10). However, under certain circumstances, apoptotic cell removal can lead to an inflammatory reaction (5, 33). Our data provide evidence that interactions of macrophages with dead neutrophils play an important role in host responses to infection with the intracellular pathogen *L. major*. We demonstrated opposite pro- and anti-inflammatory responses that depended on host genotype, and resulted in opposite in vitro and in vivo outcomes of *L. major* infection.

In the absence of neutrophils, B6 macrophages were more infected and supported increased replication of *L. major*, compared with BALB macrophages. This finding was unexpected, because B6 mice are resistant to infection in vivo. Our results might reflect the particular combination of parasite isolate and mouse strain we used. In fact, other studies compared resistant CBA and susceptible BALB strains, and reported that, in the absence of LPS and IFN- γ , parasite infection and replication in these macrophages were similar (34, 35). Interestingly, microarray analysis of macrophage responses to LPS has indicated that message for arginase I, which is required for growth of *L. major* in macrophages (18), is up-regulated in B6, compared with BALB macrophages (36).

Coculture of infected macrophages with either live or dead neutrophils resulted in similar regulation of *L. major* growth. Live inflammatory neutrophils comprise a population of senescent cells. Freshly obtained neutrophils already contained $>35\%$ apoptotic cells, as judged by annexin V staining. When left in culture, 50% of live neutrophils die after 5 h, and no viable cell can be recovered after 20 h (data not shown). Together, these results suggest that clearance of apoptotic neutrophils regulates macrophage activation. Interactions with dead BALB neutrophils triggered TGF- β production by macrophages and led to exacerbated replication of *L. major*. Increased parasite growth depended on PGE $_2$ and TGF- β . Therefore, dead BALB neutrophils drive the growth of *L. major* in macrophages in a manner similar to the growth of *T. cruzi* fueled by uptake of apoptotic lymphocytes (10). TGF- β induces ODC activity in macrophages (10), and increases polyamine synthesis, which is required for increased growth of *T. cruzi* (10). These changes could also be involved in the growth of *L. major*, because host cell ODC and ornithine production are required for intracellular *L. major* replication (18). Killed BALB neutrophils exacerbated parasite loads in draining lymph nodes in vivo. An early influx of neutrophils to infected tissue has been associated with susceptibility to infection by *L. major* (17) and *L. amazonensis* (37). Our results agree with these studies. Furthermore, our results agree with deleterious effects of PGs and TGF- β on *Leishmania major* infection in BALB mice (8, 38, 39).

Surprisingly, interactions with dead B6 neutrophils triggered TNF- α production by B6 macrophages, and led to TNF- α -dependent killing of *L. major*. These results agree with an important role of TNF- α in protecting against *L. major* infection (40, 41). In agreement with previous studies (42), we found no difference in the ability of BALB and B6 macrophages to bind and ingest syngeneic exudate neutrophils. Furthermore, both BALB and B6 macrophages exacerbated *L. major* growth in response to apoptotic Jurkat cells. However, dead BALB and B6 neutrophils induced different responses against *Leishmania*, and dead B6 neutrophils had a potent host protective effect in vivo. We sought to determine the factor responsible for the proinflammatory effects of dead B6 neutrophils. We found that it was a soluble mediator, and that it could be neutralized by a serine protease inhibitor. In agreement,

our results showed that the serine protease NE is involved in microbicidal activity. Furthermore, we demonstrated that NE is required *in vivo* for the early control of *L. major* infection in resistant B6 hosts. In agreement with these results, we found that purified NE induces leishmanicidal activity in infected macrophage monolayers, and that previous heat treatment has little or no effect on the leishmanicidal activity of NE (results not shown). NE has been implicated in tissue injury and inflammation mediated by neutrophils (43). NE and other azurophil granule proteases induce TNF- α release *in vitro* (28) and *in vivo* (44), and modulate inflammation *in vivo* (44). Moreover, purified NE induces TNF- α production by murine macrophages (28). We demonstrated that, following NE or TNF- α neutralization, killed B6 neutrophils behave like BALB neutrophils, exacerbating *L. major* replication in B6 macrophages. This result implies that the proinflammatory pathway triggered by NE is dominant over the deactivating pathway of apoptotic cell removal. The timing of signaling could be important, because soluble NE might reach the macrophage before the senescent neutrophil engages deactivating receptors. Although NE neutralization indicated that NE is involved in leishmanicidal activity, we cannot discard that other neutrophil enzymes, such as cathepsin G and myeloperoxidase, could play an additive role together with NE.

Inducible NO production is required for control of *L. major* infection in mice (45). In the absence of dead neutrophils, NO was required for macrophage control of *L. major* replication, because parasite load increased in the presence of NOS inhibitor L-NMMA. However, parasite killing induced by neutrophils was NO independent. Parasite killing could be reduced by addition of exogenous catalase, suggesting that it is mediated by oxidant injury. Both NE (46) and TNF- α (47, 48) induce oxidant species in macrophages and other cell types. The mechanism of macrophage activation by NE, however, remains unclear (28). Our data are not the first to show NO-independent control of *L. major* replication. NO-sufficient mice made deficient of TNF- α (41) or Fas (49) are unable to control *L. major* infection despite NO production. Moreover, both inducible NO synthase and phagocyte NADPH oxidase are required for proper control of *L. major* infection *in vivo* (50).

Our results with neutrophil depletion *in vivo* demonstrated that neutrophils play a deleterious role in early infection of BALB, but are protective in early infection of B6 mice. Phagocytic uptake of nonneutrophilic apoptotic cells is anti-inflammatory, and drives the growth of an intracellular pathogen (10). However, recent studies indicated that phagocytic removal of apoptotic cells can be accompanied by transient TNF- α production and by inflammatory reactions (5, 33). Our results provide evidence that, depending on host genotype, neutrophil clearance can also be proinflammatory, and can contribute to elimination of intracellular pathogens. We did not address the role of dead neutrophils at later stages of *L. major* infection. However, it is likely that responses mediated by CD4⁺ T cells play a central role as infection progresses. Interestingly, neutrophil clearance could be important for instruction of Th1/Th2 differentiation. It has been demonstrated that early depletion of neutrophils renders BALB mice resistant to *L. major* infection and shifts their immune response to Th1 type (17).

Our results could explain modulation of macrophage antimicrobial activity by exudate neutrophils in a model of mycobacterial infection (51). Our studies did not address the differences between B6 and BALB neutrophils that differentially activate macrophages. However, increased NE activity in B6 neutrophils could be involved. Neutrophils and macrophages from B6 and BALB mice could differ in the amount of serpins they produce, and the ability to neutralize serine proteases. In this regard, B6 and BALB mice express differences in α 1-antitrypsin activity affecting development of pulmonary emphysema (52). We are currently investigat-

ing whether B6 and BALB macrophages and neutrophils differ in serpin control of NE activity. Taken together, our results indicate that macrophage interactions with dead neutrophils play a previously unrecognized role in innate defense against the intracellular pathogen *L. major*. Further investigation could provide new targets for therapy against leishmaniasis and other infections.

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