Neutrophils Drive Leishmania Infection

Flávia L. Ribeiro-Gomes,1 Maria Carolina A. Moniz-de-Souza,1 Valeria M. Borges,1 Marise P. Nunes,2
Marcio Mantuano-Barradas,1 Heloisa D’Ávila,1 Patricia T. Bozza,2 Vera L. Calich,4 and George A. DosReis1

1Instituto de Biofísica Carlos Chagas Filho, Federal University of Rio de Janeiro, and 2Instituto Oswaldo Cruz, Fiocruz, Rio de Janeiro,
3Centro de Pesquisas Gonçalo Moniz, Fiocruz, Salvador, and 4Instituto de Ciências Biomédicas, University of São Paulo, São Paulo, Brazil

Apoptosis mediated by Fas ligand (FasL) initiates inflammation characterized by neutrophilic infiltration. Neutrophils undergo apoptosis and are ingested by macrophages. Clearance of dead neutrophils leads to prostaglandin- and transforming growth factor–β–dependent replication of Leishmania major in macrophages from susceptible mice. How L. major induces neutrophil turnover in a physiological setting is unknown. We show that BALB/c FasL-sufficient mice are more susceptible to L. major infection than are FasL-deficient mice. FasL promotes the apoptosis of infected resident macrophages and attracts neutrophils. Furthermore, FasL-sufficient neutrophils exacerbate L. major replication in macrophages, whereas FasL-deficient neutrophils induce parasite killing. These contrasting effects are due to delaying apoptosis and the clearance of FasL-deficient neutrophils. The transfer of neutrophils exacerbates infection in FasL-sufficient mice but reduces infection in FasL-deficient mice. Depletion of neutrophils abolishes the susceptibility of FasL-deficient mice. These data illustrate a deleterious role of the FasL-mediated turnover of neutrophils on L. major infection.

Neutrophils play a nonphagocytic immunoregulatory role in parasitic infections [1]. Indeed, neutrophils secrete reactive oxidants, cytokines, chemokines, and proteases that act on immune and inflammatory cells. However, the life span of neutrophils is tightly controlled by phagocytic clearance, and extended neutrophil survival leads to inflammation [2]. Neutrophils undergo constitutive apoptosis and are ingested by macrophages [3]. The ingestion of apoptotic neutrophils inactivates macrophages through the secretion of prostaglandin and transforming growth factor (TGF)–β [4]. Furthermore, the phagocytic clearance of dead cells drives the TGF-β–dependent replication of protozoal parasites in macrophages [5, 6]. Therefore, the turnover of inflammatory neutrophils could drive infection by intracellular pathogens that use macrophages as host cells. Neutrophils play a deleterious role in infection with Leishmania major [6, 7]. The clearance of dead neutrophils leads to prostaglandin- and TGF-β–dependent replication of L. major in macrophages [6]. However, how L. major induces the turnover of neutrophils in a physiological setting is unknown. Fas ligand (FasL) is a membrane-bound and shed surface protein that belongs to the tumor necrosis factor gene family and is the natural counterreceptor for the Fas death receptor [8]. Apoptosis initiated by FasL leads to inflammation mediated by neutrophils [9–12]. We show that BALB/c FasL–sufficient mice are more susceptible to L. major infection than are FasL–deficient mice. Infection induces FasL–dependent resident macrophage apoptosis and attracts neutrophils. Compared with FasL–sufficient neutrophils, delayed apoptosis and clearance of FasL–deficient neutrophils leads to enhanced macrophage control of parasite replication. The present data show a deleterious role of the FasL–mediated turnover of neutrophils on L. major infection.
MATERIALS AND METHODS

Mice, parasites, and infection. BALB/c mice—either wild-type (wt) or FasL-deficient gld mutant mice [11]—were from the Oswaldo Cruz Institute animal care facility. Mice were infected subcutaneously with 2 × 10⁵ L. major strain LV39 (MRHO/Sv/59/P) promastigotes. Disease was scored by footpad swelling as measured by a micrometric caliper and by parasite burden in lymph-node cells, as described elsewhere [6]. Briefly, after 41 days of infection, draining lymph nodes of individual mice were collected, and lymph-node cells were suspended at 1 × 10⁶ viable cells/mL in serum-free Dulbecco’s modified Eagle medium (DMEM). Cells were serially diluted in triplicate in supplemented Schneider medium [13] and were incubated at 26°C. Parasite load was measured by counting viable promastigotes after 7 days in culture. For the measurement of rapid cellular responses to infection, mice were injected intraperitoneally (ip) with saline or with 1 × 10⁵ promastigotes. Twenty hours later, peritoneal exudate cells were collected. Aliquots were stained by Giemsa to determine differential leukocyte counts. Cells were treated with FcBlock (BD Biosciences), stained with fluorescein isothiocyanate–labeled antibody specific for F4/80 (Caltag) and phycoerythrin-labeled antibodies specific for Mac-1 or FasL (BD Biosciences), and analyzed by flow cytometry (FCM). Cells were also adhered to plastic wells, and adherent macrophages were transferred to supplemented Schneider medium [13]. After 5 days, the intracellular load of parasites was determined as described elsewhere [14]. Parasite loads from different mice were normalized for a fixed number of 10³ macrophages, after individual proportions of macrophages were measured in stained aliquots of peritoneal exudate cells. All experiments were approved by an institutional committee for the humane care and use of animals.

Macrophage apoptosis and chemokine secretion. Peritoneal macrophages were infected in vitro with L. major promastigotes at a ratio of 1:10 for 20 h in the presence of IgG or a neutralizing antibody specific for FasL (5 μg/mL, clone MFL3; BD Biosciences) or in the presence of Fas/Fc or control CD30/Fc chimeric proteins (0.5 μg/mL; R&D Systems). Culture medium consisted of DMEM supplemented with 10% fetal calf serum (FCS). To determine the number of adhered macrophages, cultures done under glass coverslips were washed and stained with Giemsa. To analyze DNA fragmentation and chemokine secretion, cultures were done on plastic. Supernatants were assayed for fragmented DNA on agarose gels as described elsewhere [15] and for chemokines by sandwich ELISAs according to the manufacturers’ instructions (BD for macrophage inflammatory protein [MIP]–1α and R&D Systems for KC).

Neutrophils, inflammatory macrophages, and infection. Neutrophils were collected 7 h after the ip injection of thioglycollate broth into noninfected mice (Diço). Neutrophils were depleted of adherent cells [16] and further purified by Percoll density-gradient centrifugation [17], yielding cells that were >95% Gr-1 positive by FCM. Neutrophils were killed by heating for 30 min at 56°C, as described elsewhere [18], yielding cells with characteristics of late apoptotic cells—that is, condensed chromatin but permeable to trypan blue. Inflammatory macrophages were collected 4 days after the ip injection of thioglycollate into noninfected mice. Exudate cells were infected with L. major promastigotes (1:10) in DMEM–10% FCS. After 4 h, extracellular parasites and nonadherent cells were removed. Macrophages were incubated with live or killed neutrophils in 48-well vessels that contained 0.5 mL of DMEM supplemented with 1% Nutridoma-SP (Boehringer-Mannheim), instead of FCS. Hamster IgG, an antibody specific for FasL (5 μg/mL), TGF-β receptor II/Fc chimeric protein, thrombopoietin receptor/Fc chimeric protein (0.5 μg/mL; both from R&D Systems), deferoxamine (100 mmol/L; Sigma), and L-N6-(1-iminoethyl)-lysine (L-NIL; 1 mmol/L; Sigma) were added. After 3 days, cultures were washed, macrophages were transferred to Schneider medium, and the intracellular load of parasites was measured as described elsewhere [6, 14]. In some experiments, the levels of nitrite production were measured by the Griess reaction [5] in culture supernatants obtained after 2 days in culture (5 × 10³ macrophages plus 5 × 10⁴ neutrophils were cultured).

Transfer and depletion of neutrophils. Mice were infected with L. major in both footpads and received 4 × 10⁴ syngeneic neutrophils in the right footpad. After 10 days, parasite loads were determined in left and right draining lymph nodes [6]. To deplete neutrophils, mice received 5 ip injections of 100 μg of IgG or an antibody specific for Gr-1 [19], given at days 1, 2, 5, 8, and 11 after L. major infection. After 13 days, parasite loads were determined in draining lymph nodes [6].

Statistical analysis. The in vitro cultures were done in triplicate, and each experiment was repeated at least twice. Data represent the mean ± SE of representative experiments. For in vivo experiments, data show means and values from individual mice. Data derived from neutrophil recruitment and ex vivo parasite loads of macrophages were normalized by square-root transformation before statistical analysis. Data were analyzed by Student’s t test for independent samples by use of SigmaPlot for Windows (version 4.0; SPSS). Differences with P < .05 were considered to be significant.

RESULTS

Responses of FasL-deficient mice to L. major infection. We asked whether FasL regulates infection by L. major. We compared FasL-sufficient (wt) and FasL-deficient (gld) BALB/c mice for susceptibility to L. major infection. We found that gld mice had significant reductions in footpad lesion size at 9, 16, and 34 days after infection (figure 1A–1C). After 41 days, the parasite load in lymph nodes from FasL-sufficient mice was 4–5-
Neutrophils Drive Leishmania Infection

Figure 1. Fas ligand expression and increased susceptibility to Leishmania major infection. A–C, Footpad increase in wild-type (wt) and gld mice (4 animals each) after the indicated days of infection (day 9, P < .05; days 16 and 34, P < .01). D, Parasite loads in draining lymph-node cells (LNC) after 41 days of infection (P < .01). Each point represents an individual mouse.

Figure 2. Leishmania major (Lsh)–induced demise of Mac-1hi macrophages in Fas-ligand (FasL)–sufficient mice. A and B, Percentages of Mac-1hi macrophages after an intraperitoneal (ip) injection of saline PBS or Lsh in wt mice. C and D, Percentages as in panels A and B for gld mice. E, Percentages of FasL-positive cells in Mac-1hi macrophages from wt mice injected with saline PBS or Lsh. Experiment representative of 3 experiments with similar results.

c-fold higher than that in lymph nodes from FasL-deficient mice (figure 1D).

We investigated whether L. major infection induces the FasL-dependent death of resident macrophages. Indeed, the ip injection of L. major promastigotes into wt mice markedly reduced the number of peritoneal Mac-1hi macrophages, as measured by FCM (see figure 2A and 2B for a representative experiment: 22.5% ± 2.7% Mac-1hi F4/80+ cells after PBS and 8.5% ± 1.9% cells after L. major, 3 mice/group). Injection into FasL-deficient mice did not induce any loss of Mac-1hi macrophages (see figure 2C and 2D for a representative experiment: 20.1% ± 0.6% Mac-1hi F4/80+ cells after PBS and 19.3% ± 3.4% cells after L. major, 3 mice/group). Injection of L. major up-regulated FasL expression in the remaining Mac-1hi macrophages from wt mice (figure 2E), whereas Mac-1lo and Mac-1–negative cells did not up-regulate FasL expression. We investigated the death of resident peritoneal macrophages in vitro. Culture with L. major in the presence of control hamster IgG induced a 50% cell loss in adherent macrophages from wt mice after 20 h (figure 3A, left). Macrophage cell loss was completely prevented by a neutralizing antibody specific for FasL (figure 3B). Culture of resident macrophages from wt mice with L. major for 8 h increased secretion of the chemokine KC, and KC secretion could be blocked by Fas/Fc but not by a control chimeric protein (figure 3C). In contrast, culture of macrophages from gld mice with L. major did not increase KC secretion above background levels (data not shown). Furthermore, treatment of wt macrophages with L. major for 20 h induced the secretion of the chemokine MIP-1α (figure 3D), and MIP-1α secretion could be blocked with a neutralizing antibody specific for FasL (figure 3D). Injection of L. major promastigotes induced the ip extravasation of neutrophils in wt mice (figure 3E). Background ip levels of neutrophils were higher in gld mice (figure 3E). However, L. major did not increase numbers of neutrophils in gld mice (figure 3E). We also investigated the intracellular parasite load of peritoneal macrophages recov-
Figure 3. *Leishmania major* (Lsh) induction of resident macrophage apoptosis and neutrophil extravasation in Fas-ligand (FasL)–sufficient mice. A, *L. major* promastigotes induction of cell loss in macrophages from wild-type (wt) mice in vitro (*P* < .01) in the presence of hamster IgG (ham IgG) but not in the presence of antibody specific for FasL (anti-FasL). Right, No induction by Lsh of cell loss in macrophages from gld mice.

B, Prevention of DNA fragmentation in supernatants from macrophages treated with Lsh by soluble Fas/Fc but not by a control CD30/Fc construct.

C, Blocked secretion of KC elicited by Lsh in macrophages (white bar) by Fas/Fc (black bar; *P* < .05) but not by a control CD30/Fc construct (gray bar). D, Prevention of secretion of macrophage inflammatory protein (MIP)–1α induced by Lsh in macrophages an antibody specific for anti-FasL (*P* < .01).

E, Neutrophil extravasation in wt (*P* < .05) but not in gld mice. F, Higher parasite load in macrophages infected in vivo with *L. major* in wt vs. gld mice (*P* < .05). Data in A–D are representative of at least 2 independent experiments with similar results. Data in E and F are mean and values from individual mice.

**Requirement of FasL for rapid neutrophil apoptosis and increased parasite replication.** We investigated how infected macrophages control *L. major* replication after interaction with highly purified neutrophils from wt and gld mice. Inflammatory macrophages were resistant to apoptosis in the presence of parasites or neutrophils (data not shown). Inflammatory macrophages infected with *L. major* amastigotes recognized and ingested dead neutrophils (figure 4A). In agreement with previous results [6], both live and dead neutrophils from wt mice exacerbated *L. major* replication in macrophages from wt mice (figure 4B). However, live neutrophils from gld mice reduced *L. major* replication in gld macrophages (figure 4B). Dead neutrophils from gld mice exacerbated parasite replication in a manner similar to neutrophils from wt mice (figure 4B). Cell-mixing experiments demonstrated that live neutrophils, and not macrophages, regulate opposite outcomes of *L. major* infection (figure 4B).

We investigated the role of FasL in the spontaneous death of neutrophils from wt mice. In the presence of control IgG, highly purified inflammatory neutrophils rapidly died in vitro, with a *t* 1/2 of 4.8 h (figure 4C, left). After 24 h, no viable cells were left. In the presence of a neutralizing antibody specific for FasL, neutrophils still underwent spontaneous death. However, anti-FasL antibody induced a significant delay in spontaneous apoptosis that had a *t* 1/2 of 8.0 h (figure 4C, middle). Furthermore, 15% of neutrophils remained viable after 24 h. In agreement with these results, neutrophils from gld mice underwent delayed spontaneous death, compared with that in neutrophils from wt mice (figure 4C, right). Therefore, FasL significantly accelerates the rate of neutrophil apoptosis. We investigated whether the shortening of the neutrophil life span as mediated by FasL was responsible for the increased replication of *L. major* in macrophages. Indeed, an antibody specific for FasL completely prevented the exacerbation of macrophage parasite growth that had been induced by live neutrophils (figure 4D). Anti-FasL antibody had no effect on inflammatory macrophages alone or on the deleterious effect promoted by...
dead neutrophils (figure 4D). Next, we compared the rate of clearance of live neutrophils from wt and gld mice by infected macrophages from wt mice (figure 4E). In the presence of infected macrophages, the spontaneous death of neutrophils was delayed, and viable cells were recovered after 20 h in culture. However, the recovery of neutrophils from gld mice was significantly higher than that of neutrophils from wt mice (figure 4E), which indicates that Fasl accelerates the clearance of neutrophils by macrophages.

Macrophage interactions with neutrophils from wt and gld mice were mechanistically and molecularly distinct. Parasite growth induced by neutrophils from wt mice required cell contact with macrophages, but parasite killing induced by live neutrophils from gld mice did not. Furthermore, parasite growth induced by neutrophils from wt mice could be blocked with a soluble TGF-β receptor chimeric construct (figure 4F, left). Parasite killing induced by neutrophils from gld mice did not involve TGF-β (figure 4F, right). Parasite growth driven by neutrophils from wt mice was independent of the production of both NO and reactive oxygen species (figure 4G, left). Parasite killing induced by neutrophils from gld mice was dependent of oxygen species but dependent on NO production, given that killing was prevented by the addition of the NO synthase inhibitor L-NIL (figure 4G, right). To investigate whether neutrophils from gld mice induce increased NO secretion, macrophages from wt mice were cultured with neutrophils from either wt or gld mice, and the levels of nitrites in culture were measured by the Griess reaction. The results of 1 experiment, which were reproduced in a repeat experiment, confirmed that neutrophils from gld mice induce increased NO production by
macrophage/neutrophil cultures (macrophages alone from wt mice, 2.22 ± 0.12 μmol/L nitrites; macrophages plus neutrophils from wt mice, 6.95 ± 0.12 μmol/L nitrites; macrophages plus neutrophils from gld mice, 23.01 ± 4.49 μmol/L nitrites [P < .05 for neutrophils from wt vs. gld mice]; neutrophils alone from wt mice, 1.40 ± 0.20 μmol/L nitrites; and neutrophils alone from gld mice, 2.22 ± 0.08 μmol/L nitrites). These data suggest that extended neutrophil survival controls parasite replication through the release of soluble mediators that promote NO production in macrophages.

**In vivo infection driven by FasL expression by neutrophils.**

We investigated the role of FasL in the function of neutrophils in vivo. We transferred inflammatory neutrophils to the right footpad of wt and gld mice that had been injected with *L. major* in both footpads. The transfer of neutrophils from wt mice markedly exacerbated *L. major* infection in wt recipients (figure 5A). However, the transfer of neutrophils from gld mice reduced the parasite burden in gld recipients (figure 5B). We also depleted wt and gld mice of neutrophils. In mice treated with control IgG, parasite loads were 4-fold higher in wt mice, compared with those in gld mice (figure 5C). Neutrophil depletion markedly reduced parasite loads in wt mice (8-fold) but marginally increased parasite loads in gld mice (figure 5C). As a result, after neutrophil depletion, parasite loads were 5-fold lower in wt mice, compared with those in gld mice (figure 5C). Therefore, neutrophils are responsible for the divergent phenotypes of wt and gld mice. Only neutrophils expressing FasL are deleterious to the host.

**DISCUSSION**

Our results have demonstrated that the turnover of neutrophils as mediated by FasL is exploited by *L. major* to drive infection in susceptible hosts. The phagocytic removal of apoptotic neutrophils inactivates macrophages through the secretion of prostaglandin and TGF-β [4]. Furthermore, interactions with dead neutrophils lead to prostaglandin- and TGF-β–dependent replication of *L. major* in macrophages [6]. Therefore, the turnover of inflammatory neutrophils could drive infection by *L. major*. However, how *L. major* induces neutrophil turnover in a physiological setting is unknown. Because the apoptosis induced by FasL attracts neutrophils [9–12], we investigated the role of FasL in infected, susceptible mice.

Our results have indicated that the expression of FasL increases susceptibility to *L. major* infection at 2 distinct steps of neutrophil turnover. First, *L. major* induces the FasL-dependent apoptosis of Mac-1+ resident macrophages, concomitant with chemokine secretion and neutrophil extravasation. A previous study showed similar results after the injection of vesicles that contained FasL into healthy mice [12]. Our data have demonstrated that the increased secretion of KC and MIP-1α promoted by *L. major* is dependent on FasL. However, previous studies have demonstrated that FasL engagement in macrophages leads to the secretion of additional cytokines and chemokines, such as interleukin-1β, MIP-2, and MIP-1β [12]. The chemokines important for neutrophil recruitment in our model were not determined. Furthermore, resident macrophages exposed to *L. major*, but not control macrophages, expressed FasL. Further experiments are necessary to characterize the mechanism of FasL expression that is induced by *L. major*.

Second, our results have demonstrated that FasL accelerates the rate of constitutive neutrophil death and that this accelerated death is essential for *L. major* replication within macrophages. Previous studies have implicated FasL in neutrophil apoptosis, either directly [20] or indirectly through the secretion of soluble FasL by macrophages [21]. Our results agree with an autocrine role of FasL in neutrophil apoptosis [20]. The tissue life span determines the inflammatory properties of neutrophils [2]. Delays in apoptosis prolongs the functional longevity of neutrophils [22], leading to neutrophil accumulation and inflammation [23, 24]. However, the onset of apoptosis down-regulates

---

**Figure 5.** Neutrophils (polymorphonuclear cells [PMNs]) expressing Fas ligand (FasL) and exacerbation of *Leishmania major* (Lsh) infection. A and B, Local transfer of syngeneic neutrophils and exacerbation of infection by *L. major* in draining lymph-node cells (LNC) of wild-type (wt) mice (A; *P < .01*), but reduction of infection in gld mice (B; *P < .01*). Data are from individual mice and were amplified for gld mice (inset). C, Greater susceptibility of FasL-sufficient (wt) mice to *L. major* infection than that of FasL-deficient (gld) mice (parasite loads, *P < .05*); and increased susceptibility is abolished by treatment with a depleting antibody specific for neutrophils (anti-PMN) but not by control IgG (rat IgG). **P < .01.**
the proinflammatory activities of neutrophils [25, 26]. Contrary to neutrophils from wt mice, live neutrophils from gld mice underwent delayed apoptosis, which resulted in the delayed clearance of neutrophils and increased macrophage control over parasite replication. In agreement with this, neutralizing anti-FasL antibody delayed neutrophil death in wt mice and markedly reduced parasite replication in macrophages.

In agreement with the in vitro results, granulocyte depletion by treatment with anti–Gr-1 antibody abolished the increased susceptibility of wt mice to infection, which suggests a deleterious role of neutrophils in vivo. Although neutrophil depletion with anti–Gr-1 has been used in several studies, we cannot disregard the involvement of other myeloid cell populations that express the Gr-1 antigen [27, 28]. Parasite loads in gld mice treated with anti–Gr-1 were higher than those in wt mice. One possibility to explain this finding would be that, in the absence of neutrophils, functional abnormalities of T lymphocytes in gld mice [29] are responsible for the deficient control of L. major infection.

Recently, an alternative deleterious effect of neutrophils has been described in human cells [30]. Neutrophils infected with L. major undergo apoptosis and are ingested by macrophages, which leads to the transfer of parasites to macrophages [30]. In the present study, we did not investigate this mechanism. Instead, we used uninfected neutrophils cultured with infected macrophages. It is likely that both mechanisms coexist at the site of infection. However, the efficiency of parasite transfer from dying neutrophils remains to be investigated in mice.

In contrast to our results, studies in mice genetically resistant to L. major have indicated a protective role of FasL. [31, 32]. Recently, we [6] and others [33] have demonstrated distinct populations of type 1 and type 2 neutrophils that direct macrophage activation to microbicidal or alternatively activated states, respectively. Contrary to that of neutrophils in BALB/c mice, the clearance of neutrophils in B6 mice is proinflammatory and leishmanicidal [6]. These findings could explain a protective role of FasL in resistant strains [31, 32]. Our data indicate immunoregulatory roles of FasL in parasite infection that include the promotion of resident macrophage apoptosis, neutrophil recruitment, and rapid phagocytic removal of senescent neutrophils. Apoptosis of resident macrophages as mediated by FasL could represent a general signal that alerts the immune system of the presence of an invading pathogen. These findings could be helpful in the design of new therapies for L. major infection in genetically susceptible individuals.

Acknowledgments

We thank Christina Takiya, for photomicrographs, and Eduardo Pinto, for help with L. major infection (both from Federal University of Rio de Janeiro).

References

31. Huang FP, Xu D, Esfandiari EO, Sands W, Wei XQ, Liew FY. Mice defective in Fas are highly susceptible to Leishmania major infection despite elevated IL-12 synthesis, strong Th1 responses, and enhanced nitric oxide production. J Immunol 1998; 160:4143–7.