Cytokines, Signaling Pathways, and Effector Molecules Required for the Control of *Leishmania (Viannia) braziliensis* in Mice

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Cutaneous leishmaniasis is caused by protozoan parasites of the genus *Leishmania*. The mechanisms of pathogen control have been established primarily in the mouse model of *Leishmania major* infection, but they may not hold true for other *Leishmania* species associated with cutaneous disease. Here, we analyzed the role of cytokines, signaling components, and effector molecules in the control of New World cutaneous leishmaniasis due to *L. braziliensis*. Unlike *L. major*, *L. braziliensis* caused small, nonulcerative, and self-healing skin swelling in C57BL/6 mice, as well as BALB/c mice. In contrast to the results obtained for *L. mexicana*, mice deficient for interleukin-12 or its key signaling molecule, signal transducer and activator of transcription 4, rapidly succumbed to severe visceral leishmaniasis. Infection of tumor necrosis factor knockout mice with *L. braziliensis* led to progressive, nonhealing skin lesions with erosions and hemorrhagic ulcerations, but in contrast to the results with *L. major*, only 20 to 30% of the mice developed fatal visceral disease. As seen with *L. major*, mice with a deleted inducible nitric oxide synthase gene (iNOS<sup>−/−</sup>) were unable to contain *L. braziliensis* in the skin, whereas the control of the parasite in the spleen remained unimpaired. Unlike what happens in *L. major* infections, NADPH oxidase had no impact on the course of disease in *L. braziliensis*-infected mice. These results not only define essential components of a protective immune response to *L. braziliensis* but also illustrate that the requirements for the control of cutaneous leishmaniasis vary between different parasite species.

Parasites of the protozoan genus *Leishmania* are transmitted by sandflies to mammalian hosts, including humans, in which they elicit a spectrum of diseases that range from cutaneous to mucocutaneous and visceral leishmaniasis (48). One key factor that determines the clinical manifestation and course of infection is the parasite species. Thus, local cutaneous leishmaniasis is caused by *Leishmania* (subgenus *Leishmania*) major and *L. (L.) tropica* in Europe and the Near and Far East and by *Leishmania* (subgenus *Viannia*) braziliensis, *L. (V.) guyanensis*, and *Leishmania* (L.) *mexicana* in the Americas, whereas the prototypic species associated with visceral leishmaniasis are *Leishmania* (L.) *donovani*, *Leishmania* (L.) *infantum*, and *L. chagasi* (18, 66) (below the subgenus is omitted in the names of the *Leishmania* species).

During the past 30 years experimental cutaneous infections of mice with *L. major* have been used widely to elucidate the cell types, cytokines, signal transduction cascades, and antileishmanial effector mechanisms that are necessary for the control of parasites replicating in myeloid cells, as well as for the clinical resolution of disease (9–11, 35, 54, 57). Although in vivo the defense against most species of *Leishmania* investigated to date follows the rule of CD4<sup>+</sup> T-cell- and gamma interferon (IFN-γ)-mediated activation of macrophages (5, 13, 19, 20, 61, 65), several studies revealed unexpected differences in the components of the immune system that are required for effective control of certain *Leishmania* species. For example, whereas IFN-γ, interleukin-12 (IL-12), and its signal transducer, signal transducer and activator of transcription 4 (STAT4), were essential for the development of a self-healing immune response to *L. major* (5, 24, 39, 59, 65), IFN-γ and STAT4, but not IL-12, were crucial for preventing disease progression in *L. mexicana*-infected mice (13). In another study with *L. mexicana*-infected mice endogenous IL-12 was found to be required for parasite control only during the late phase of infection (>10 weeks) (1). Similarly, IFN-γ<sup>−/−</sup> mice infected with *L. amazonensis* were as resistant as their wild-type counterparts during the first 10 weeks of infection and developed huge skin lesions only after this time (49). In *L. major*-infected mice inducible nitric oxide synthase (iNOS) was indispensable for the control of the parasite during the acute and chronic phases of infection in the skin and draining lymph nodes, whereas NADPH oxidase was required for parasite containment in the spleen (7). In *L. donovani*-infected mice, in contrast, iNOS, but not NADPH oxidase, was critical for the resolution of visceral infection (45). Thus, the paradigms of protection and pathogenesis established in the *L. major* mouse model do not necessarily hold true for New World cutaneous leishmaniasis or visceral leishmaniasis (40).

*L. braziliensis* is the most common cause of local cutaneous leishmaniasis in South America. Over the years, only a few
groups have started to investigate the immunological param-
eters that determine the course of *L. braziliensis* infections in mice (16, 19, 20, 36, 46, 55). Cutaneous footpad or ear infec-
tions with *L. braziliensis* lead to transient, self-healing skin swel-
ing in both BALB/c and C57BL/6 mice (16, 19, 20, 37, 55, 62). This contrasts with the well-studied *L. major* mouse model, in which C57BL/6 mice contain the infection, whereas BALB/c mice usually develop progressive, nonhealing skin le-
sions and lethal visceral disease after challenge with *L. major* (10, 57). The control of *L. braziliensis* was shown to be depend-
ent on the generation of IFN-γ (19, 20). Also, lymph node cells from *L. braziliensis*-infected BALB/c mice produced sig-
ificantly less IL-4 upon restimulation in vitro than the corre-
sponding cells from *L. major*-infected BALB/c mice (19). The development of self-healing *L. braziliensis* skin lesions was ac-
companied by the expression of a broad spectrum of chemo-
kines that are known to attract neutrophils, monocytes/macro-
phages, NK cells, and CD4+ and CD8+ T cells (62).

In the present study we aimed to further characterize the protective immune response to *L. braziliensis* using BALB/c and C57BL/6 mice. We focused on analysis of cytokines, signal transduction factors, and antimicrobial effector mechanisms that had not yet been investigated in this model. Our results reveal that IL-12, STAT-4, tumor necrosis factor (TNF), and iNOS, but not NADPH oxidase, are critical for the control of *L. braziliensis* in vivo and in vitro.

**MATERIALS AND METHODS**

**Mice.** Female C57BL/6 and BALB/c mice were from Charles River Breeding Laboratories (Sulzfeld, Germany). Breeding pairs of mice with a disrupted iNOS gene (33) (iNOS–/–; 11th generation backcross to C57BL/6 mice), with a deleted gp91phox NADPH oxidase gene (50) (gp91phox−/−; 11th generation backcross to C57BL/6 mice), or lacking a STAT4 gene (29) (STAT4–/–; 11th generation backcross to BALB/c mice) were from the Jackson Laboratories (Ann Arbor, MI). Breeding pairs of C57BL/6 mice deficient for TNF (31) (TNF−/−) were kindly provided by H. Körner (University ofTownsville, Australia). Mice deficient for the IL-12 p35 subunit (39) (IL-12p35−/−; fifth generation backcross to BALB/c mice) and mice deficient for both the IL-12 p35 and IL-12 p40 subunits (IL-12p35/p40−/−; fifth generation backcross to C57BL/6 mice) were bred by one of us (G.A.) and were kindly provided by H. Mossmann (Max Planck Institute of Immunobiology, Freiburg, Germany), respectively. All mice used were bred and maintained in the specific-pathogen-free animal facilities of the Institute of Clinical Microbiology, Immunology and Hygiene (University of Erlangen) or the Institute of Medical Microbiology and Hygiene (University of Freiburg) in accordance with the guidelines for animal research in Germany. The in vivo experiments were approved by the local governments of Middle Franco-
nia and Freiburg.

**Parasites and parasite antigen preparation.** The *L. braziliensis* strain used in this study (MHOM/BR/84/BS-327) was originally isolated from a biopsy of a lymph node of a 12-year-old child from Ceará with the bubonic form of cutane-
ous leishmaniasis, which is very common in northeast Brazil and is characterized by an enlarged regional lymph node ("bubo") draining the skin lesion (4, 58). The strain was initially passaged in hamsters and was specifically identified by using monoclonal antibodies and PCR (27, 64). The *L. major* strain (MHOM/IL/S1/FE010N) (60) was isolated from a cutaneous ulcer of a patient in Israel with classical localized cutaneous leishmaniasis.

**L. braziliensis and *L. major* promastigotes were propagated in vitro in RPMI 1640 medium plus 10% fetal calf serum on Novy-Nicolle-MacNeal blood agar slants for no more than five passages.** The virulence of *L. braziliensis* and *L. major* was maintained by regular in vivo passages in iNOS−/− C57BL/6 and wild-type BALB/c mice, respectively. Nondividing *L. braziliensis* promastigotes were pre-
pared by γ-irradiation (150 kilorads, 137Cs source) and were controlled for the inability to replicate by limiting dilution analysis (25).

**L. braziliensis** antigen and *L. major* antigen were prepared from stationary-
phase promastigotes, which were resuspended in phosphate-buffered saline (PBS) and subjected to five cycles of rapid freezing and thawing as described previously (56).

**Infection of mice and determination of parasite burden.** Eight- to 16-week-old age- and sex-matched mice were infected in the skin of the right hind footpad with different doses of stationary-phase *L. major* or *L. braziliensis* promastigotes from a low in vitro passage (≤5 passages) in 50 μl PBS. At each time point during infection, the swelling of the infected and uninfected footpads was measured with a metric caliper, and the measurements were related to each other using the following formula: percent increase in footpad thickness = [(thickness of infected right hind footpad in millimeters − thickness of uninfected left hind footpad in millimeters)/ thickness of uninfected left hind footpad in millimeters] × 100 (56, 60). In all experiments described here the thicknesses of the uninfected footpads ranged from 1.70 to 1.95 mm during the 50 to 150 days of infection. Bilateral infections were performed in some experiments in order to allow limiting dilution analysis, immunohistology analysis, and cytokine analysis of a single infected mouse. The number of parasites in the tissue was determined by limiting dilution analysis (60, 63). The suspensions of the tissue (footpad, lymph node cells, spleen cells) were subjected to 16 or 24 fivefold serial dilutions with 12 replicate wells per dilution step. The wells were assessed for growth of *L. major* or *L. braziliensis* promastigotes by light microscopy and by measuring the absorbance at 405 nm with an enzyme-linked immunosorbent assay (ELISA) reader (MR 5000; Dynex). The number of parasites per gram of mouse tissue was determined by Poisson statistics using the χ² minimization method (60). Significance was as-
sumed when the 95% confidence intervals did not overlap.

**Immunohistology of tissue sections.** Five- to 7-μm sections from lymph nodes and spleens were prepared with a cryostat microtome (model HM500 O; Fa. Microm, Walldorf, Germany), thawed on slides coated with Fre-Marker (Science Services, Munich, Germany) and stained with PAP PEN (Science Services) and DAB (Dako). Staining was dried, fixed in acetone (for 10 min at −20°C), and briefly washed in PBS-0.05% Tween 20. Endogenous peroxidase and nonspecific binding sites were blocked with Tris-buffered saline-0.15% H2O2 and with PBS-0.1% saponin-1% bovine serum albumin-20% fetal calf serum, respectively. Detection of iNOS, *L. bra-
ziliensis* (using an antisera raised against *L. mexicana* [51, 53]), *L. major* (using a rabbit anti-*L. major* antiseraer [6]), and cell types by immunoperoxidase stain-
ing (using a-aminonaphthylcarbozol as a substrate) and hematoxylin counter-
staining was performed as described previously (60).

**Culture, infection, and activation of macrophages.** Thiglycolate-elicited peri-
toneal exudate macrophages were prepared 4 days after intraperitoneal injection of 2 ml of 4% Brewer’s thiglycolate broth (7). Macrophages were cultured in RPMI 1640 medium containing 2 mM glutamine, 10 mM HEPES, 13 mM NaHCO3, 50 μM 2-mercaptoethanol, 100 μg/ml penicillin, 100 μg/ml strepto-
mycin, and 2.5 or 5% fetal bovine serum (Sigma, Deisenhofen, Germany).

For analysis of macrophage-mediated killing of *Leishmania*, macrophages were seeded into eight-well LabTek tissue culture peroxidase chambers (Nalge Nunc International, Naperville, IL) at a density of 2 × 105 to 5 × 105 macro-
phages/well and allowed to adhere for 2 h. The resulting macrophage monolayers were incubated with or without cytokines (IFN-γ [20 ng/ml]; obtained from BioSource, Camarillo, CA) for 14 days. Subsequently, the monolayers were fixed with Tris-buffered saline-0.15% H2O2 and with PBS-0.1% saponin-1% bovine serum albumin-20% fetal calf serum, respectively. Detection of iNOS, *L. bra-
ziliensis* (using an antisera raised against *L. mexicana* [51, 53]), *L. major* (using a rabbit anti-*L. major* antiseraer [6]), and cell types by immunoperoxidase stain-
ing (using a-aminonaphthylcarbozol as a substrate) and hematoxylin counter-
staining was performed as described previously (60).

**Culture and stimulation of total lymph node cells or purified lymph node CD4+ T cells.** At different time points during *L. major* or *L. braziliensis* infections single-cell suspensions were obtained from popliteal lymph node cells. In some experiments CD4+ T cells were purified (purity, ≥90%) from total lymph node cells by using anti-CD4 MicroBeads and magnetic cell sorting technology (Miltenyi Biotec, Bergisch-Gladbach, Germany). The cells were seeded into complete RPMI 1640 medium supplemented with 10% fetal bovine serum at a density of 2 × 107 cells/well (total lymph node cells) or 1 × 107 cells/well (CD4+ T cells) in 96-well plates and allowed to adhere at 37°C in 5% CO2-95% humidified air in medium alone or medium with *Leishmania* antigen (*L. braziliensis* antigen or *L. major* antigen; parasite/cell ratio, 1.25:1), concanavalin A (5 μg/ml; Sigma), recombinant murine IL-12 (5 ng/ml; R&D Systems), or IL-18 (10 ng/ml; R&D Systems) or combinations of these preparations. When purified CD4+ T cells...
were stimulated with *Leishmania* antigen, total spleen cells (3 x 10^6 cells/well) or magnetic cell sorting-purified splenic CD11c+ cells (purity, ≥80%; 1 x 10^5 cells/well) from naïve syngeneic mice were added as antigen-presenting cells. Supernatants of the cell cultures were collected after 72 h, and the levels of IFN-γ were measured by a capture ELISA (sensitivity, 20 pg/ml; BD Biosciences and R&D Systems).

**Quantitative analysis of tissue mRNA expression by real-time PCR.** Total RNA from lymph node tissue was extracted with Triol (Invitrogen, Karlsruhe, Germany), and 10 µg of RNA was reverse transcribed using a high-capacity cDNA archive kit (Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s recommendations. Subsequent reverse transcription (RT)-PCR was performed with an ABI Prism 7900 sequence detector (Applied Biosystems) using Taqman universal master mixture and Assays-on-Demand (Applied Biosystems), which include intron-overlapping forward and reverse primers and the 6-carboxyfluorescein-labeled probe for the target gene, respectively. The following assays were used: murine hypoxanthine guanine phosphoribosyltransferase 1 (Mm00446905_m1), murine IL-4 (Mm00445259_m1), murine IL-10 (Mm00439616_m1), murine IL-13 (Mm00434204_m1), murine IL-12p35 (Mm00475988_m1), murine IL-12p40 (Mm00431701_m1), murine IFN-γ (Mm00801778_m1), TNF (Mm00432858_m1), iNOS (Mm00440485_m1), and arginase 1 (Mm00475988_m1). Each cDNA was amplified and measured in triplet with 100 ng of cDNA per well in a 15-µl reaction mixture and the following cycle conditions: one cycle of 2 min at 50°C (RT reaction), 10 min at 95°C (activation of the polymerase and denaturation of the DNA), and 40 cycles of 15 s at 95°C (denaturation) and 60 s at 60°C (annealing and extension). The PCR results were analyzed with the SDS 2.1 software (Applied Biosystems), and the amount of mRNA of each gene of interest was calculated and the fold difference relative to the housekeeping gene by the formula: relative expression = 2 ^ [(CT(target) - CT(housekeeping))] / n.d., no parasites detected. The results of one of two independent experiments are shown.

**RESULTS**

Comparative analysis of *L. major* and *L. braziliensis* infections in the mouse model. In the first set of experiments we investigated the clinical courses of infection in *L. major*- and *L. braziliensis*-infected BALB/c and C57BL/6 mice. As expected, *L. major* caused progressive skin swelling and ulcers in BALB/c mice, whereas the lesions in C57BL/6 mice did not ulcerate and regressed in all infected mice (Fig. 1). In contrast, both BALB/c and C57BL/6 mice infected with *L. braziliensis* developed only small, nodular lesions (ca. 20 to 35% increase in footpad thickness) that completely resolved within 4 to 6 weeks, confirming previous findings with the same or different strains of *L. braziliensis* (19, 20, 62). There was no significant difference between the lesion sizes in *L. braziliensis*-infected BALB/c and C57BL/6 mice (Fig. 1).

Analysis of the parasite burden by limiting dilution analysis revealed the presence of *L. braziliensis* in the local lesions, in the draining lymph nodes, and in the spleens of acutely infected wild-type mice (Fig. 2 and data not shown). A direct comparison of *L. braziliensis*-infected mice and *L. major*-infected BALB/c mice revealed that during the early phase of infection (day 11) the parasite loads in the skin lesions and the draining lymph nodes were only slightly higher in *L. major*-infected mice, whereas at day 30 *L. major*-infected lesions and lymph node harbored at least 10^4-fold more parasites than the corresponding *L. braziliensis*-infected tissues (Fig. 2).

At later time points during infection *L. braziliensis* could be grown only from the popliteal lymph nodes (see the data for BALB/c mice on day 71 in Fig. 6). Despite complete clinical resolution of the infection, we observed long-term persistence of *L. braziliensis* parasites in the draining lymph nodes of both C57BL/6 and BALB/c mice, which has not been reported before. In four independent experiments with limiting dilution analyses at six different time points (days 90, 96, 106, 116, 245, and 254 of infection), the numbers of parasites per gram of lymph node tissue were between 2,076 (95% confidence interval, 600 to 3,525) and 204,000 (95% confidence interval, 111,280 to 301,179). Thus, similar to *L. major* (60), *L. braziliensis* is capable of evading elimination in mice.

In order to gain insight into the immunological basis for the differential courses of infection in *L. major* - and *L. braziliensis*-infected BALB/c mice, we analyzed the draining lymph nodes and the spleens of infected mice for the expression of cytokines and arginine-metabolizing pathways of macrophages that are associated with a nonprotective or protective immune response.

Analysis of cytokine mRNA levels by real-time RT-PCR during the early (day 11 or day 13), clinically acute (day 30), or...
late (day 76) phase of infection revealed 2- to 4-fold-higher expression of IFN-γ mRNA, 2- to 5-fold-lower expression of IL-4 mRNA, 4- to 5-fold-lower expression of IL-10 mRNA, and 3- to 20-fold-lower expression of IL-13 mRNA in the popliteal lymph nodes of *L. braziliensis*-infected BALB/c mice compared to *L. major*-infected BALB/c mice (Fig. 3 and data not shown). In accordance with the mRNA results, total lymph node cells, purified CD4+ lymph node T cells, or total spleen cells from *L. braziliensis*-infected mice released significantly more IFN-γ protein upon restimulation in vitro with IL-12 plus IL-18, concanavalin A, or *Leishmania* antigen than the corresponding cells from *L. major*-infected mice released (Fig. 4 and data not shown). Despite this IFN-γ shift, the lymph nodes from *L. braziliensis*-infected BALB/c mice did not express higher levels of IL-12 or iNOS mRNA than the *L. major*-infected tissues (Fig. 3 and data not shown). However, the expression of arginase I, which is driven by Th2-like cytokines (43), was at least 1,000-fold higher in *L. major*-infected lymph nodes at day 30 and day 76 than in *L. braziliensis*-infected lymph nodes (Fig. 3 and data not shown).

These results suggest that in *L. braziliensis*-infected BALB/c mice increased responsiveness to IL-12/IL-18 and *Leishmania* antigen leads to enhanced production of IFN-γ and reduced expression of IL-4, IL-10, and IL-13. This, in turn, causes a higher iNOS/arginase I ratio in *L. braziliensis*-infected mice than in *L. major*-infected mice, which is associated with rapid control of the parasites (Fig. 2) and a curative course of the infection.

**IL-12 and STAT4 are required for control of *L. braziliensis* in vivo.** IFN-γ is essential for the cure of infections with *L. braziliensis*, because anti-IFN-γ treatment or deletion of the IFN-γ gene was previously shown to lead to rapidly progressive dermal lesions that required euthanasia of the mice (19, 20). In contrast, *L. braziliensis*-infected IL-12p40-deficient mice were reported to develop skin lesions that were twice as large as those of C57BL/6 wild-type mice but then persisted for the total observation period of 12 weeks without clinical progression or causing lethality, whereas the lesions of wild-type mice completed resolved (20). Considering the unexpectedly different outcomes of infection in IFN-γ−/− mice and IL-12p40−/− mice (which lack both IL-12 and IL-23), we re-investigated the role of IL-12 for the control of *L. braziliensis*.
We analyzed the course of *L. braziliensis* infection in (i) BALB/c wild-type mice and IL-12p35<sup>−/−</sup> mice (BALB/c background), which lack only IL-12, and (ii) C57BL/6 wild-type mice and IL-12p35/p40<sup>−/−</sup> mice (C57BL/6 background), which are deficient for IL-12 and IL-23. The BALB/c and C57BL/6 control mice developed only small and rapidly healing skin lesions, confirming that they share a “resistant” phenotype in the *L. braziliensis* infection model (Fig. 1). In contrast, in both IL-12p35<sup>−/−</sup> and IL-12p35/p40<sup>−/−</sup> mice the local infection with 2<sup>×</sup>10<sup>6</sup> *L. braziliensis* promastigotes not only led to large (albeit nonulcerating) cutaneous lesions with prominent infiltrates of neutrophils and macrophages and high parasite loads in the skin and draining lymph nodes but also led to rapid visceralization of the parasite (with huge numbers of *Leishmania* in the spleen) and death of the mice between days 50 and 80 of infection (Fig. 5A and B and 6 and data not shown). The same phenotype was observed in BALB/c mice lacking STAT4, the key signal transducing molecule that becomes activated upon binding of IL-12 to the IL-12 receptor (Fig. 5B and 6).

The numbers of iNOS-positive cell clusters in the lymph nodes of *L. braziliensis*-infected BALB/c IL-12p35<sup>−/−</sup> and BALB/c STAT4<sup>−/−</sup> mice were drastically reduced compared to the numbers in BALB/c control mice (Fig. 7 and data not shown). Thus, similar to control of *L. major* (29, 38, 39) but unlike control of *L. mexicana* (13), the control of *L. braziliensis* in vivo depended not only on the activity of STAT4 but also on the presence of IL-12. In C57BL/6 mice that lacked IL-23 in addition to IL-12 (IL-12p35/p40<sup>−/−</sup> mice), *L. braziliensis* also caused visceral and fatal disease.
iNOS-dependent, but NADPH oxidase-independent, control of *L. braziliensis* in vivo. iNOS and phagocyte NADPH oxidase (Phox) are the two central antileishmania effector pathways in mice (9). However, for certain organs, therapeutic interventions, stages of infection, or strains of parasites the expression of iNOS or Phox was found to be dispensable or insufficient for clearance of *L. major* or *L. donovani* (3, 7, 44, 45). We therefore analyzed the role of iNOS and Phox in the control of *L. braziliensis* in vitro and in vivo.

Thioglycolate-elicited peritoneal macrophages of both C57BL/6 wild-type and C57BL/6 gp91phox<sup>−/−</sup> mice efficiently killed intracellular *L. braziliensis* after activation by IFN-γ or IFN-γ plus TNF for 72 h. In iNOS<sup>−/−</sup> macrophages cultured with medium alone the number of intracellular *L. braziliensis* parasites was smaller than the number in wild-type macrophages, which correlated with reduced initial uptake of *L. braziliensis* promastigotes (data not shown). However, activation of iNOS<sup>−/−</sup> macrophages by IFN-γ or IFN-γ/TNF failed to elicit any further reduction in the infection rate or in the number of intracellular *Leishmania* cells per infected macrophage, as seen in cytokine-stimulated wild-type or gp91phox<sup>−/−</sup> cells (Fig. 8A and B).

iNOS mRNA and protein were strongly expressed in the lymph nodes of C57BL/6 mice following infection with *L. braziliensis* (data not shown). C57BL/6 iNOS<sup>−/−</sup> mice developed severe, ulcerating, and nonhealing skin lesions upon infection with *L. braziliensis* promastigotes (Fig. 5A). The parasite loads in the footpad and in the draining lymph node were increased by factors of approximately 10<sup>4</sup> and 10<sup>2</sup>, respectively, but parasite spreading to the spleen (visceral leishmaniasis) and death of the mice did not occur (Fig. 6 and data not shown).

The *L. braziliensis*-induced dermal lesions in C57BL/6 gp91phox<sup>−/−</sup> mice were small, self-healing, and indistinguishable from those of C57BL/6 controls (Fig. 5A). The tissue parasite loads were similar in the two strains of mice (Fig. 6).

From these data we concluded that iNOS, but not Phox, is essential for the control of *L. braziliensis*.

**TNF protects *L. braziliensis*-infected mice against nonhealing cutaneous lesions.** In the absence of TNF *L. major* caused nonhealing cutaneous lesions (15) or fatal visceral disease (52, 68). As the overall expression of iNOS protein was unaltered in *L. major*-infected TNF<sup>−/−</sup> mice (52, 68), an iNOS-independent protective effect of TNF against *Leishmania* can be assumed. We therefore investigated the function of TNF in *L. braziliensis*-infected C57BL/6 mice.

In C57BL/6 TNF<sup>−/−</sup> mice *L. braziliensis* led to an unusual local inflammation with erosive skin lesions (loss of the epidermis) at the site of infection, hemorrhagic ulcerations, and impairment of the mobility of the ankle, knee, and hip joints (Fig. 9 and data not shown). During the early phase of infec-
tion (day 50) this severe clinical phenotype was clearly associated with strongly increased parasite loads in the tissues (Table 1). Immunohistology analysis of draining lymph nodes at days 37, 50, and 116 after infection revealed that TNF deficiency was associated with a more diffuse distribution of iNOS-positive cells but not with a reduction in the overall level of iNOS protein expression (data not shown). Two of 10 TNF−/− mice from three independent experiments that were infected with *L. braziliensis* and monitored for more than 48 days developed visceral leishmaniasis and died within 60 to 80 days after infection (data not shown). The remainder of the mice (after day 80) ultimately controlled the parasites locally and systemically, but despite very low parasite numbers in the tissues these mice were still unable to heal the skin lesions (Table 1 and data not shown). These findings contrast with previous results for C57BL/6 TNF−/− mice infected with our *L. major* strain (MHOM/IL/81/FE/BN), all of which rapidly succumbed to severe visceral disease in the absence of ulcerative skin lesions (68), but they are reminiscent of the nonlethal chronic cutaneous disease seen in C57BL/6 TNF−/− mice after infection with a different strain of *L. major* (MHOM/IL/80/FRIEDELIN) (52).

In light of the discrepancy between low parasite load and nonhealing cutaneous disease we tested whether the antiparasitic immune response itself rather than the replicating parasite is responsible for the severe pathology seen in *L. braziliensis*-infected mice. To this end, we injected C57BL/6 wild-type and TNF−/− mice with irradiated and nonreplicating but viable *L. braziliensis* promastigotes. Nonhealing skin lesions developed in TNF−/− mice infected with live, replicating parasites but not after injection of irradiated and nonreplicating but viable *L. braziliensis* promastigotes (Fig. 9).

These data demonstrate that in *L. braziliensis*-infected mice TNF is crucial for the coordinated healing of skin lesions induced by viable, replicating parasites and contributes to the suppression of parasite spread to visceral organs.

**DISCUSSION**

Cutaneous leishmaniasis is caused by Old and New World members of the protozoan genus *Leishmania*. The immunological mechanisms of pathogen control have been established primarily in the mouse model of *L. (L.) major* infection, but these mechanisms might not occur with other *Leishmania* species associated with cutaneous disease. There have been a few previous analyses that defined IFN-γ and IL-12p40 as components of a protective immune response against *L. braziliensis* (19, 20), but the present report is the first report that provides detailed information on the cytokines, signaling pathways, and effector molecules that are required for the resolution of an *L. braziliensis* infection in vivo. Our results demonstrate that IL-12, STAT4, iNOS, and TNF are all essential for healing of a local infection with *L. braziliensis*, whereas the NADPH oxidase is dispensable. IL-12 and STAT4 were necessary to prevent visceralization of the parasite. iNOS, in contrast, exerted only local antileishmanial effects in the skin and draining lymph node. Another novel result of our study was the observation that despite complete resolution of *L. braziliensis*-induced lesions, both C57BL/6 and BALB/c mice failed to fully eliminate parasites from draining lymph nodes.

Below we discuss differences between the immune responses to *L. braziliensis*, *L. major*, and *L. mexicana* that became apparent in the present study and previously published studies.

**Leishmania and Th1/Th2 cytokines.** In accordance with previous studies (19, 20, 62), BALB/c mice developed only small and transient skin lesions upon infection with *L. braziliensis*, whereas inoculation of this mouse strain with *L. major* led to the known progressive and nonhealing ulcers. This differential course of infection might be at least partially related to the strong expression of IL-12-inducing antigens in *L. braziliensis*, such as eukaryotic ribosomal elongation and initiation factor 4e (12). In line with this notion, we observed increased expression of IFN-γ mRNA and protein (Fig. 3 and 4). The comparable or only slightly different expression of IL-12 mRNA (data not shown) and iNOS mRNA (Fig. 3) in *L. braziliensis*- and *L. major*-infected BALB/c mice does not bring into question the existence of a Th1 shift in the *L. braziliensis*-infected BALB/c mice. First, the number of cells expressing IL-12 p40 protein in

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**TABLE 1. Tissue parasite loads in C57BL/6 TNF−/− and TNF−/− mice infected with *L. braziliensis***

<table>
<thead>
<tr>
<th>Days after infection</th>
<th>Footpad Load in TNF−/− mice</th>
<th>Lymph node Load in TNF−/− mice</th>
<th>Spleen Load in TNF−/− mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>ND b</td>
<td>1.3 × 10^6 (3.4 × 10^5 to 2.3 × 10^6)</td>
<td>ND b</td>
</tr>
<tr>
<td>116</td>
<td>ND b</td>
<td>2.1 × 10^6 (6 × 10^5 to 3.5 × 10^6)</td>
<td>ND b</td>
</tr>
</tbody>
</table>

The parasite load is expressed as the number of viable parasites per gram of tissue, which was determined by limiting dilution analysis in C57BL/6 and TNF−/− mice after infection with nonirradiated, replicating *L. braziliensis* promastigotes. No parasites were detected in C57BL/6 TNF−/− and TNF−/− mice infected with irradiated, nonreplicating *L. braziliensis* (not shown). The values in parentheses are the 95% confidence intervals.

b ND, no parasites detected.
the lymphoid tissue of *Leishmania*-infected mice is very small (23, 41). In the draining lymph nodes of *L. major*- or *L. braziliensis*-infected mice the expression level of IL-12p40 mRNA was at the limit of detection using real-time RT-PCR, whereas IL-12p35 mRNA was constitutively expressed at rather high levels in uninfected mice (data not shown). mRNA analyses of whole organs are therefore unlikely to detect differences that result from the upregulation or induction of IL-12p35 or IL-12p40 mRNA in a small number of lymph node cells. Second, the expression levels of mRNA transcripts do not necessarily correlate with differences in the protein level. The latter argument is especially true for iNOS, which is subject to strong translational regulation by arginase (22) (see below).

The most striking difference revealed by the real-time RT-PCR analysis was the significantly lower expression of IL-4, IL-10, and IL-13 mRNA in the lymph nodes of *L. braziliensis*-infected BALB/c mice during the early and acute phases of infection (Fig. 3 and data not shown). This correlated with at least 10-fold-lower expression of arginase I at day 30 of infection, leading to a much higher iNOS/arginase I ratio after infection with *L. braziliensis* than after infection with *L. major*. As arginase I inhibits the translation of iNOS mRNA and the production of NO in macrophages via degradation of arginine (8, 22) and favors the growth of *Leishmania* in vivo (28, 32), we postulate that the rapid control of the parasites and the healing of the skin lesions in *L. braziliensis*-infected BALB/c mice are causally related to the absence of upregulation of arginase I in these mice.

We found that, similar to infections with *L. major* (38, 39, 59), infections with *L. braziliensis* led to visceral disease and death in mice that were unable to produce bioactive IL-12p70 (BALB/c IL-12p35−/− mice) or to express the STAT4 signal transducer (BALB/c STAT4−/− mice). In *L. braziliensis*-infected C57BL/6 IL-12p35/p40−/− mice, which lack bioactive IL-12p70 as well as IL-23 (an IL-12 family member [p19/p40 heterodimer] that can exert IFN-γ-independent proinflammatory effects (17, 26)), we observed the same lethal phenotype. Thus, we could not confirm previous results obtained with C57BL/6 IL-12p40−/− mice (also devoid of IL-12p70 and IL-23) that developed persistent and nonhealing, but nonprogressive, skin lesions after infection with *L. braziliensis* without causing any lethality (20).

Our data further demonstrate that *L. braziliensis* differs strikingly from *L. mexicana*. The latter *Leishmania* species was found to be controlled in vivo in a STAT4-dependent manner, whereas IL-12p40 (IL-12/IL-23) was partially or completely dispensable (1, 13).

**Leishmania**, iNOS, and Phox. Infection of C57BL/6 wild-type, iNOS−/−, or Phox−/− mice with *L. major* revealed organ-specific control of the parasite by iNOS or NADPH oxidase (Phox). In the skin and draining lymph node iNOS was essential for the containment of *L. major*, whereas in the spleen (in which the expression of iNOS was low) parasite replication was primarily controlled by Phox (7, 21). In *L. braziliensis* infections, neither C57BL/6 iNOS−/− mice nor C57BL/6 gp91phox−/− mice exhibited an increased parasite burden in the spleen compared to wild-type mice (Fig. 6). This finding suggests that reactive nitrogen and reactive oxygen intermediates are each sufficient to control *L. braziliensis* in the spleen. In the case of *L. mexicana* iNOS deficiency also caused nonhealing skin lesions with increased parasite loads (13). To date, no published data are available on the role of Phox in the control of *L. mexicana* or *L. amazonensis*.

**Leishmania** and TNF. TNF has at least two functions during infections with *L. braziliensis*. During the acute phase of infection it contributes to the control of the parasite, which is reflected by the increased parasite numbers in the skin, lymph nodes, and spleens of TNF−/− mice (Table 1) and by the death of some (20 to 30%) of the mice. These findings contrast with the findings for *L. major*-infected mice, in which TNF was absolutely essential for suppression of parasite visceralization and host survival (68). The second, immunoregulatory function of TNF in *L. braziliensis*-infected mice can be readily recognized during the chronic phase of infection, when the skin lesions of TNF−/− mice do not heal despite efficient control of the parasites (Fig. 9 and Table 1). The mechanism underlying the presumed tissue-repairing function of TNF in *L. braziliensis*-infected mice is currently unknown. One possibility is that TNF is required for wound-healing processes. However, the majority of previous studies suggested that TNF does not induce but inhibits angiogenesis, collagen deposition, and epithelialization of skin lesions, because wound healing was clearly accelerated in TNF−/− or TNF receptor p55−/− mice (42, 67). In Brazilian patients with mucocutaneous leishmaniasis increased levels of TNF were found in the sera (14) and in tissue lesions (2), and treatment with the TNF inhibitor pentoxifylline (combined with antileishmanial pentavalent antimony) led to rapid epithelialization of the mucosal tissues (34). This finding contrasts with the nonhealing dermal erosions seen in *L. braziliensis*-infected TNF−/− mice. As another explanation for the nonhealing phenotype of TNF−/− mice, one could imagine that deletion of the TNF cassette caused an alteration in the major histocompatibility complex (MHC) locus (which is also located on chromosome 17), resulting in impaired antigen presentation. Although we cannot formally exclude the possibility of an impact of the TNF gene targeting on the MHC locus, several aspects argue against this possibility. First, the TNF locus is not in the immediate vicinity of the TNF gene targeting regions (it is 70 kb proximal of H-2D and approximately 1 Mb from I-A, I-E, and H-2K) (30). Second, previous experiments with *L. major* revealed a delay but no defect in antigen-dependent T-cell activation in TNF−/− mice (68). Third, if antigen presentation was severely impaired in *L. braziliensis*-infected TNF−/− mice, a strikingly increased parasite burden in the nonhealing skin lesions should be seen, which is not the case.

In TNF−/− mice the formation of primary B-cell follicles and germinal centers and the maturation of the humoral immune response are absent and strongly impaired, respectively (47). Accordingly, the levels of parasite-specific immunoglobulin G1 and immunoglobulin G2a serum antibodies were significantly reduced in *L. major*-infected TNF−/− mice compared to the levels in infected wild-type controls. On the assumption that anti-*Leishmania* antibodies contribute to the induction of a protective T-cell response (69), the reduced parasite control in *L. braziliensis*-infected TNF−/− mice during the acute phase of infection might be related to a lack of antibodies. This, however, does not explain the absence of healing of the chronic skin lesions.

In conclusion, in the present study we further defined the
immunological requirements for the control of *L. braziliensis* in vivo and thereby unraveled differences between *L. braziliensis*, *L. mexicana*, and *L. major*. Our observation that *L. braziliensis*-induced skin lesions do not heal in the absence of TNF suggests that anti-TNF treatment strategies in mucocutaneous leishmaniasis patients might fail if the concentration of TNF in the tissue falls below a critical level.

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