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Neutrophils and Macrophages Cooperate in Host Resistance against *Leishmania braziliensis* Infection[1]

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Neutrophils play an active role in the control of infections caused by intracellular pathogens such as *Leishmania*. In the present study, we investigated the effect of neutrophil depletion at the time of *Leishmania braziliensis* infection of BALB/c mice and how neutrophils interact with the infected macrophage to promote parasite elimination. The in vivo depletion of neutrophils led to a significant increase in parasite load and enhanced the Th1-Th2 immune response in this experimental model of infection. BALB/c mice coinoculated with both parasites and live neutrophils displayed lower parasite burdens at the site of infection and in the draining lymph nodes. In vitro, we observed that live neutrophils significantly reduced the parasite load in *L. braziliensis*-infected murine macrophages, an effect not observed with *Leishmania major*. *L. braziliensis* elimination was dependent on the interaction between neutrophils and macrophages and was associated with TNF-α as well as superoxide production. Furthermore, cooperation between neutrophils and macrophages toward parasite elimination was also observed in experiments performed with *L. braziliensis*-infected human cells and, importantly, with two other New World *Leishmania* species. These results indicate that neutrophils play an important and previously unappreciated role in *L. braziliensis* infection, favoring the induction of a protective immune response. ***The Journal of Immunology,*** **2009, 183: 8088–8098.**

New World cutaneous leishmaniasis, caused by *Leishmania braziliensis* (Lb),[3] is distinguished from other leishmaniasis by its chronicity, latency, and tendency to metastasize in the human host (1). During infection with Lb, development of clinical lesions coincides with an influx of inflammatory cells such as macrophages, eosinophils, and neutrophils (2, 3). Neutrophils are among the first cells to be recruited to the infection site and are important in preventing pathogen survival via oxidant- and protease-dependent mechanisms (4, 5). Neutrophils also provide an important link between innate and adaptive immunity during parasitic infections (6, 7).

The role of neutrophils in infection with *Leishmania* has primarily been studied using experimental models of cutaneous leishmaniasis caused by *Leishmania major*. In resistant mice, transient depletion of neutrophils has been shown to lead to an increase in the parasite load (8, 9). However, neutrophil depletion in susceptible mice (BALB/c) has been shown to lead to the opposite effect, as demonstrated by an increase in parasite elimination (10, 11). It was proposed that interactions of apoptotic neutrophils with *L. major*-infected macrophages from susceptible mice induced an anti-inflammatory response, indicated by the presence of de-activating mediators such as TGF-β and PGE_2 (10). Moreover, neutrophils infected with *L. major* display distinct phenotypes that could, in turn, influence the development of the host's immune response (12). However, the control of cutaneous leishmaniasis caused by *L. major* and Lb differs (13) and the role of neutrophils during infection with Lb has not been investigated to date.

We have previously shown that BALB/c mice infected in the ear dermis with Lb develop cutaneous lesions at the site of inoculation (14). Histological analysis of ear sections demonstrated a constant recruitment of neutrophils to the inoculation site. Given that resolution of an infection by *Leishmania* species that cause cutaneous disease is marked by the ability of the innate immune system to assist in wound healing, as indicated by the intradermal model of infection, we have investigated the role of neutrophils in American cutaneous leishmaniasis caused by Lb. Our data provide evidence that in vivo neutrophil depletion enhances parasite multiplication, whereas coinoculation of neutrophils with Lb leads to the opposite effects. Moreover, contact-dependent mechanisms that enable parasite elimination during the interaction of Lb-infected macrophages with live neutrophils are associated with TNF-α and superoxide production. Our observations were also extended to experiments performed with human cells and with two other New World *Leishmania* species: *Leishmania amazonensis* and *Leishmania chagasi*.

Materials and Methods

Mice

Female BALB/c and C57BL/6 mice were obtained from the Centro de Pesquisas Gonçalo Moniz/Fundação Oswaldo Cruz Animal Facility. Mice lacking TNF-α receptor I ($p55^{-/-}$; C57BL/6 background) were a gift from

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[3] Abbreviations used in this paper: Lb, *L. braziliensis*; PMN, polymorphonuclear neutrophil; NAC, *N*-acetylcysteine; dLN, draining lymph node; WT, wild type.

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Dr. L. Quercia-Vieira (Universidade Federal de Minas Gerais, Belo Horizonte, Brazil). All mice were maintained under pathogen-free conditions and were used in experiments at 6–8 wk of age. Experiments were performed using methods approved by the Animal Care and Utilization Committee from Centro de Pesquisas Gonçalo Moniz/Fundação Oswaldo Cruz.

Parasite culture

Lb (strain MHOM/BR/01/BA788) (14), *L. amazonensis* (strain MHOM/BR/87/BA125) (15), *L. major* (MHOM/JL/80/Friedlin), and *L. chagasi* (MHOM/BR/00/BA262) promastigotes were grown in Schneider medium (Sigma-Aldrich) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% heat-inactivated FCS (all from Invitrogen). Metacyclic Lb parasites were prepared as described in Spath and Beverley (16).

Neutrophil depletion, intradermal inoculation, and lesion measurement

For neutrophil depletion, mice were treated with RB6-8C5 (17) (a gift from Dr. A. Sher, Laboratory of Parasitic Diseases/National Institutes of Health) directed against Ly-6G and Ly-6C (Gr-1), an Ag present on the surface of granulocytes. RB6-8C5 or control rat IgG was given i.p. at a dose of 500 μg at -1, 3, 6, 9, and 12 days after Lb inoculation. This treatment led to a transient neutrophil depletion that returned to normal levels 4 days after Ab injection, as determined by analysis of H&E-stained blood smears. At day 0, control and depleted mice were infected with stationary phase Lb promastigotes (10^5 parasites in 10 μl of saline) by inoculation into the right ear dermis using a 27.5-gauge needle. Alternatively, mice were coinoculated with 10^5 Lb and 5×10^5 live neutrophils in the right ear dermis. Lesion size was monitored on a weekly basis for 10 wk using a digital caliper (Thomas Scientific). Parasite load was determined using a quantitative limiting dilution assay as described previously (18).

Histological analysis

Infected ears were removed postmortem, at 2, 5, and 10 wk after infection, and fixed in 10% formaldehyde. After 12–24 h of fixation, tissues were processed and embedded in paraffin and 5-μm sections were stained with H&E. Neutrophils within Lb-infected and within PBS-inoculated ear sections were detected by microscopic analysis (magnification, $\times$100). The morphometric analysis was made on five different sections per animal in an area of 0.01 mm^2. Samples from four different animals, from experimental and control groups, were analyzed in each of the three time points.

In vitro stimulation and cytokine detection

RB6-8C5-treated and control mice were infected with Lb promastigotes as described above. Two weeks after infection, mice were euthanized and single-cell suspensions of draining lymph nodes (dLN) and infected ears were prepared aseptically. Briefly, lymph nodes were homogenized in RPMI 1640 medium and cells were resuspended in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10% FCS (all from Invitrogen), and 0.05 M 2-ME. The ventral and dorsal sheets of the infected ears were separated and incubated for 1 h at 37°C/5% CO_2 in RPMI 1640 medium containing Liberase CI enzyme blend (50 μg/ml) (Roche). Ears were processed using a Medimachine (BD Biosciences) and cell suspensions were washed by centrifugation. Cells were adjusted to a final concentration of 5×10^5/well of a 96-well plate and were incubated with or without stationary phase Lb promastigotes (five parasites to one cell) or with Con A (10 μg/ml; Amersham Pharmacia Biotech) for 24 h. Culture supernatants were harvested and frozen at -20°C until analysis. Cytokine production was detected using a Th1/Th2 cytokine cytometric bead array (BD Biosciences), which detects murine IL-2, IL-4, IL-5, IFN-γ, and TNF-α, following the manufacturer's instructions. Data were acquired and analyzed using a FACSort flow cytometer (BD Immunocytometry Systems) and FCAP Array version 1.0.1 software.

Murine neutrophils

Exudate neutrophils were obtained 7 h after i.p. injection of 1 ml of 3% thioglycolate broth (Sigma-Aldrich). Exudate cells were then washed and incubated in RPMI 1640 at 37°C for 1 h in 25-ml flasks to remove adherent cells (10). Nonadherent cells were stained with anti-Gr-1 (BD Pharmingen) to assess purity and were subsequently analyzed by flow cytometry using CellQuest software and a FACSort flow cytometer (BD Immunocytometry Systems). Gr-1^+ cells were routinely >80% pure, while <4% stained positive for propidium iodide. In some experiments, neutrophils (3×10^6 cells/ml) were exposed to UV radiation (10) or were fixed with 4% paraformaldehyde for 20 min. UV-treated neutrophils were ~70–90% annexin V positive, were determined to be propidium iodide negative using FACS analysis, and presented pyknotic nuclei when assayed by light microscopy. All cultures were performed in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% v/v of Nutridoma-SP (Roche).

Macrophages, infection, and cocultures

Resident macrophages were obtained after peritoneal injection of 5 ml of RPMI 1640. Peritoneal exudate cells (3×10^5cells/ml) were plated onto glass coverslips placed within the wells of a 24-well plate containing RPMI 1640 medium supplemented with 10% FCS, 2 mM/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen). The cells immediately received 3×10^6 cells/ml Lb promastigotes and were incubated at 37°C in complete RPMI 1640 medium. After 24 h, monolayers were extensively washed to remove extracellular parasites and nonadherent cells. All cultures were then incubated in RPMI 1640 medium supplemented with 1% Nutridoma-SP (Roche), 2 mM/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen). In experiments performed with *L. amazonensis, L. chagasi,* and *L. major*, monolayers were washed after 4 h of infection. UV-treated or live neutrophils were added in a neutrophil:macrophage ratio of 10:1. For determination of bound or internalized neutrophils, cocultures were performed as described above. After 1 h, monolayers were washed and coverslips were stained with H&E. Phagocytosis of apoptotic neutrophils induces concurrent ingestion of extracellular fluid into spacious phagosomes (19). Ingested neutrophils were identified as surrounded by a large vacuole. The remaining associated neutrophils were scored as bound. Monolayers were assessed for the percentage of neutrophils (polymorphonuclear neutrophils (PMN)) bound to or ingested by either infected or uninfected macrophages. In some experiments, live neutrophils were added either in the same compartment or separated by a cell-permeable culture insert (MilliCell inserts, 0.4 μm; Sigma-Aldrich). Cultures were kept for 6 days at 37°C in 5% CO_2. Extracellular parasites were absent throughout this period. Infected macrophages were also cocultured with 10-fold excess Jurkat T cells instead of PMN. In some experiments, cocultures of infected macrophages and PMN were performed in the presence of *N*-acetylcysteine (NAC, 10 nM; Sigma-Aldrich). In this case, NAC was added at the same time as neutrophils.

Parasite load

After 6 days, glass coverslips containing infected macrophages cocultured with neutrophils were washed, stained with H&E, and analyzed using light microscopy. The number of intracellular amastigotes was counted in 200 macrophages. The results are shown as the percentage of infected macrophages or the amastigote number per 100 macrophages. Alternatively, after 3 days, infected macrophage monolayers were extensively washed and the medium was replaced with 0.5 ml of Schneider medium (Sigma-Aldrich) supplemented with 10% FCS. The monolayers were cultured at 26°C for an additional 3 days and the number of promastigotes was determined by use of a hemocytometer.

Cytokine production

Supernatants from control macrophages or from the coculture of infected macrophages and live neutrophils were collected 6 h later and immediately assayed for TNF-α (BD Biosciences) by sandwich ELISA according to the manufacturer's instructions.

NO and superoxide detection

NO production in the supernatant of the cocultures of infected macrophages and live neutrophils was evaluated using the Griess method, measuring its stable reaction product, nitrite (NO_2^-) (20). Superoxide production was determined by adding hydroxylamine (0.5 mM; Sigma-Aldrich) (21, 22) to cocultures of infected macrophages and live neutrophils. Hydroxilamine converts superoxide into nitrite, which can then be quantitated by performing the Griess reaction, as described above. Background levels of nitrite generated by the release of NO were determined in parallel, without the addition of hydroxylamine.

Human neutrophil collection and coculture with autologous macrophages

Human blood was obtained from healthy volunteers from Hemocentro do Estado da Bahia, BA, Brazil. Human neutrophils and PBMCs were obtained as described previously (15). Macrophages were cultured on glass coverslips for 4 h after being infected with Lb at a parasite:cell ratio of 2:1. Wells were extensively washed to remove extracellular parasites and nonadherent cells, leaving adherent macrophages. Next, live or UV-treated neutrophils (15) were added to infected macrophage cultures at a neutrophil:macrophage ratio of 5:1 in RPMI 1640 medium supplemented with

1% Nutridoma-SP (Roche), 2 mM/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen). Cocultures were performed on glass coverslips and then harvested after 3 days, fixed with ethanol, and stained with H&E. In some experiments, coculture of Lb-infected macrophages was performed in the presence of 6 μg/ml anti-human TNF-α (R&D Systems) or in the presence of purified IgG1 isotype, which served as a control.

Statistical analysis

The data are presented as the mean ± SD of the mean. The significance of the results was calculated using nonparametric statistical tests: Mann-Whitney (two-sided *t* test), for comparisons between two groups or Kruskal-Wallis, followed by Dunn's multiple comparison test, for comparisons between three groups. Analyses were conducted using Prism (GraphPad software) and a $p < 0.05$ was considered significant.

Results

In vivo neutrophil depletion enhances parasite growth while neutrophil coinoculation enhances parasite death

It has been previously shown that neutrophils play a critical role in controlling *L. major* (9) and *Leishmania donovani* infection (23). Upon inoculation of Lb promastigotes into the ear dermis of BALB/c mice, neutrophils can be detected throughout the duration of clinical disease (14). To determine the number of neutrophils to the site of Lb infection, ear sections were obtained at 2, 5, and 10 wk after parasite inoculation and were stained with H&E. These sections were compared with samples obtained from PBS-inoculated mice. The numbers of neutrophils present in Lb-infected and in PBS-inoculated ear sections was determined by microscopic analysis. As shown in Fig. 1*A*, neutrophils can be detected in Lb-infected mice (Lb) at the three time points analyzed, whereas in control sections (PBS), neutrophils were not observed (Fig. 1*A* and supplemental Fig. 1).[4] These results confirm our previous observations (14) and suggest that neutrophils are associated with lesion healing and pathogen control. Based on these results, we investigated whether transient neutrophil depletion altered the course of Lb infection in BALB/c mice. As shown in Fig. 1*B*, neutrophil depletion with mAb RB6-8C5 led to a significant increase in ear thickness, observed as early as 1 wk after parasite inoculation, an effect that persisted until 4 wk later. Afterward, ear thickness did not differ between RB6-8C5-treated mice and the control group. Importantly, RB6-8C5 treatment also resulted in an increase in parasite load at the inoculation site (Fig. 1*C*) and in dLN (Fig. 1*D*). These determinations were made at 2 wk after parasite inoculation, when lesion sizes between RB6-8C5-treated and control mice differed significantly. We also investigated whether a similar outcome would be observed following infection with metacyclic promastigotes since stationary phase parasites may be susceptible to neutrophil killing but may nonetheless contribute to infection in the absence of neutrophils, in vivo. Importantly, when purified Lb metacyclics were used to infect RB6-treated mice, we observed that parasite load at the ear dermis and in dLN was also significantly higher in the PMN-depleted group when compared with control mice (supplemental Fig. 2), similar to results obtained with Lb stationary promastigotes (Fig. 1, *C* and *D*). Therefore, it appears that neutrophils are necessary to contain parasite growth at the initial phase of Lb infection and this effect was observed with either stationary phase or metacyclic promastigotes.

Conversely, ear thickness of mice coinoculated with live neutrophils and Lb promastigotes (PMN plus Lb) was significantly smaller than that of control mice at 4 wk after infection (Fig. 1*A*). At this time point, mice coinoculated with PMN plus Lb also displayed a significantly smaller parasite load both at the inoculation site (Fig. 1*E*) and in dLN (Fig. 1*F*). These results suggest that

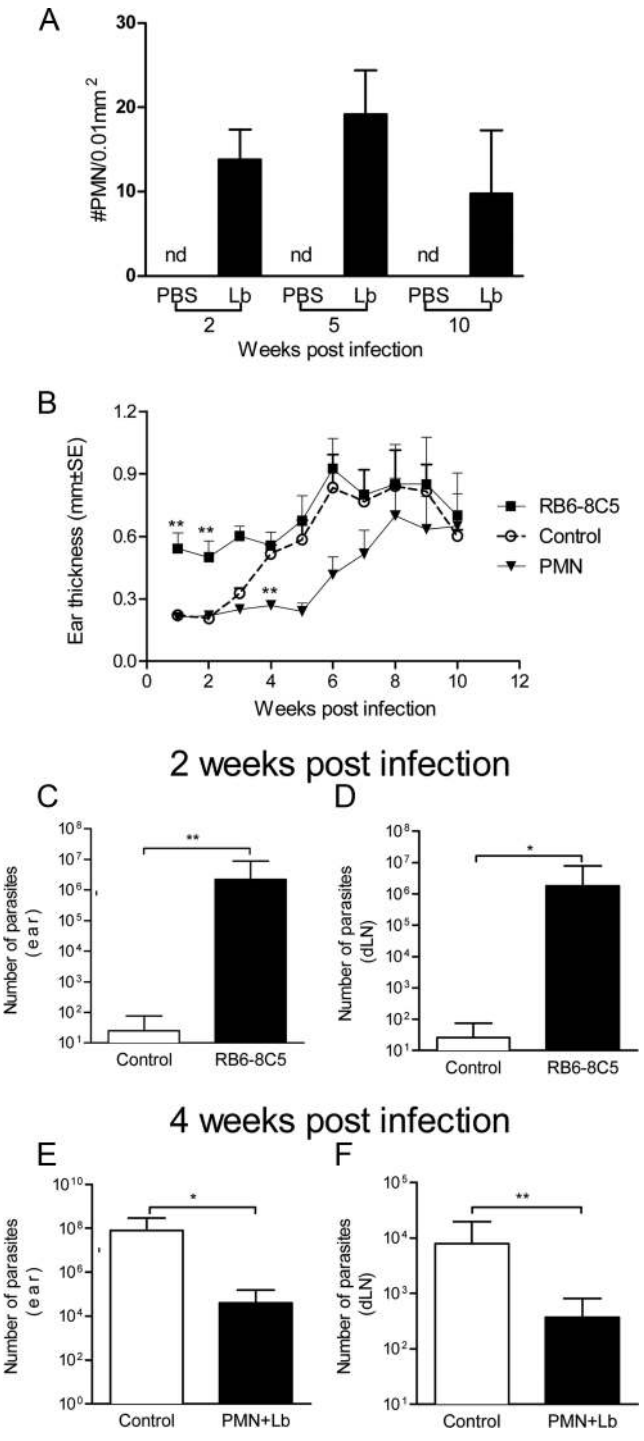

FIGURE 1. In vivo neutrophil recruitment and effects of neutrophil depletion or neutrophil coinoculation on Lb infection. *A*, BALB/c mice were infected with 10^5 Lb or inoculated with PBS. Mice were euthanized at 2, 5, and 10 wk following infection or PBS inoculation. Ear sections were stained with H&E and the presence of neutrophils was evaluated by microscopic analysis. *B*, BALB/c mice (four to six per group) were injected with mAb RB6-8C5 (■), with control rat IgG (○), or with live syngeneic exudate neutrophils (▲) and were infected with Lb. Lesion size was measured weekly. *C* and *D*, Two weeks after infection, parasite load in control and in RB6-treated mice was determined at the infection site and in the dLN. *E* and *F*, Four weeks after infection, parasite load in control and in mice coinoculated with neutrophils and Lb (PMN + Lb) was determined at the infection site and in dLN. The data shown are from a single experiment representative of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$.

[4] The online version of this article contains supplemental material.

neutrophils provide protective functions during Lb infection of BALB/c mice.

We then evaluated cytokine production in RB6-8C5-treated and control mice. At 2 wk after infection, cell suspensions prepared from the lesion site and from dLN were restimulated in vitro with Con A. RB6-8C5-treated mice displayed higher production of IL-2 ($p < 0.05$), IFN-γ ($p < 0.05$), and TNF-α in cell cultures from both ear (Fig. 2, *A*,*C*, and *E*) and dLN (Fig. 2, *B*, *D*, and *F*) when compared with control mice. Additionally, cell cultures from ears and dLN of RB6-8C5-treated mice also showed a significantly higher ($p < 0.05$) concentration of both IL-4 and IL-5 (Fig. 2, *G–J*, respectively) when compared with controls. Similar results were observed when cell cultures were restimulated with Lb promastigotes (supplemental Fig. 3). These data suggest that the cytokine response observed in PMN-depleted mice infected with Lb is associated with increased inflammation/ear thickness (Fig. 1*B*) as well as with higher parasite loads (Fig. 1, *C* and *D*).

Live neutrophils induce the killing of Lb within infected macrophages

Based on the continuous neutrophil recruitment during Lb infection coupled to the protective role played by PMN in vivo, we hypothesized that neutrophils modulate macrophage function, promoting parasite killing. First, we determined the kinetics of parasite elimination during coculture of resident BALB/c macrophages infected with either stationary or metacyclic Lb and exudate neutrophils. As indicated in Fig. 3, the presence of live neutrophils in these cocultures led to an initial reduction in the percentage of infected macrophages (Fig. 3*A*) and the number of intracellular amastigotes (Fig. 3*B*) as early as 2 h after initiation of the cocultures. After 12 h, the presence of exudate neutrophils continued to gradually decrease the percentage of infected cells (Fig. 3*A*) and the number of amastigotes per infected cell (Fig. 3*B*). This decrease became significant after 6 days of coculture for experiments performed with either stationary or metacyclic Lb promastigotes.

It was previously shown that whether dead or alive, neutrophils markedly exacerbated *L. major* replication in BALB/c macrophages, but almost completely eliminated *L. major* from C57BL/6 macrophages (10). Herein, we confirmed that addition of live neutrophils exacerbated *L. major* infection (Fig. 4*A*) and parasite replication within BALB/c macrophages (Fig. 4*B*). However, during coculture of Lb-infected BALB/c macrophages with live neutrophils, a significant reduction in both the percentage of infected cells (Fig. 4*A*) and in the parasite load (Fig. 4*B*) was observed. Similar results were obtained when parasite load was determined by in vitro growth of parasites (Fig. 4*C*). Interestingly, the coculture of Lb-infected BALB/c with UV-induced apoptotic neutrophils did not significantly enhance infection (Fig. 4, *A–C*), as seen with *L. major* (10). In addition, the coculture of Lb-infected BALB/c macrophages with neutrophils obtained from C57BL/6 mice also led to a significant decrease in both the percentage of infected cells (Fig. 4*D*) and in the parasite load (Fig. 4*E*). Conversely, the presence of UV-treated neutrophils from B6 mice did not alter the number of Lb-infected macrophages or the number of amastigotes per infected cell (Fig. 4, *D* and *E*). These results indicate that neutrophils collaborate with macrophages to control Lb multiplication, differently from what was observed with *L. major*.

Lb killing requires contact and is associated with the production of TNF-α and superoxide

To investigate possible differences in macrophage reaction to preserved or activated stimuli, we cocultured infected BALB/c macrophages with paraformaldehyde-treated or control neutrophils. The killing effect observed in Fig. 4, *A* and *B*, was dependent on

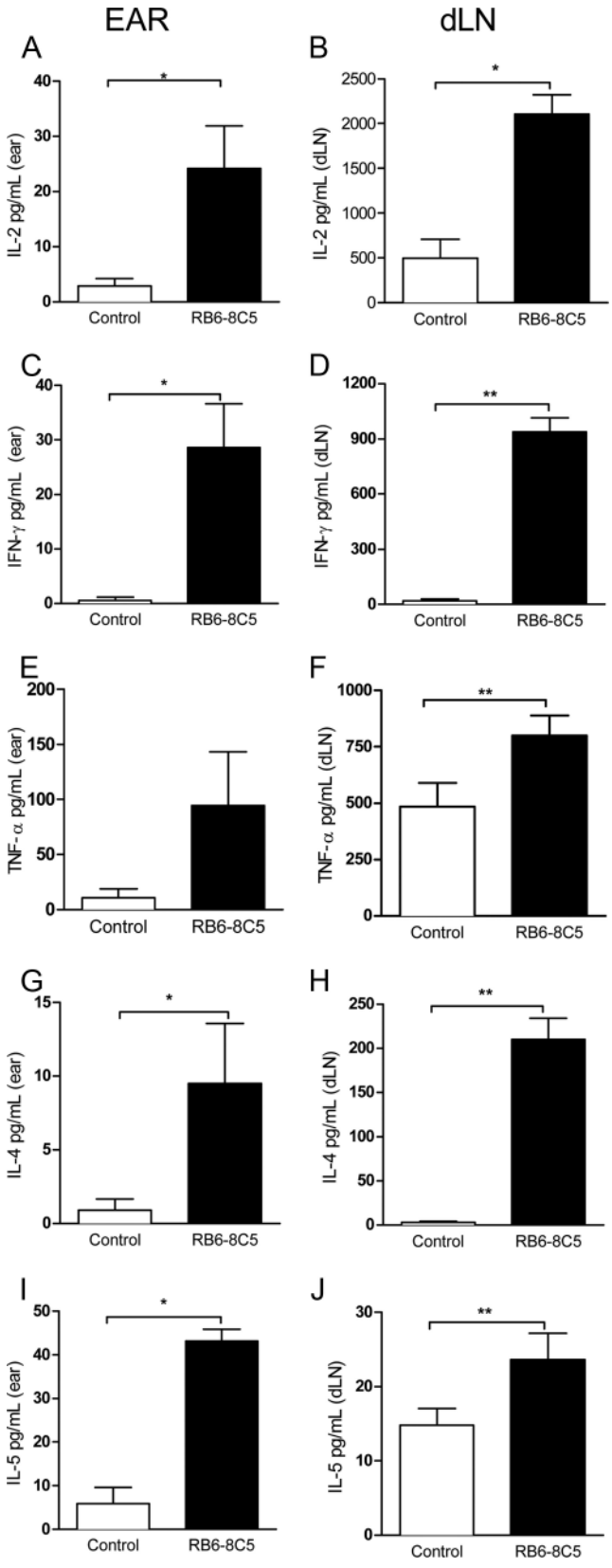

FIGURE 2. Cytokine production upon neutrophil depletion and Lb infection. Control rat IgG-treated (□) or RB6-8C5-treated (■) BALB/c mice (four to six per group) were infected with Lb in the ear dermis. Two weeks after infection, ear (*A*, *C*, *E*, *G*, and *I*) and dLN (*B*, *D*, *F*, *H*, and *J*) were obtained and cell cultures were stimulated in vitro with Con A. Supernatants were harvested 24 h later and cytokine production was determined using a murine Th1-Th2 cytometric bead array. The data shown are from a single experiment representative of two independent experiments. *, $p < 0.05$ and **, $p < 0.01$.

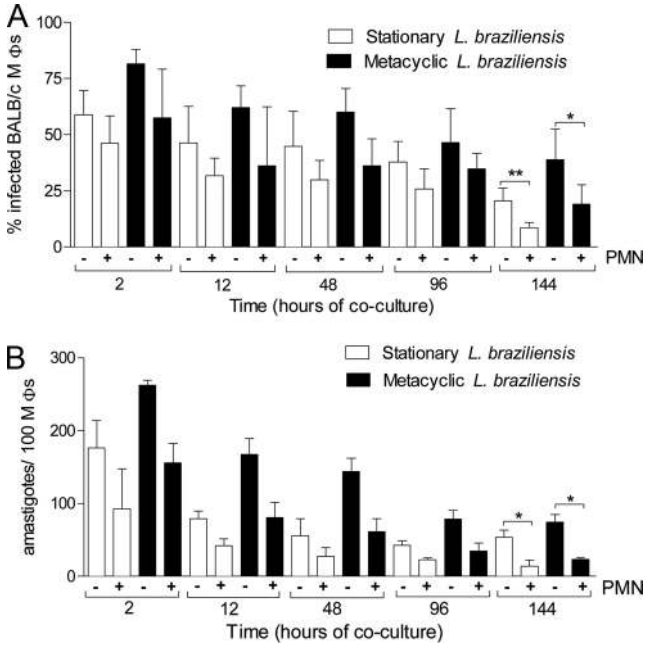

FIGURE 3. Kinetics of Lb killing after coculture with neutrophils. Resident BALB/c macrophage monolayers were infected with stationary Lb promastigotes or with metacyclic Lb promastigotes and cultured for 2, 24, 48, 96, and 144 h alone (−PMN) or with a 10-fold excess of live syngeneic exudate neutrophils (+PMN). Glass coverslips were stained with H&E and assessed for the percentage of infected macrophages (*A*) and the number of amastigotes per 100 cells (*B*) using light microscopy. The data are presented as the mean ± SD. The data shown are from a single experiment representative of three independent experiments. ∗, $p < 0.05$ and ∗∗, $p < 0.01$.

interactions with live neutrophils since fixed cells failed to significantly alter either the percentage of infected macrophages (Fig. 5*A*) or the number of amastigotes per infected macrophage (Fig. 5*B*). Infection with Lb also did not alter the percentage of neutrophils bound or ingested by BALB/c macrophages (Fig. 5*C*). The killing of Lb required a physical interaction between infected macrophages and live neutrophils. Separation of neutrophils using a semipermeable membrane failed to decrease the percentage of infected macrophages (Fig. 5*D*) and the number of amastigotes per infected macrophage (Fig. 5*E*). Importantly, this microbicidal effect was associated with an increase in TNF-α production. Cocultures performed in the presence of live PMN led to a significant increase in TNF-α production (Fig. 6*A*). TNF-α production in cocultures performed across a semipermeable membrane (Fig. 6*A*) was not significantly higher than the concentration detected in control cultures (none).We then investigated whether the absence of TNF-α signaling, using cells from p55 TNFR knockout mice, blocked the killing effect. Wild-type (WT) Lb-infected B6 macrophages cocultured with WT B6 PMN were able to decrease the percentage of infected cells (Fig. 6*B*) and the number of amastigotes per infected cell (Fig. 6*C*). On the contrary, coculture of infected WT macrophages with PMN from p55 TNFR knockout C57BL/6 mice ($p55^{-/-}$) failed to induce parasite killing (Fig. 6, *B* and *C*). Moreover, coculture of Lb-infected $p55^{(-/-)}$ macrophages with WT PMN also led to a significant reduction in the percentage of infected cells (Fig. 6*D*) and in the number of amastigotes (Fig. 6*E*). In contrast, coculture of Lb-infected $p55^{(-/-)}$ macrophages with $p55^{(-/-)}$ PMN reversed these effects (Fig. 6, *D* and *E*). These

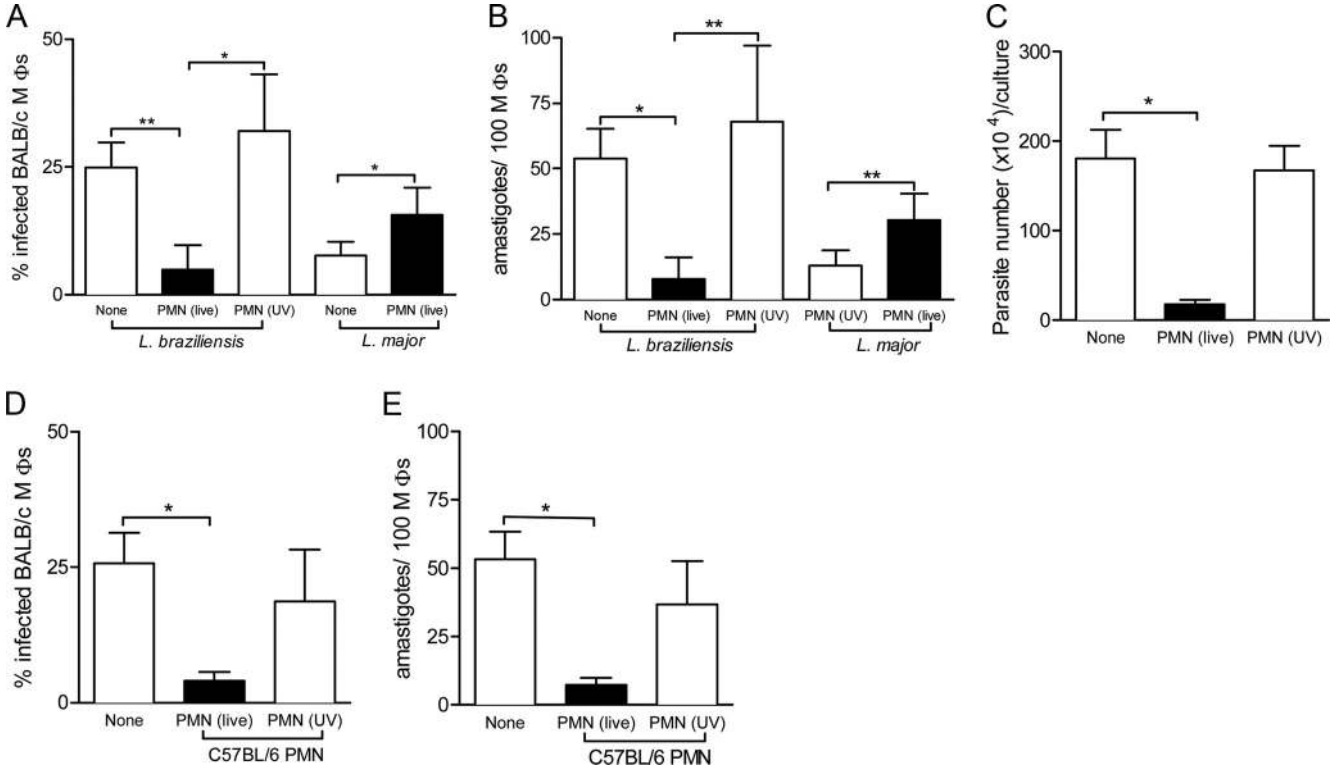

FIGURE 4. Live neutrophils reduce the parasite load of Lb-infected macrophages but not of *L. major*-infected macrophages. Resident BALB/c macrophage monolayers were infected with Lb or with *L. major* and cultured alone (none) or with a 10-fold excess of live or UV-treated syngeneic exudate neutrophils (PMN). Glass coverslips were stained with H&E and assessed for the percentage of infected macrophages (*A*) and the number of amastigotes per 100 macrophages (*B*) using light microscopy or via culture in Schneider medium (*C*). Alternatively, experiments were performed with Lb-infected BALB/c macrophages cocultured alone (none) or with a 10-fold excess of live or UV-treated exudate C57BL/6 neutrophils (PMN). Glass coverslips were stained and assessed for the percentage of infected cells (*D*) and the number of amastigotes per 100 macrophages (*E*). The data shown are from a single experiment representative of three independent experiments. ∗, $p < 0.05$ and ∗∗, $p < 0.01$.

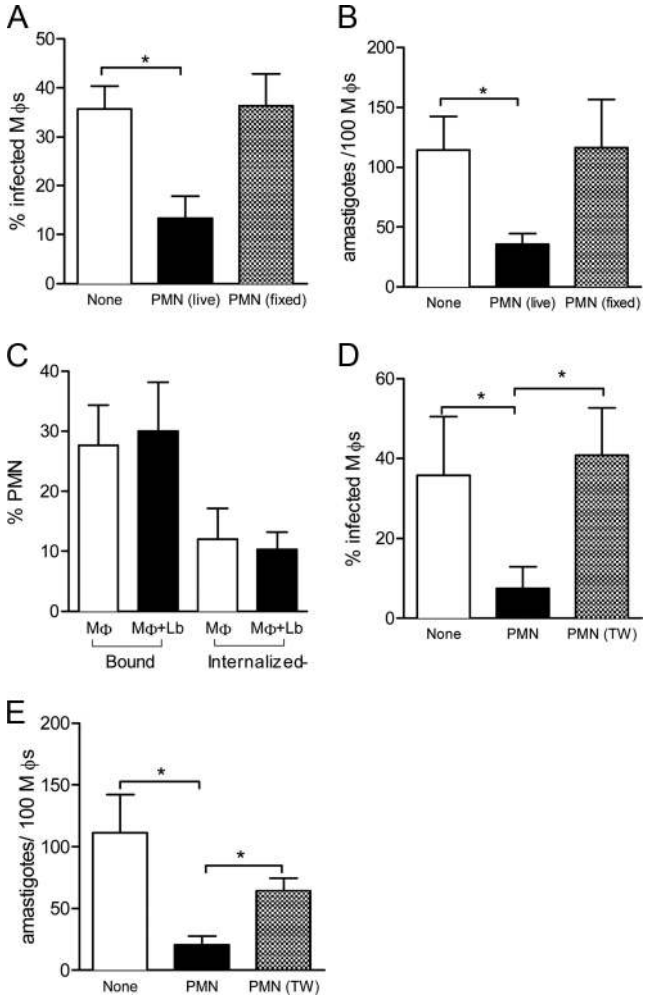

FIGURE 5. Reduction of Lb infection by neutrophils is contact dependent. Resident BALB/c macrophage monolayers were infected with Lb and cultured alone (none) or with a 10-fold excess of live or fixed exudate neutrophils (PMN). Glass coverslips were fixed and stained with H&E and assessed for the percentage of infected cells (*A*), the number of amastigotes per 100 cells (*B*) using light microscopy, and for (*C*) the percentage of neutrophils bound or internalized by control (macrophage (Mϕ)) or Lb-infected macrophages (Mϕ + Lb). Lb-infected macrophages were cultured with PMN in the same compartment (PMN) or separated by culture inserts (PMN-TW). The percentage of infected cells (*D*) and the number of amastigotes per 100 cells were assessed (*E*). The data shown are from a single experiment representative of three independent experiments. *, $p < 0.05$ and **, $p < 0.01$.

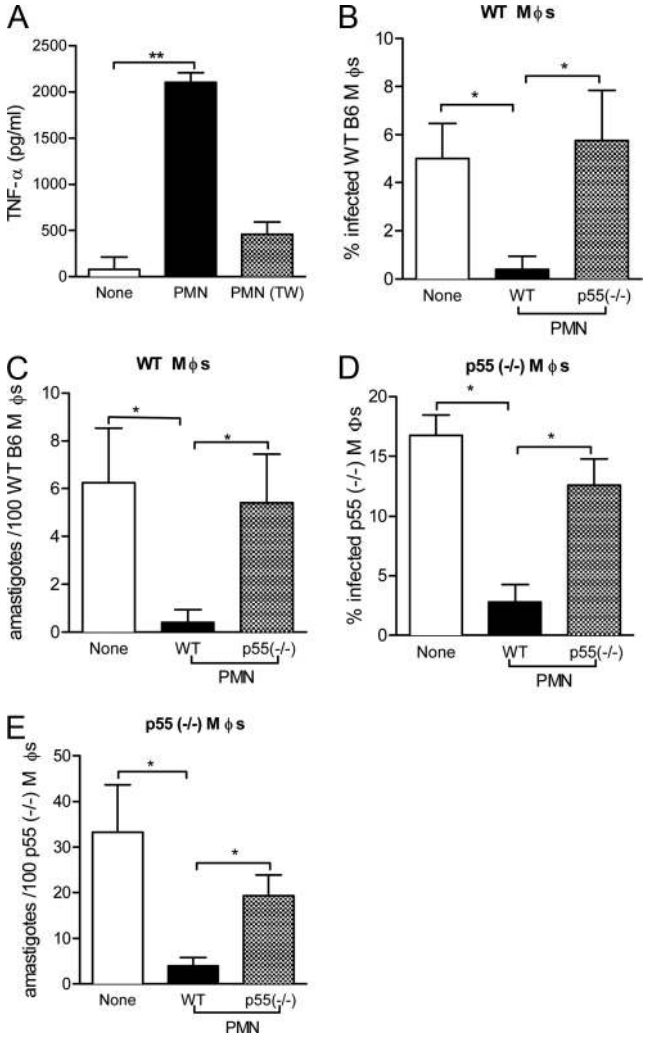

FIGURE 6. Reduction of Lb infection by neutrophils is associated with TNF-α production. Resident BALB/c macrophage (Mϕ) monolayers were infected with Lb and were cultured alone (none) or with a 10-fold excess of exudate neutrophils (PMN) in the same compartment (PMN) or separated by cultured inserts (PMN-TW). Supernatants were collected and assayed for the presence of TNF-α (*A*). Lb-infected C57BL/6 macrophages were cultured alone (none), with live WT C57BL/6 PMN or with live PMN from mice lacking TNF-α receptor I ($p55^{(-/-)}$). Glass coverslips were fixed and stained with H&E and assessed for the percentage of infected cells (*B*) and the number of amastigotes per 100 cells (*C*). Alternatively, Lb-infected macrophages from $p55^{(-/-)}$ mice were cultured alone (none), with live WT C57BL/6 PMN, or with live $p55^{(-/-)}$ PMN. The percentage of infected cells (*D*) and the number of amastigotes per 100 cells were assessed (*E*). The data shown are from a single experiment representative of two independent experiments. *, $p < 0.05$ and **, $p < 0.01$.

results indicate that TNF-α is important in eliminating Lb from infected macrophages upon contact with live neutrophils.

Two important macrophage-derived oxidants are critical in controlling *Leishmania* infection: superoxide (O_2^-) (22, 24) and NO (25). Increased superoxide production is associated with phagocytosis and to demonstrate that Lb killing is dependent upon neutrophil phagocytosis, infected macrophages were cocultured with Jurkat T cells. The decrease in the percentage of infected cells (Fig. 7A) and in the number of amastigotes per infected cell (Fig. 7*B*) was restricted to the presence of neutrophils and not observed in the presence of Jurkat T cells. Coculture of infected macrophages with live neutrophils led to an increase in NO production, which was observed 72 h after initiation of the coculture (Fig. 7*C*). However, addition of live neutrophils to cultures of Lb-infected macrophages also induced a significant increase in superoxide production, observed as early as 2 h after initiation of the coculture (Fig. 7*D*). To confirm that superoxide anion is important in eliminating parasites from Lb-infected BALB/c macrophages upon coculture with neutrophils, we performed experiments in the presence of NAC, a superoxide scavenger (26). Addition of NAC to cocultures of Lb-infected macrophages and live PMN reversed the killing effect exerted by the addition of neutrophils as seen in the percentage of infected cells (Fig. 7*E*). Moreover, cocultures performed with PMN and NAC also induced a significant increase in the number of amastigotes (Fig. 7*F*). Therefore, we can conclude that the effect exerted by the addition of live PMN to Lb-infected macrophages is associated with superoxide.

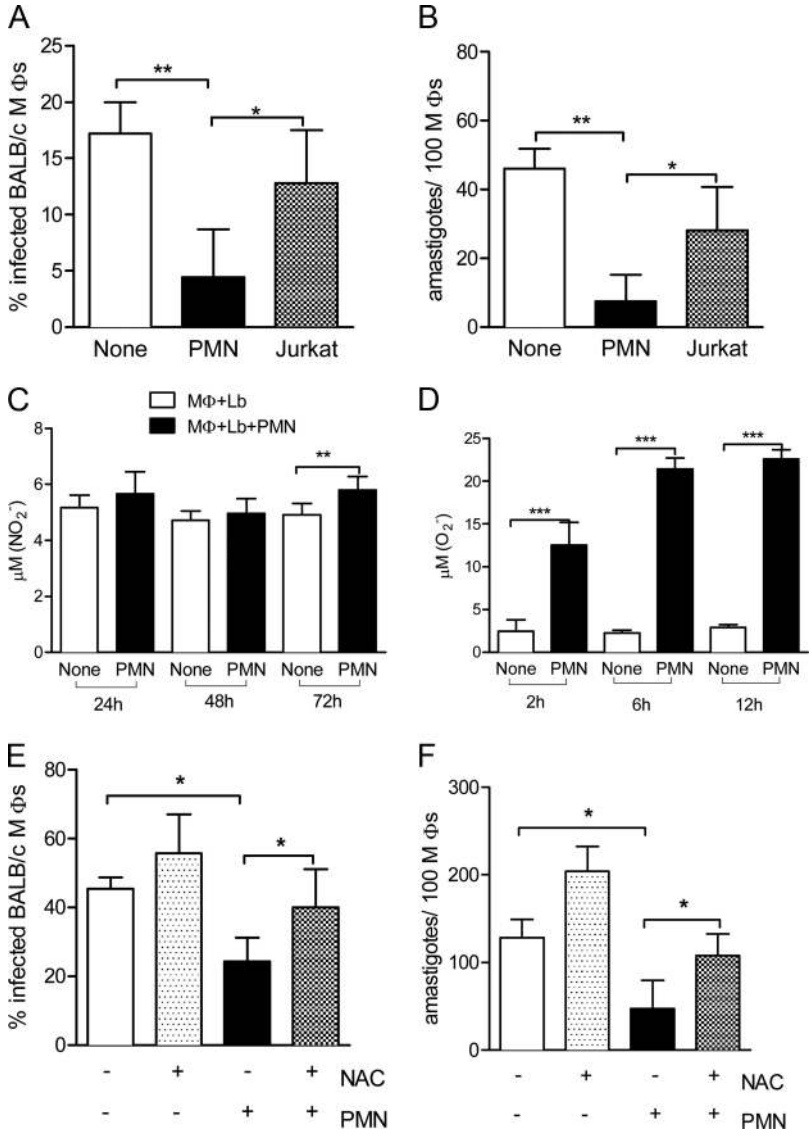

FIGURE 7. Lb elimination from infected macrophages is associated with neutrophil phagocytosis and with superoxide production. Resident BALB/c macrophage (Mϕ) monolayers were infected with Lb and were cultured alone (none) and in the presence of live exudate neutrophils (PMN) or in the presence of 10-fold excess Jurkat T cells. Glass coverslips were fixed and stained with H&E and assessed for the percentage of infected cells (*A*) and the number of amastigotes per 100 cells (*B*) using light microscopy. Alternatively, resident BALB/c macrophage monolayers were infected with Lb and were cultured alone (none) or in the presence of live exudate neutrophils (PMN). Supernatants were collected at different time points, and nitrite was measured using the Griess reaction (*C*). Supernatants were assayed for superoxide production as described in *Materials and Methods* (*D*). Lb-infected macrophages were cultured alone (□), in the presence of NAC (▨), in the presence of live exudate neutrophils (■), or in the presence of both NAC and PMN (▩). Glass coverslips were fixed, stained with H&E, and assessed for the percentage of infected cells (*E*) and the number of amastigotes per 100 cells (*F*) using light microscopy. The data shown are from a single experiment representative of three independent experiments. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

Human neutrophils reduce the parasite load in Lb-infected macrophages

We next investigated whether human neutrophils were also able to influence parasite loads in Lb-infected human macrophages. Coculture of Lb-infected macrophages and neutrophils in the presence of anti-TNF-α led to a significant increase in the percentage of infected cells when compared with control cultures (PMN vs PMN plus anti-TNF-α; Fig. 8*C*). The killing effect was maintained when cocultures were performed in the presence of control IgG (PMN plus anti-TNF-α vs PMN plus IgG; Fig. 8*C*). Similarly, the addition of anti-TNF-α to the cocultures led to a significant increase in the number of amastigotes (PMN vs PMN plus anti-TNF-α; Fig. 8*D*), an effect not observed upon addition of control Ab (PMN plus anti-TNF-α vs PMN plus IgG). Collectively, these results indicate that neutrophil action toward parasite elimination in Lb-infected human macrophages is also mediated by TNF-α, as seen previously.

Because we observed that live neutrophils induce *L. braziliensis* killing, we investigated whether this effect was also extended to experimental systems involving other New World *Leishmania* species. Importantly, the coculture of BALB/c macrophages infected with either *L. amazonensis* or *L. chagasi* promastigotes, etiological agents of visceral and cutaneous leishmaniasis in the Americas, respectively, also led to a significant decrease in the percentage of infected cells (Fig. 9*A*) and in the number of intracellular amastigotes (Fig. 9*B*).

Discussion

Neutrophils, by interacting with monocytes, dendritic cells, T cells, and B cells, through cell-cell contact or secreted products, drive inflammatory responses involved in host defense and tissue repair (7). Neutrophils have been shown to play an active role in the control of infections with specific and distinct pathogens such as *Legionella* (17), *Toxoplasma* (27), *Mycobacterium* (28), *Entamoeba* (29), *Histoplasma* (30), and *Cryptosporidium* (31). BALB/c mice infected in the ear dermis with *L. braziliensis* develop cutaneous lesions at the site of inoculation and histological analyses of infected ear sections have demonstrated a constant recruitment of neutrophils to the inoculation site (14). Since in this experimental model ear lesions heal spontaneously, we hypothesized that neutrophils exert a protective effect. Data presented herein collectively indicate that live neutrophils play an important yet unresolved role during experimental infection with Lb: in vivo neutrophil depletion increased lesion size and parasite load, whereas coinoculation of neutrophils with *L. braziliensis* led to

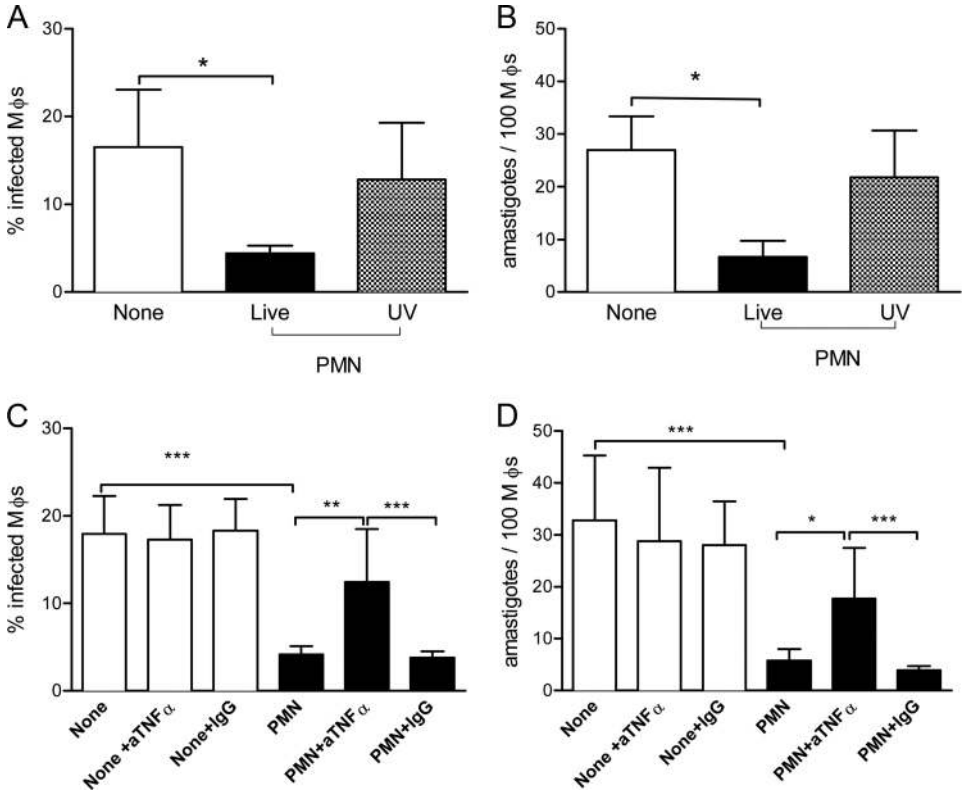

FIGURE 8. Lb elimination from human macrophages is mediated by live neutrophils and is associated with the production of TNF-α. Human macrophages were infected with Lb and cultured alone (none) or in the presence of live or UV-treated human neutrophils (PMN). Glass coverslips were fixed and stained with H&E after 6 days and assessed for the number of infected cells (*A*) and the number of amastigotes per 100 cells (*B*) using light microscopy. Alternatively, the cocultures of human macrophages and live neutrophils were performed in the presence of anti-TNF-α (PMN + anti-TNFα) or in the presence of an isotype control (PMN + IgG). Glass coverslips were fixed and stained with H&E after 6 days and assessed for the percentage of infected cells (*C*) and the number of amastigotes per 100 cells (*D*) using light microscopy. The data shown were obtained from eight individuals. *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$.

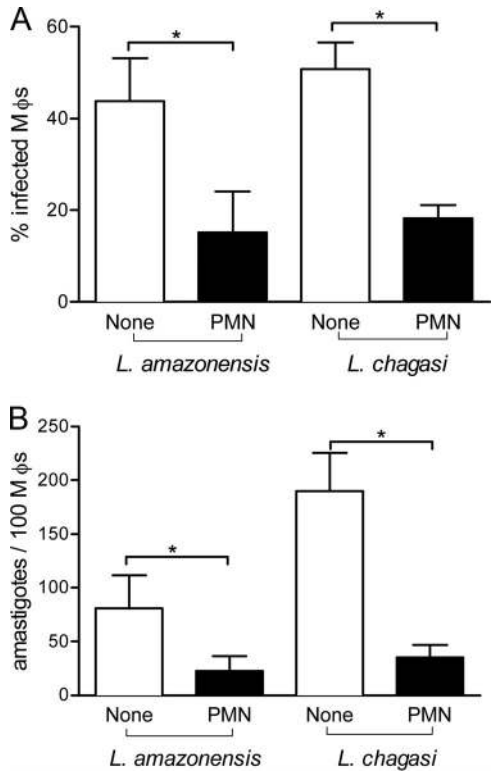

FIGURE 9. Live neutrophils reduce the parasite load of *L. amazonensis*- and *L. chagasi*-infected macrophages. Resident BALB/c macrophage monolayers were infected with *L. amazonensis* or *L. chagasi* and cultured alone (none) or in the presence of live syngeneic exudate neutrophils. Glass coverslips were fixed and stained with H&E and assessed for the number of infected cells (*A*) and the number of amastigotes per 100 cells (*B*) using light microscopy. The data shown are from a single experiment representative of two independent experiments. *, $p < 0.05$.

opposite effects. Although neutrophils may exert a direct role toward killing of Lb, we have addressed the mechanisms by which neutrophils could modulate macrophage function. Indeed, we observed that parasite elimination within Lb-infected macrophages was dependent on interaction with live neutrophils and was associated with the production of both TNF-α and superoxide.

Our previous findings regarding neutrophil recruitment during Lb infection of BALB/c mice (14) were confirmed in the present study. Neutrophil depletion before Lb inoculation led to a significant increase in parasite load and to accelerated spreading of the pathogen to the dLN, suggesting that neutrophils play an important role in the establishment of an early resistance to Lb. The Ab used for in vivo neutrophil depletion (RB6-8C5) binds to Ly6G, which is present on neutrophils, and to Ly6C, which is expressed on neutrophils, dendritic cells, and subpopulations of lymphocytes and monocytes. Therefore, it is possible that such cell populations are also involved in early containment of Lb infection of BALB/c mice. Moreover, in vivo neutrophil depletion enhanced the Th1-Th2-type immune response in BALB/c mice rather than changing the pattern of the cytokine response. We propose that the responses observed in PMN-depleted mice infected with Lb are associated with increased inflammation/ear thickness (IL-2, IFN-γ, and TNF-α) as well as with higher parasite loads (IL-4 and IL- 5). In contrast to our findings, transient depletion of neutrophils in *L. major*-infected BALB/c mice inhibited the development of a Th2 immune response (11), associated with susceptibility to *L. major* infection, and the decreased Th2 response was paralleled with partial resolution of footpad lesions. It was later confirmed that neutrophils play a deleterious role during early infection of BALB/c mice with *L. major* (10).

More recently, in the elegant work published by Peters et al. (32), they showed that *L. major* parasites take advantage of the rapid neutrophil influx induced by sand fly bites and that neutrophil depletion reduced the establishment of productive infections. In light of the present literature, we believe our results

emphasize striking differences between the murine models of Old World (*L. major*) vs New World (Lb) cutaneous leishmaniasis, considering the opposite role played by neutrophils in these two experimental infections. We can also attribute such differences to the important genetic and biological diversity between these two *Leishmania* species (33), which ultimately is reflected in the outcome of infection of BALB/c mice: *L. major* leads to uncontrolled parasite proliferation, whereas Lb infection generates a cutaneous lesion that heals spontaneously.

During infection with *Leishmania* parasites, the development of clinical lesions coincides with the influx of inflammatory cells such as macrophages, eosinophils, and neutrophils (2, 3, 34). In Lb infection, patients displaying lymphadenopathy without ulceration exhibited a higher proportion of neutrophils and eosinophils in lymph node aspirates (35). It is possible that neutrophils are able to directly kill Lb promastigotes in vivo through, for example, the formation of neutrophil extracellular traps, as recently documented with *L. amazonensis* (36). Nonetheless, the macrophage is considered a major effector cell of the immune response during cutaneous leishmaniasis since it is able to produce inflammatory cytokines that are ultimately responsible for parasite killing and elimination. Indeed, the interaction among *L. major*-infected B6 macrophages and dead neutrophils induces parasite elimination, which was associated with neutrophil elastase (10). However, the coculture of *L. major*-infected BALB/c macrophages and dead neutrophils resulted in parasite multiplication and production of TGF-β and PGE_2. Indeed, mature neutrophils have a short life span, undergo constitutive apoptosis, and are removed by phagocytosis, all of which lead to the functional deactivation of macrophages (37). In the present study, we confirmed that interaction of *L. major*-infected macrophages with live neutrophils favors parasite multiplication. In sharp contrast, coculture of Lb-infected macrophages with live inflammatory neutrophils resulted in a significant decrease in parasite load. Of note, we also observed an initial reduction in the number of amastigotes within Lb-infected macrophages in the absence of neutrophils. This parasite elimination without activation may reflect the mixed population of metacyclic and nonmetacyclic parasites present in stationary cultures (38). In this context, metacyclics present in the stationary inoculum may be more efficient at infecting macrophages and sustaining the infection, whereas nonmetacyclics may be more susceptible to intracellular killing and elimination. Indeed, when we used metacyclic Lb, we observed a higher number of amastigotes per infected macrophage. The reduction in parasite load during coculture with live neutrophils was independent of the BALB/c or B6 genetic background since neutrophils from these two strains of mice were equally efficient at inducing parasite killing. The microbicidal effect upon addition of live neutrophils was not observed when the cocultures were conducted with fixed neutrophils or when the cocultures of neutrophils and Lb-infected macrophages were carried in the presence of a semipermeable membrane. The latter indicates that the killing effect exerted by neutrophils is contact dependent and most likely is mediated by constitutively expressed membrane-bound products.

The cocultures of neutrophils and Lb-infected macrophages induced the production of TNF-α, an effect that was also not observed when cells were separated across a semipermeable membrane. We then investigated whether absence of TNF-α signaling would affect parasite elimination from infected macrophages. Cocultures experiments were performed in the presence of macrophages and neutrophils obtained from p55 TNFR knockout ($p55^{-/-}$) C57BL/6 mice. Lack of TNF-α signaling in cocultures with $p55^{(-/-)}$ PMN abrogated the killing effect. On the contrary, the presence of WT PMN led to parasite elimination when coculture was performed with Lb-infected $p55^{(-/-)}$ macrophages. Although activated neutrophils secrete TNF-α (39), the cytokine source could also be the infected macrophage. Coculture of *L. major*-infected macrophages from C57BL/6 mice with dead neutrophils was capable of promoting parasite killing and the secretion of TNF-α (10). Moreover, inflammatory macrophages from C57BL/6 mice induce a cell contact-dependent apoptotic effect of neutrophils through membrane-expressed TNF-α, an effect that is enhanced in the presence of *L. major* (40). Taking these findings together, we suggest that a similar process occurs during Lb infection in BALB/c mice and that this collaboration between macrophages and neutrophils results in the control of the inflammatory process as indicated by lesion healing. Nonetheless, other mechanisms of cross-talk between infected macrophages and neutrophils have been described previously, such as the transfer of lactoferrin (41) and myeloperoxidase (42) and, more recently, the presence of neutrophil granules that colocalized with intracellular mycobacteria (43). It was also shown that the CD28/CD80-CD86 interaction on neutrophil/macrophages induces IFN-γ production, restricting the growth of *L. major* (44). Importantly, the mechanisms leading to cure and resistance to *Leishmania* infection are associated with macrophage activation, resulting from TNF-α- and IFN-γ-induced production of free radicals and ultimately with destruction of the intracellular parasites (45, 46).

During the initial stages of infection with *Leishmania*, superoxide is produced as part of the respiratory burst of macrophages in response to phagocytosis (24). Unlike superoxide, which is produced during phagocytosis, NO is generated after macrophage activation by IFN-γ and TNF-α. NO plays an important role in controlling the established murine leishmaniasis infection (25). The coculture of neutrophils and Lb-infected macrophages led to a striking up-regulation in the production of superoxide, observed as early as after 2 h of incubation. In contrast, NO production induced by the presence of live neutrophils remained unchanged and at minimal levels (below 10 μM), although NO detection was significantly higher after 72 h of coculture. In agreement with this, experiments performed with *L. chagasi* promastigotes indicated that the kinetics of superoxide vs NO formation by infected macrophages is different (47). Since NO is generated after macrophage activation by IFN-γ and TNF-α, we can speculate that TNF-α secretion induced by contact with live neutrophils generates a respiratory burst, as previously demonstrated (48), and the resulting superoxide production leads to Lb elimination from infected macrophages. Parasite killing was dependent on phagocytosis of neutrophils since coculture of infected macrophages with Jurkat cells did not decrease parasite load. Furthermore, the addition of NAC, a superoxide scavenger (26), also blocked the effect exerted by coculture with live neutrophils. Macrophages are also "primed" for enhanced superoxide release following pretreatment with neutrophil serine proteases such as cathepsin G and neutrophil elastase (49). Interestingly, neutrophil elastase activates human macrophages, leading to TNF-α secretion (50), and activates murine macrophages infected with *L. major* through TLR4 ligation (51). The protective role played by neutrophils in vitro, in the murine Lb infection system, was also extended to human cells. More importantly, in these experiments, neutralization of TNF-α secretion led to a significant increase in parasite load similar to previous findings obtained during the coculture of *L. amazonensis*-infected human macrophages infected and necrotic neutrophils (15).

Experiments performed with two other New World *Leishmania* species, namely, *L. amazonensis* and *L. chagasi*, yielded similar results. Interestingly, in vivo neutrophil depletion in mice infected with *L. donovani* (23, 52) and *Leishmania infantum* (53) led to an increase in parasite burden, confirming our existing data with *L. chagasi,* which is phylogenetically related to *L. infantum*, and suggesting that the parasite species is a crucial determinant of the outcome of infection. Again, a recent comparative study indicated that the genomes of *L. major*, Lb, and *L. infantum* are highly similar in content and organization but that there are species-specific genes and mechanisms that are able to distinguish one species from another (33).

Based on these findings, we suggest that clearance of neutrophils in BALB/c mice infected with Lb generates a proinflammatory environment and that neutrophils play a significant role in parasite elimination through collaboration with macrophages. We believe our study brings an important contribution toward elucidating aspects of the host-parasite relationship associated with resistance to Lb, a major cause of disease in South America.

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Disclosures

The authors have no financial conflict of interest.

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