INFECTION AND IMMUNITY, Feb. 2008, p. 532–541 Vol. 76, No. 2
0019-9567/08/$08.00+0 doi:10.1128/IAI.01388-07

Neutrophils Contribute to Development of a Protective Immune Response during Onset of Infection with *Leishmania donovani*▽

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Received 16 October 2007/Returned for modification 10 November 2007/Accepted 21 November 2007

Neutrophils are key components of the inflammatory response and as such contribute to the killing of microorganisms. In addition, recent evidence suggests their involvement in the development of the immune response. The role of neutrophils during the first weeks post-infection with *Leishmania donovani* was investigated in this study. When *L. donovani*-infected mice were selectively depleted of neutrophils with the NIMP-R14 monoclonal antibody, a significant increase in parasite numbers was observed in the spleen and bone marrow and to a lesser extent in the liver. Increased susceptibility was associated with enhanced splenomegally, a delay in the maturation of hepatic granulomas, and a decrease in inducible nitric oxide synthase expression within granulomas. In the spleen, neutrophil depletion was associated with a significant increase in interleukin 4 (IL-4) and IL-10 levels and reduced gamma interferon secretion by $CD4^+$ and $CD8^+$ T cells. Increased production of serum IL-4 and IL-10 and higher levels of *Leishmania*-specific immunoglobulin G1 (IgG1) versus IgG2a revealed the preferential induction of Th2 responses in neutrophil-depleted mice. Altogether, these data suggest a critical role for neutrophils in the early protective response against *L. donovani*, both as effector cells involved in the killing of the parasites and as significant players influencing the development of a protective Th1 immune response.

The best-characterized function of neutrophils or polymorphonuclear neutrophils is their preeminent role in the phagocytosis and killing of invading microorganisms via the generation of oxygen intermediates and the release of lytic enzymes stored in their granules. Neutrophils, by promoting tissue injury, also contribute to the initiation of inflammation, an essential step in the launching of immunity. In addition to their being key components of the inflammatory response, an immunoregulatory role for neutrophils during microbial infection has recently been identified via the secretion of cytokines and chemokines (reviewed in reference 16), which were shown to contribute to the recruitment and activation of antigen-presenting cells (APCs). Thus, neutrophils are now recognized as important decision shapers during the early phases of the immune response (reviewed in reference 45).

The role of neutrophils in infections with *Leishmania* has been mainly studied using the murine model of cutaneous leishmaniasis induced by the subcutaneous injection of *Leishmania major*. Transient depletion of neutrophils prior to infection with *L. major* had a significant influence on the number of parasites surviving at the site of *L. major* inoculation in both resistant and susceptible mice. In strains of mice resistant to infection, such as C57BL/6 and C3H/HeJ, depletion of neutrophils by the injection of a monoclonal antibody (MAb) depleting either neutrophils (NIMP-R14) or both neutrophils and eosinophils (RB6-8C5) at the time of infection and/or during the first week of infection led to an increase in the parasite load at the site of infection (21, 35, 49, 58). These results revealed a protective role for neutrophils in *L. major*-resistant strains of mice, at least during the first weeks of infection. In sharp contrast, in the *L. major*-susceptible BALB/c strain of mice, the absence of neutrophils during the first week of infection had the opposite effect, reducing significantly the parasite number within the draining lymph nodes and at the site of infection (49, 58). In addition, the absence of neutrophils during the first days of infection modified the development of the immune response to a more $CD4^+$ Th1 resistant type (less interleukin 4 [IL-4] and more gamma interferon [IFN-γ]) (58). However, using different strains of *L. major* and a MAb depleting both eosinophils and neutrophils, increased parasite numbers were measured in BALB/c mice (21, 35). The difference in treatment outcomes could be related to the use of different strains of *L. major* and/or the use of monoclonal antibodies with different specificities (eosinophils and neutrophils versus neutrophils alone).

Visceral leishmaniasis is a systemic disease which is mainly caused by infection with *Leishmania donovani*, *Leishmania infantum*, or *Leishmania chagasi*. These parasites infect and multiply within macrophages of the liver, spleen, and bone marrow. To investigate the organ-specific immune response developing in human visceral leishmaniasis, most studies, including the present one, use experimental murine models of infection involving intravenous injection of amastigotes (reviewed in reference 32). In most strains of mice, including BALB/c and C57BL/6 mice, one can distinguish an acute but ultimately resolving infection in the liver and a chronic infection in the spleen characterized by splenomegally and parasite persistence (reviewed in reference 23). The protective immune

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▽ Published ahead of print on 3 December 2007.

response observed in the liver includes the formation of granulomas contributing to the clearance of the parasite. Cytokines, such as IFN-γ, IL-12, and low levels of tumor necrosis factor (TNF), contribute to parasite control within the hepatic granulomas (5, 24, 25, 44). In the spleen, pathology is associated with excess TNF and granuloma formation is markedly delayed or absent. IL-10 plays a central role in susceptibility to *L. donovani* infection, both in the liver and in the spleen (40, 42). In human visceral leishmaniasis, the balance of IFN-γ, IL-4, and IL-10 correlates with either protection (IFN-γ) or susceptibility to infection (increased production of IL-4 and IL-10; decreased or deficient production of IFN-γ, IL-12, and IL-2) (4, 5, 14). Recently a low-dose dermal experimental model of visceral leishmaniasis was also described (1) that should open new opportunities to further study visceralization and immune responses that may be closer to those occurring in humans.

The role of neutrophils in visceral leishmaniasis is as yet not well defined. Human neutrophils have been shown to ingest *L. donovani* and *L. infantum* (19, 47, 54), and in vitro experiments revealed that the killing of *L. donovani* by neutrophils is performed by the H_2O_2-peroxidase-halide system (47). In experimental models of visceral leishmaniasis, depletion of both neutrophils and eosinophils during the first week of infection did not alter parasite killing in the liver but induced a significant increase in the splenic parasite load following *L. infantum* infection (54), and enhanced parasite growth in both the liver and the spleen during *L. donovani* infection was noted (55). Since both eosinophils and neutrophils were depleted in these studies, the exact role of neutrophils remains to be established. In addition, in these reports the regulatory role of neutrophils in the development of an immune response was not investigated.

Consequently, in this study, we investigated the immunomodulatory role of neutrophils in *L. donovani*-infected BALB/c mice depleted selectively of neutrophils during the first 2 weeks of infection using the neutrophil-specific depleting NIMP-R14 MAb. Depletion of neutrophils significantly increased the parasite load in the spleen and bone marrow and to a lesser extent in the liver, demonstrating an important role for neutrophils in early protection against *L. donovani* infection. This observation was reinforced by impaired granuloma development and inducible nitric oxide synthase (iNOS) expression in the liver. Most strikingly, in the absence of neutrophils, the development of the *L. donovani*-induced immune response was altered, as revealed by an increase in serum IL-10 and IL-4 and in splenic IL-10 and IL-4 mRNA transcripts, and a decrease in $CD4^+$ and $CD8^+$ T cells producing IFN-γ, suggesting that in the absence of neutrophils, the development of a protective $CD4^+$ Th1 immune response is impaired. Altogether, these results demonstrate that neutrophils play a key role in both resistance to infection with *L. donovani* and the development of the subsequent immune response.

MATERIALS AND METHODS

Mice and parasites. Age- and sex-matched in-house inbred BALB/c mice (20 to 25 g, in-house female) were used in this study. Commercially obtained Golden Syrian hamsters (*Mesocricetus auratus*) were used for maintenance of *L. donovani* strains (Harlan Olac, Bicester, United Kingdom). *L. donovani* strain LV82 was used (13), and mice were infected on day 0 by intravenous injection (tail vein, no anesthetic) with 1×10^7 to 2×10^7 *L. donovani* amastigotes. Animal experiments were carried out in accordance with UK Home Office regulations.

Neutrophil depletion. Mice (n = 4/treatment) were treated with affinity column-purified NIMP-R14 MAb (36), given intraperitoneally at a dose of 250 μg, 0, 3, 6, 9, and 12 days postinfection, and sacrificed at day 14. This MAb recognizes a 25- to 30-kDa protein present on the neutrophil membrane and was reported to deplete neutrophils selectively in vivo (18, 22, 27, 39, 57, 62, 63). As controls, mice were injected with a similar regimen of a MAb against the Vα3.2 chain of the T-cell receptor, a chain that is absent in BALB/c mice (gift of R. MacDonald, Ludwig Institute for Cancer Research, Epalinges, Switzerland). Mice were also treated with phosphate-buffered saline (PBS) as an additional control. As reported previously (52) and as measured in initial experiments, depletion of neutrophils within the blood and the spleen of BALB/c mice was verified by fluorescence-activated cell sorting (FACS) and on Giemsa-stained blood smears. For FACS analysis, peripheral blood was collected in PBS containing heparin. Erythrocytes were lysed, and Fc receptors were blocked using a purified anti-mouse CD16/32 (clone 2.4G2) antibody directed against FcγII/III receptors. Neutrophils were stained with a rat anti-mouse Ly6G MAb (1A8) coupled with phycoerythrin (PE) (BD Pharmingen), and cells were analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA). Six hours after the injection of 250 μg of the NIMP-R14 MAb, neutrophils were depleted in the blood to 0.01% of the circulating cells and to 0.1% of the splenic cell population. Three days post-injection of the MAb, neutrophils slowly started to reenter the blood (0.6 to 0.7%). Consequently, we chose to inject the neutrophil-depleting MAb every 3 days up to day 12. The efficacy of neutrophil depletion was monitored in the blood of each mouse used in the experiments on Giemsa-stained smears at the day of infection and every 3 days until sacrifice. Effective neutrophil depletion was maximal during the first days after infection, but as reported previously (55), the replenishment of the neutrophil pool increased rapidly, rendering their elimination difficult after 12 days of infection.

Quantitation of parasites and granuloma counting. To determine parasite burdens in the spleen, liver, and bone marrow, impression smears were prepared on individual glass microscope slides for each mouse at the time of sacrifice. The slides were fixed in methanol for 2 min and then stained with a 10% Giemsa solution (BDH; VWR International Ltd., United Kingdom) for 20 min before being left to dry. The spleens and livers were weighed to assess the levels of splenomegally and hepatomegally induced by infection and for calculation of parasite burdens as Leishman-Donovan units (LDU). The number of parasites/1,000 host nuclei for each sample was determined at magnification ×1,000. Results are expressed as LDU, which represent the number of amastigotes per 1,000 host nuclei, multiplied by the organ weight. For granuloma counting, sections of liver were removed and fixed in neutral buffered formalin, processed, and stained with hematoxylin and eosin (Fisher Scientific, Loughborough, United Kingdom). Granulomas were scored as infected Kupffer cells (parasitized macrophages), immature (developing granuloma consisting of $CD4^+$ and $CD8^+$ T cells and monocytes surrounding infected Kupffer cells), mature (more developed), or sterile (parasite-free granuloma), as previously described (41).

Cytokine production in serum and culture supernatants. Enzyme-linked immunosorbent assays (ELISAs) were used to determine IL-4, IL-10, and IL-12p40 levels in the serum of *L. donovani*-infected mice. Blood was collected from mice 14 days after infection and stored for 5 h at 4°C. Clotted blood was centrifuged at 13,000 rpm for 10 min and the resulting serum collected and stored at −20°C. IFN-γ was determined by ELISA with the supernatants of spleen cells. Splenocytes (5×10^5) were cultured in the presence or absence of 5 μg/ml of concanavalin A (ConA) (Sigma, Poole, United Kingdom) and harvested 72 h later. All ELISA reagents were from Insight Biotechnology.

Determination of antibody response. *L. donovani*-specific immunoglobulin G1 (IgG1) and IgG2a levels were determined as previously described, using serum harvested at the termination of the experiment (12).

Intracellular staining for IFN-γ. Analysis of intracellular cytokine expression was performed at 14 days postinfection with splenocytes stimulated in the presence of 50 ng/ml phorbol myristate acetate (Sigma-Aldrich) and 500 ng/ml of ionomycin (Sigma-Aldrich) for 4 h with the addition of brefeldin A during the last 2 h, as previously described (57). The antimouse antibody PerCP-labeled anti-$CD4^+$, APC-labeled anti-$CD8^+$, and PerCP- and APC-labeled IgG isotype controls were used to stain the cell surface, and the PE-labeled anti-mouse IFN-γ and PE-labeled IgG isotype control were used for intracellular staining. All antibodies were obtained from BD Biosciences (Oxford, United Kingdom). Cells were analyzed with the FACSCanto flow cytometer (BD, Oxford, United Kingdom). The FACsDiva software was used to analyze results.

mRNA isolation, cDNA synthesis, and quantitative reverse transcription-PCR. Fourteen days after infection with *L. donovani*, mRNA was isolated from the spleens and livers of all three treatment groups (BALB/c mice treated with

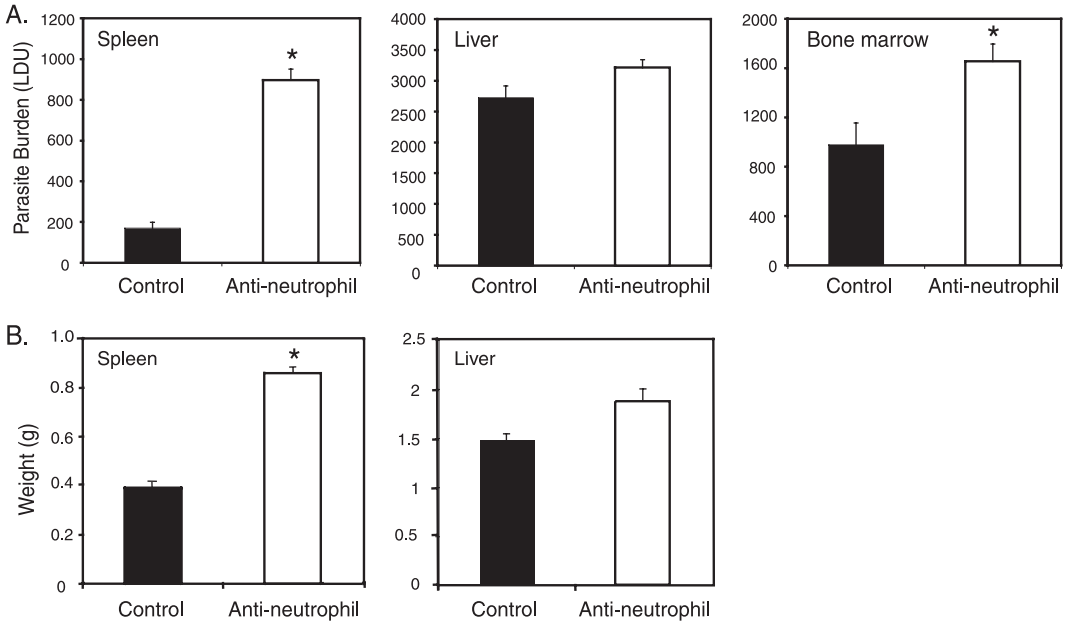

FIG. 1. Role of neutrophils in the early control of *L. donovani* growth. (A) Parasite burdens following infection with *L. donovani* were measured in the spleen, liver, and bone marrow at day 14 postinfection. Mice were treated with 250 μg of either a control MAb or an antineutrophil MAb (NIMP-R14) on days 0, 3, 6, 9, and 12 postinfection. A significant increase in parasite burdens in the spleens and bone marrow of neutrophil-depleted mice compared to controls was observed ($P < 0.0006$ and $P < 0.0001$, respectively). (B) Spleens and livers were removed and weighed 14 days postinfection. A significant increase ($P < 0.0001$) in spleen weight was observed in the neutrophil-depleted group compared to control MAb-treated mice.

PBS, the control MAb, or the neutrophil-depleted MAb). mRNA was also isolated from organs of naive uninfected mice as controls for basal levels of transcription. mRNA was isolated using the RNeasy minikit from Qiagen, and cDNA was synthesized using SuperScript II reverse transcriptase (Invitrogen, Paisley, United Kingdom), following the protocol of the kit. The random nonamers (Microsynth, Balgach, Switzerland) were used. cDNA was purified using the QIAquick PCR purification kit from Qiagen. For quantitative reverse transcription-PCR, SYBR green was used and runs were performed with a LightCycler system (Roche) as described previously (57). The PCR primers were as previously described (20, 57). Triplicate measurements of samples from four individual mice per experiment were performed. The quantity of amplified products was determined by the comparative threshold cycle method using the formula $2^{-\Delta\Delta C_T}$. Each cytokine analyzed was normalized to the value of the hypoxanthine phosphoribosyltransferase endogenous control, represented as arbitrary values. Positive samples for each cytokine were run in parallel to set the graph scale.

Immunohistology. Histological analysis of spleens and livers was performed 14 days after infection with *L. donovani* in groups of BALB/c mice treated with PBS, a control MAb, or the neutrophil-depleting NIMP-R14 MAb. Infected organs were collected and embedded in paraffin, and sections from four mice per group were processed for iNOS staining. The polyclonal rabbit anti-mouse iNOS MAb (Calbiochem) was used to reveal the enzymatic activity, as previously described (17).

Statistical analysis. Parasite data from in vivo experiments were analyzed using one-way analysis of variance (using the $\log_{10}$ transformed parasite burden for the spleen and liver data). Differences between treatments were analyzed using Fisher's protected-least-significant-difference test, using the Statview version 5.01 software package. Cytokine, antibody, and mRNA data were analyzed using the two-tailed *t* test for unpaired data.

RESULTS

Neutrophil depletion during the first 2 weeks of infection with *L. donovani* significantly enhances parasite growth in spleen and bone marrow and to a lesser extent in liver of BALB/c mice. Wild-type BALB/c mice were treated with the NIMP-R14 MAb, selectively depleting neutrophils. Mice were injected with 250 μg of MAb on days 0, 3, 6, 9, and 12 postinfection with *L. donovani*. As a control, another group of *L. donovani*-infected mice was treated similarly with PBS or a control MAb. Since no significant differences were noted between mice treated with PBS or with a control MAb, only the data obtained with the control MAb are shown. Fourteen days after infection, parasite burdens were significantly increased in the spleens of neutrophil-depleted mice, with mean LDU values fivefold higher than those for mice treated with control MAb (Fig. 1A). Parasite burdens were also increased (1.8-fold) within the bone marrow of neutrophil-depleted mice compared to that of mice treated with a control MAb (Fig. 1A). In the liver, depletion of neutrophils induced a small increase in the parasite load, which was statistically significant in one out of three independent experiments (Fig. 1A; also data not shown).

Splenomegally associated with parasite persistence is a characteristic of visceral leishmaniasis and is observed in experimental *L. donovani* infection. Depletion of neutrophils was associated with a statistically significant increase in spleen weight (2.5-fold) in *L. donovani*-infected mice compared to that in infected mice treated with a control MAb (Fig. 1B). No statistically significant difference was measured in liver weight in three independent experiments (Fig. 1B; also data not shown).

Altogether, these results demonstrate that during the first weeks of infection with *L. donovani*, neutrophils contribute actively to parasite killing in the spleen and bone marrow and to a lesser extent in the liver.

Liver granuloma formation is delayed and nitric oxide production decreased in neutrophil-depleted mice. The formation

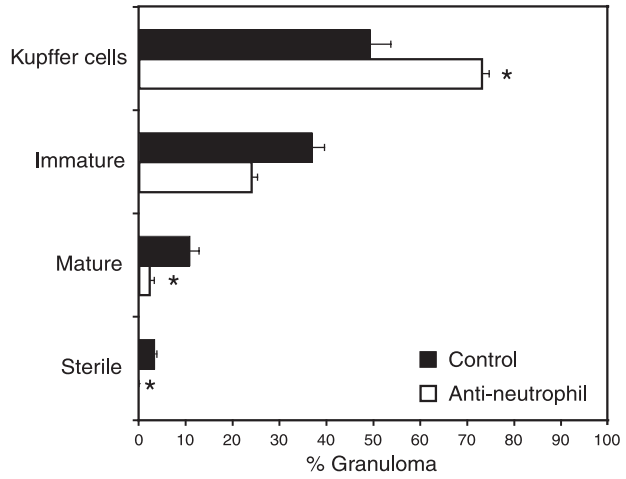

FIG. 2. Liver granuloma formation in *L. donovani*-infected BALB/c mice 14 days postinfection. Granuloma maturation was assessed at day 14 postinfection on paraffin-embedded liver sections from control MAb-treated or antineutrophil MAb (NIMP-R14)-treated mice. Sections were stained with hematoxylin and eosin, and granuloma formation was determined at magnification ×1,000. A significant decrease in mature ($P < 0.012$) and sterile ($P < 0.006$) granulomas was observed for neutrophil-depleted mice compared to results for mice treated with the control MAb, while levels of infected Kupffer cells were significantly increased ($P < 0.016$).

of hepatic granulomas is one of the key elements in the control of *L. donovani* growth within the liver. To assess if neutrophils could influence this process, liver tissues from *L. donovani*-infected mice, depleted of neutrophils or not depleted, were analyzed 14 days after infection. We first performed a quantitative analysis of granuloma formation 14 days postinfection, at which time the parasite burden was not significantly different between antineutrophil-treated mice and control mice. The prepared slides were stained with hematoxylin and eosin, and the density of hepatic granulomas, assessed by counting the number of granulomas per microscopic field (×100), was similar in control and neutrophil-depleted mice (207.5 ± 29.2 versus 160.3 ± 5.45, respectively). However, there were differences in the maturation status of granulomas in control and neutrophil-depleted mice. The ongoing infection was evaluated by scoring the presence of infected Kupffer cells, immature granulomas (Kupffer cells surrounded by a few inflammatory cells without organization), mature granulomas characterized by their organized structure, and sterile granulomas in which no *L. donovani* parasites subsisted. A significantly higher percentage of infected Kupffer cells was found for mice treated with the neutrophil-depleting MAb than for mice treated with the control MAb (73% versus 49%; $P < 0.016$), and a significantly lower percentage of mature and sterile granulomas was present in livers of neutrophil-depleted mice than in livers of mice treated with a control MAb ($P < 0.012$ and 0.006, respectively) (Fig. 2). Thus, depletion of neutrophils during the first days after infection with *L. donovani* delays the maturation of hepatic granulomas.

IFN-γ induces iNOS, which catalyzes NO products that have been demonstrated to be crucial in the killing of *L. donovani* amastigotes within hepatic granulomas (43). Immunohistochemistry was performed to determine if the decreased production of mature granulomas observed in the livers of neutrophil-depleted mice was associated with a reduction in hepatic iNOS levels. Fourteen days postinfection, livers from BALB/c mice treated with a control MAb showed intense iNOS staining at all parasite foci. Staining for iNOS within hepatic granulomas of mice treated with the neutrophil-depleting MAb was significantly less (Fig. 3). These results correlate with the apparent lesser effectiveness of hepatic granulomas observed in neutrophil-depleted mice. Nevertheless, parasite burdens in the livers of neutrophil-depleted mice were only mildly increased over those for control mice on day 14 postinfection.

Depletion of neutrophils during the first weeks of infection with *L. donovani* alters the development of the immune response. The effect of neutrophil absence on the cytokine microenvironment within parasitized organs was further evaluated within the spleens of neutrophil-depleted mice, where a significant increase in the parasite burden was measured (Fig. 1A). Cytokine mRNA levels for mice depleted of neutrophils were analyzed 14 days postinfection and compared to those for *L. donovani*-infected mice treated with a control MAb. Uninfected mice treated with the control or the neutrophil-depleting MAb of untreated were also included to have a transcription baseline for the evaluated cytokines (Fig. 4). The basal mRNA levels for IFN-γ, IL-12p40, TNF, IL-10, and IL-4 did not vary in uninfected mice treated with PBS, the control

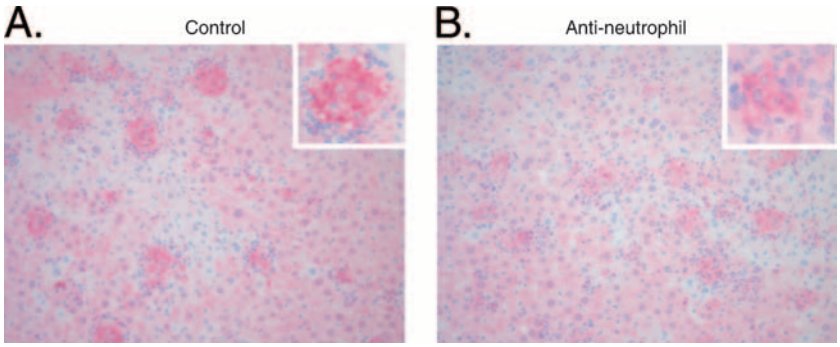

FIG. 3. Decreased iNOS in hepatic granulomas of neutrophil-depleted mice 14 days postinfection. iNOS-stained liver sections from 14-day-infected BALB/c mice treated with a control MAb (A) or the neutrophil-depleting MAb (B). Sections were counterstained with hematoxylin and eosin. Original magnification, ×100; insert magnification, ×400.

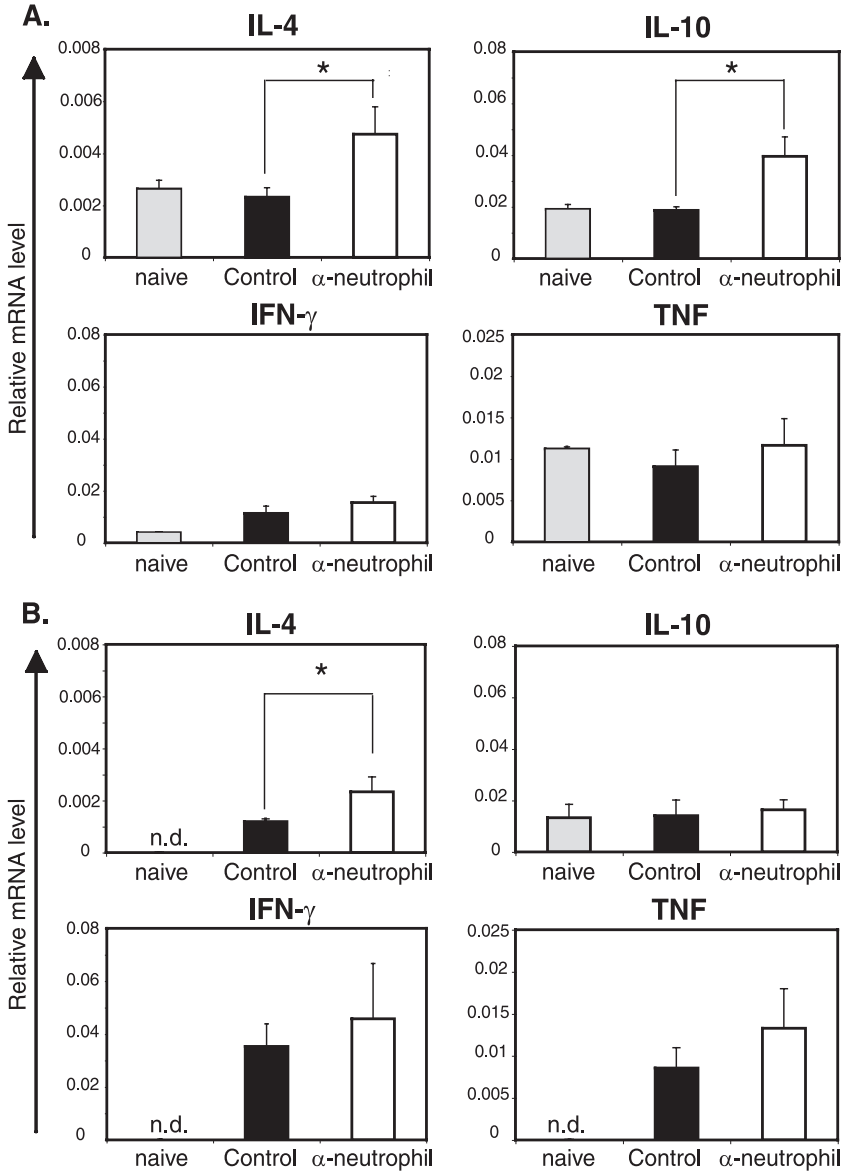

FIG. 4. Cytokine mRNA expression in spleen and liver. Quantitative real-time PCR was performed on spleen (A) or liver (B) of *L. donovani*-infected mice treated with PBS, control MAb, or the NIMP-R14 neutrophil-depleting MAb (α-neutrophil) 14 days postinfection. Basal levels of mRNA were analyzed with spleen cells from uninfected mice similarly treated (naive). Values of IFN-γ, TNF, IL-4, and IL-10 mRNA were normalized to endogenous levels of HPRT mRNA and are represented as arbitrary units. Each bar is the mean of triplicate measurement for three or four spleens or livers. Data are representative of three distinct experiments. n.d., not detectable.

MAb, or the neutrophil-depleting MAb (data not shown). We therefore represented the values obtained for uninfected mice treated with the control MAb as a baseline level of transcription (Fig. 4A, naive).

Fourteen days after infection with *L. donovani*, the levels of IL-10 mRNA were not increased by infection but significantly higher levels of IL-10 mRNA were measured for neutrophil-depleted mice than for mice treated with control MAb ($P < 0.005$) (Fig. 4A). Infection also induced an increase in IL-4 mRNA, which was also significantly higher for neutrophil-depleted mice ($P < 0.02$, compared to results for mice injected with control MAb) (Fig. 4A). Only small differences in splenic TNF mRNA levels were measured between infected mice and naive mice (uninfected), while IFN-γ mRNA levels were slightly increased following infection, with minor differences between the two infected groups (Fig. 4A). The levels of IL-12p40 mRNA were low and not significantly induced following infection (data not shown). Thus, the major effect of neutrophil depletion on the transcription of the cytokines tested for *L. donovani*-infected mice is the enhancement of splenic IL-4 and IL-10 mRNAs.

Since the immune responses generated in the spleen and the liver differ, transcription of liver cytokines was also analyzed for uninfected mice and the three groups of *L. donovani*-infected mice. As shown in Fig. 4B, infection also increased IL-4 mRNA levels in neutrophil-depleted mice ($P < 0.02$ com-

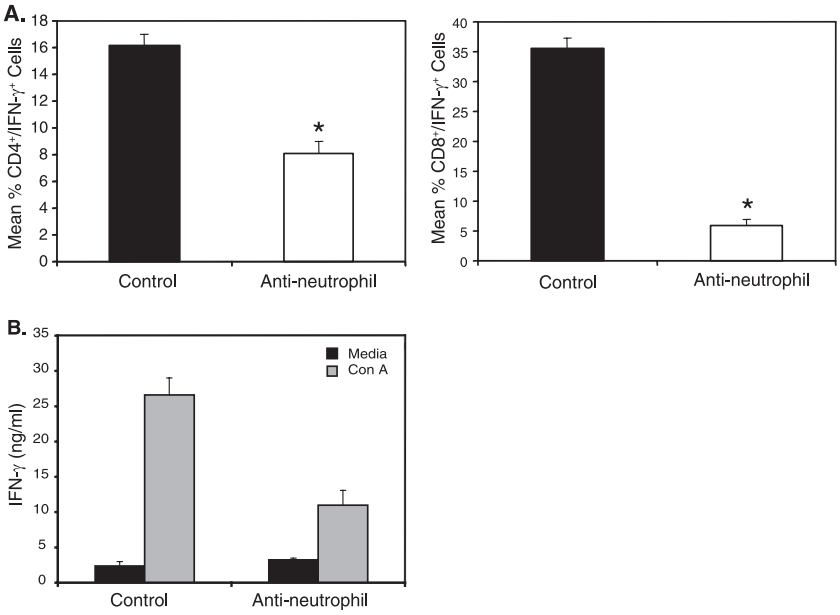

FIG. 5. Analysis of IFN-γ production by splenic T cells of neutrophil-depleted mice. (A) The number of IFN-gamma-producing $CD4^+$ and $CD8^+$ spleen cells was measured in control MAb-treated and antineutrophil MAb-treated mice at day 14 postinfection. Cells were restimulated with phorbol myristate acetate-ionomycin and gated on $CD4^+$ and $CD8^+$ T cells, and the FACSDiva software was used to analyze the results. The results are representative of three experiments performed. A significant reduction in $CD4^+$ IFN-γ^+ cells ($P < 0.001$) and $CD8^+$ IFN-γ^+ cells ($P < 0.0005$) for antineutrophil-treated mice compared to results for control mice was observed. (B) Cytokine levels in supernatants of splenocytes stimulated or not with ConA for *L. donovani*-infected mice depleted of neutrophils or not depleted. Data are the means ± standard errors for splenocyte cultures from four mice, representative of three experiments.

pared to results for mice treated with the control MAb). The levels of IL-10 mRNA were slightly increased or similar in all groups (Fig. 4B; also data not shown). Thus, neutrophil depletion had a comparatively milder effect on IL-10 cytokine mRNA levels in the liver than in the spleen. High levels of IFN-γ and TNF-α were induced by infection, but no statistically significant difference was measured for neutrophil-depleted mice compared to results for controls. An increase in IL-12p40 was also measured in the livers of *L. donovani*-infected mice but with no difference in the three groups (data not shown).

IL-10 can inhibit the production of cytokines such as IFN-γ indirectly at both the transcriptional and posttranscriptional levels. Therefore, the secretion of IFN-γ by splenic $CD4^+$ and $CD8^+$ T cells was determined for control and neutrophil-depleted mice by intracellular staining and analysis by FACS. The percentage of both $CD4^+$ and $CD8^+$ T cells secreting IFN-γ was dramatically reduced in the spleens of neutrophil-depleted mice compared to the values obtained from mice treated with control MAb (Fig. 5A). In addition, a significantly smaller amount of IFN-γ was produced by splenic cells isolated 14 days after infection and stimulated in vitro with ConA. A significant decrease in IFN-γ was observed in spleen cells from neutrophil-depleted mice compared to levels for controls ($P < 0.005$), as measured by ELISA (Fig. 5B).

Neutrophil depletion modifies serum cytokines and isotype switching in *L. donovani*-infected mice. To evaluate the effect of neutrophil depletion on the secretion of serum cytokines, we compared the serum levels of IL-4 and IL-10 in *L. donovani*-infected mice depleted of neutrophils or not depleted (Fig. 6A). Fourteen days after infection, neutrophil depletion resulted in a sharp increase in IL-4 and IL-10 levels over control values (Fig. 6A). Similar levels of IL-12p40 and IFN-γ were measured in the serum of infected mice depleted of neutrophils or not depleted (data not shown). The increase in IL-10 and IL-4 in the serum correlates with the higher levels of transcription of these cytokines' mRNA measured within the spleen (Fig. 4).

Since the balance of cytokines determines the IgG isotypes of Th responses, *L. donovani*-specific IgG1 and IgG2a antibody levels, characteristic of a Th2 or Th1 type of response, respectively, were assessed in neutrophil-depleted and control mice. The levels of IgG2a were similarly low and did not differ significantly between the serum of mice depleted of neutrophils or not depleted (Fig. 6B). In contrast, the levels of IgG1, associated with the production of IL-4, were significantly increased in neutrophil-depleted mice (Fig. 6B), in line with the elevated levels of IL-4 detected in the serum of these mice.

Altogether, these results demonstrate that the absence of neutrophils during the first days after infection affects the development of the immune response. A shift from an IFN-γ dominant response (Th1) to a $CD4^+$ Th2 type of response was observed, suggesting that neutrophils control the balance of these cytokines early following infection with *L. donovani*.

DISCUSSION

Neutrophils have been associated with efficient innate defense mechanisms in experimental models of infections with bacteria, fungi, and protozoa, including *Listeria* (3), *Legionella pneumophila* (59), *Mycobacterium* (48, 50), *Candida albicans* (52, 53), *Toxoplasma gondii* (7, 8, 37), and *Trypanosoma cruzi*

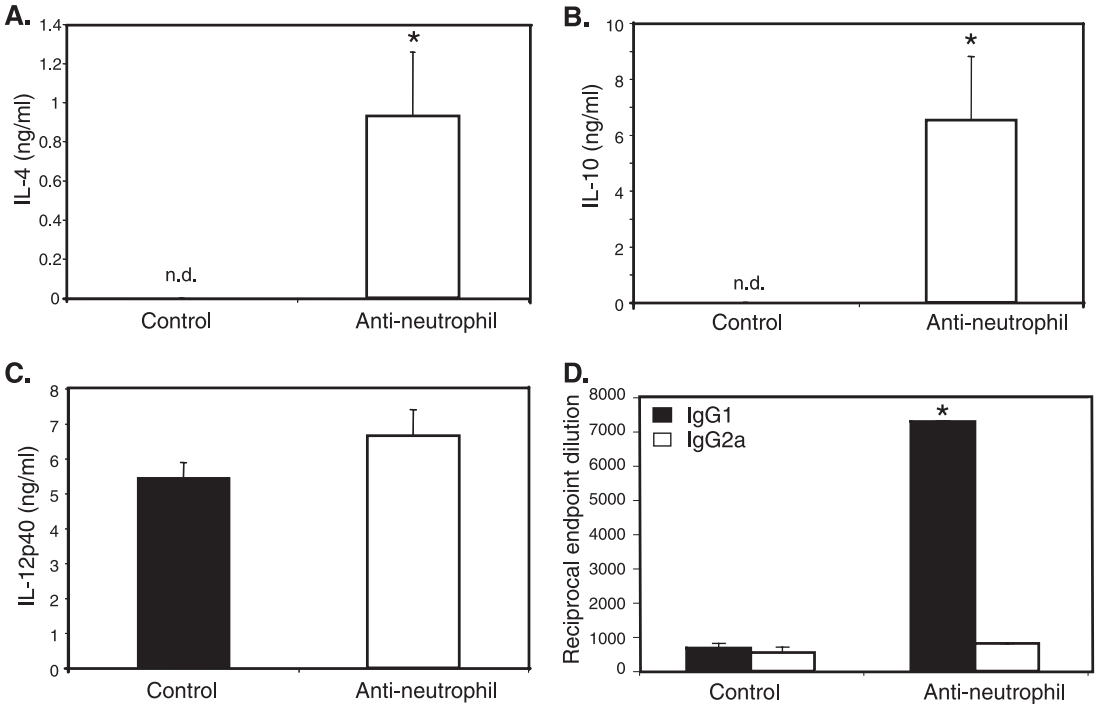

FIG. 6. Increased IL-4 and IL-10 cytokine levels and increased IgG1 levels in serum of neutrophil-depleted mice 14 days postinfection. (A to C) *L. donovani*-infected mice were treated with neutrophil-depleting MAb or a control MAb and serum cytokine levels analyzed 14 days postinfection. Serum levels of IL-4 (A), IL-10 (B), or IL-12 (C) were analyzed by ELISA. Results are the means ± standard errors of the cytokine levels for four to six mice per group from one representative experiment of two performed. n.d., not detectable. (D) Parasite-specific IgG1 and IgG2a levels were measured by ELISA in the serum of mice treated with control MAb or antineutrophil MAb at day 14 postinfection. A significant increase in IgG1 levels ($P < 0.0001$) in the NIMP-R14-treated group over levels for control MAb-treated mice was observed.

(in BALB/c mice). With these models of infection, the presence of neutrophils was associated with resistance to infection, and depletion of neutrophils by a MAb prior to infection resulted in increased susceptibility to these microorganisms (37, 51, 52). In sharp contrast, in the *L. major*-susceptible BALB/c strain of mice, the absence of neutrophils during the first week of infection had the opposite effect, reducing significantly the parasite number within the draining lymph nodes and at the site of infection (49, 58). However, in the current study, depletion of neutrophils caused disease exacerbation 14 days after infection with *L. donovani*, with significant increases in parasite burdens in the spleen and the bone marrow of infected mice, although the procedure did not result in a consistent increase in liver parasite burdens. The organ-specific differences in effects may be linked to the relative contributions of different cell types at these sites. The liver contains 80 to 90% of the tissue macrophages in the body, with Kupffer cells making up approximately 35% of the nonparenchymal liver cells in a mouse; therefore, neutrophils are likely to have less of an influence on parasite killing at this site (10).

Upon infection, human neutrophils were reported to have a delay in the killing of *L. major* (61), leading to the hypothesis that during the first hours postinfection, neutrophils were providing a safe environment for *L. major*, allowing its subsequent silent entry into macrophages (34). Conversely, human neutrophils and mouse neutrophils, respectively, have been reported to kill *L. donovani* and the related parasite *L. infantum* rapidly, within an hour postinfection (47, 54). However, a recent publication indicates that while most parasites are rapidly killed, some *L. donovani* promastigotes nevertheless survive in dog neutrophils, being targeted to nonlytic compartments of the cell (29). The transient survival of a few *L. donovani* parasites within neutrophils could potentially provide a safe transient niche for the parasite and allow its silent entry into macrophages, as was suggested for *L. major*. Thus, in the absence of neutrophils, the alteration of the immune response developing during the first days of infection could also result from a distinct kinetics of parasite entry into macrophages.

Neutrophils are key components of the inflammatory response, and an immunoregulatory role for neutrophils was recently demonstrated with several infectious disease models through the generation of chemokines and cytokines (2, 7, 11, 20, 49, 52, 60), creating a microenvironment in which parasite-specific T cells can be primed. Thus, the cytokines and chemokines produced by neutrophils can influence the development of immune responses to various microorganisms. In the present study, neutrophil depletion during early *L. donovani* infection was associated with the preferential development of a $CD4^+$ Th2 response, demonstrated by an increase in *L. donovani*-specific IgG1 and an increase in serum IL-4 and IL-10 compared to results for control mice. The modification of the *L. donovani*-driven immune response early after infection reported here demonstrates an immunoregulatory role for neu-

trophils in the early phase of infection, mainly in the spleens and with a lesser impact in the livers of infected mice.

Transcription of IL-4 and IL-10 was increased in spleens of neutrophil-depleted mice. No significant decrease in IFN-γ mRNA was observed in the spleens of neutrophil-depleted *L. donovani*-infected mice; however, reduced levels of IFN-γ secreted by $CD4^+$ and $CD8^+$ splenic T cells was observed, with significant consequences for the control of the parasite burden. IL-10 has been reported to inhibit cytokine secretion at both the transcriptional and posttranscriptional levels through mechanisms including the inhibition of accessory cell functions and/or the induction of the synthesis of inhibitory factors preventing cytokine secretion (6, 9, 26; reviewed in reference 28). Thus, the decrease in IFN-γ secretion may result from IL-10 inhibitory action.

The regulation of IL-10 by neutrophils is of special interest, as IL-10 has been reported to be a major immunosuppressive cytokine during *L. donovani* infection both in humans and mice. In humans, increased production of IL-10 and IL-4 has been measured following infection with *L. donovani* (15, 33, 56). The increase in IL-10 in the spleens of neutrophil-depleted mice appears crucial in mediating the negative effects associated with the absence of neutrophils (increase in splenic weight and parasite burden and possibly negative regulation of IFN-γ). In contrast to IL-10 levels, which are increased selectively in the spleen, an increase in IL-4 mRNA is observed both in the spleens and in the livers of *L. donovani*-infected neutrophil-depleted mice. However, the levels of IL-4 mRNA measured in the liver are significantly lower than those measured in the spleen, and that may be correlated with the milder effect of neutrophil depletion observed on liver weight and parasite burden.

In *L. donovani*-infected mice, the inhibitory role of IL-10 in the immune responses has been demonstrated using IL-10 blockade, IL-10-transgenic, and IL-10-deficient mice (40, 42). High levels of IL-10 may induce the alternative activation of macrophages, favoring parasite growth within granulomas (30). The increased source of IL-10 measured in neutrophil-depleted *L. donovani*-infected mice is not yet defined, since this cytokine can be secreted by many cells, including Th2 cells, T-regulatory cells, Th1 cells, dendritic cells, macrophages, neutrophils, and B cells. However, a recent study performed with *L. donovani*-infected patients revealed that IL-10-suppressive effects are mediated by $CD4^+$ Th1 IL-10-secreting cells (46).

The protection conferred by neutrophils in the livers of *L. donovani*-infected BALB/c mice, although less pronounced than that in the spleens, includes help in the maturation of functional hepatic granulomas; as in the absence of neutrophils, a significant delay in granuloma formation was measured. In addition, in the absence of neutrophils, the levels of iNOS within granulomas were reduced, suggesting a contribution for neutrophils in the induction of hepatic iNOS. Following infection with *L. donovani*, neutrophils are mainly present in the spleen, but a small number of infiltrating neutrophils have been found in the liver early after infection (38, 55). Neutrophils store iNOS in their primary granules; thus, a contribution of neutrophil-derived iNOS is possible. Indeed, in acetaminophen-induced liver injury, neutrophils have been reported to be an important source of iNOS, and depletion of neutrophils was shown to attenuate iNOS expression and the generation of nitrotyrosine, a marker of NO tissue damage (31). An indirect role for neutrophils in the regulation of the microbicidal functions of the granulomas is also possible through the secretion of cytokines and chemokines activating the host protective immune response (reviewed in reference 16).

Overall, the results presented in this study demonstrate that following infection with *L. donovani*, neutrophils play a critical role in the regulation of the balance of cytokines present at the sites of parasite infection, contributing to the microenvironment driving the development of *L. donovani*-specific immunity. In addition, neutrophils are critical effector cells in the onset of infection, contributing to killing of *L. donovani*. Thus, neutrophils favor host protection during the early development of the immune response against *L. donovani*.

ACKNOWLEDGMENTS

We thank P. Launois for helpful discussions and the Microscopy and Imaging core facility (MIM) at the BIL (Epalinges) for histology work.

This work was supported by grants from the Royal Society and the Swiss National Foundation for Scientific Research (320000-116197 to F.T.-C.).

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Editor: W. A. Petri, Jr.