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Neutrophils have a protective role during early stages of *Leishmania amazonensis* infection in BALB/c mice

L. M. A. SOUSA¹, M. B. H. CARNEIRO¹, M. E. RESENDE¹, L. S. MARTINS², L. M. DOS SANTOS¹, L. G. VAZ¹, P. S. MELLO¹, D. M. MOSSER³, M. A. P. OLIVEIRA², and L. Q. VIEIRA¹

¹Departamento de Bioquímica e Imunologia, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil

²Instituto de Patologia Tropical, Universidade Federal de Goiás, Goiânia, GO, Brazil

³Maryland Pathogen Research Institute, University of Maryland, College Park, MD, USA

SUMMARY

Neutrophils are involved in the early stages of immune responses to pathogens. Here, we investigated the role of neutrophils during the establishment of *Leishmania amazonensis* infection in BALB/c and C57BL/6 mice. First, we showed an accumulation of neutrophils between 6 and 24 h post-infection, followed by a reduction in neutrophil numbers after 72 h. Next, we depleted neutrophils prior to infection using RB6-8C5 or 1A8 mAb. Neutrophil depletion led to faster lesion development, increased parasite numbers and higher arginase activity during the first week of infection in BALB/c mice, but not in C57BL/6 mice. Increased susceptibility was accompanied by augmented levels of anti-*L. amazonensis* IgG and increased production of IL-10 and IL-17. Because IL-10 is a mediator of susceptibility to *Leishmania* infection, we blocked IL-10 signalling in neutrophil-depleted mice using anti-IL-10R. Interestingly, inhibition of IL-10 signalling abrogated the increase in parasite loads observed in neutrophil-depleted mice, suggesting that parasite proliferation is at least partially mediated by IL-10. Additionally, we tested the effect of IL-17 in inflammatory macrophages and observed that IL-17 increased arginase activity and favoured parasite growth. Taken together, our data indicate that neutrophils control parasite numbers and limit lesion development during the first week of infection in BALB/c mice.

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Correspondence: Leda Q. Vieira, Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antonio Carlos 6627, 31270-901 Belo Horizonte, MG, Brazil (lqvieira@icb.ufmg.br).

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AUTHORS CONTRIBUTIONS

Sousa, L.M.A. performed most of the experiments, designed the research study, analysed the data and wrote the article. Carneiro, M.B.H. helped perform and analyse the data from the *in vivo* studies. Resende, M.E. and Vaz, L. G. helped perform the *in vivo* studies. Mello, P. S. performed experiments required by referees. Martins, L.S. performed experiments using peritoneal macrophages. Dos Santos, L.M. helped perform and analyse all flow cytometry analysis. Mosser, D.M. contributed essential tools, designed experiments using BMM/φ and critically revised the article. Oliveira, M.A.P. designed experiments using peritoneal macrophages and critically revised the article. Vieira, L.Q. designed the research study, analysed the data, critically revised several drafts of the article and submitted the article.

Keywords

IL-10; immune response; Leishmania amazonensis; neutrophils

INTRODUCTION

Neutrophils provide the first line of defence against infections through their capacity to kill parasites by phagocytosis, formation of neutrophil extracellular traps (NETs) and release of the content of their granules (lytic enzymes, antimicrobial peptides) (1, 2). In addition to their role in innate immunity, immunomodulatory roles for neutrophils during microbial infection have been identified (3–8). Neutrophils have been reported to produce a number of cytokines and chemokines, and to contribute to the recruitment and activation of different cell types, including dendritic cells (3, 7, 8), and influence the outcome of the subsequent T cell-dependent immunity (3).

The role of neutrophils in infections with *Leishmania* has been mostly studied in the murine model of cutaneous leishmaniasis produced by subcutaneous injection of *Leishmania major* (9–11). This model of infection is very useful to study the immune response associated with lesion healing in C57BL/6 mice, which are resistant to infection and develop Th1 responses, and also the events related to the susceptibility to the parasite, as seen in BALB/c mice, which develop a Th2 response (12). Transient depletion of neutrophils prior to infection with *L. major* in BALB/c mice prevented the early burst of IL-4 in draining lymph nodes, leading to a decreased Th2 response and partial resolution of the lesions (11). In contrast, when a different strain of *L. major* and a different antibody to deplete neutrophils were used, exacerbated lesions and increased parasite burdens were found in BALB/c mice (9). Thus, in different studies, neutrophils were implicated in susceptibility (11, 13) or resistance (9, 10) to *L. major* in BALB/c mice. On the other hand, depletion of neutrophils in mice resistant to *L. major* led to a slight increase in lesion size during the first weeks of infection, although it did not change the final outcome of the disease, which for this mouse strain would be complete healing of lesions (9–11, 13).

In contrast to *L. major* infection, most of the lineages of mice are susceptible to *Leishmania amazonensis* (including C57BL/6 mice), presenting chronic nonhealing lesions. In *L. amazonensis* infection, resistance and susceptibility are not strictly associated with the development of a Th1 and a Th2 immune response, respectively. Many factors seem to be associated with susceptibility to *L. amazonensis*. First, the parasite is able to inhibit the maturation of DCs and subsequent production of IL-12 by these cells (14). This may prevent the development of a strong Th1 response (15, 16). In effect, it has been shown that T cells have a pathogenic role during *L. amazonensis* infection (17, 18). Surprisingly, it has been demonstrated that IFN- γ has an ambiguous role during the infection of macrophages with *L. amazonensis in vitro*; depending on the concentration of the cytokine, it may cause parasite death or parasite growth (19). In addition to the failures in acquired immune response, effector functions of macrophages are also compromised. The parasite has developed adaptive strategies to escape host defences including the activation of NF- κ B repressor complex, which suppresses iNOS expression by infected macrophages, favouring the

establishment and success of *L. amazonensis* infection (20). Besides, macrophage killing of *L. amazonensis* amastigotes requires high levels of nitric oxide and also superoxide (21, 22).

Although many aspects of the immune response to *L. amazonensis* have been unveiled, little is known about the early events after *L. amazonensis* infection. *In vitro* interaction between apoptotic or necrotic human neutrophils and macrophages infected with *L. amazonensis* has recently been studied (23). In that work, it was demonstrated that the interaction with apoptotic neutrophils favoured increase in parasite burden inside macrophages through a mechanism dependent on PGE₂ and TGF- β , whereas the uptake of necrotic neutrophils induced elimination of the parasite, which was dependent on neutrophil elastase and TGF- α (23). Another recent work has shown that the interaction between murine neutrophils and *L. amazonensis*-infected macrophages contributes to parasite elimination through the production of TGF- α and PAF (24). However, these studies do not address the role of neutrophils during *L. amazonensis* infection *in vivo*. Therefore, the aim of this study was to investigate the role of neutrophils during the early stages of *L. amazonensis* infection. We demonstrate that neutrophils migrate to the site *L. amazonensis* infection during the first 24 h post-infection and that they control both parasite load and lesion development during the first week after infection in BALB/c mice, but not in C57BL/6 mice. We also show that in the absence of neutrophils, there was early augmented secretion of IL-10 that mediated the increase in parasite burden but not lesion development. IL-17 was also increased early in infection and it favoured arginase activity and *Leishmania* growth in inflammatory macrophages. These data suggest that neutrophils restrain the early development of disease in *L. amazonensis*-infected BALB/c mice.

MATERIALS AND METHODS

Mice, parasites and infections

Female BALB/c and C57BL/6 mice were purchased from CEBIO (Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, MG, Brazil) or from the animal facility at the Instituto de Patologia Tropical e Saúde Pública (Universidade Federal de Goiás, Goiânia, GO, Brazil). Animals were kept in a conventional animal care facility at controlled temperature, light/dark cycles and environmental barriers. All mice were between 6 and 8 weeks old at the time of infection. All of the experimental procedures were conducted according to the guidelines of the Animal Research Ethical Committee (CETEA) of the University of Minas Gerais. This project was approved under the protocol CETEA 051/09. *Leishmania amazonensis* (IFLA/BR/67/PH8) promastigotes were isolated monthly from footpad lesions of infected BALB/c mice and maintained *in vitro* as proliferating promastigotes. Parasites were cultured in Grace's Insect Medium (Gibco® Life Technologies, Grand Island, NY, USA) supplemented with 20% heat-inactivated foetal bovine serum (Cultilab, Campinas, SP, Brazil), 2 mM L-glutamine, 100 U/mL penicillin and 100 μ g/mL streptomycin (Gibco) at 25°C. Infective stage metacyclic promastigote forms of *L. amazonensis* were isolated from stationary cultures (4 days old) as described previously (25) and washed twice in PBS before they were used for infection. Mice were inoculated intradermally into the ear dermis with 1×10^6 metacyclic *L. amazonensis* parasites in a volume of 10 μ L PBS. The evolution of the infection was monitored daily until the 7th day

post-infection or weekly by measuring the thickness of the ear lesion with a digital calliper (Starrett, São Paulo, SP, Brazil).

Ab treatment

To deplete neutrophils, monoclonal antibodies (mAb) RB6-8C5, an IgG2b rat anti-mouse Ly6G/Ly6C, and 1A8, an IgG2a rat anti-mouse Ly6G, were employed. The hybridoma secreting RB6-8C5 was a gift from Dr. Glória Maria Collet de Araújo Lima (Universidade Federal de Goiás, Goiânia, GO, Brazil). The antibody was purified from hybridoma culture supernatants by ammonium sulphate precipitation. Antibody 1A8 was kindly provided by Dr. David L. Sacks (National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA). To deplete neutrophils, 500 µg of RB6-8C5 mAb or 1 mg of 1A8 mAb was administered by intraperitoneal injections 16 h prior to infection. As control antibody, we used 500 µg of rat IgG purified from rat serum by ammonium sulphate precipitation. For the inhibition of IL-10 signalling, mice were inoculated intraperitoneally every 3 days with 500 µg monoclonal anti-IL-10 receptor, also provided by Dr. David L. Sacks, as was the isotype control. For the blockage of Fcγ receptor, BALB/c mice were inoculated i.p. with 200 µg of 2.4G2 mAb on days 2, 4 and 6 post-infection. Antibody 2.4G2 was a gift from Dr. Luís Carlos Crocco Afonso (Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil). It was demonstrated that 2.4G2 persists on cells for at least 4–6 days after a single injection (200 µg) (26). As an unrelated control, we used rat IgG, purified from rat serum by ammonium sulphate precipitation.

Measurement of myeloperoxidase activity

The numbers of neutrophils within lesions were estimated using the myeloperoxidase (MPO) assay (27, 28). At the indicated time points, ear tissues were weighed and homogenized in pH 4.7 buffer (0.1 NaCl; 0.02 M Na₃PO₄; 0.015 M Na₂EDTA) and centrifuged at 10 000× g for 10 min at 4°C. The pellets were then diluted in NaCl 0.2% followed by NaCl 1.6% containing glucose 5% and homogenized. After another centrifugation at 10 000× g for 10 min at 4°C, the pellets were diluted in 0.05 M sodium phosphate buffer (pH 4.5) containing 0.5% hexa-1,6-bis-decyltrimethylammonium bromide (HTAB; Sigma-Aldrich, St. Louis, MO, USA). The suspensions were freeze-thawed three times using liquid nitrogen and finally centrifuged at 10 000× g for 15 min at 4°C. Myeloperoxidase activity in the resulting supernatant was assayed by adding 25 µL 3,3',5,5'-tetramethylbenzidine (TMB; Sigma-Aldrich), prepared in dimethyl sulfoxide (DMSO; Merck, Darmstadt, Germany) in a final concentration of 1.6 mM, to 25 µL of the supernatant from tissue sample and incubating for 5 min at 37°C. Then, 100 µL H₂O₂ in a final concentration of 0.003% v/v, dissolved in sodium phosphate buffer (pH 5.4), was added to the mixture of supernatant and TMB, and the microplate was incubated for 5 min at 37°C. The reaction was terminated by adding 100 µL 1 M H₂SO₄ and was quantified colorimetrically at 450 nm in a spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). Neutrophil numbers in each sample were calculated from a standard curve of neutrophils obtained from the peritoneal cavity of 5% casein-treated animals and processed the same way. The results were expressed as relative number of neutrophils per mg of wet tissue.

Estimation of parasitic load and measurement of arginase activity

Parasite burden was determined by performing a limiting dilution analysis as previously described (29). In brief, the homogenates of infected lesion and draining lymph nodes were serially diluted (1 : 4 serial dilutions) in Graces Insect Medium supplemented with 20% FCS and observed 5–7 days later for promastigote growth. Parasite numbers are expressed as the negative log₁₀ dilution at which parasite growth was observed. Results are expressed as mean log₁₀ of the inverse of the titre. Arginase activity in homogenates of infected lesions was assayed as described previously (30) with few modifications. About 35 µL lesion homogenate was incubated in 24-well plates with 50 µL Triton X-100 and plates were shaken for 30 min. Arginase was activated with 50 µL MnCl₂ (10 mM) and 50 µL of TRIS-HCl (50 mM, pH 7.5) at 55°C for 10 min. Then, 50 µL of the samples was transferred to a fresh 24-well plate containing 25 µL of L-arginine (0.5 mM, pH 9.7) and incubated for 60 min at 37°C. The reaction was stopped by the addition of 400 µL of a mixture of H₂SO₄ : H₃PO₄ : H₂O (1 : 3 : 7). Subsequently, 25 µL of 9% 1-phenyl-1,2-propanodione-2-oxime in ethanol was added and the plates were incubated at 95°C for 45 min for colour development. Reaction mixtures were read at 540 nm in a spectrophotometer (Molecular Devices). One unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 µmol of urea/min. A standard curve was performed using urea and the detection limit for the assay was 270 µM of urea.

Lymph node cell preparation and the culture for the detection of cytokine production by ELISA

Retromaxillary lymph nodes from infected mice were removed and made into single-cell suspensions. Cells were washed, counted and suspended at the concentration of 5×10^6 cells/mL in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated FBS (Cultilab), 25 mM HEPES, 0.05 mM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) were plated. Cells were re-stimulated in the presence of 50 µg protein/mL *L. amazonensis* antigen (freeze-thawed preparation of stationary-phase promastigotes of *L. amazonensis*). Culture supernatants were collected after 72 h and tested for their IFN-γ, IL-4, IL-10 and IL-17 contents by capture enzyme-linked immunosorbent assay (ELISA). IFN-γ was quantified using rat monoclonal anti-mouse IFN-γ (clone R46A2) as capture antibody and rabbit polyclonal anti-rat anti-IFN-γ as detector antibody. For the detection of IL-4, monoclonal anti-IL-4 (clone 11B11) and anti-IL-4 biotinylated (clone BVD6) were used as capture and detector antibody, respectively. The threshold of sensitivity in both assays was 15.6 pg/mL. All these antibodies were kindly provided by Dr. Luís Carlos Crocco Afonso (Universidade Federal de Ouro Preto, Ouro Preto, MG, Brazil). IL-10 production was measured using *Mouse IL-10 ELISA Set* kit (BD Biosciences Pharmingen, San Jose, CA, USA), as indicated by the manufacturer (sensitivity 31.25 pg/mL). IL-17 production was determined using *Mouse IL-17A (homodimer) ELISA Ready-SET-Go!* Kit (eBioscience, San Diego, CA, USA) according to the procedures supplied by the manufacturer (sensitivity 7.8 pg/mL).

Measurement of *Leishmania*-specific serum IgG

Serum obtained from BALB/c mice infected with *L. amazonensis* was used to detect IgG1 and IgG2a by ELISA. Plates were coated with freeze-thawed *L. amazonensis* culture promastigotes (10 mg of protein/mL) in carbonate buffer (0.05 M, pH 9.6) overnight at 4°C. After this incubation, plates were washed and blocked with 5% FBS in PBS for 1 h at 37°C. Serum samples were diluted 1 : 50, added to the plates and incubated for 2 h at 37°C. After washing the plates, rat anti-mouse IgG1 and IgG2a directly conjugated with peroxidase (Southern Biotechnology, Birmingham, AL, USA) were diluted 1 : 2000 in 5% FBS in PBS and added to the plates. After 1 h of incubation at 37°C, plates were washed and ELISAs were revealed with 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS; Sigma-Aldrich) and stopped with 1% SDS. The reaction was read at 405 nm in a spectrophotometer (Molecular Devices).

Immunolabelling and flow cytometry

At 1, 3 and 7 days after infection, mice were euthanized and their ears were collected. The ears were rinsed in 70% ethanol with vigorous shaking and allowed to dry. Using a pair of fine forceps, the ventral and dorsal dermal sheets were separated and transferred dermal side down on 750 µL of RPMI containing 125 µg/mL Liberase TL (Roche Diagnostics, Mannheim, Germany), 0.5 mg/mL deoxyribonuclease I (Sigma-Aldrich), 24 mM NaHCO₃, 10 mM HEPES, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 50 U/mL penicillin and 50 µg/mL streptomycin. The sheets were incubated for 1 h and 30 min at 37°C and 5% CO₂. The reaction was terminated by adding 15 µL of EDTA (0.5 M, pH 8) and 750 µL of RPMI containing 3% FBS. Digested ear sheets were homogenized and then filtered using a 70-µm-pore-size Falcon cell strainer (BD Biosciences). The single-cell suspensions were washed, counted and 10⁶ cells were distributed in 96-well plates. Cells were incubated for 15 min with an anti-Fc-γIII/II (CD16/32) receptor antibody clone 2.4G2 and a combination of one or two of the following antibodies in PBS containing 1% BSA: anti-Ly6G-PE, clone 1A8; anti-CD3-FITC, clone 145-2C11; anti-CD4-PerCP, clone RM4-5. All the antibodies used were purchased from BD Biosciences Pharmingen, unless specified otherwise. For intracellular staining, cells were fixed for 20 min in 2% paraformaldehyde (Sigma-Aldrich) and then washed twice with permeabilization buffer PermWash (BD Biosciences Pharmingen). After washing, cells were incubated for 20 min with anti-Foxp3-PE, clone FJK-16s (eBioscience) and its corresponding isotype control. After staining, the cells were washed and then diluted in PBS containing 1% BSA. The data were immediately collected using a FAC-SCan (Becton Dickinson, Mountain View, CA, USA). For each sample, 5 × 10⁴ events were analysed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Bone marrow-derived macrophages stimulation and infection

Bone marrow-derived macrophages (BMM/φ) were prepared as previously described (31). Briefly, bone marrow was flushed from the femurs and tibias of mice and cells were plated in Petri dishes in DMEM/F12 supplemented with 10% FBS, penicillin/streptomycin, glutamine and 15% conditioned medium from the supernatant of M-CSF secreting L929 (LCM) fibroblasts (complete medium). Cells were fed on day 2, and complete medium was replaced on day 6. Cells were used at 7–10 days for experiments, and 5 × 10⁵ macrophages

per well were plated overnight in a 48-well plate in DMEM/F12. Cells were then washed and stimulated with 10 ng/mL LPS alone or with LPS in combination with immune complexes (IC). Immune complexes were prepared by mixing a 10-fold molar excess of rabbit anti-OVA IgG (Cappel, Durham, NC, USA) to OVA (Worthington, Lakewood, NJ, USA) for 30 min at room temperature, as described previously (32). Macrophages were infected with stationary-phase promastigotes of *L. amazonensis* at a parasite-to-macrophage ratio of 5 : 1. Culture supernatants from monolayers of control and stimulated macrophages were assayed by ELISA for cytokine production 24 h after stimulation. Cytokines were measured by sandwich ELISA using the following antibody pair from BD Pharmingen according to the manufacturers instructions: IL-10, JESS-2A5 and JES5-16E3.

Peritoneal macrophage stimulation and infection

Thyoglicolate-elicited peritoneal macrophages were collected from BALB/c mice 5 days after i.p. injection of 2 mL 3% thyoglicolate (DIFco Laboratories, Detroit, MI, USA). Cell suspensions were washed twice with PBS and cultivated at 1×10^6 cells/well in 24-well plates (TPP, Trasadingen, Switzerland). Nonadherent cells were removed by washing in PBS after 2-h incubation at 37°C and adherent cells were cultured in 1 mL of RPMI 1640 medium (Sigma-Aldrich), supplemented with 10% foetal bovine serum (Cripion, Andradina, SP, Brazil), 10 mM Hepes (Sigma-Aldrich), 100 U/mL penicillin (Sigma-Aldrich) and 100 µg/mL streptomycin (Sigma-Aldrich). Macrophages were stimulated with 10 ng/mL of IL-17 (Invitrogen, Carlsbad, CA, USA) and then were infected with *L. amazonensis* promastigotes at a parasite-to-macrophage ratio of 5 : 1. After 48 h, macrophage monolayers were assayed for arginase activity. For *in vitro* infection, cell suspensions were washed twice with PBS and incubated at 2×10^5 cells/well in 16-well chamber slides (Laboratory-Tek; Nunc Nalge International, Rochester, NY, USA). Nonadherent cells were removed by washing in PBS after 2-h incubation at 37°C. Monolayers were stimulated overnight with 10 ng/mL of IL-17 (Invitrogen) and infected with 1×10^6 *L. amazonensis* promastigotes. After 24 h, coverslips were washed to remove unbound parasites. Coverslips were stained with Instant Prov kit (Newprov, Pinhais, PR, Brazil) after 24 and 72 h of culture for the evaluation of parasite growth.

Statistical procedures

Results were expressed as mean $\pm$ SE for each group. In the experiments of neutrophil migration, two-way ANOVA followed by Bonferroni post-tests were performed to determine the differences between times and groups. To establish differences between three or more groups, one-way ANOVA was used followed by Tukeys multiple comparison test. To determine the differences between two groups, Mann–Whitney test was performed using a two-tailed distribution. All the statistic analyses were conducted using GraphPad Prism version 4 for Windows (GraphPad Software, La Jolla, CA, USA). Differences were considered to be statistically significant if $P < 0.05$.

RESULTS

Kinetics of neutrophil recruitment to the site of infection and effectiveness of treatment with RB6-8C5 in preventing neutrophil accumulation

Before evaluating the role of neutrophils during *L. amazonensis* infection, we determined the kinetics of neutrophil migration to the site of infection in response to parasite inoculation. For this purpose, BALB/c and C57BL/6 mice were infected in the ear dermis with 1×10^6 metacyclic forms of *L. amazonensis* and at the indicated times, MPO activity, which correlates with neutrophil accumulation, was evaluated. As seen in Figure 1(a) left panel, neutrophils were found both in BALB/c and C57BL/6 mice as early as 6 h post-infection. In both groups of mice, we found maximum MPO activity between 12 and 24 h after infection. After 72 h of infection, we observed a decrease in MPO activity. Although we found similar kinetics of MPO activity in the ears of both BALB/c and C57BL/6 mice, the magnitude of MPO activity at 24-h post-infection was higher in BALB/c mice compared to C57BL/6 mice. To confirm these data, we performed flow cytometry analysis to examine the presence of neutrophils (Ly6G⁺ cells) in the ears during infection. The cells were isolated from the ears after 1, 3 and 7 days post-infection and stained for Ly6G (Figures 1b,c). Our data showed that the kinetics of the accumulation of neutrophils in the infected ears correlates well with the MPO activity, confirming that there is substantial migration of neutrophils into the ears during the first day of infection but their numbers decrease drastically after the third day.

To understand the role of neutrophils in the early stages of infection, we performed neutrophil depletion using monoclonal antibody RB6-8C5. After testing different doses of the antibody, we selected an intraperitoneal injection of 0.5 mg of RB6 16 h before infection. This dose was the lowest dose able to induce 99% depletion for at least 48 h in our hands. Treatment with monoclonal antibody RB6 was very effective in inhibiting neutrophil accumulation at the site of infection (Figures 1a right panel, b, c), whereas treatment with rat IgG did not affect MPO activity (Figure 1d). Treatment with RB6 completely abrogated the accumulation of neutrophils in the site of inoculation on the first day post-infection (Figure 1c). Three days after infection, the number of neutrophils in the RB6-treated group increased, reaching the levels found in the nontreated group. After 7 days, the percentages and absolute numbers of neutrophils at the site of infection in the neutrophil-depleted group became higher than in the nontreated group. We were also interested in determining whether the tissue damage caused by the introduction of a needle in the ear dermis was sufficient to cause neutrophil accumulation. Our results indicated that needle injury induced only a slight neutrophil accumulation at 24 h and MPO activity at this time point was not different from naïve controls or RB6-treated animals ($P > 0.05$, Tukeys multiple comparison test, Figure 1a right panel). On the other hand, infection with *L. amazonensis* induced a strong increase in MPO activity when compared with PBS-injected mice ($P < 0.001$ by Tukeys multiple comparison test). Our data indicate that infection with *L. amazonensis* causes the rapid recruitment of neutrophils.

Neutrophils limit the early development of lesions in BALB/c mice infected with *L. amazonensis*

To evaluate the effect of neutrophil depletion on the course of *L. amazonensis* infection, BALB/c and C57BL/6 mice were treated with 0.5 mg of RB6 16 h prior to infection with 1×10^6 metacyclic forms of *L. amazonensis*, and the progression of the lesions was measured weekly. As seen in Figure 2(a), treatment with RB6 resulted in accelerated lesion development in BALB/c infected with *L. amazonensis* during the first week post-infection; however, as the disease progressed, the course of infection was comparable between the two groups. On the other hand, neutrophil depletion did not affect the course of *L. amazonensis* infection in C57BL/6 mice (Figure 2a).

Although we found differences only in the first week after infection with *L. amazonensis* in BALB/c mice, these differences were consistent ($P < 0.0001$, Mann–Whitney test) and they led us to further investigate these early stages of infection. Thus, we followed the course of infection daily during the first week of infection and found that there was an increase in the ear thickness as soon as 2 days post-infection in the RB6-treated groups (Figure 2b). While in neutrophil-sufficient mice there was almost no increase in ear thickness during the first week of infection, neutrophil-depleted mice presented nearly a twofold increase in the ear thickness after 7 days of infection (Figure 2b). On the other hand, treatment with rat IgG, as an unrelated control antibody, did not affect the course of infection in BALB/c mice (Figure 2b insert). These data indicate that the presence of neutrophils at the time of infection is important for the early control of lesion development in BALB/c mice during infection with *L. amazonensis*, because neutrophil depletion exacerbated lesions in these mice. On the other hand, the depletion of neutrophils had no effect on the course of infection in C57BL/6 mice, even at early time points (Figure 2b).

Next, we verified what would happen if we depleted neutrophils during the entire first week of infection. To do so, we treated BALB/c and C57BL/6 mice on days –1, 1, 3, 5 and 7 after infection with *L. amazonensis* and monitored ear thickness biweekly (Figure 2c). Depletion of neutrophils during the first week of infection affected the course of infection in BALB/c mice. Neutrophil-depleted mice presented increased lesions compared to neutrophil-sufficient mice until 8 weeks post-infection, when the experiment was terminated. These results support the idea that neutrophils have a protective role during *L. amazonensis* infection in BALB/c mice. Differences between the depletion of neutrophil for 2 days and neutrophil depletion for an entire week may indicate that the wave of neutrophil migration on day seven post-infection observed in mice that received a single injection of RB6 (Figure 1c) seems to protect mice from further exacerbation of lesion development. Regarding C57BL/6 mice, even the depletion of neutrophils for one entire week was not enough to affect lesion development, confirming that neutrophils do not play an important role in *L. amazonensis* infection in C57BL/6 mice.

Neutrophils are required for the early control of parasite burden in *L. amazonensis*-infected BALB/c mice

Because neutrophils limit lesion development during the first week of infection, our next goal was to evaluate the involvement of these cells in limiting parasite burdens during these

early stages of infection. To do so, we estimated the parasite loads by performing a limiting dilution analysis in the infected ears and draining lymph nodes, in the presence or in the absence of neutrophils. We found that neutrophil-depleted BALB/c mice presented a 10-fold increase in parasite loads at the site of infection and draining lymph nodes 7 days post-infection compared to neutrophil-sufficient BALB/c mice (Figure 3a,c). These data indicated that neutrophils could be restraining parasite growth. To confirm this hypothesis, we assessed parasite loads in BALB/c mice 1 day post-infection. We observed that mice infected in the absence of neutrophils presented approximately 10 times more parasites both in the ear and in the draining lymph nodes than neutrophil-sufficient mice 1 day after infection (Figure 3g,h). Therefore, neutrophils seem to limit not only lesion development but also parasite growth in BALB/c mice.

Arginase I is an important factor in the pathogenesis of leishmaniasis (33, 34). This enzyme hydrolyses L-arginine into urea and ornithine, which is the building block for the production of polyamines that are important for the proliferation of *Leishmania*. To determine whether there was a correlation between parasite proliferation and arginase activity, we quantified arginase activity in the infected ears of neutrophil-depleted and neutrophil-sufficient mice. Interestingly, infection with *L. amazonensis* results only in a slight increase in arginase activity when compared to noninfected ears. In accordance with the higher parasite titres observed, neutrophil depletion provoked a marked increment in arginase activity at the site of inoculation 1 week post-infection with *L. amazonensis* (Figure 3e). Thus, we found a direct correlation between an increase in parasite loads and an increase in arginase activity in BALB/c mice infected with *L. amazonensis* and depleted of neutrophils. In C57BL/6 mice, neutrophil depletion did not alter parasite burdens nor arginase activity during the first week of infection (Figure 3b,d,f) and these data are in agreement with the fact that neutrophil depletion did not affect the course of *L. amazonensis* infection in C57BL/6 mice.

Neutrophil depletion in BALB/c mice caused enhanced secretion of IL-17 and IL-10 by draining lymph node cells during recall responses

Our next goal was to evaluate the effect of neutrophil depletion in the early production of cytokines by lymph node cells. For this purpose, we performed *in vitro* re-stimulation with *L. amazonensis* antigen of draining lymph node cells obtained from neutrophil-sufficient and neutrophil-deficient mice 3 and 7 days after infection, and we evaluated the secretion of IL-4, IFN- γ , IL-10 and IL-17 by ELISA. We found that infection with *L. amazonensis* resulted in the production of high levels of both IL-4 (≈ 1 ng/mL) and IFN- γ (≈ 5 ng/mL) during the first week of infection. However, treatment with RB6 did not affect the production of IL-4 or IFN- γ by lymph node cells (data not shown). When we evaluated the production of IL-17, we found that lymph node cells produced high levels of IL-17 at 3 days after infection (Figure 4a), but the levels of IL-17 diminished drastically at 7 days post-infection (data not shown). Depletion of neutrophils produced an increase in IL-17 production at 3 days post-infection (Figure 4a). It is important to emphasize that even unstimulated lymph node cells obtained from neutrophil-depleted mice secreted high levels of IL-17. Finally, the production of IL-10 was assessed. Lymph node cells stimulated with *L. amazonensis* antigen produced high levels of IL-10 at both 3 and 7 days after infection. When mice were depleted of neutrophils, the secretion of IL-10 was further increased

(Figure 4b). Therefore, neutrophil depletion increases the secretion of IL-10 and IL-17 by draining lymph node cells during recall responses.

Treatment with 1A8 antibody yields similar results as treatment with RB6-8C5 antibody

RB6-8C5 antibody has been extensively used to deplete neutrophils in mice. Although RB6-8C5 binds with high affinity to Ly6G, which is specifically expressed in neutrophils, it also binds with lower affinity to Ly6C, which is expressed on neutrophils, dendritic cells and subpopulations of lymphocytes and monocytes. On the other hand, 1A8 mAb binds specifically to Ly6G (35). To confirm that the events observed so far were associated with specific neutrophil depletion, we treated BALB/c mice with 1 mg of 1A8 mAb 16 h prior to the infection with *L. amazonensis*. Although more specific, antibody 1A8 is not as efficient as RB6 (RB6 depletes 99% of neutrophil, while 1A8 depletes 85% of neutrophils) (36). Our data demonstrate that treatment with both antibodies induced similar results. Depletion of neutrophils with 1A8 mAb resulted in increased lesion size (Figure 5a), greater parasite loads (Figure 5b), higher arginase activity (Figure 5c) and increased secretion of IL-10 during *in vitro* re-stimulation of lymph node cells (Figure 5d). These data validate our experiments performed with RB6-8C5 antibody.

Neutrophil depletion results in increased titres of anti-*Leishmania* IgG1 and IgG2a in the serum of infected BALB/c mice

Antibody production plays an important role in immune response to *L. amazonensis*. To determine whether the presence of neutrophils at the time of infection could interfere with the early production of *L. amazonensis*-specific antibodies, we obtained serum from infected BALB/c mice sufficient or deficient in neutrophils and measured the levels of anti-*L. amazonensis* IgG1 and IgG2a (Figure 6). After 7 days of infection, we could detect both anti-*L. amazonensis* IgG1 and IgG2a. The levels of IgG2a were higher than the titres of IgG1 and the IgG2a/IgG1 ratio was 11.04. The depletion of neutrophils increased the titres of both of these immunoglobulins in the serum (Figure 6). After neutrophil depletion, the IgG2a/IgG1 ratio was reduced from 11.04 to 1.53. Therefore, the presence of neutrophils at the time of infection influences the production of both anti-*Leishmania* IgG1 and IgG2a in BALB/c mice.

The presence of neutrophils at the site of infection modulates the accumulation of regulatory T cells

Because regulatory T cells have proven to play an important role in restraining early pathogenic responses during *L. amazonensis* infection, we assessed Treg accumulation at the site of infection in the presence or in the absence of neutrophils. Interestingly, we found that neutrophil depletion affected the accumulation of regulatory T cells (CD3⁺ CD4⁺ Foxp3⁺ cells, Tregs) in the infected ears 7 days post-infection. Uninfected ears presented high percentages of Tregs in the CD3⁺ CD4⁺ population. The percentage of Tregs decreased during infection with *L. amazonensis*. Moreover, in the absence of neutrophils, the reduction in the percentage of Tregs was even greater (Figure 7). Altogether, our results suggest that neutrophils modulate the accumulation of regulatory T cells at the site of infection.

Increased production of IL-10 induced by neutrophil depletion is in part dependent on the increase in IgG levels and drives parasite growth

We have already demonstrated that neutrophil depletion is associated with faster lesion development, higher arginase activity and parasite burdens. In addition, we found increased titres of anti-*Leishmania* IgG1 and IgG2a after neutrophil depletion, which may induce the production of IL-10 by macrophages (37), and enhanced the secretion of IL-10 by lymph node cells stimulated with *L. amazonensis* antigen. The elevated levels of IL-10 observed in neutrophil-depleted mice could favour parasite proliferation and consequent lesion progression (38). Also, because IL-10 is an inducer of arginase activity (39), this could be the mechanism by which IL-10 could be favouring parasite growth. Therefore, our next goal was to investigate whether the increase in IgG levels was favouring IL-10 production in neutrophil-depleted mice, using two different approaches. First, we performed an *in vitro* experiment with BMM/φ in which we stimulated the cells with LPS or LPS plus IC, which are known to induce IL-10 production, and then we infected these cells with *L. amazonensis* promastigotes. In this experiment, we worked with BMM/φ because macrophages represent the definitive host for *Leishmania*, and we used promastigote forms of *Leishmania* because there is some evidence that neutrophils can deliver viable and motile parasites to macrophages (40). Interestingly, infection with *L. amazonensis* induced even higher levels of IL-10 in both LPS and LPS+IC-stimulated macrophages (Figure 8a). Our results suggest that not only IC are able to induce IL-10 production *in vitro*, but also promastigote forms of *L. amazonensis* are also able to regulate the production of cytokines in LPS-stimulated macrophages, leading to the secretion of high levels of IL-10. These results indicate that the production of IL-10 can be at least in part induced by stimulation with IC. To verify whether this is true *in vivo*, we blocked Fcγ receptor signalling using 2-4G2 mAb on days 2, 4 and 6 post-infection. Treatment with 2-4G2 inhibited IL-10 production induced by neutrophil depletion (Figure 8b). Our results suggest that the increased levels of IgG observed in neutrophil-depleted mice may be inducing IL-10 production.

Our following goal was to determine whether the increase in IL-10 observed in neutrophil-depleted mice was driving parasite proliferation and lesion development. To test this hypothesis, we treated neutrophil-depleted mice with anti-IL-10R (0.5 mg) on the day of infection and every 3 days for 7 days (Figure 8). Treatment with anti-IL-10R exacerbated lesion development further during the first week of infection (Figure 8c). Additionally, it enhanced arginase activity (Figure 8d). However, blockage of IL-10 abrogated the increase in parasite loads induced by neutrophil depletion (Figure 8e). Therefore, our results suggest that IL-10 drives the parasite proliferation observed in neutrophil-depleted mice through a mechanism that seems to be independent of arginase-1. On the other hand, we observed that the inhibition of IL-10 leads to exacerbation of lesion size, suggesting that IL-10 restrains lesion development.

IL-17 favours arginase activity and *Leishmania* growth in inflammatory macrophages

We further investigated the effect that the elevated levels of IL-17 in neutrophil-depleted mice could have on macrophages. IL-17 is produced by a broad range of cells from innate and adaptive immune system, and it acts on different cell types promoting the production of chemokines that recruit and activate neutrophils. More recently, it was demonstrated that

inflammatory macrophages express high levels of IL-17R (41). Because macrophages are the main host cell for *Leishmania* parasites, we investigated whether IL-17 was playing a role in supporting parasite growth. We showed that IL-17 does not change the phagocytic activity of macrophages, but favours intracellular parasite proliferation (Figure 9a). Additionally, IL-17 increased arginase activity in inflammatory macrophages (Figure 9b).

DISCUSSION

In this work, we demonstrate that neutrophils mediate protection during the early stages of *L. amazonensis* infection in BALB/c mice, controlling both parasite loads and lesion development. It is well established that neutrophils provide the first line of defence against infections and it has been demonstrated that these cells are associated with resistance to infections with bacteria such as *Listeria monocytogenes* (42) and *Mycobacterium tuberculosis* (43); to fungi such as *Candida albicans* (44); and to protozoa such as *Toxoplasma gondii* (3) and some species of *Leishmania* (9, 10, 45–47). During *L. braziliensis* infection, neutrophil depletion enhanced parasite proliferation, whereas co-inoculation of neutrophils with the parasite increased parasite death (46). Also, early in *Leishmania infantum* infection, neutrophils contributed to parasite killing (45). Furthermore, neutrophils had a critical role in early protective immune response to *Leishmania donovani* by acting both in parasite killing and as an important cell influencing the development of a Th1 response (47). The role of neutrophils during *L. major* infection is still controversial. *In vitro*, it was shown that *L. major* is taken up by neutrophils. Some parasites can survive inside these cells, which eventually become apoptotic and are internalized by macrophages. The phagocytosis of apoptotic neutrophils avoids macrophage activation, allowing a silent entry of the parasite into macrophages (48). Based on these *in vitro* studies, it was hypothesized that neutrophils would serve as Trojan horses through which *L. major* would enter silently into macrophages (48). Actually, some *in vivo* studies corroborate this hypothesis, by showing that neutrophil depletion reduced the number of viable *L. major* parasites at the site of infection (40) and controlled lesion size (11). However, in other studies, neutrophil depletion in *L. major*-infected BALB/c mice led to exacerbated lesions and increased parasite burden, arguing against the Trojan horse hypothesis (9, 10). Therefore, the role of neutrophils will depend upon the characteristics of the microenvironment where the infection takes place (genetic background of the host and site of infection) and upon the parasite species.

In our study, we found neutrophil accumulation at the site of infection as early as 6 h post-infection and neutrophils were found in high numbers between 6 and 24 h. After 72 h, numbers of neutrophils reduced dramatically. Interestingly, although neutrophils had distinct roles in response to different species of *Leishmania*, we found similar kinetics of neutrophil migration to the ears in response to *L. amazonensis* compared to the reported in the literature for *L. major* (36) and *L. donovani* infections (49).

In this study, we used RB6-8C5 mAb to deplete neutrophils. Although this antibody has been extensively used to deplete neutrophils, there is an intense debate on the specificity of the antibody. RB6 binds with high affinity to Ly6G, which is primarily expressed by granulocytes (50), and with low affinity to Ly6C, which is expressed at the highest level by

monocytes in the bone marrow and at intermediate levels by bone marrow neutrophils (51, 52). It has been shown that the treatment with RB6 depletes not only neutrophils but also Ly6C⁺ monocytes, while treatment with 1A8 mAb, which reacts only with Ly-6G, depletes specifically neutrophils (35). In our model of infection, we have shown that treatment with either antibody produced larger lesions, increased parasite loads and arginase activity and resulted in higher levels of IL-10 production in BALB/c mice infected with *L. amazonensis*. In accordance with our results, Soehnlein *et al.* (2008) have also shown that treatment with either RB6 or 1A8 mAb produced similar results. Additionally, they found that treatment with RB6 did not affect the number of circulating monocytes and the pattern of monocyte extravasation was identical to that found when they used 1A8 mAb (53). Slight differences between treatment with RB6 or 1A8 may be a result of different efficiencies of the antibodies. RB6 depletes 99% of neutrophil, while 1A8 depletes 85% of neutrophils (36). Another possibility is that part of the effect of RB6 is due to the depletion of Ly6C⁺ monocytes. However, we strongly believe that the events observed in our study are associated with the depletion of neutrophils and are unlikely to be due to the depletion of other cell population that express Ly-6C.

Depletion of neutrophils altered significantly the early stage of infection in BALB/c mice. In the presence of neutrophils, there was almost no evidence of lesions during the first week post-infection. However, when neutrophils were depleted, we observed an increase in ear thickness as early as 2 days after infection. After one week, it was possible to see a well-defined lesion in the neutrophil-depleted mice, but not in the neutrophil-sufficient mice. However, this increased susceptibility in neutrophil-depleted mice was transient, because the final course of infection was not altered. But when we depleted neutrophils for an entire week, we found more dramatic effect on the course of infection. BALB/c mice presented increased lesions until the eighth week post-infection. The development of lesions was accompanied by a 10-fold increase in parasite loads in the ears and draining lymph nodes 1 week post-infection. More interestingly, differences in parasite numbers were detected as early as 1 day after infection. Taken together, these data suggest that neutrophils have a protective role by controlling parasite numbers and lesion development during the early stages of *L. amazonensis* infection in BALB/c mice. Indeed, neutrophils are able to efficiently eliminate *Leishmania* either via phagocytosis of opsonized parasites followed by the production of reactive oxygen species (54) or via the release of neutrophil extracellular traps, which can kill *L. amazonensis in vitro* (55). Phagocytosis and killing of *Leishmania* by neutrophils were also demonstrated *in vivo*. Neutrophils harbouring one or more parasites in several degrees of degradation were detected during the early stages of infection with *L. infantum* (45) and *L. major* (10). Additionally, during *L. infantum* infection, neutrophils contained the greatest proportion of intracellular parasites between 6 to 24 h and neutrophil depletion slowed the clearance of extracellular promastigotes (49). Neutrophils have also been implicated in the *in vivo* clearance of *L. amazonensis*: the deficiency of type I IFN receptor resulted in a persistent infiltration of neutrophils, which was associated with parasite clearance (56).

Although we showed that neutrophil depletion enhanced susceptibility to *L. amazonensis* in BALB/c mice at early time points, it did not affect the course of *L. amazonensis* infection in

C57BL/6 mice. Differences in the involvement of neutrophils during *Leishmania* infection in BALB/c and C57BL/6 mice have been reported previously (11,13). There are many potential reasons for the different outcomes in the two mouse strains. First, the numbers of neutrophils in the lesions are much higher in BALB/c compared to C57BL/6 (Figure 1). In BALB/c mice, neutrophils represent approximately 90% of the cells infiltrating the lesion, whereas in C57BL/6 mice they were only 50% of the cells (57). Second, *Leishmania* may induce distinct phenotypes of neutrophils from BALB/c and C57BL/6 mice (6). Finally, C57BL/6 mice present other mechanisms of resistance that might overcome the absence of neutrophil-dependent resistance mechanisms, such as higher NK cell activity (58, 59) and earlier migration of activated macrophages compared to BALB/c mice (60).

High levels of IL-17 were produced by both unstimulated and *L. amazonensis*-stimulated lymph node cells obtained from neutrophil-depleted mice after 3 days of infection. During the early stages of inflammatory processes, natural Th17 cells produce IL-17, which is known to regulate neutrophil functions (61). Interestingly, the phagocytosis of apoptotic neutrophils by macrophages inhibits IL-17 expression (62). Therefore, it is possible that the increase in the secretion of IL-17 by lymph node cells occurs merely because in the absence of neutrophils, the negative feedback for the secretion of IL-17 is not present. Therefore, the production of IL-17 becomes uncontrolled until enough neutrophils reach peripheral tissues. Whatever the reason for higher IL-17 production, this cytokine had a clear effect *in vitro* upon macrophages: higher arginase activity. Ornithine, a product of arginase I, is a substrate for the synthesis of polyamines, which promote *Leishmania* growth (39). *in vitro*, macrophages cultivated with IL-17 not only increased arginase activity, but also allowed better growth of parasites (Figure 9).

IL-10 is involved in susceptibility to different *Leishmania* species (38, 63). In our work, we observed increased levels of IL-10 associated with higher parasite loads in neutrophil-depleted mice. Using IL-10R mAb to block IL-10 signalling, we show that the increase in parasite numbers observed in neutrophil-depleted mice was dependent on IL-10 signalling. Therefore, IL-10 favoured *L. amazonensis* survival or proliferation. Because IL-10 is one of the main cytokines involved in the induction of arginase activity, we hypothesized that IL-10 could be favouring parasite proliferation by the induction of arginase I activity. Neutrophil-depleted mice presented increased number of parasites, associated with higher levels of IL-10 and augmented arginase activity. Unexpectedly, blockage of IL-10R did not reduce arginase activity. In fact, inhibition of signalling through the IL-10R further increased arginase activity at the site of infection. Therefore, the mechanism by which IL-10 favours the survival of the parasite does not seem to be dependent on arginase activity. It is possible that IL-10 could be favouring parasite proliferation by deactivating macrophages through the inhibition of the production of TGF- α , nitric oxide and IL-12. Treatment with exogenous IL-10 was shown to inhibit IFN- γ -induced production of TGF- α and of nitric oxide as well as mycobacterial growth inhibition (64).

In our study, although blockade of IL-10 decreased parasite numbers, it increased lesion size. For *L. major*, the blockage of IL-10 receptor also reduced parasite loads and slightly increased lesions (65). In addition, IL-10 knockout mice infected with *L. major* showed higher lesions and smaller parasite numbers (65). No differences in lesion sizes were

observed between IL-10 knockout mice and wild-type mice during *L. amazonensis* infection (66). However, smaller numbers of parasites were found. Close examination of these data reveals slightly larger lesions at 1 week of infection. We believe that these data are due to two well-described roles of IL-10: anti-inflammatory, regulating cell migration (67), and the antagonistic effect on IFN- γ - and TGF- α -dependent macrophage activation (68, 69). Belkaid *et al.* (65) have also elegantly provided evidence for a lack of correlation of lesion sizes and parasite numbers, especially early in infection.

Because we found increased production of anti-*L. amazonensis* IgG1 and IgG2a associated with higher IL-10 levels in neutrophil-depleted mice, we speculated that the increase in anti-*Leishmania* IgG could be triggering IL-10 production. In different models of *Leishmania* infection, the ligation of Fc γ R by *Leishmania*-bound IgG promotes IL-10 production (37, 70–72). Moreover, it was clearly demonstrated that IgG IC contribute to disease progression by inducing activated macrophages to produce IL-10 (70, 73). In this work, we have confirmed that IC can induce the production of high levels of IL-10 by *L. amazonensis*-infected macrophages *in vitro*. We have also shown that blockage of Fc γ R inhibited the increase in IL-10 production caused by neutrophil depletion. Thus, the increased levels of anti-*L. amazonensis* IgG observed in neutrophil-depleted mice seem to be favouring the production of IL-10 by macrophages, which supports parasite proliferation. But how could neutrophils affect the levels of *L. amazonensis*-specific antibodies? Neutrophils are able to influence the availability of antigens during immune response to protein antigens by competition. Depletion of neutrophils increased the titres of antibody produced in response to protein antigen (74). Indeed, we found extracellular promastigotes in the ears of neutrophil-depleted mice 24 h post-infection, but not in neutrophil-sufficient ones (data not shown). Therefore, we believe that in the absence of neutrophils, more parasites are available for the induction of anti-*L. amazonensis* IgG production.

Relatively high percentages of regulatory T cells are found in *L. amazonensis*-infected ears during the first 3 weeks post-infection (75). Naturally occurring regulatory T cells develop in the thymus and regulate self-reactive T cells in the periphery. Natural T cells are present in the host before pathogen exposure and can be rapidly recruited in response to self-antigens exposed during infection. Interestingly, the presence of regulatory T cells at the site of injury during the early stages of infection restrains the early development of lesions during *L. amazonensis* infection (75). These data are in agreement with our results because we found accelerated lesion development associated with lower percentage of regulatory T cells in neutrophil-depleted mice. Therefore, our data suggest that the presence of neutrophils at the time of infection contributes to the maintenance of regulatory T cells at the site of infection and these cells may be restraining the early development of lesions in BALB/c mice infected with *L. amazonensis*.

In conclusion, in this work, we have shown that neutrophils control parasite numbers during *L. amazonensis* infection in BALB/c mice. In the absence of neutrophils, we found increased levels of *L. amazonensis* IgG. IgG IC are detrimental during *Leishmania* infection and they probably exert their negative effects by the induction of IL-10 production by macrophages. Here, we have shown that blockage of Fc γ R signalling leads to decreased secretion of IL-10 in neutrophil-depleted mice. Also, we showed that the increased production of IL-10

observed in the absence of neutrophils favours parasite proliferation. However, although we found increased arginase activity associated with high parasite numbers, the mechanism of parasite multiplication does not seem to be dependent of arginase activity. Our results also suggest a dual role for IL-10 during the early stages of *L. amazonensis* infection: on one hand, IL-10 favours parasite survival; on the other hand, due to its anti-inflammatory effects, IL-10 protects mice from developing exacerbated lesions. Neutrophil depletion also increased IL-17 production, and we found that macrophages stimulated with IL-17 were more permissive to parasite growth and presented higher arginase activity. Finally, we have shown that neutrophils not only control parasite numbers, but also control lesion development, which can be at least in part attributed to the maintenance of higher percentages of regulatory T cells, observed in neutrophil-sufficient mice. Thus, the presence of neutrophils at the onset of infection ensures parasite elimination, control of antibody and cytokine production and maintenance of Treg cells at the site of infection, favouring control of parasite numbers and of lesion development.

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Figure 1.

Kinetics of neutrophil recruitment to the site of *Leishmania amazonensis* infection. (a) *Left Panel* BALB/c and C57BL/6 mice were injected intradermally with 1×10^6 *L. amazonensis* metacyclic promastigotes. At the indicated time points, the ears were collected and myeloperoxidase (MPO) activity was assessed. Data represent mean $\pm$ SE from at least three independent experiments. Statistical differences were observed between groups (BALB/c and C57BL/6) and times (two-way ANOVA). * $P < 0.001$ (Bonferroni post-tests). (a) *Right Panel* MPO activity in ears isolated from BALB/c and C57BL/6 mice that received

the following treatments: (1) noninfected animals; (2) PBS inoculated intradermally into the ears; (3) infection with 1×10^6 *L. amazonensis* metacyclic promastigotes; (4) treatment with 0.5 mg of anti-Ly6G/Ly6C antibody clone RB6-8C5 (RB6) 16 h before infection with 1×10^6 metacyclic promastigote forms of *L. amazonensis*. Twenty-four hours post-infected animals were euthanized, ears were collected and MPO activity was assessed. Data represent mean $\pm$ SE from 4 to 6 mice/group. Results are representative of at least three independent experiments. $\#P < 0.001$, $*P < 0.01$ (one-way ANOVA followed by Tukeys multiple comparison test). (b, c) BALB/c mice were treated with RB6 (0.5 mg) 16 h before infection with 1×10^6 *L. amazonensis* metacyclic promastigotes. At the indicated time points, animals were euthanized and ears were collected and processed for immunophenotyping by flow cytometry. The kinetics of neutrophil accumulation at the site of infection is represented by the percentage of Ly6G⁺ cells in the live cell population at the indicated time points (b). The absolute number of neutrophils at the site of infection in BALB/c mice is shown in (c). In (d), BALB/c mice were either left untreated or they were treated with 0.5 mg of RB6 or rat IgG 16 h before infection with 1×10^6 *L. amazonensis*. After 24 h, MPO activity was assessed. Data represent mean $\pm$ SE from 5 to 6 mice/group. Results are representative of two independent experiments. $*P < 0.05$ (Mann–Whitney test).

Figure 2.

Course of infection after the depletion of neutrophils in BALB/c and C57BL/6 mice. BALB/c and C57BL/6 mice were injected intradermally with 1×10^6 *Leishmania amazonensis* metacyclic promastigotes. The groups treated with RB6 received one i.p. injection of RB6 (0.5 mg) 16 h before infection (a, b). Ear thickness was monitored weekly (a). In (b), lesion development was monitored daily during the first week post-infection. The insert in (b) shows that treatment with 0.5 mg of rat IgG 16 h prior to infection does not affect the course of infection in BALB/c mice. In (c), BALB/c and C57BL/6 mice were infected as described above and the groups treated with RB6 received i.p. injection of RB6 (0.5 mg) on days -1, 1, 3, 5 and 7, and ear thickness was monitored biweekly. Data represent mean $\pm$ SE from 5 to 10 mice/group. Results are representative of at least three independent experiments. * $P < 0.05$ (Mann-Whitney test).

Figure 3.

Neutrophil depletion increases parasite burden and arginase activity in BALB/c mice. BALB/c (a, c, e, g, h) and C57BL/6 (b, d, f) mice were treated with 0.5 mg of RB6, and 16 h later, they were infected intradermally into the ears with 1×10^6 *Leishmania amazonensis* metacyclic promastigotes. One week after infection, mice were euthanized and ears were collected for the assessment of parasite burdens by limiting dilution analysis (a, b) and arginase activity (e, f). Lymph nodes were also collected for the assessment of parasite burdens (c, d). The dotted line in (e) and (f) represents the arginase activity in ears of noninfected mice. Parasite burdens are represented as the negative log of the titre. Data represent mean $\pm$ SE from 5 to 6 mice/group. Results are representative of at least three independent experiments. Parasite burdens in the ears (g) and in the draining lymph nodes (h) were also assessed by limiting dilution analysis 1 day post-infection (p.i.). Data represent mean $\pm$ SE from 5 to 6 mice/group. Results are the mean of two independent experiments. * $P < 0.05$ (Mann–Whitney test).

Figure 4.

Effect of neutrophil depletion on the secretion of IL-10 and IL-17 by draining lymph node cells during recall responses. BALB/c mice were treated with 0.5 mg of RB6, and 16 h later, they were infected intradermally into the ears with 1×10^6 *Leishmania amazonensis* metacyclic promastigotes. Three and 7 days post-infection draining lymph node cells were harvested and stimulated with *L. amazonensis* antigen (Ag). After 72 h, supernatants were collected for the measurement of IL-17 (a) and IL-10 (b) levels by enzyme-linked immunosorbent assay. Data represent mean $\pm$ SE from 5 to 6 mice/group. Results are representative of at least two independent experiments. * $P < 0.05$ (Mann–Whitney test).

Figure 5.

Treatment with 1A8 mAb yields similar results as treatment with RB6-8C5 mAb. BALB/c mice were treated with 1 mg of 1A8 mAb, and 16 h later, they were infected intradermally into the ears with 1×10^6 *Leishmania amazonensis* metacyclic promastigotes. Seven days after infection, ear thickness was measured (a); then, mice were euthanized and ears were collected for the assessment of parasite burdens by limiting dilution analysis (b) and arginase activity (c); draining lymph node cells were harvested and stimulated with *L. amazonensis* antigen (Ag). After 72 h, supernatants were collected for the measurement of IL-10 levels by enzyme-linked immunosorbent assay (d). Data represent mean $\pm$ SE from 5 to 6 mice/group. * $P < 0.05$ (Mann–Whitney test).

Figure 6.

IgG1 and IgG2a in the serum of neutrophil-depleted infected BALB/c mice. BALB/c mice were treated with 0.5 mg of RB6, and 16 h later, they were infected intradermally into the ears with 1×10^6 *Leishmania amazonensis* metacyclic promastigotes. Seven days after infection, animals were anesthetized and blood was collected from the femoral vein. After centrifugation, serum was collected and used for the detection of anti-*L. amazonensis* IgG1 and IgG2a by enzyme-linked immunosorbent assay. Data represent mean $\pm$ SE from 5 to 6 mice/group. Results are representative of two independent experiments. * $P < 0.05$ (Mann–Whitney test).

Figure 7.

Neutrophil depletion alters the percentage of Tregs at the site of infection during the first week post-infection. BALB/c mice were treated with 0.5 mg of RB6, and 16 h later, they were infected intradermally into the ears with 1×10^6 *Leishmania amazonensis* metacyclic promastigotes. After 7 days of infection, animals were euthanized and ears were collected and processed for immunophenotyping by flow cytometry. Accumulation of Treg cells in the infected ears 7 days post-infection is represented by the percentage of CD3⁺ CD4⁺ Foxp3⁺ cells gated in the population of CD3⁺ CD4⁺ cells in the ear, which represents 1–2% of the cells at the site of infection. The dotted line represents the percentage of Treg cells in ears of noninfected mice. Data represent mean $\pm$ SE from 5 to 6 mice/group. Results are representative of two independent experiments. * $P < 0.05$ (Mann–Whitney test).

Figure 8.

The increase in IL-10 production is in part dependent on the increase in IgG levels and drives parasite growth in neutrophil-depleted mice. (a) Bone marrow-derived macrophages were infected with stationary-phase promastigotes of *Leishmania amazonensis* at 5 : 1 (parasite/macrophage) ratio in the presence or in the absence of LPS (10 ng/mL) or LPS + immune complexes. After 24 h, supernatants were harvested and IL-10 levels were determined by enzyme-linked immunosorbent assay (ELISA). Values are expressed as the means SD. Results represent the mean of two independent experiments performed in triplicates. * $P < 0.01$ (Mann–Whitney test). (b) For blockage of Fc γ receptor, BALB/c mice received i.p. injections of 2.4G2 (0.2 mg) or rat IgG on days 2, 4 and 6 after infection with 1×10^6 *L. amazonensis* metacyclic promastigotes. The groups treated with RB6 received one i.p. injection of RB6-8C5 (0.5 mg) 16 h before infection. Seven days post-infection, draining lymph node cells were harvested and stimulated with *L. amazonensis* antigen (Ag). After 72 h, supernatants were collected for the measurement of IL-10 levels by ELISA. Data

represent mean $\pm$ SE from 6 mice/group. $*P < 0.05$ (Mann–Whitney test). (c–g) For the inhibition of IL-10, BALB/c mice were infected with 1×10^6 *L. amazonensis* metacyclic promastigotes and were inoculated i.p. every 3 days with 0.5 mg of anti-IL-10 receptor monoclonal antibody (c, d, e, f). As a control, mice were treated i.p. every 3 days with 0.5 mg of rat IgG (d, e, g). Ear thickness was monitored daily (c). $*P < 0.001$ between control and RB6-treated group; $\#P < 0.001$ between control and RB6 + anti-IL-10R-treated group; $\$P < 0.001$ between RB6 and RB6 + anti-IL-10R-treated groups (one-way ANOVA followed by Tukeys multiple comparison test). (d) Ear thickness after 1 week of infection. *ns* indicates no statistic difference was observed between RB6-treated and RB6+ rat IgG-treated groups. $*P < 0.05$ between the indicated group and all the other groups (one-way ANOVA followed by Tukeys multiple comparison test). One week after infection, mice were euthanized and ears were collected for the assessment of arginase activity (e) and parasite burdens by limiting dilution analysis (f, g). Data represent mean $\pm$ SE from 5 to 6 mice/group. Results are representative of two independent experiments. $*P < 0.05$ (Mann–Whitney test).

Figure 9.

IL-17 increases arginase activity and favours parasite growth in inflammatory macrophages. Peritoneal macrophages were collected from BALB/c mice 5 days after i.p. injection of thioglycolate and cultured in the presence of 10 ng/mL of IL-17, 100 ng/mL of LPS and 5 : 1 parasites/macrophage. Phagocytosis was investigated 24 h after addition of parasites, and intracellular parasite growth was analysed after 72 h (a). After 48 h, cells were assayed for arginase activity (b). * $P < 0.05$ (one-way ANOVA followed by Tukeys multiple comparison test).