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

Fernanda O. Novais,* Rômulo C. Santiago,* André Báfrica,† Ricardo Khouri,* Lilian Afonso,* Valéria M. Borges,*§ Cláudia Brodskyn,*§§ Manoel Barral-Netto,*§§ Aldina Barral,†§§ and Camila I. de Oliveira2*‡

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 PGE2 (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...
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 μg/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 RB6-8C5 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 coinculated with 10^6 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, ×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, and 10% heat-inactivated FCS (all from Invitrogen). Metacyclic Lb parasites were prepared as described in Spath and Beverley (16).

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, nitrate (NO₃⁻) (20). Superoxide production was determined by adding hydroxylamine (0.5 mM; Sigma-Aldrich) (21, 22) to cocultures of infected macrophages and live neutrophils. Hydroxylamine converts superoxide into nitrite, which can then be quantified 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 non-adherent 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 10% FCS. Neutrophils were extensively washed to remove extracellular parasites and non-adherent cells. All cultures were then incubated in RPMI 1640 medium supplemented with 1% Nutridoma-SP (Roche).
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 antihuman 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 were determined by microscopic analysis. As shown in Fig. 1A, 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. 1A 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. 1B, 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. 1C) and in dLN (Fig. 1D). 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. 1A). At this time point, mice coinoculated with PMN plus Lb also displayed a significantly smaller parasite load both at the inoculation site (Fig. 1E) and in dLN (Fig. 1F). These results suggest that

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. 1B) 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 replication within cocultures 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 cells (Fig. 3A) and the number of intracellular amastigotes (Fig. 3B) 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. 3A) and the number of amastigotes per infected cell (Fig. 3B). 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. 4A) and parasite replication within BALB/c macrophages (Fig. 4B). However, during coculture of Lb-infected BALB/c macrophages with live neutrophils, a significant reduction in both the percentage of infected cells (Fig. 4A) and in the parasite load (Fig. 4B) was observed. Similar results were obtained when parasite load was determined by in vitro growth of parasites (Fig. 4C). 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. 4D) and in the parasite load (Fig. 4E). 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
interactions with live neutrophils since fixed cells failed to significantly alter either the percentage of infected macrophages (Fig. 5A) or the number of amastigotes per infected macrophage (Fig. 5B). Infection with Lb also did not alter the percentage of neutrophils bound or ingested by BALB/c macrophages (Fig. 5C). 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. 5D) and the number of amastigotes per infected macrophage (Fig. 5E). 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. 6A). TNF-α production in cocultures performed across a semipermeable membrane (Fig. 6A) 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. 6B) and the number of amastigotes per infected cell (Fig. 6C). 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. 6D) and in the number of amastigotes (Fig. 6E). In contrast, coculture of Lb-infected p55+/− macrophages with p55−/− PMN reversed these effects (Fig. 6, D and E).
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 (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.

7D). 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 (F). The data shown are from a single experiment representative of two independent experiments. *, p < 0.05 and **, p < 0.01.
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. Co-culture of Lb-infected macrophages and neutrophils in the presence of anti-TNF-α/H9251 led to a significant increase in the percentage of infected cells (PMN vs PMN plus anti-TNF-α/H9251; Fig. 8C). The killing effect was maintained when cocultures were performed in the presence of control IgG (PMN vs PMN plus IgG; Fig. 8C). 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. 8D), 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. 9A) and in the number of intracellular amastigotes (Fig. 9B).

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 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.](http://www.jimmunol.org/content/full/8094)
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, in contrast to their role in other protozoan infections (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 PGE₂. 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. Coculture 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 myeloperoxidase (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 pre-treatment 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, 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 *Leishma*-nia 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

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