Interactions with apoptotic but not with necrotic neutrophils increase parasite burden in human macrophages infected with *Leishmania amazonensis*

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Abstract: Neutrophils are involved in the initial steps of most responses to pathogens. In the present study, we evaluated the effects of the interaction of apoptotic vs. necrotic human neutrophils on macrophage infection by *Leishmania amazonensis*. Phagocytosis of apoptotic, but not viable, neutrophils by *Leishmania*-infected macrophages led to an increase in parasite burden via a mechanism dependent on TGF-β1 and PGE_2. Conversely, infected macrophages' uptake of necrotic neutrophils induced killing of *L. amazonensis*. Leishmanicidal activity was dependent on TNF-α and neutrophilic elastase. Nitric oxide was not involved in the killing of parasites, but the interaction of necrotic neutrophils with infected macrophages resulted in high superoxide production, a process reversed by catalase, an inhibitor of reactive oxygen intermediate production. Initial events after *Leishmania* infection involve interactions with neutrophils; we demonstrate that phagocytosis of these cells in an apoptotic or necrotic stage can influence the outcome of infection, driving either parasite survival or destruction. *J. Leukoc. Biol.* 84: 389–396; 2008.

Key words: *apoptosis · necrosis · phagocytosis · TGF-β1 · NE*

INTRODUCTION

Neutrophils are among the first cells to be recruited to the infection site and are important in controlling the host defense through oxidant and protease-dependent mechanisms [1–3]. They also provide an important link between innate and adaptive immunity during parasite infections [4, 5]. Neutrophils interact with monocytes, dendritic cells, T and B cells through cell-cell contact or secreted products, driving inflammatory responses involved in host defense and tissue repair [5].

Neutrophils have a short life span and are constitutively programmed to die by apoptosis. Apoptotic neutrophils are removed by macrophages, accelerating the resolution of inflammation [6]. Clearance of apoptotic cells by macrophages releases anti-inflammatory mediators such as TGF-β1 and PGE_2 that block macrophage activation [7–9]. Apoptotic cells that are not removed by phagocytosis progress to a secondary necrosis stage [6]. In contrast to apoptotic cells, ingestion of necrotic cells induces macrophage activation through production of proinflammatory mediators [10]. Phagocytosis of lysed neutrophils has the ability to stimulate MIP-2, IL-8, TNF-α, and IL-10 production by human macrophages [11].

The suppressive environment induced by the phagocytosis of apoptotic cells leads to increased growth of intramacrophagic pathogens such as *Trypanozoma cruzi* [12], HIV-1 virus [13], and *Coxiella burnetti* [14]. Phagocytosis of apoptotic lymphocytes by *T. cruzi*-infected macrophages increases the parasite burden through the production of TGF-β1, PGE_2, and polyamines [12].

Neutrophils have been implicated in the immunopathogenesis of murine leishmaniasis [15, 16]. The uptake of apoptotic inflammatory neutrophils has a direct effect on the host response to infection by *L. major* in BALB/c and C57BL/6 mice, driving susceptibility, or resistance to infection, respectively. Moreover, the leishmanicidal activity induced by neutrophils in C57BL/6 mice is dependent on neutrophilic elastase (NE), reactive oxygen species, and TNF-α production [15]. Infection of human neutrophils by *L. major* inhibits their spontaneous apoptosis [17]; at the same time, neutrophils can also serve as a vector for *Leishmania* entry into macrophages [18]. However, the role of human neutrophils in the outcome of *Leishmania* infection is not completely understood.

In the present study, we investigated the effect on *L. amazonensis*–infected macrophages of phagocytosis of apoptotic vs. necrotic human neutrophils. We observed that apoptotic, but not viable, neutrophils increased the parasite burden through a mechanism dependent on TGF-β1 and PGE_2. Conversely, interaction of necrotic neutrophils with *L. amazonensis*-infected macrophages decreased the infection rate as well as the para-

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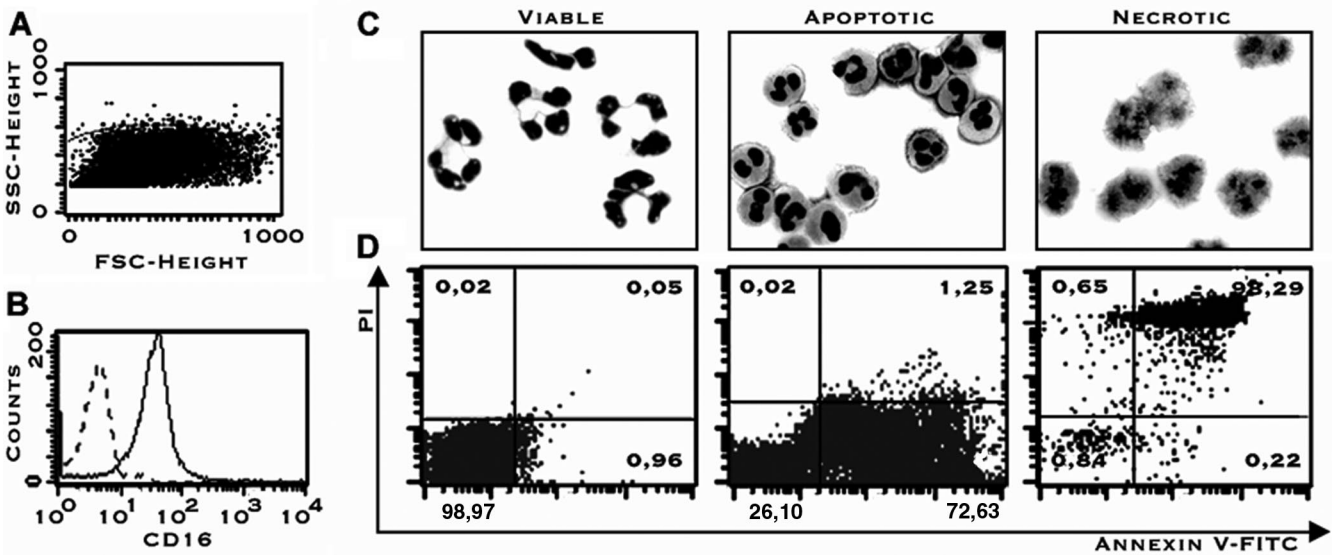

Fig. 1. Characterization of apoptotic, necrotic, and viable neutrophils. Neutrophils were obtained as described in Material and Methods and were analyzed by light microscopy and FACS analysis. (A) Forward and side scatter characterization of viable neutrophils. (B) Their staining by human anti-CD16 antibody.(full line) and isotype control (dashed line). (C) Charcaterization of viable, apoptotic, and necrotic neutrophils by light microscopy, the cells were stained using Diff-Quick (Magnification ×1000). (D) Characterization of viable, apoptotic, and necrotic neutrophils by flow cytometry, staining the cells with PI and Annexin V.

site burden. The inhibition of parasite replication by uptake of necrotic cells was mediated by TNF-α and by secretion of NE. These findings identify novel mechanisms regulating innate immunity during human infection by *Leishmania*.

MATERIALS AND METHODS

Cell culture

Human blood was obtained from healthy volunteers from Hemocentro do Estado da Bahia, BA, Brazil. This study was approved by the Research Ethics Committee of FIOCRUZ-Bahia.

Human neutrophils were isolated by centrifugation using PMN medium according to the manufacturer's instructions (Robbins Scientific Corporation, Sunnyvale, CA, USA). Briefly, the blood was centrifuged for 30 min at 300 *g* at room temperature. Neutrophils were collected and washed three times at room temperature at 200 *g*. They were analyzed by flow cytometry (FACS) as shown in **Fig. 1A** (forward vs. side scatter) and labeled with human anti-CD16 (Fig. 1B). PBMC were isolated by passage over Ficoll-Hypaque gradients (Sigma-Aldrich, St. Louis, MO, USA). PBMC were washed three times, resuspended at a concentration of 5×10^6 cells per ml in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA), plated in a 24-well tissue culture plate (Corning Incorporation, Costar, NY, USA), and incubated at 37°C and 5% CO_2 for 30 min. Nonadherent cells were removed; adherent cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Ogden, UT, USA), 2 mM/ml L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen) for 7 days.

Leishmania culture and macrophage infection

L. amazonensis (MHOM/BR/87/BA125) promastigotes were cultured at 27°C in DMEM medium (Invitrogen), supplemented with 10% SBF, 2 mM/ml L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Invitrogen, Carlsbad, CA). Macrophages were cultured on glass coverslips for 4 h after being infected with *L. amazonensis* in the early stationary phase at a parasite-to-cell ratio of 2:1. Wells were then washed to remove extracellular parasites.

Apoptotic, necrotic, and viable neutrophils

Apoptotic neutrophils were obtained by ultraviolet irradiation exposure (245 nm) for 10 min [15]. Afterward, they remained in RPMI 1640 medium at 37 C and 5% CO_2 for 2 h before use. Apoptotic neutrophils prepared in this way presented pyknotic nuclei when assayed by light microscopy (Fig. 1C) and were ~70-90% Annexin-V positive and PI negative by FACS analysis (Fig. 1D) Necrotic neutrophils were obtained after 10 freeze-thaw cycles, as described previously [12, 19]. Using this experimental approach, necrotic cells stained with Diff-Quick and observed under the microscope maintained their integrity (Fig. 1C). Besides that, staining with Trypan blue were permeable to Trypan blue. Necrotic neutrophils were also detectable by forward and side scatter parameter in the FACS analysis and had more than 98% Annexin V and PI-positive stain (Fig. 1D). Viable neutrophils were kept on ice until their use as an experimental control. They showed multilobular nuclei (Fig. 1C) and ~95% of cells were Annexin-V and PI negative by FACS analysis (Fig. 1D). Some experiments used apoptotic and necrotic Jurkat cells obtained as described above.

Coculture of *L. amazonensis*-infected macrophage with neutrophils

Macrophage cultures received early stationary phase of *L. amazonensis* promastigotes and, after 4 h, monolayers were extensively washed to remove extracellular parasites and nonadherent cells, leaving adherent macrophages. After this period, viable, apoptotic or necrotic neutrophils were added to infected macrophage cultures at a neutrophil to macrophage ratio of 3:1 in RPMI medium supplemented with 1% Nutridoma-SP (Roche, Indianapolis, IN, USA), 2 mM/ml L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen). Cocultures were performed on glass coverslips and then harvested after 4, 24, 48, 72, and 96 h, fixed with ethanol, and stained with hematoxylin-eosin (HE). Intracellular amastigotes were counted in 200 macrophages. Results are shown as parasite number per 100 macrophages (parasite burden) and as a percentage of infected macrophages. In some experiments, infected macrophages were cocultured with apoptotic or necrotic neutrophils in the presence of neutralizing antibody: 6 μg/ml of anti-human-TGF-β1 (R&D Systems, Minneapolis, MN, USA), 6 μg/ml of anti human-TNF-α (R&D Systems), or purified mouse IgG1 isotype control (BD PharMingen, San Diego, CA, USA). In some cases, cultures were performed in the presence of 50 μg/ml of anti-human neutrophil elastase (Calbiochem, La Jolla, CA, USA), anti-IgG rabbit control (50 μg/ml) (R&D Systems);1 μM/ml of indomethacin (Sigma-Aldrich); 10 μg/ml of NE inhibitor (Calbiochem) or 50 μg/ml of antipain, a nonspecific inhibitor of serine protease as a control (Sigma-Aldrich).

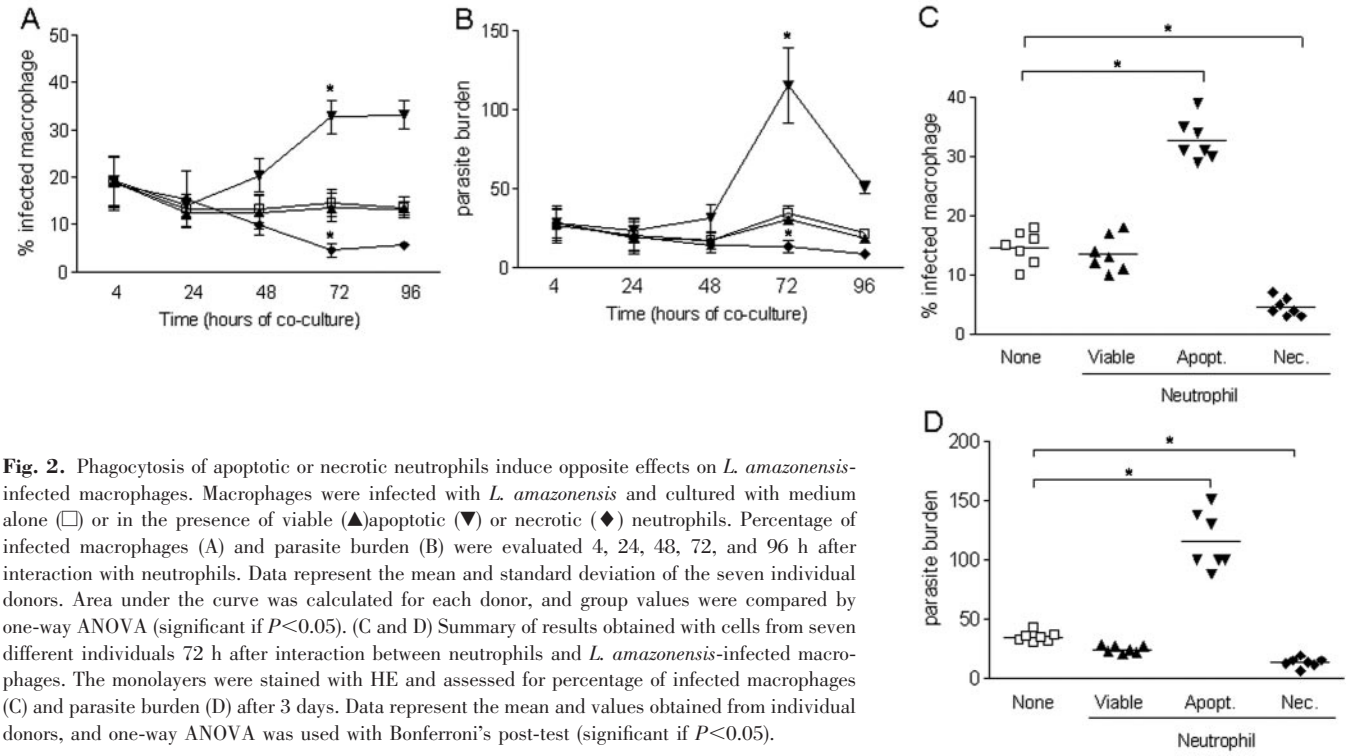

Fig. 2. Phagocytosis of apoptotic or necrotic neutrophils induce opposite effects on *L. amazonensis*-infected macrophages. Macrophages were infected with *L. amazonensis* and cultured with medium alone (□) or in the presence of viable (▲)apoptotic (▼) or necrotic (♦) neutrophils. Percentage of infected macrophages (A) and parasite burden (B) were evaluated 4, 24, 48, 72, and 96 h after interaction with neutrophils. Data represent the mean and standard deviation of the seven individual donors. Area under the curve was calculated for each donor, and group values were compared by one-way ANOVA (significant if $P<0.05$). (C and D) Summary of results obtained with cells from seven different individuals 72 h after interaction between neutrophils and *L. amazonensis*-infected macrophages. The monolayers were stained with HE and assessed for percentage of infected macrophages (C) and parasite burden (D) after 3 days. Data represent the mean and values obtained from individual donors, and one-way ANOVA was used with Bonferroni's post-test (significant if $P<0.05$).

Cytokine production

Supernatants from control macrophages or cocultures of infected macrophage and apoptotic or necrotic neutrophils were collected after 6 h of culture and then immediately assayed for TNF-α (BD Bioscience, San Diego, CA, USA), according to the manufacturer's instructions. After 24 h of culture, the supernatant was collected, cleared by centrifugation, acidified, and assayed for total TGF-β1 (Promega, Madison, WI) by sandwich ELISA, according to the manufacturer's instructions.

NO evaluation

Nitric oxide production in the supernatant of cocultures of infected macrophages and necrotic neutrophils was evaluated by the Griess method, as described elsewhere [15]. The parasite burden was assessed in control cultures or in cultures with 1 nM of N^{ω}-nitro L-arginine (L-NNA) (Sigma), an inducible nitric oxide synthase inhibitor. After 72 h of incubation, coverslips were stained with HE and macrophage infection was determined by optical microscopy, as described previously.

Superoxide evaluation

We verified the superoxide production by chemiluminescence (CL) reaction in infected macrophages, either alone or cocultured with necrotic neutrophils. One thousand (1000) U/ml of catalase (Sigma) was added to some cultures as a control of superoxide inhibition. Cells cultured (1×10^6 cells, 2 ml in 35-mm dishes) were used for CL measurement in a photon-counting device composed of a gallium arsenide photomultiplier tube (Hamamatsu R943) thermoelectrically cooled to −20°C. Samples were placed in dishes, sealed with cling film, and maintained at 37°C in a thermostatic light-sealed chamber. After this, their CL emissions were collected by reflections off a concave mirror and focused onto the photomultiplier tube [20]. Therefore, 2 h after interaction between infected macrophages and necrotic neutrophils, we verified the superoxide production by chemoluminescence reaction. Superoxide production measured throughout was recorded by luminescence after the addition of 20 μM lucigenin to the culture for 30 min.

To identify the dependency of superoxide production on the parasite burden, 1000 U/ml of catalase (Sigma) was added to the cultures. After 72 h of incubation, coverslips were stained with HE, and macrophage infection was determined by optical microscopy as described previously.

Statistical analysis

To take into account all values of the kinetics of interaction between macrophages and neutrophils, we calculated the area under the curve of each donor and compared group values by one-way ANOVA. Parametric data were analyzed by one-way ANOVA Bonferroni post-test using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA). Differences with $P < 0.05$ were considered significant.

RESULTS

Interactions with apoptotic or necrotic neutrophils regulate replication of *L. amazonensis* in human macrophages

Viable, apoptotic, and necrotic neutrophils were cocultured with *L. amazonensis*-infected macrophages. Initially, we evaluated the percentage of infected cells after different extents of coculture time, in order to determine the best time point to analyze the interaction between infected macrophages and neutrophils. As shown in **Fig. 2A**, after 4 h of infection, ~20% of cells were infected in all experimental conditions. The addition of apoptotic neutrophils to the culture led to a significant increase in the percentage of cells infected; this effect was maintained until 96 h. On the other hand, the presence of necrotic neutrophils caused a significant decrease in the number of infected cells after 72 and 96 h of coculture. These effects were observed only in the presence of apoptotic and necrotic neutrophils, whereas the addition of viable neutrophils did not cause any changes in the infection rate of macrophages. Similar results were observed for the parasite burden (Fig. 2B). Therefore, on the basis of these results, we decided to use the time point of 72 h (3 days) for the remaining experiments in this study. Figure 2, C and D shows the percentage of infected

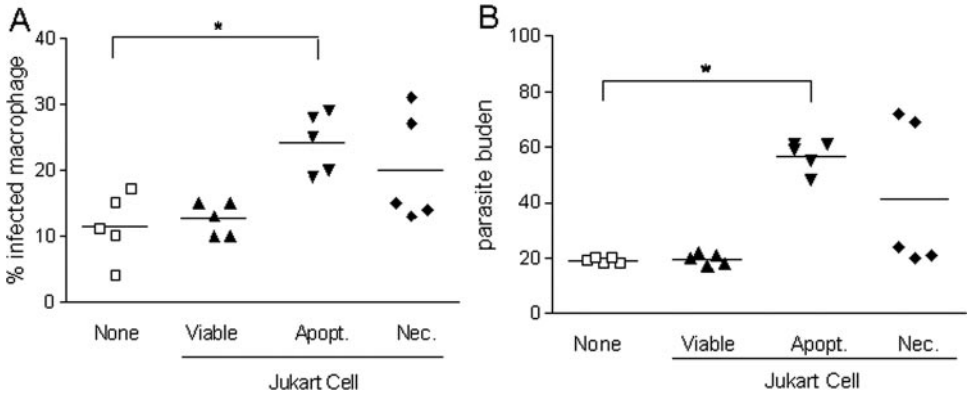

Fig. 3. Phagocytosis of apoptotic or necrotic Jurkat T lymphocytes drive parasite growth. Macrophages were infected with *L. amazonensis* and cultured in medium alone (□) or in the presence of viable (▲), apoptotic (▼), or necrotic (♦) Jurkat cells. The monolayers were stained with HE and assessed for the percentage of infected macrophages (A) and for parasite burden (B) after 3 days. Each point represents a different donor, and each bar represents the mean. For statistical analysis, one-way ANOVA was used with Bonferroni's post-test (significant if $P<0.05$).

macrophages, as well as the parasite burden obtained in macrophages infected by *L. amazonensis*, in the absence or in the presence of viable, apoptotic and necrotic neutrophils after 3 days of coculture. Variations were observed among the different individuals analyzed. The interaction of infected macrophages with apoptotic neutrophils significantly exacerbated infection, as indicated by the increase in both the percentage of infected macrophages (Fig. 2C) and parasite burden (Fig. 2D). On the other hand, no differences were observed when the cocultures were prepared with viable neutrophils (Fig. 2, C and D). The interaction between infected macrophages and necrotic neutrophils significantly decreased the percentage of infected cells (Fig. 2C) and parasite burden (Fig. 2D).

To determine whether this effect was dependent on specific interactions between macrophages and neutrophils, we repeated the same experiments with viable, apoptotic, and necrotic Jurkat T cells [15]. Only apoptotic Jurkat T cells induced an increase in the percentage of infected macrophages, as well as in parasite burden (**Fig. 3, A and B**), whereas the phagocytosis of necrotic Jurkat T cells by infected macrophages altered neither the percentage of infected cells nor the parasite burden, suggesting that the specific interaction of infected macrophages with necrotic neutrophils is responsible for the effects observed earlier.

As previously shown, the phagocytic ability of *Leishmania*-infected macrophages is greatly reduced in the mouse model [21]. To rule out the possibility that our results could be influenced by different rates of phagocytosis and/or adherence of neutrophils between infected and noninfected macrophages, we quantified the binding and ingestion of viable and apoptotic neutrophils by these cells. As shown in **Fig. 4, A and B**, no significant differences were observed in the ingestion and binding of viable or apoptotic neutrophils by infected or non-infected macrophages.

Another potential explanation for our results is that phagocytosis of viable, apoptotic or necrotic neutrophils resulted in macrophage loss. To rule out this possibility, we counted the numbers of uninfected and infected macrophages at 4, 24, 48, and 72 h after addition of viable, apoptotic and necrotic neutrophils in 10 randomly chosen fields of fixed cultures. We observed that there were no significant differences in the loss of macrophages at different timepoints (data not shown).

Role of TGF-β1 and PGE_2 during interactions with apoptotic neutrophils

To evaluate the possible involvement of TGF-β1 in the increase of infection observed after interactions with apoptotic neutrophils, we measured TGF-β1 levels in supernatants after 24 h of coculture. TGF-β was found when macrophages were cocultured with apoptotic neutrophils, but not with necrotic cells (**Fig. 5A**). Additionally, we cultured apoptotic neutrophils and infected macrophages with anti-human-TGF-β1 antibody. As shown in Fig. 5B, the addition of the neutralizing antibody decreased the parasite burden and percentage of infection. We also evaluated the role of PGE_2 in this phenomenon. Indomethacin, a cyclooxygenase inhibitor, led to an inhibition of both parasite burden (Fig. 5B) and percentage of infection (not shown) when added to the co-culture of apoptotic

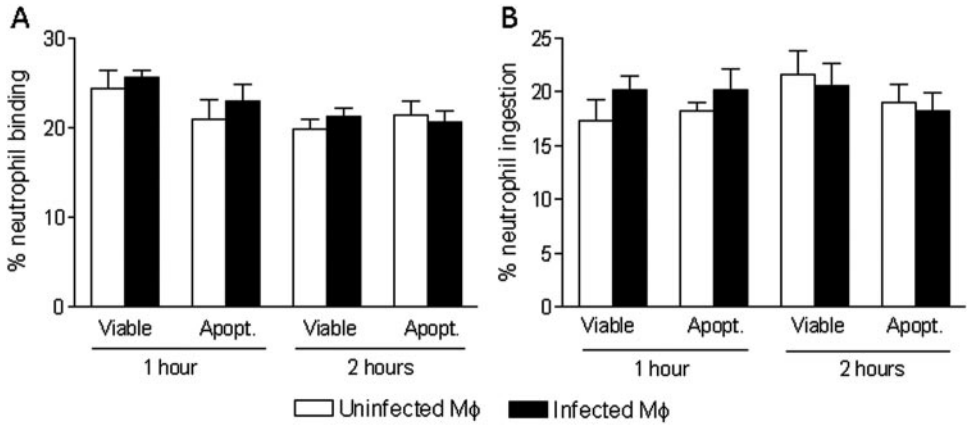

Fig. 4. Binding and ingestion of neutrophils by infected and uninfected macrophages. Macrophages were infected (or not) with *L. amazonensis* and cultured with viable and apoptotic neutrophils for 1 and 2 h. The monolayers were stained with HE and assessed for the percentage of neutrophil binding (A) and ingestion (B). Data represent the mean and standard deviation of five individual donors. For statistical analysis, one-way ANOVA with Bonferroni's post test was used, and no significance was found among the different groups analyzed.

neutrophils and infected macrophages. To rule out the possibility that indomethacin could be toxic to the macrophages; we incubated macrophages with this product at a concentration of 1 μM/ml and evaluated the viability of cells. No differences were observed in the viability of cells incubated with or without indomethacin (data not shown). These results indicate that TGF-β1 and PGE-2 are involved in exacerbated replication of *Leishmania*-infected macrophages cocultured with apoptotic neutrophils.

Role of TNF-α and NE in the interaction between necrotic neutrophils and infected macrophages

To verify whether TNF-α was involved in the decrease of parasite burden induced by necrotic neutrophils, we evaluated TNF-α levels in the supernatants of cocultures of infected macrophages and necrotic neutrophils after 6 h of coculture. The results showed a higher level of TNF-α in the presence of necrotic neutrophils, but not in the presence of apoptotic cells, suggesting the participation of this cytokine in mediating the decrease in the rate of macrophage infection (**Fig. 6A**). Accordingly, the addition of anti-human TNF-α antibody to cultures of infected-macrophages and necrotic neutrophils led to an increase both in parasite burden (Fig. 6B) and percentage of infection (data not shown). Although the infection of macrophages by *L. amazonensis* also led to a low production of TNF-α, the addition of neutralizing anti-TNF-α antibody did not significantly change the parasite burden observed in the absence of antibody (Fig. 6B). Thus, production of TNF-α is involved in the control of parasite replication by necrotic neutrophils.

NE induces TNF-α production by human macrophages exposed to lysed neutrophils [11]. To test whether NE was involved in the leishmanicidal effect observed with necrotic neutrophils, we added anti-human-NE and NE-specific inhibitor to cocultures of infected macrophages and necrotic neutrophils. As controls, we used antipain, a protease inhibitor unable to inhibit NE. As shown in Fig. 5, C and D, the addition of either anti-NE neutralizing antibody or NE inhibitor, respectively, restored the parasite burden observed in the absence of necrotic neutrophils, suggesting the participation of NE in intracellular induction of parasite destruction.

The mechanism of parasite destruction is dependent on superoxide

We evaluated whether the decrease in macrophage infection induced by TNF-α and NE was due to secretion of NO. The addition of necrotic neutrophils did not alter the spontaneous production of NO by infected human macrophages at early or late coculture time points (**Fig. 7A**). To confirm these data, we also added L-NNA (N^W-nitro-L-arginine), an inhibitor of NO production, to the cocultures; no differences were noted in the ability of necrotic neutrophils to reduce macrophage infection (Fig. 7B). Therefore, parasite destruction appears to be mediated by an NO-independent mechanism.

To test whether parasite killing was mediated by superoxides, we measured the production of superoxide by chemiluminescence using lucigenin as described in Materials and Methods. After 2 h of interaction with necrotic neutrophils and *Leishmania*-infected macrophages, the superoxide production was measured for 30 min. As shown in **Fig. 8A**, superoxide levels increased significantly in the cultures, as evaluated by photon emission. No differences in the superoxide production were observed after exposing infected macrophages to necrotic neutrophils for 24 h (data not shown). We also simultaneously added catalase, an inhibitor of reactive oxygen intermediate production and necrotic neutrophils to the coculture. The addition of catalase decreased the superoxide production (Fig. 8A) and reversed the microbicidal effect of necrotic neutrophils, leading to a significant increase in parasite burden (Fig. 8B) and the percentage of infected macrophages (data not shown), suggesting that superoxide is involved in parasite killing.

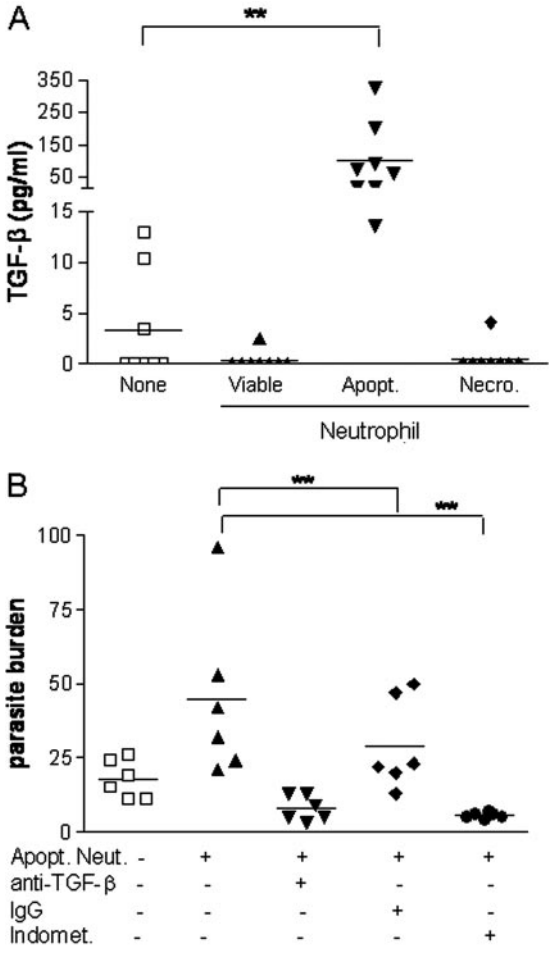

Fig. 5. TGF-β induces growth of *L. amazonensis* in human macrophages. (A) Infected macrophage monolayers were cultured with medium alone (□), with viable (▲), apoptotic (▼), or necrotic neutrophils (♦). Supernatants were collected 24 h later and assayed for the presence of TGF-β1. (B) Infected macrophages were cultured in medium alone (□) or with apoptotic neutrophils (▲), either in the presence of anti-TGF-β1 (▼), isotype control (♦), or indomethacin (●). The monolayers were stained with HE, and the parasite burden was assessed after 3 days. Each point represents a different donor, and each bar represents the mean. For statistical analysis, one-way ANOVA with Bonferroni's post-test (significant if $P<0.05$).

DISCUSSION

Our results have demonstrated that interactions with apoptotic or necrotic neutrophils cause significant changes in the para-

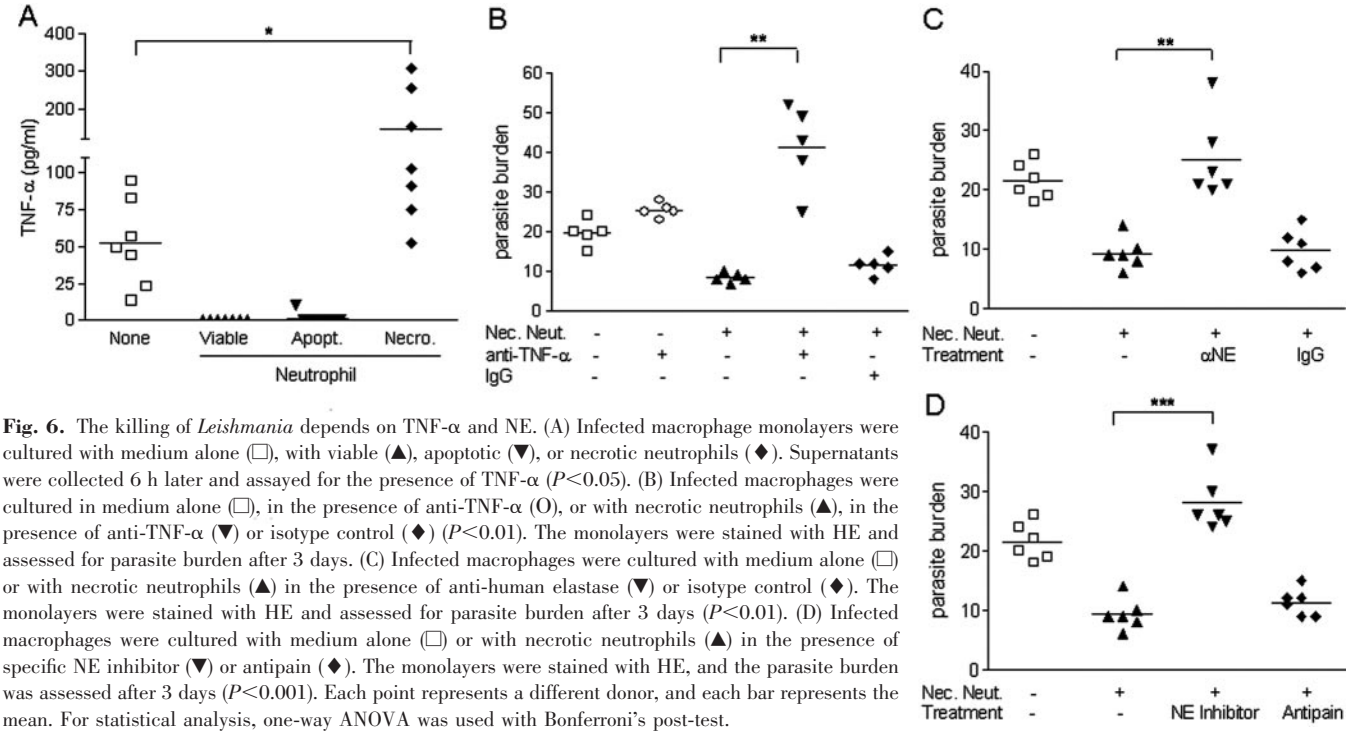

Fig. 6. The killing of *Leishmania* depends on TNF-α and NE. (A) Infected macrophage monolayers were cultured with medium alone (□), with viable (▲), apoptotic (▼), or necrotic neutrophils (♦). Supernatants were collected 6 h later and assayed for the presence of TNF-α ($P<0.05$). (B) Infected macrophages were cultured in medium alone (□), in the presence of anti-TNF-α (O), or with necrotic neutrophils (▲), in the presence of anti-TNF-α (▼) or isotype control (♦) ($P<0.01$). The monolayers were stained with HE and assessed for parasite burden after 3 days. (C) Infected macrophages were cultured with medium alone (□) or with necrotic neutrophils (▲) in the presence of anti-human elastase (▼) or isotype control (♦). The monolayers were stained with HE and assessed for parasite burden after 3 days ($P<0.01$). (D) Infected macrophages were cultured with medium alone (□) or with necrotic neutrophils (▲) in the presence of specific NE inhibitor (▼) or antipain (♦). The monolayers were stained with HE, and the parasite burden was assessed after 3 days ($P<0.001$). Each point represents a different donor, and each bar represents the mean. For statistical analysis, one-way ANOVA was used with Bonferroni's post-test.

site burden of *L. amazonensis*-infected human macrophages. The increase in parasite burden and percentage of infected macrophages induced by apoptotic neutrophils was dependent on TGF-β1 and PGE_2. In contrast, the decrease in intramacrophagic parasite burden after interaction with necrotic neutrophils was dependent on TNF-α and NE and was mediated by reactive oxygen species.

Our results also demonstrated that the infected macrophages phagocytosed apoptotic and necrotic neutrophils. Although previous studies suggested the existence of differences in the phagocytic capacity of *Leishmania*-infected macrophages [21, 22], we did not observe this in our experiments. *L. amazonensis*-infected human macrophages performed similarly to uninfected counterparts in their ability to bind and engulf viable or apoptotic neutrophils. These results suggest that changes observed in parasite burden and percentages of infected macrophages are related to intracellular signaling mechanisms triggered by interactions with apoptotic or necrotic neutrophils, rather than being attributable to the extent of phagocytosis. Although a small number of macrophages were infected by *L. amazonensis* and at the same time ingested apoptotic or necrotic PMNs, we observed significant alterations in the parasite burden in the presence of these cells. It is possible that mediators released in the culture by macrophages ingesting apoptotic or necrotic neutrophils are responsible for the observed effects.

TGF-β induced by phagocytosis of apoptotic cells inhibit proinflammatory cytokine production through autocrine and paracrine mechanisms [7, 8]. This cytokine is also implicated in the *Leishmania* infection as a parasite escape mechanism [23] Suppression of proinflammatory cytokine release induced by the clearance of apoptotic cells favored the replication of *L. major* in susceptible BALB/c mice, both in vitro and in vivo. This mechanism required TGF-β1 and PGE_2 production [15]. *L. amazonensis* amastigotes expose phosphatidylserine and mimic apoptotic cells through TGF-β1 secretion and mouse macrophage deactivation [24].

The ability to produce TGF-β1 has been determined as a potential virulence mechanism responsible for infection of

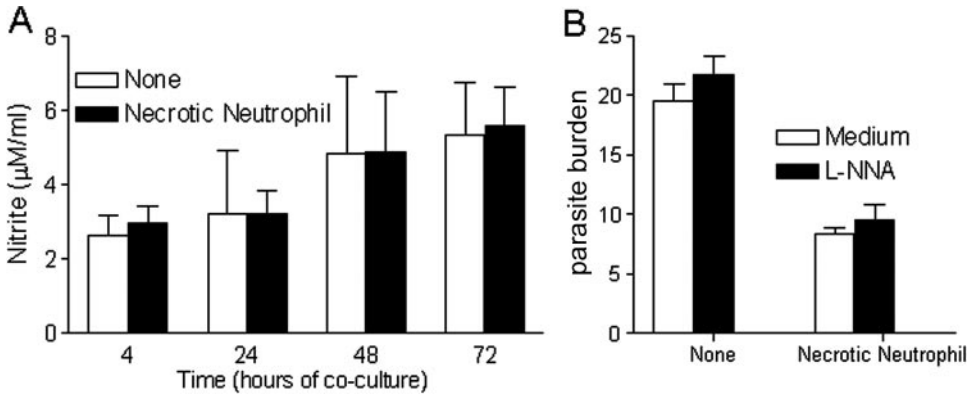

Fig. 7. Mechanism of parasite destruction is independent of NO. (A) Infected macrophages were cultured alone (open bar) or in the presence of necrotic PMN (solid bar) for 4, 24, 48, and 72 h. Supernatants were collected and assayed for NO by the Griess method. (B) Infected macrophages were cultured alone or with necrotic neutrophils, in the absence (open bars) or presence (closed bars) of L-NNA. The monolayers were stained with HE and assessed for parasite burden after 3 days. Data represent the mean and standard deviation of five individual donors. For statistical analysis, one-way ANOVA was used with Bonferroni's post-test, and no significance was found among the different groups analyzed.

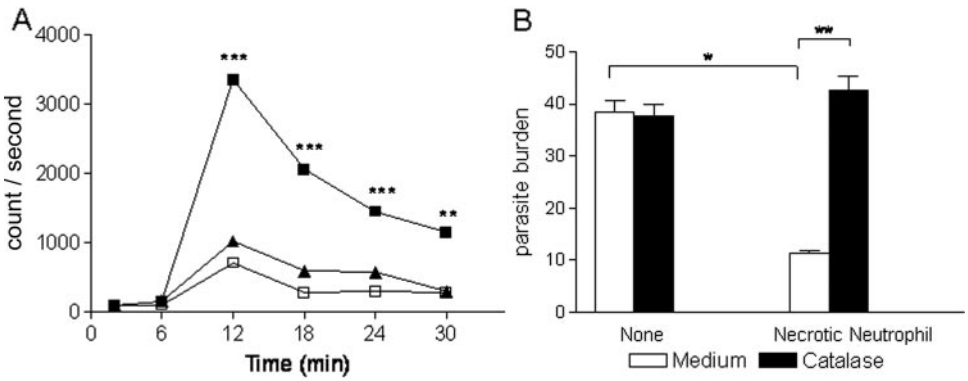

Fig. 8. Mechanism of parasite killing is dependent on region of interest. (A) Superoxide levels were measured by chemoluminescence in supernatant of *L. amazonensis*-infected macrophage cultured alone (□) or in the presence of necrotic neutrophils (■) or necrotic neutrophils and catalase (1,000 U/ml) (▲) for 2 h. The superoxide measurement was performed for 30 min after the addition of 20 μM lucigenin (**, $P<0.01$; ***, $P<0.001$). (B) Infected macrophages were cultured alone or with necrotic neutrophils, in the absence (open bars) or presence (solid bars) of catalase (1,000 U/ml). The monolayers were stained with HE, and the parasite burden assessed after 3 days. Data represent the mean and standard deviation of five individual donors. For statistical analysis, one-way ANOVA was used with Bonferroni's post-test. (*, $P<0.05$; **, $P<0.01$).

humans with *L. amazonensis* [25]. In this report, we have used this species of parasite, which is responsible for two distinct forms of cutaneous leishmaniasis: localized cutaneous leishmaniasis, represented in most cases by a single ulcerated skin lesion, and rare cases of diffuse cutaneous leishmaniasis, characterized by heavily parasitized lesions and host anergic response [26]. High levels of TGF-β1 were observed in diffuse cutaneous lesions compared with localized cutaneous lesions [27]. In this case, it is possible that continual clearance of apoptotic neutrophils and the subsequent macrophage deactivation induced by TGF-β1 favors high parasite burdens in *L. amazonensis* infections. In this regard, our results showed that interaction of apoptotic human neutrophils with *L. amazonensis*-infected macrophages resulted in release of TGF-β1 and neutralizing antibody anti-TGF-β1 reversed the increased parasite replication. Apoptotic neutrophils showed undetectable levels of TGF-β in the supernatant harvested after 18 h of culture [7], suggesting that the source of this cytokine in our model could be uninfected or infected macrophages that ingested apoptotic cells.

We should also consider that other factors resulting from the sand fly bite, such as heme [28] or inflammatory factors [29], may influence the rate of neutrophil apoptosis. Cocco and Ucker showed that macrophages bind and engulf apoptotic and necrotic cells to similar extents and with similar kinetics [30]. However, the mechanisms of recognition and uptake of these two classes of dying cells are controversial. While some reports suggest that this process occurs via distinct and noncompeting mechanisms [11, 30], others provide evidence for a common set of engulfment genes to mediate removal of both apoptotic and necrotic cell corpses [31, 32]. Both the kinetics of this process and its consequences to the outcome of *Leishmania* infection are unknown.

Recruitment of inflammatory neutrophils to lesion sites could influence this process, since NE is constitutively released from viable activated neutrophils [33]. Lysed human neutrophils induce macrophage activation by NE secretion and TNF-α production [7]. NE is implicated in tissue injury and inflammation [34], and it binds specifically to macrophages, resulting in increased chemokine production [35]. Our results support these past studies by suggesting that TNF-α and NE are implicated in the parasite destruction following the interaction of necrotic neutrophils and *L. amazonensis*-infected macrophages. NE could induce an increase in TNF-α, and then this cytokine could be responsible for the destruction of intracellular parasites. Alternatively, NE could act on macrophages through toll-like receptors [15, 35–37], activating their respiratory burst and thereby diminishing the intracellular parasite number.

In our study, we used Jurkat T lymphocytes to demonstrate that the effect observed with neutrophils was related to the content of those granules [11, 15] released into the environment, which induce macrophage activation. Indeed, necrotic Jurkat T lymphocytes did not interfere with parasite burden, suggesting that microbicidal activity induced by necrotic neutrophils was dependent on protease release. The strong inhibitory effects of a neutralizing antibody specific to NE and of a NE inhibitor suggest that NE is a major contributor in activating the macrophages and decreasing the burden of *L. amazonensis*. Although this finding further suggests an important role for release of NE in a macrophage proinflammatory response, we cannot rule out possible roles played by other molecules present in neutrophil granules [3, 38] in microbicidal mechanisms against *Leishmania*.

Production of NO is important for the control of *Leishmania* infection in mice [39], but the role of NO in human infection is controversial [40–42]. Our results showed that the presence of necrotic neutrophils did not induce any differences in NO production by *L. amazonensis*-infected human macrophages. On the other hand, superoxide was produced in cocultures of infected macrophages and necrotic neutrophils. The addition of catalase inhibited the superoxide production and reversed the necrotic neutrophil-induced decrease in parasite burden. Interestingly, in resistant C57BL/6 mice, neutrophils induced the killing of *L. major* by infected macrophages via a mechanism that requires TNF-α, NE, and ROI, but not NO [15]. However, our culture system mimics only the initial events of infection and we cannot rule out the participation of NO at later phases of infection.

In this report, we verified that the interaction of apoptotic or necrotic neutrophils with *L. amazonensis*-infected macrophages drives the initial parasite burden in these cells, which could determine the outcome of infection. This work contributes to the understanding of the mechanisms involved in the innate immunity response against *Leishmania* and may contribute to the development of new therapeutic strategies against this disease.

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REFERENCES

1. Faurschou, M., Borregaard, N. (2003) Neutrophil granules and secretory vesicles in inflammation. *Microbes Infect.* **5,** 1317–1327.
2. Laskay, T., van Zandbergen, G., Solbach, W. (2003) Neutrophil granulocytes–Trojan horses for *Leishmania major* and other intracellular microbes? *Trends Microbiol.* **11,** 210–214.
3. Nauseef, W. M. (2007) How huamn neutrophils kill and degrade microbes: an integrated view. *Immunol. Rev.* **219,** 88–102.
4. Appelberg, R. (2007) Neutrophils and intracellular pathogens: beyond phagocytosis and killing. *Trends Microbiol.* **15,** 87–92.
5. Nathan, C. (2006) Neutrophils and immunity: challenges and opportunities. *Nat. Rev. Immunol.* **6,** 173–182.
6. Savill, J., Haslett, C. (1995) Granulocyte clearance by apoptosis in the resolution of inflammation. *Semin. Cell Biol.* **6,** 385–393.
7. Fadok, V. A., Bratton, D. L., Konowal, A., Freed, P. W., Westcott, J. Y., Henson, P. M. (1998) Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. *J. Clin. Invest.* **101,** 890–898.
8. Freire-de-Lima, C. G., Xiao, Y. Q., Gardai, S. J., Bratton, D. L., Schiemann, W. P., Henson, P. M. (2006) Apoptotic cells, through transforming growth factor-beta, coordinately induce anti-inflammatory and suppress pro-inflammatory eicosanoid and NO synthesis in murine macrophages. *J. Biol. Chem.* **281,** 38376–38384.
9. Voll, R. E., Herrmann, M., Roth, E. A., Stach, C., Kalden, J. R., Girkontaite, I. (1997) Immunosuppressive effects of apoptotic cells. *Nature* **390,** 350–351.
10. Savill, J., Fadok, V. (2000) Corpse clearance defines the meaning of cell death. *Nature* **407,** 784–788.
11. Fadok, V. A., Bratton, D. L., Guthrie, L., Henson, P. M. (2001) Differential effects of apoptotic versus lysed cells on macrophage production of cytokines: role of proteases. *J. Immunol.* **166,** 6847–6854.
12. Freire-de-Lima, C. G., Nascimento, D. O., Soares, M. B., Bozza, P. T., Castro-Faria-Neto, H. C., de Mello, F. G., DosReis, G. A., Lopes, M. F. (2000) Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. *Nature* **403,** 199–203.
13. Lima, R. G., Van Weyenbergh, J., Saraiva, E. M., Barral-Netto, M., Galvao-Castro, B., Bou-Habib, D. C. (2002) The replication of human immunodeficiency virus type 1 in macrophages is enhanced after phagocytosis of apoptotic cells. *J. Infect. Dis.* **185,** 1561–1566.
14. Zamboni, D. S., Rabinovitch, M. (2004) Phagocytosis of apoptotic cells increases the susceptibility of macrophages to infection with *Coxiella burnetii* phase II through down-modulation of nitric oxide production. *Infect. Immun.* **72,** 2075–2080.
15. Ribeiro-Gomes, F. L., Otero, A. C., Gomes, N. A., Moniz-De-Souza, M. C., Cysne-Finkelstein, L., Arnholdt, A. C., Calich, V. L., Coutinho, S. G., Lopes, M. F., DosReis, G. A. (2004) Macrophage interactions with neutrophils regulate *Leishmania major* infection. *J. Immunol.* **172,** 4454–4462.
16. Tacchini-Cottier, F., Zweifel, C., Belkaid, Y., Mukankundiye, C., Vasei, M., Launois, P., Milon, G., Louis, J. A. (2000) An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with *Leishmania major*. *J. Immunol.* **165,** 2628–2636.
17. Aga, E., Katschinski, D. M., van Zandbergen, G., Laufs, H., Hansen, B., Muller, K., Solbach, W., Laskay, T. (2002) Inhibition of the spontaneous apoptosis of neutrophil granulocytes by the intracellular parasite *Leishmania major*. *J. Immunol.* **169,** 898–905.
18. Van Zandbergen, G., Klinger, M., Mueller, A., Dannenberg, S., Gebert, A., Solbach, W., Laskay, T. (2004) Cutting edge: neutrophil granulocyte serves as a vector for *Leishmania* entry into macrophages. *J. Immunol.* **173,** 6521–6525.
19. Kandil, H., Bachy, V., Williams, D. J., Helmi, R., Gotch, F. M., Ibrahim, M. A. (2005) Regulation of dendritic cell interleukin-12 secretion by tumour cell necrosis. *Clin. Exp. Immunol.* **140,** 54–64.
20. Meimaridou, E., Jacobson, J., Seddon, A. M., Noronha-Dutra, A. A., Robertson, W. G., Hothersall, J. S. (2005) Crystal and microparticle effects on MDCK cell superoxide production: oxalate-specific mitochondrial membrane potential changes. *Free Radic. Biol. Med.* **38,** 1553–1564.
21. Olivier, M., Tanner, C. E. (1987) Susceptibilities of macrophage populations to infection in vitro by *Leishmania donovani*. *Infect. Immun.* **55,** 467–471.
22. Bray, R. S., Heikal, B., Kaye, P. M., Bray, M. A. (1983) The effect of parasitization by *Leishmania mexicana mexicana* on macrophage function in vitro. *Acta Trop.* **40,** 29–38.
23. Barral-Netto, M., Barral, A., Brownell, C. E., Skeiky, Y. A., Ellingsworth, L. R., Twardzik, D. R., Reed, S. G. (1992) Transforming growth factor-beta in leishmanial infection: a parasite escape mechanism. *Science* **257,** 545–548.
24. Wanderley, J. L., Moreira, M. E., Benjamin, A., Bonomo, A. C., Barcinski, M. A. (2006) Mimicry of apoptotic cells by exposing phosphatidylserine participates in the establishment of amastigotes of *Leishmania* (L) *amazonensis* in mammalian hosts. *J. Immunol.* **176,** 1834–1839.
25. Barral, A., Teixeira, M., Reis, P., Vinhas, V., Costa, J., Lessa, H., Bittencourt, A. L., Reed, S., Carvalho, E. M., Barral-Netto, M. (1995) Transforming growth factor-beta in human cutaneous leishmaniasis. *Am. J. Pathol.* **147,** 947–954.
26. Barral, A., Costa, J. M., Bittencourt, A. L., Barral-Netto, M., Carvalho, E. M. (1995) Polar and subpolar diffuse cutaneous leishmaniasis in Brazil: clinical and immunopathologic aspects. *Int. J. Dermatol.* **34,** 474–479.
27. Diaz, N. L., Arvelaez, F. A., Zerpa, O., Tapia, F. J. (2006) Inducible nitric oxide synthase and cytokine pattern in lesions of patients with American cutaneous leishmaniasis. *Clin. Exp. Dermatol.* **31,** 114–117.
28. Arruda, M. A., Rossi, A. G., de Freitas, M. S., Barja-Fidalgo, C., Graca-Souza, A. V. (2004) Heme inhibits human neutrophil apoptosis: involvement of phosphoinositide 3-kinase, MAPK, and NF-κB. *J. Immunol.* **173,** 2023–2030.
29. Lee, A., Whyte, M. K., Haslett, C. (1993) Inhibition of apoptosis and prolongation of neutrophil functional longevity by inflammatory mediators. *J. Leukoc. Biol.* **54,** 283–288.
30. Cocco, R. E., Ucker, D. S. (2001) Distinct modes of macrophage recognition for apoptotic and necrotic cells are not specified exclusively by phosphatidylserine exposure. *Mol. Biol. Cell* **12,** 919–930.
31. Chung, S., Gumienny, T. L., Hengartner, M. O., Driscoll, M. (2000) A common set of engulfment genes mediates removal of both apoptotic and necrotic cell corpses in *C. elegans*. *Nat. Cell Biol.* **2,** 931–937.
32. Gregory, C. D., Devitt, A. (2004) The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? *Immunology* **113,** 1–14.
33. Rainger, G. E., Rowley, A. F., Nash, G. B. (1998) Adhesion-dependent release of elastase from human neutrophils in a novel, flow-based model: specificity of different chemotactic agents. *Blood* **92,** 4819–4827.
34. Lee, W. L., Downey, G. P. (2001) Leukocyte elastase: physiological functions and role in acute lung injury. *Am. J. Respir. Crit. Care Med.* **164,** 896–904.
35. Ishihara, K., Yamaguchi, Y., Okabe, K., Ogawa, M. (1999) Neutrophil elastase enhances macrophage production of chemokines in receptor-mediated reaction. *Res. Commun. Mol. Pathol. Pharmacol.* **103,** 139–147.
36. Greene, C. M., Carroll, T. P., Smith, S. G., Taggart, C. C., Devaney, J., Griffin, S., O'Neill, S., Mc, J., Elvaney, N. G. (2005) TLR-induced inflammation in cystic fibrosis and non-cystic fibrosis airway epithelial cells. *J. Immunol.* **174,** 1638–1646.
37. Tsujimoto, H., Ono, S., Majima, T., Kawarabayashi, N., Takayama, E., Kinoshita, M., Seki, S., Hiraide, H., Moldawer, L. L., Mochizuki, H. (2005) Neutrophil elastase, MIP-2, and TLR-4 expression during human and experimental sepsis. *Shock* **23,** 39–44.
38. Borregaard, N., Sorensen, O. E., Theilgaard-Monch, K. (2007) Neutrophil granules: a library of innate immunity proteins. *Trends Immunol.* **28,** 340–345.
39. Murray, H. W., Nathan, C. F. (1999) Macrophage microbicidal mechanisms in vivo: reactive nitrogen versus oxygen intermediates in the killing of intracellular visceral *Leishmania donovani*. *J. Exp. Med.* **189,** 741–746.
40. Erel, O., Kocyigit, A., Bulut, V., Gurel, M. S. (1999) Reactive nitrogen and oxygen intermediates in patients with cutaneous leishmaniasis. *Mem. Inst. Oswaldo Cruz* **94,** 179–183.
41. Gantt, K. R., Goldman, T. L., McCormick, M. L., Miller, M. A., Jeronimo, S. M., Nascimento, E. T., Britigan, B. E., Wilson, M. E. (2001) Oxidative responses of human and murine macrophages during phagocytosis of *Leishmania chagasi*. *J. Immunol.* **167,** 893–901.
42. Serarslan, G., Yilmaz, H. R., Sogut, S. (2005) Serum antioxidant activities, malondialdehyde and nitric oxide levels in human cutaneous leishmaniasis. *Clin. Exp. Dermatol.* **30,** 267–271.