Infection and Immunity

Toward a Novel Experimental Model of Infection To Study American Cutaneous Leishmaniasis Caused by *Leishmania braziliensis*

Tatiana R. de Moura, Fernanda O. Novais, Fabiano Oliveira, Jorge Clarêncio, Almério Noronha, Aldina Barral, Claudia Brodskyn and Camila I. de Oliveira

Infect. Immun. **2005, 73(9):5827. DOI: 10.1128/IAI.73.9.5827-5834.2005.**

INFECTION AND IMMUNITY, Sept. 2005, p. 5827–5834 Vol. 73, No. 9
0019-9567/05/$08.00+0 doi:10.1128/IAI.73.9.5827–5834.2005

Toward a Novel Experimental Model of Infection To Study American Cutaneous Leishmaniasis Caused by *Leishmania braziliensis*

Tatiana R. de Moura,[1]† Fernanda O. Novais,[1]† Fabiano Oliveira,[1] Jorge Clarêncio,[1] Almério Noronha,[1] Aldina Barral,[1,2] Claudia Brodskyn,[1,3] and Camila I. de Oliveira[1]*

Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, 121, Salvador, BA 40295-001,[1] Faculdade de Medicina da Bahia, Universidade Federal da Bahia, Praça XV de Novembro S/N, Largo do Terreiro de Jesus, Salvador, BA 40025-010,[2] and Departamento de Biointeração, Instituto de Ciências da Saúde, Universidade Federal da Bahia, Av. Reitor Miguel Calmon, S/N, Salvador, BA 40110-160,[3] Brazil

Received 28 January 2005/Returned for modification 8 March 2005/Accepted 25 March 2005

***Leishmania* spp. cause a broad spectrum of diseases collectively known as leishmaniasis. *Leishmania braziliensis* is the main etiological agent of American cutaneous leishmaniasis (ACL) and mucocutaneous leishmaniasis. In the present study, we have developed an experimental model of infection that closely resembles ACL caused by *L. braziliensis*. In order to do so, BALB/c mice were infected in the ear dermis with 10^5 parasites and distinct aspects of the infection were evaluated. Following inoculation, parasite expansion in the ear dermis was accompanied by the development of an ulcerated dermal lesion which healed spontaneously, as seen by the presence of a scar. Histological analysis of infected ears showed the presence of a mixed inflammatory infiltrate consisting of both mononuclear and polymorphonuclear cells. In draining lymph nodes, parasite replication was detected throughout the infection. In vitro restimulation of draining lymph node cells followed by intracellular staining showed an up-regulation in the production of gamma interferon (IFN-γ) and in the frequency of IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells. Reverse transcription-PCR of ears and draining lymph node cells showed the expression of CC chemokines. The dermal model of infection with *L. braziliensis* herein is able to reproduce aspects of the natural infection, such as the presence of an ulcerated lesion, parasite dissemination to lymphoid areas, and the development of a Th1-type immune response. These results indicate that this model shall be useful to address questions related to the concomitant immunity to reinfection and parasite persistence leading to mucocutaneous leishmaniasis.**

Protozoan parasites of the genus *Leishmania* cause a broad spectrum of diseases, collectively known as leishmaniasis, that occur predominantly in tropical and subtropical regions. The leishmaniases are transmitted by different species of sand flies, and depending on the *Leishmania* species involved and the genetic makeup or immunological status of the host, different clinical manifestations of the disease are observed. In this sense, Old World cutaneous leishmaniasis caused by *Leishmania major* is usually benign; infection of human hosts leads to the development of a localized cutaneous lesion that eventually heals, leading to the generation of lifelong immunity. In contrast, New World cutaneous leishmaniasis caused by *Leishmania braziliensis* is distinguished from other leishmaniases by its chronicity, latency, and tendency to metastasize in the human host (10). In New World cutaneous leishmaniasis, a single ulcer with elevated borders and a necrotic center is frequently observed and a chronic inflammatory response develops despite the paucity of parasites. In 1 to 5% of patients, mucocutaneous leishmaniasis may develop due to the ability of this parasite species to persist within lesion scars after spontaneous or chemotherapy-mediated healing and to its ability to metastasize to the nasal mucosa (33, 44). In this case, extensive tissue destruction is observed as a result of the potent cell-mediated immune response triggered by parasite replication, a hallmark of mucosal leishmaniasis (30). More rarely, parasite invasion of the bloodstream results in disseminated skin lesions (15).

Murine models of cutaneous leishmaniasis are a valuable tool in dissecting mechanisms related to disease pathogenesis, resistance to a secondary infection, and vaccine development (reviewed in references 11, 21, and 42). The *L. major* murine model of infection has generally relied on the inoculation of large numbers of parasites (10^5 to 10^7) into subcutaneous sites, typically the mouse footpad. Under these conditions, the C57BL/6 mouse strain develops localized cutaneous lesions which spontaneously resolve and it is protected from disease upon a second inoculation with *L. major.* BALB/c mice, on the contrary, develop severe and uncontrolled lesions that lead to progressive disease. Therefore, upon experimental infection with *L. major*, distinct aspects of the clinical spectrum of human cutaneous leishmaniasis can be reproduced and, in this sense, C57BL/6 mice represent the resistant phenotype and BALB/c mice represent the susceptible phenotype.

However, in the natural course of *Leishmania* transmission, a small number of infective metacyclic parasites are introduced into the skin of the vertebrate host by the sand fly. Accordingly, Belkaid and colleagues have established a dermal experimental model of infection that closely resembles the natural infection (5, 6). In this situation, infection is initiated in the skin, which is organized in dermal and epidermal compartments each containing specialized cells able to deal with exposure to the hostile agents present in the external environment (reviewed in

* Corresponding author. Mailing address: Centro de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, 121, Salvador, BA 40295-001, Brazil. Phone: 55-71-356 8785, ext. 211. Fax: 55-71-356 8785, ext. 261. E-mail: camila@cpqgm.fiocruz.br.

† T. R. de Moura and F. O. Novais contributed equally to this work.

reference 24) after the injection of a small number of parasites. This type of experimental model has recently been established for another New World *Leishmania* species, namely, *L. amazonensis* (16, 36). However, in contrast to *L. amazonensis* and *L. major*, considerable less experimental work has been conducted with *L. braziliensis* in spite of the serious health problem posed by this parasite species in South America. This is probably due to the facts that *L. braziliensis* does not easily grow in vitro and that its conversion into metacyclics under standard culture conditions is inefficient, requiring that a large parasite inoculum (10^7 parasites) be used to successfully achieve infection (27). Importantly, most mouse strains appear to be resistant to infection with *L. braziliensis* (14, 35, 43). This phenotype has been associated with the inability of *L. braziliensis* to inhibit the Th1-type immune response developed after infection (18). In the present work, an experimental model of American cutaneous leishmaniasis, caused by *L. braziliensis*, has been established in BALB/c mice taking into account features of the natural course of transmission, such as the inoculation of a lower number of parasites (10^5) into a dermal site. The resulting clinical outcome is similar to that observed in the human host, particularly in terms of lesion ulceration, parasite persistence, and immune response.

MATERIALS AND METHODS

Mice. Female BALB/c mice were obtained from the Centro de Pesquisas Gonçalo Moniz/Fundaçáo Oswaldo Cruz Animal Facility, where they were maintained under pathogen-free conditions. Mice were used for experiments at 6 to 8 weeks of age by methods approved by The Animal Care and Utilization Committee from CPqGM/FIOCRUZ.

Parasite culture, intradermal inoculation, and lesion measurement. *L. braziliensis* strain MHOM/BR/01/BA788 was isolated from a patient with cutaneous leishmaniasis from the state of Bahia (northeastern Brazil) after brief (2–4) passages in culture medium. This isolate was identified as *L. braziliensis* by using PCR (12) and monoclonal antibodies (31). Promastigotes were grown in Schneider medium (Sigma Chemical Co., St. Louis, MO) supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 10% heat-inactivated fetal calf serum (all from Life Technologies, Rockville, MD), and 2% sterile human urine. Stationary-phase promastigotes (10^5 parasites in 10 μl of saline) were inoculated into the right ear dermis of age-matched BALB/c mice using a 27.5-gauge needle. Lesion size was monitored weekly for 10 weeks using a digital caliper (Thomas Scientific, Swedesboro, NJ).

Parasite load estimate. Parasite load was determined using a quantitative limiting-dilution assay as described previously (48). Briefly, infected ears and retromaxillar draining lymph nodes (LNs) were aseptically excised at 2, 4, 6, 8, and 10 weeks postinfection and homogenized in Schneider medium (Sigma Chemical Co., St. Louis, MO). The homogenates were serially diluted in Schneider medium supplemented as before and seeded into 96-well plates containing biphasic blood agar (Novy-Nicolle-McNeal) medium. The number of viable parasites was determined from the highest dilution at which promastigotes could be grown out after up to 2 weeks of incubation at 25°C.

Histological analysis. Infected ears were removed postmortem at 5 and 9 weeks postinfection and fixed in 10% formaldehyde. After 12 to 24 h of fixation, tissues were processed and embedded in paraffin and 5-μm-thick sections were stained with hematoxylin and eosin stain and analyzed by light microscopy.

Intracellular cytokine detection by flow cytometry. Reagents for staining cell surface markers and intracellular cytokines were purchased from BD Biosciences, San Diego, CA. Single-cell suspensions of draining LNs were prepared aseptically at 2, 4, 6, and 8 weeks postinfection with *L. braziliensis* or after intradermal injection of phosphate-buffered saline (PBS). The cells were suspended in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 10% fetal calf serum (all from Life Technologies, Rockville, MD), and 0.05 M 2-mercaptoethanol dispensed into 96-well plates. Cells were activated in the presence of anti-CD3 (10 μg/ml) and anti-CD28 (10 μg/ml) for 12 h at 37°C in 5% CO_2. After incubation for 4 h at 37°C in the presence of 10 μg/ml Brefeldin A (Sigma Chemical Co., St. Louis, MO), cells were collected and incubated with anti-Fc receptor antibody (2.4G2). Each labeling was carried out on 10^6 cells for 30 min on ice in a 100-μl volume. Cells were double stained simultaneously with anti-mouse surface CD4 (L3T4) and CD8 (53-6.7) conjugated to fluorescein isothiocyanate and Cy-Chrome, respectively. For the intracellular staining of cytokines, cells were permeabilized using Cytofix/Cytoperm (BD Biosciences) and were incubated with the following anti-cytokine antibodies conjugated to phycoerythrin: for gamma interferon (IFN-γ), XMG1.2; for interleukin-4 (IL-4), BVD4-1D11; for IL-5, TRFK-5; and for IL-10, JES5-16E3. The isotype controls used were rat immunoglobulin G2b (A95-1) and rat immunoglobulin G2a (R35-95). After staining, cells were fixed in 1% paraformaldehyde and, for each sample, 10^4 cells were analyzed. Data were collected and analyzed using CELLQuest software and a FACSort flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA).

Cytokine detection by ELISA. For measurement of in vitro cytokine production, single-cell suspensions of draining LNs were prepared aseptically at 2, 4, 6, and 8 weeks postinfection. The cells were diluted to 5×10^6 cells/ml in RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 10% fetal calf serum (all from Life Technologies, Rockville, MD), and 0.05 M 2-mercaptoethanol and dispensed into 96-well plates with or without stationary-phase *L. braziliensis* promastigotes (five parasites to one cell) or concanavalin A (10 μg/ml) (Amersham Pharmacia Biotech, Piscataway, NJ). Cultures were incubated at 37°C in 5% CO_2. Supernatants were harvested at 48 h and assayed for IL-4 and IL-10 or at 72 h and assayed for IFN-γ. Cytokine presence was determined by an enzyme-linked immunosorbent assay (ELISA) using commercial kits (BD Biosciences, San Diego, CA). Cytokine production of lymph node cells from mice injected with PBS was below the detection level of the kits used: for IFN-γ, 55 pg/ml; for IL-4, 5.5 pg/ml; for IL-10, 3 pg/ml.

RNA isolation and chemokine detection by RT-PCR. Infected ears and draining LNs were excised at 2, 4, 6, and 8 weeks postinfection, and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) as specified by the manufacturer. Synthesis of the first-strand cDNA was performed on 1 μg of total RNA using the SuperScript III First-Str Synthesis System for reverse transcription (RT)-PCR (Invitrogen, Carlsbad, CA). The resulting cDNA was employed in PCR using primers for CCL2/monocyte chemoattractant protein 1 (MCP-1), CCL3/macrophage inflammatory protein 1α (MIP-1α), CCL4/MIP-1β, CCL5/RANTES, and β-actin as described previously (47). Amplification products were visualized in 6% polyacrylamide gels stained with ethidium bromide.

Statistical analysis. The data are presented as means ± standard errors of the means. The significance of the results was calculated by a nonparametric one-way analysis of variance test (Kruskal-Wallis) using Prism (GraphPad Software, San Diego, CA), and a P value of <0.05 was considered significant.

RESULTS

BALB/c mice inoculated with *L. braziliensis* in the ear dermis develop an ulcerated lesion. To establish a natural model of American cutaneous leishmaniasis caused by *L. braziliensis*, parasites were inoculated in the ear dermis of BALB/c mice. In preliminary experiments, we observed that the inoculation of 10^5 stationary-phase promastigotes was able to induce ulcerated lesions in the majority of infected mice. After intradermal inoculation, lesion size was examined weekly over a period of 10 weeks. Infected mice developed a detectable lesion at week 3 (Fig. 1A). Lesion size progressed steadily and reached a maximum of 1 mm at week 5. Thereafter, lesion size regressed and complete ear scarring was observed at week 9 postinfection. Ear induration or lesion recurrences were not observed up to 9 months postinfection (data not shown). The dermal lesion observed at week 5 was nodular and ulcerated, with elevated borders and a sharp crater (Fig. 1B) similar to the dermal lesion observed in patients with cutaneous leishmaniasis caused by *L. braziliensis*.

Parasite load in the ear dermis differs from the parasite load in draining LNs of *L. braziliensis*-infected mice. To investigate if there was a correlation between lesion development and parasite replication, parasite load was estimated at both the inoculation site and draining LNs. In the ear dermis (Fig. 2), parasite expansion was observed at week 2 and progressed

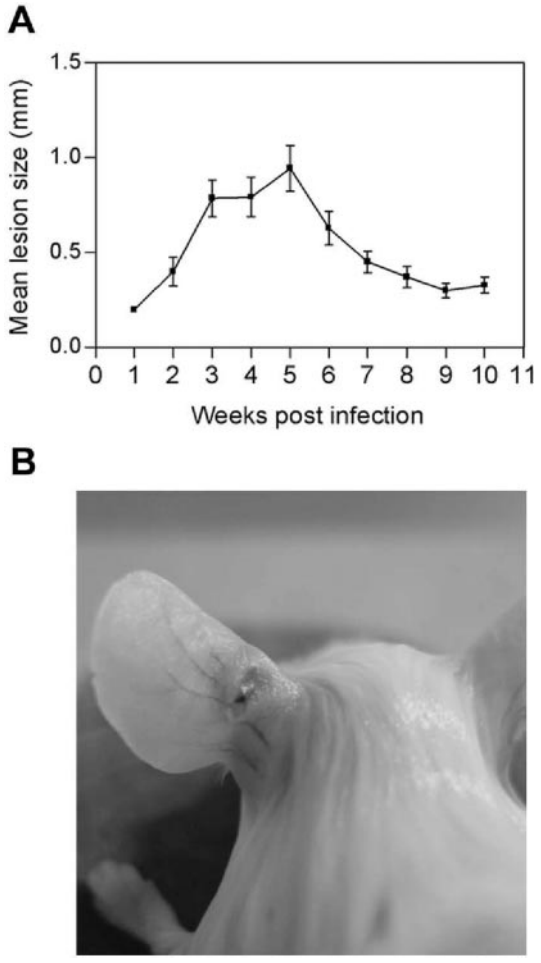

FIG. 1. Inoculation of *L. braziliensis* into the ear dermis of BALB/c mice leads to the development of an ulcerated lesion. (A) Mice were infected with 10^5 *L. braziliensis* promastigotes, and the course of lesion development was monitored for 10 weeks. Lesion sizes (in millimeters) are expressed as means and standard errors of the means from three independent experiments, each performed with five mice. (B) Photomicrograph of a mouse ear at 5 weeks postinfection demonstrating lesion ulceration.

steadily up to week 6, at which time a 1,000-fold increase ($\sim 10^8$ parasites/ear) was detected and lesion size reached its peak. From week 6 onward, the parasite load decreased and, after week 8, parasites were no longer detected in the ear dermis by either the limiting dilution assay or immunohistochemistry using an anti-*Leishmania* antibody (data not shown). At this time, healed lesion showed the presence of a scar; ear thickness, however, remained above 0.2 mm, the baseline thickness for a noninfected ear (Fig. 1A). In draining LNs (Fig. 2), parasite load reached a peak of $\sim 10^4$ parasites/ear at week 2 and remained at this level throughout the infection. Interestingly, we observed an increase in the cell number of lymph nodes draining the infection site: 1×10^7 cells at 2 weeks postinfection compared to 3.6×10^7 cells at 6 weeks postinfection, indicating that enlargement of the regional lymph nodes as seen in the natural infection (4) was also reproduced in this experimental model.

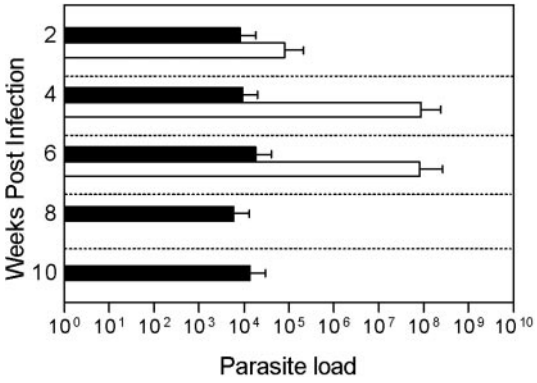

FIG. 2. Parasite load estimate in BALB/c mice after intradermal inoculation of *L. braziliensis*. Mice were infected with 10^5 *L. braziliensis* promastigotes. Ear (open bars) and draining lymph node (solid bars) parasite loads were determined at 2, 4, 6, 8, and 10 weeks postinfection via a limiting dilution assay. The data represent the means and standard errors of the means from three independent experiments, each performed with five mice.

Inflammatory response changes during lesion evolution in *L. braziliensis*-infected BALB/c mice, and this correlates with the control in parasite load. To analyze the inflammatory response in the ear lesions, histological aspects were evaluated. Following inoculation of the ear dermis with 10^5 *L. braziliensis* promastigotes, lesions exhibited a progressive increase in the inflammatory infiltrate. Five weeks postinfection (Fig. 3A), the inflammatory response was composed of polymorphonuclear cells, numerous macrophages, most of them heavily parasitized, and foam cells (Fig. 3B). Vascular changes and tissue necrosis were observed in parallel with ulcer formation. At 9 weeks postinfection, initial deposition of collagen could be seen, indicating the beginning of the cicatrisation process (Fig. 3C). The inflammatory infiltrate consisted mainly of macrophages, epithelioid cells with scarce foam cells, lymphocytes, and parasites. Few plasma cells and polymorphonuclear cells were observed (Fig. 3D)

IFN-γ is constantly produced by $CD4^+$ and $CD8^+$ T cells in draining LN of *L. braziliensis*-infected mice. Since parasite killing is associated with IFN-γ production, draining LN cells were stained for intracellular cytokine expression and analyzed by flow cytometry. The steady-state frequencies of cytokine-positive cells were determined using lymph node cells from mice inoculated with PBS. The frequency of $CD4^+$ T cells producing IFN-γ and antiinflammatory cytokines is shown in Fig. 4. The frequency of IFN-γ-producing $CD4^+$ T cells increased from week 2 to week 6 postinfection and decreased by week 8. This decrease was statistically significant compared to week 6 ($P < 0.05$) (Fig. 4). The frequency of IL-4-producing $CD4^+$ T cells increased from weeks 2 to 4 postinfection and decreased by week 8. Statistically significant differences in IL-4 production were observed between weeks 4 and 8 ($P < 0.05$) (Fig. 4). IL-5 and IL-10 secretion by $CD4^+$ T cells did not change significantly throughout the infection, although the percentage of IL-10-secreting $CD4^+$ T cells was higher than the frequency of IL-5-secreting cells (Fig. 4). We also analyzed the frequency of cytokine-producing $CD8^+$ T cells (Fig. 5). We observed an increase in the production of IFN-γ by $CD8^+$ T cells from week 2 to weeks 6 and 8 postinfection ($P < 0.01$) and

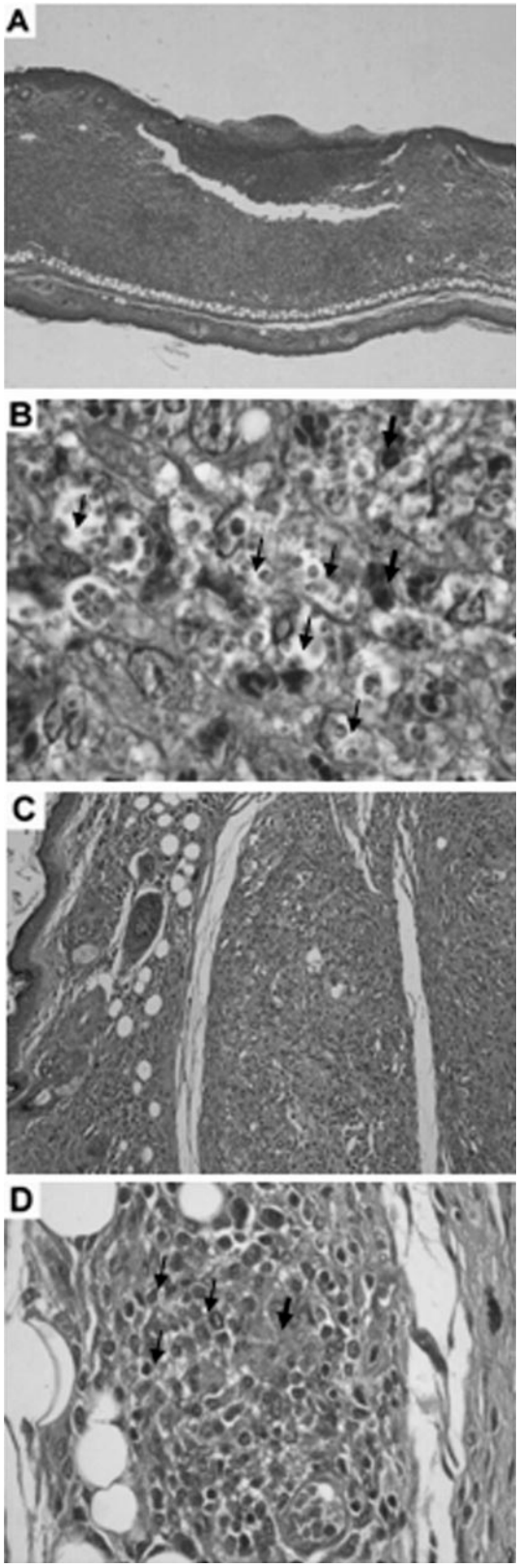

FIG. 3. Histological aspects of ear lesions in BALB/c mice after intradermal inoculation of *L. braziliensis*. BALB/c mice (five mice per group) were infected with 10^5 *L. braziliensis* promastigotes. Ears were removed at 5 (A and B) and 9 (C and D) weeks postinfection and stained with hematoxylin and eosin stain. (A) Ulcerated lesion showing extensive fibrinoid necrosis and inflammatory infiltrate extending up to the epidermis. Magnification, ×40. (B) Parasitized macrophages are indicated by thin arrows, and polymorphonuclear cells are indicated by broad arrows. Magnification, ×1,000. (C) Epidermal reconstitution accompanies the healing process. Magnification, ×100. (D) Macrophages are indicated by thin arrows, and epithelioid cells are indicated by broad arrows. Magnification, ×400.

from week 4 to week 6 ($P < 0.05$). The highest expression of IFN-γ was at week 6, and surprisingly, this expression was maintained at week 8, differently from IFN-γ-producing $CD4^+$ T cells. Moreover, the mean expression of IFN-γ by $CD8^+$ T cells was higher than that by $CD4^+$ T cells (7% versus 3.8%). Also in contrast to $CD4^+$ T cells, the highest frequency of IL-4-, IL-5-, and IL-10-producing $CD8^+$ T cells was observed at week 6 postinfection and decreased by week 8.

To determine whether the expression of intracellular cytokines in CD3-CD28-activated draining LN cells correlated with protein expression in *L. braziliensis*-stimulated draining LN cells, the levels of IFN-γ, IL-4, and IL-10 in LN cell culture supernatants were determined by ELISA (Fig. 6). Lesion development and parasite multiplication in draining LNs were associated with the production of IFN-γ, IL-4, and IL-10. Accordingly, cytokine secretion was highest 2 weeks postinfection and thereafter, secretion of IL-4 and IL-10 decreased whereas secretion of IFN-γ remained high until 8 weeks postinfection.

CC chemokines are expressed in ears and draining LNs of *L. braziliensis*-infected mice. To study the expression of CC ckemokines, RNA from infected ears and draining LNs was probed by RT-PCR. The steady-state cytokine expression was determined using ear and lymph node cells from uninfected mice. In the ear dermis, CCL2/MCP-1 was detected at 2 and at 4 weeks postinfection (Fig. 7A), which correlated with the peak lesion size and parasite load (Fig. 1A and 2). CCL5/RANTES expression in the ear was detected at 4 and 6 weeks postinfection (Fig. 7A). Expression of either CCL3/MIP-1α or CCL4/MIP-1β was not detected at this site. In draining LNs, CCL2/MCP-1 expression was detected from 2 to 6 weeks postinfection whereas CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES were expressed throughout the infection (Fig. 7B).

DISCUSSION

Currently, experimental models to study cutaneous leishmaniasis caused by *L. braziliensis* involve the inoculation of high numbers of parasites into the mouse footpad (14, 22, 35). In the footpad model, mice develop a transient lesion that resolves spontaneously due to the development of a strong Th1-type immune response following infection (18). In an attempt to reproduce the natural biology of *Leishmania* transmission, Belkaid et al. established a dermal model of infection in which low numbers of *L. major* promastigotes are inoculated into the ear dermis of mice (5). In the present study, we have established a similar model using *L. braziliensis*. Following inoculation of parasites into the ear dermis, BALB/c mice develop a cutaneous lesion similar to that developed by patients with American cutaneous leishmaniasis: localized and ulcerated, with elevated borders and a sharp crater (23, 28),

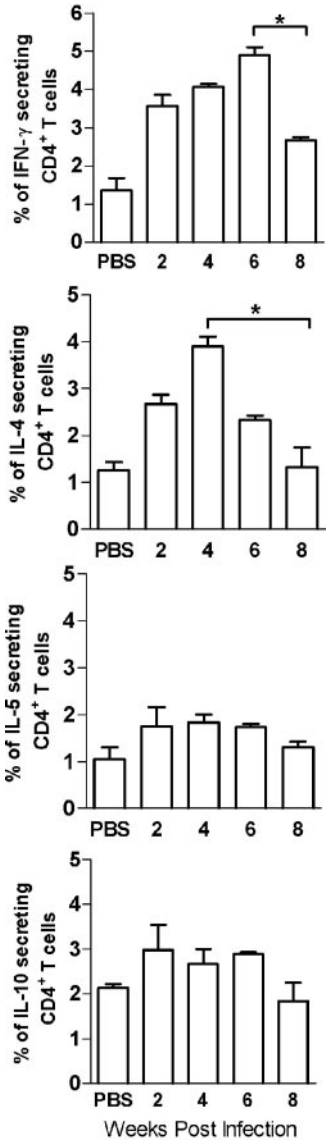

FIG. 4. Intracellular cytokine production by $CD4^+$ T cells from BALB/c mice after intradermal inoculation of *L. braziliensis*. Mice were inoculated with PBS or with 10^5 *L. braziliensis* promastigotes. At 2, 4, 6, and 8 weeks postinfection, draining lymph nodes were pooled and cells were preincubated with Brefeldin A for 4 h before being stained. Data represent the percentages of cells with signals for the particular cytokine that were greater than the background signals established using isotype controls. The data represent the means and standard errors of the means from three independent experiments, each performed with five mice per group. *, $P \leq 0.05$.

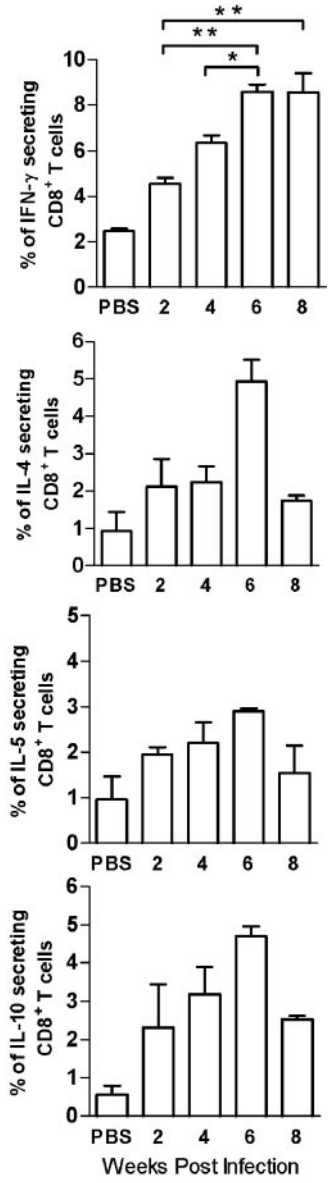

FIG. 5. Intracellular cytokine production by $CD8^+$ T cells from BALB/c mice after intradermal inoculation of *L. braziliensis*. Mice were inoculated with PBS or with 10^5 *L. braziliensis* promastigotes. At 2, 4, 6, and 8 weeks postinfection, draining lymph nodes were pooled and cells were preincubated with Brefeldin A for 4 h before being stained. Data represent the percentages of cells with signals for the particular cytokine that were greater than the background signals established using isotype controls. The data represent the means and standard errors of the means from three independent experiments, each performed with five mice per group. *, $P \leq 0.05$; **, $P < 0.01$.

reinforcing the notion that the site of infection plays an important role in disease outcome (3).

The conversion of *L. braziliensis* into metacyclic parasites is very inefficient in standard culture conditions, and although *L. braziliensis* metacyclic purification has been reported (37), we failed in obtaining agglutinated parasites for intradermal infection. In this study, dermal lesions were observed following the inoculation of 10^5 stationary-phase parasites whereas previous studies using this same parasite strain showed that the inoculation of 10^7 parasites into the footpads of BALB/c mice leads to, comparatively, mild increases in footpad thickness (22).

In the footpad model, BALB/c mice infected with *L. braziliensis* develop a local inflammation consisting of epithelioid macrophages and polymorphonuclear cells (19, 22). These same cell populations were recruited to the ear dermis. However, a marked recruitment of neutrophils was observed during the infection. The participation of neutrophils in cutaneous leishmaniasis lesions has been reported elsewhere (2, 26). In

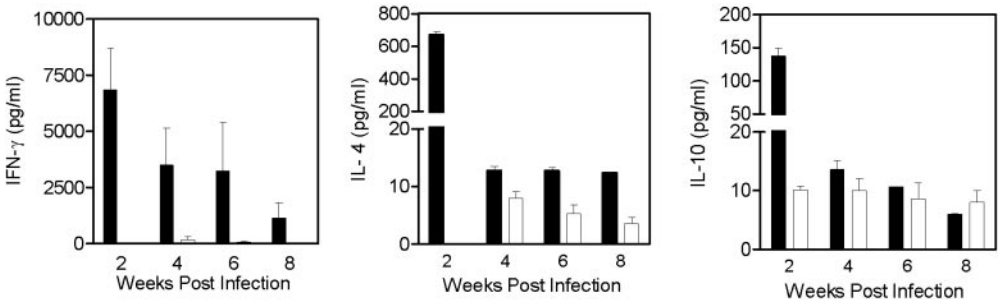

FIG. 6. Cytokine production in BALB/c mice after intradermal inoculation of *L. braziliensis*. Mice were infected with 10^5 *L. braziliensis* promastigotes. At 2, 4, 6, and 8 weeks postinfection, draining lymph node cells were collected and stimulated (solid bars) or not (open bars) with *L. braziliensis* for 48 h (IL-4 and IL-10) or 72 h (IFN-γ). Cytokine levels in supernatants were determined by ELISA. The data represent the means and standard errors of the means from three independent experiments, each performed with five mice per group.

resistant C57BL/6 mice, neutrophils prevent the growth of *L. major* and neutrophil depletion exacerbates infection (41). The cross-linking of CD28 on neutrophils results in the release of IFN-γ which, in turn, restricts parasite replication within macrophages (50). In *L. major*-infected BALB/c mice, however, the transient depletion of neutrophils leads to a decreased Th2-type immune response and partial resolution of footpad lesions (46). Therefore, it is possible that neutrophils actively played a protective role in our experimental model, as seen with *L. major*-infected B6 mice. However, we have yet to determine how this protection is mediated.

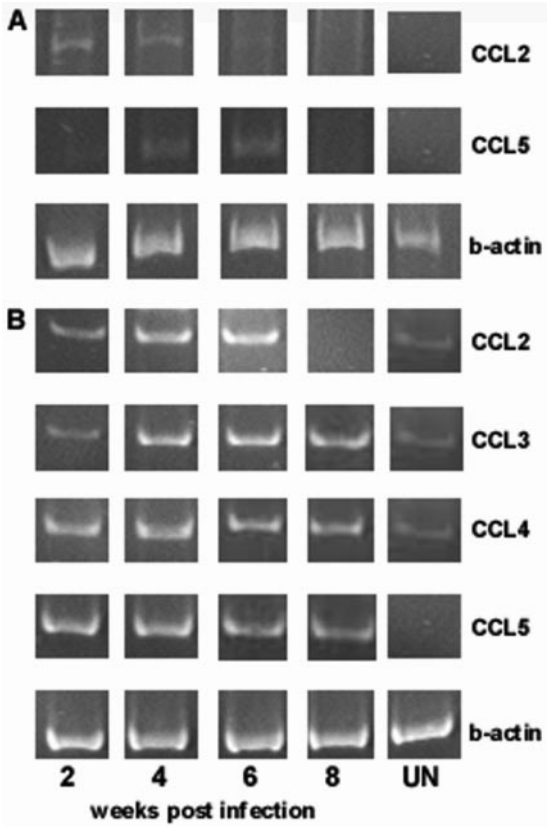

FIG. 7. Chemokine mRNA expression in BALB/c mice after intradermal inoculation of *L. braziliensis*. Mice were infected with 10^5 *L. braziliensis* promastigotes. At 2, 4, 6, and 8 weeks postinfection, total RNA was obtained from pooled ears (A), draining lymph nodes (B), and uninfected mice (UN). Total RNA was used in RT-PCR for the amplification of CCL2/MCP-1, CCL3/MIP1-α, CCL4-MIP1-β, CCL5/RANTES, and β-actin. Data shown are from a single experiment representative of three separate experiments, each performed with five mice.

In experimental models of cutaneous leishmaniasis, resistance is dependent on the development of a predominant Th1 response leading to parasite growth control, lesion healing, and the development of protective immunity (29, 40, 42). Failure to mount this type of response results in progressive disease. Accordingly, BALB/c mice are relatively resistant to infection with *L. braziliensis* due to the potent Th1 immune response that develops following infection characterized by high levels of IFN-γ and low levels of IL-4 and IL-10 (18). Using the footpad model of infection, it has been shown that *Leishmania*-reactive $CD8^+$ T cells are found in higher numbers in resistant mice (49). The role of $CD8^+$ T cells has recently been reexamined in the context of dermal infection with *L. major*, where lesion development was paralleled by parasite killing at the ear dermis and this outcome was associated with the accumulation of $CD8^+$ T cells in the skin and with their capacity to release IFN-γ upon stimulation (8). In the present study, infection of the ear dermis in mice led to a strong production of IFN-γ, secreted by both $CD4^+$ and $CD8^+$ T cells. The early presence of IFN-γ correlated with the control of parasite replication in the ear dermis and, consequently, lesion healing. Since no overt pathology was observed, we conclude that the proinflammatory effects of IFN-γ were correctly balanced by the presence of IL-4, IL-5, and IL-10, which were also detected during infection. Importantly, this immune response was effective at eliminating parasites from the ear dermis; however, parasite persistence was observed within draining lymph nodes.

Using the footpad model, we recently showed that this particular strain of *L. braziliensis* induces the expression of CCL2/MCP-1, CCL3/MIP-1α, and CXCL1/KC in BALB/c mice (47). CCL2/MCP-1α and CCL3/MIP-α are potent chemoattractants for monocytes (20, 25), and their expression in the ear and draining LNs of *L. braziliensis*-infected mice may recruit host cells, promoting parasite multiplication and lesion development. Later during infection, CCL2/MCP-1 expression in the ear dermis may be related with lesion healing since this chemokine participates in the induction of leishmanicidal activities in murine macrophages (9). CCL3/MIP-1α, CCL4/MIP-1β, and CCL5/RANTES expression in draining LNs may

contribute to the rapid development of a Th1-type immune response which, interestingly, cannot eliminate the parasites present at this same site. On the other hand, it is possible that *L. braziliensis* is interfering with the apoptosis of host cells, as has been shown in the infection of macrophages with *Leishmania donovani* promastigotes (34).

Parasite persistence may be related either to immune evasion or immunosuppression. It has been shown that both mice and humans continue to harbor parasites in the lymphoid tissue after spontaneous or chemotherapy-mediated healing (1, 39, 44). In the *L. major* model of infection, persisting parasites were associated with LN fibroblasts which, compared to macrophages, have a reduced ability to kill intracellular *L. major*. Moreover, it has been demonstrated that human skin fibroblasts are able to uptake *Leishmania* parasites (13, 17, 45). Although we have not determined which cell type is harboring *L. braziliensis* within LNs, fibroblasts seem a likely target. In terms of immunosuppression, participation of regulatory T cells in *L. major* persistence has also been described (7, 32). Regulatory T cells may therefore play a beneficial role by controlling overt pathology and ascertaining immunity to reinfection. Importantly, we observed that healed mice are immune to a challenge infection with *L. braziliensis* in the ear dermis (data not shown). Protection against homologous and heterologous challenge has been observed elsewhere following an *L. braziliensis* primary infection (27, 38).

The study presented here shows that BALB/c mice infected in the ear dermis with *L. braziliensis* develop ulcerated lesions that heal spontaneously. Lesion regression is safeguarded by the development of a powerful Th1 immune response, where $CD8^+$ T cells may play an important role in parasite elimination. However, this cellular immune response is unable to control parasite replication in draining LNs. Since the dermal inoculation of *L. braziliensis* showed herein actively reproduces many aspects of the natural infection, this model now enables us to address the basic immunological mechanisms of persistence leading to either mucocutaneous or, most importantly, protective immunity.

ACKNOWLEDGMENTS

We thank Manoel Barral-Netto and Maria Jania Teixeira for critical reading of the manuscript. We thank Margarida Pompeu and Washington L. C. Dos-Santos for help with histological analysis.

This work is supported by grants from FAPESB, PAPES/FIOCRUZ, and CNPq. T.R.d.M. was supported by a CNPq fellowship; F.O.N. and C.I.d.O. were supported by FAPESB fellowships. A. Barral and C. Brodskyn are senior investigators from CNPq and Instituto de Investigação em Imunologia (iii).

REFERENCES

1. **Aebischer, T., S. F. Moody, and E. Handman.** 1993. Persistence of virulent *Leishmania major* in murine cutaneous leishmaniasis: a possible hazard for the host. Infect. Immun. **61:**220–226.
2. **Andrade, Z. A., S. G. Reed, S. B. Roters, and M. Sadigursky.** 1984. Immunopathology of experimental cutaneous leishmaniasis. Am. J. Pathol. **114:** 137–148.
3. **Baldwin, T. M., C. Elso, J. Curtis, L. Buckingham, and E. Handman.** 2003. The site of *Leishmania major* infection determines disease severity and immune responses. Infect. Immun. **71:**6830–6834.
4. **Barral, A., M. Barral-Netto, R. Almeida, A. R. de Jesus, G. Grimaldi Junior, E. M. Netto, I. Santos, O. Bacellar, and E. M. Carvalho.** 1992. Lymphadenopathy associated with *Leishmania braziliensis* cutaneous infection. Am. J. Trop. Med. Hyg. **47:**587–592.
5. **Belkaid, Y., S. Kamhawi, G. Modi, J. Valenzuela, N. Noben-Trauth, E. Rowton, J. Ribeiro, and D. L. Sacks.** 1998. Development of a natural model of cutaneous leishmaniasis: powerful effects of vector saliva and saliva preexposure on the long-term outcome of *Leishmania major* infection in the mouse ear dermis. J. Exp. Med. **188:**1941–1953.
6. **Belkaid, Y., S. Mendez, R. Lira, N. Kadambi, G. Milon, and D. Sacks.** 2000. A natural model of *Leishmania major* infection reveals a prolonged "silent" phase of parasite amplification in the skin before the onset of lesion formation and immunity. J. Immunol. **165:**969–977.
7. **Belkaid, Y., C. A. Piccirillo, S. Mendez, E. M. Shevach, and D. L. Sacks.** 2002. CD4+CD25+ regulatory T cells control *Leishmania major* persistence and immunity. Nature **420:**502–507.
8. **Belkaid, Y., E. Von Stebut, S. Mendez, R. Lira, E. Caler, S. Bertholet, M. C. Udey, and D. Sacks.** 2002. CD8+ T cells are required for primary immunity in C57BL/6 mice following low-dose, intradermal challenge with *Leishmania major*. J. Immunol. **168:**3992–4000.
9. **Bhattacharyya, S., S. Ghosh, B. Dasgupta, D. Mazumder, S. Roy, and S. Majumdar.** 2002. Chemokine-induced leishmanicidal activity in murine macrophages via the generation of nitric oxide. J. Infect. Dis. **185:**1704–1708.
10. **Bittencourt, A., and A. Barral.** 1991. Evaluation of the histopathological classifications of American cutaneous and mucocutaneous leishmaniasis. Mem. Inst. Oswaldo Cruz **86:**51–56.
11. **Brodskyn, C., C. I. de Oliveira, A. Barral, and M. Barral-Netto.** 2003. Vaccines in leishmaniasis: advances in the last five years. Expert Rev. Vaccines **2:**705–717.
12. **Castilho, T. M., J. J. Shaw, and L. M. Floeter-Winter.** 2003. New PCR assay using glucose-6-phosphate dehydrogenase for identification of *Leishmania* species. J. Clin. Microbiol. **41:**540–546.
13. **Chang, K. P.** 1978. *Leishmania* infection of human skin fibroblasts in vitro: absence of phagolysosomal fusion after induced phagocytosis of promastigotes, and their intracellular transformation. Am. J. Trop. Med. Hyg. **27:** 1084–1096.
14. **Childs, G. E., L. K. Lightner, L. McKinney, M. G. Groves, E. E. Price, and L. D. Hendricks.** 1984. Inbred mice as model hosts for cutaneous leishmaniasis. I. Resistance and susceptibility to infection with *Leishmania braziliensis*, *L. mexicana*, and *L. aethiopica*. Ann. Trop. Med. Parasitol. **78:**25–34.
15. **Costa, J. M., P. D. Marsden, E. A. Llanos-Cuentas, E. M. Netto, E. M. Carvalho, A. Barral, A. C. Rosa, C. C. Cuba, A. V. Magalhaes, and A. C. Barreto.** 1986. Disseminated cutaneous leishmaniasis in a field clinic in Bahia, Brazil: a report of eight cases. J. Trop. Med. Hyg. **89:**319–323.
16. **Courret, N., T. Lang, G. Milon, and J. C. Antoine.** 2003. Intradermal inoculations of low doses of *Leishmania major* and *Leishmania amazonensis* metacyclic promastigotes induce different immunoparasitic processes and status of protection in BALB/c mice. Int. J. Parasitol. **33:**1373–1383.
17. **Dedet, J. P., A. Ryter, E. Vogt, P. Hosli, and L. P. da Silva.** 1983. Uptake and killing of *Leishmania mexicana amazonensis* amastigotes by human skin fibroblasts. Ann. Trop. Med. Parasitol. **77:**35–44.
18. **DeKrey, G. K., H. C. Lima, and R. G. Titus.** 1998. Analysis of the immune responses of mice to infection with *Leishmania braziliensis*. Infect. Immun. **66:**827–829.
19. **Donnelly, K. B., H. C. Lima, and R. G. Titus.** 1998. Histologic characterization of experimental cutaneous leishmaniasis in mice infected with *Leishmania braziliensis* in the presence or absence of sand fly vector salivary gland lysate. J. Parasitol. **84:**97–103.
20. **Fahey, T. J., III, K. J. Tracey, P. Tekamp-Olson, L. S. Cousens, W. G. Jones, G. T. Shires, A. Cerami, and B. Sherry.** 1992. Macrophage inflammatory protein 1 modulates macrophage function. J. Immunol. **148:**2764–2769.
21. **Gumy, A., J. A. Louis, and P. Launois.** 2004. The murine model of infection with *Leishmania major* and its importance for the deciphering of mechanisms underlying differences in Th cell differentiation in mice from different genetic backgrounds. Int. J. Parasitol. **34:**433–444.
22. **Indiani de Oliveira, C., M. J. Teixeira, C. R. Teixeira, J. Ramos de Jesus, A. Bomura Rosato, J. Santa da Silva, C. Brodskyn, M. Barral-Netto, and A. Barral.** 2004. *Leishmania braziliensis* isolates differing at the genome level display distinctive features in BALB/c mice. Microbes Infect. **6:**977–984.
23. **Jones, T. C., W. D. Johnson, Jr., A. C. Barretto, E. Lago, R. Badaro, B. Cerf, S. G. Reed, E. M. Netto, M. S. Tada, T. F. Franca, et al.** 1987. Epidemiology of American cutaneous leishmaniasis due to *Leishmania braziliensis braziliensis*. J. Infect. Dis. **156:**73–83.
24. **Kupper, T. S., and R. C. Fuhlbrigge.** 2004. Immune surveillance in the skin: mechanisms and clinical consequences. Nat. Rev. Immunol. **4:**211–222.
25. **Leonard, E. J., and T. Yoshimura.** 1990. Human monocyte chemoattractant protein-1 (MCP-1). Immunol. Today **11:**97–101.
26. **Lima, G. M., A. L. Vallochi, U. R. Silva, E. M. Bevilacqua, M. M. Kiffer, and I. A. Abrahamsohn.** 1998. The role of polymorphonuclear leukocytes in the resistance to cutaneous Leishmaniasis. Immunol. Lett. **64:**145–151.
27. **Lima, H. C., G. K. DeKrey, and R. G. Titus.** 1999. Resolution of an infection with *Leishmania braziliensis* confers complete protection to a subsequent challenge with *Leishmania major* in BALB/c mice. Mem. Inst. Oswaldo Cruz **94:**71–76.
28. **Llanos Cuentas, E. A., C. C. Cuba, A. C. Barreto, and P. D. Marsden.** 1984. Clinical characteristics of human *Leishmania braziliensis braziliensis* infections. Trans. R. Soc. Trop. Med. Hyg. **78:**845–846.
29. **Louis, J., H. Himmelrich, C. Parra-Lopez, F. Tacchini-Cottier, and P. Lau-**

nois. 1998. Regulation of protective immunity against *Leishmania major* in mice. Curr. Opin. Immunol. **10:**459–464.
30. **Marsden, P. D.** 1986. Mucosal leishmaniasis ("espundia" Escomel, 1911). Trans. R. Soc. Trop. Med. Hyg. **80:**859–876.
31. **McMahon-Pratt, D., E. Bennett, and J. R. David.** 1982. Monoclonal antibodies that distinguish subspecies of *Leishmania braziliensis*. J. Immunol. **129:**926–927.
32. **Mendez, S., S. K. Reckling, C. A. Piccirillo, D. Sacks, and Y. Belkaid.** 2004. Role for CD4(+) CD25(+) regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity. J. Exp. Med. **200:**201–210.
33. **Mendonca, M. G., M. E. de Brito, E. H. Rodrigues, V. Bandeira, M. L. Jardim, and F. G. Abath.** 2004. Persistence of *Leishmania* parasites in scars after clinical cure of American cutaneous leishmaniasis: is there a sterile cure? J. Infect. Dis. **189:**1018–1023.
34. **Moore, K. J., and G. Matlashewski.** 1994. Intracellular infection by *Leishmania donovani* inhibits macrophage apoptosis. J. Immunol. **152:**2930–2937.
35. **Neal, R. A., and C. Hale.** 1983. A comparative study of susceptibility of inbred and outbred mouse strains compared with hamsters to infection with New World cutaneous leishmaniases. Parasitology **87:**7–13.
36. **Norsworthy, N. B., J. Sun, D. Elnaiem, G. Lanzaro, and L. Soong.** 2004. Sand fly saliva enhances *Leishmania amazonensis* infection by modulating interleukin-10 production. Infect. Immun. **72:**1240–1247.
37. **Pinto-da-Silva, L. H., M. Camurate, K. A. Costa, S. M. Oliveira, N. L. da Cunha-e-Silva, and E. M. Saraiva.** 2002. *Leishmania (Viannia) braziliensis* metacyclic promastigotes purified using Bauhinia purpurea lectin are complement resistant and highly infective for macrophages in vitro and hamsters in vivo. Int. J. Parasitol. **32:**1371–1377.
38. **Porrozzi, R., A. Teva, V. F. Amaral, M. V. Santos da Costa, and G. Grimaldi, Jr.** 2004. Cross-immunity experiments between different species or strains of *Leishmania* in rhesus macaques (Macaca mulatta). Am. J. Trop. Med. Hyg. **71:**297–305.
39. **Ramirez, J. L., and P. Guevara.** 1997. Persistent infections by *Leishmania (Viannia) braziliensis*. Mem. Inst. Oswaldo Cruz **92:**333–338.
40. **Reiner, S. L., and R. M. Locksley.** 1995. The regulation of immunity to *Leishmania major*. Annu. Rev. Immunol. **13:**151–177.
41. **Ribeiro-Gomes, F. L., A. C. Otero, N. A. Gomes, M. C. Moniz-De-Souza, L. Cysne-Finkelstein, A. C. Arnholdt, V. L. Calich, S. G. Coutinho, M. F. Lopes, and G. A. DosReis.** 2004. Macrophage interactions with neutrophils regulate *Leishmania major* infection. J. Immunol. **172:**4454–4462.
42. **Sacks, D., and N. Noben-Trauth.** 2002. The immunology of susceptibility and resistance to *Leishmania major* in mice. Nat. Rev. Immunol. **2:**845–858.
43. **Samuelson, J., E. Lerner, R. Tesh, and R. Titus.** 1991. A mouse model of *Leishmania braziliensis braziliensis* infection produced by coinjection with sand fly saliva. J. Exp. Med. **173:**49–54.
44. **Schubach, A., F. Haddad, M. P. Oliveira-Neto, W. Degrave, C. Pirmez, G. Grimaldi, Jr., and O. Fernandes.** 1998. Detection of *Leishmania* DNA by polymerase chain reaction in scars of treated human patients. J. Infect. Dis. **178:**911–914.
45. **Schwartzman, J. D., and R. D. Pearson.** 1985. The interaction of *Leishmania donovani* promastigotes and human fibroblasts in vitro. Am. J. Trop. Med. Hyg. **34:**850–855.
46. **Tacchini-Cottier, F., C. Zweifel, Y. Belkaid, C. Mukankundiye, M. Vasei, P. Launois, G. Milon, and J. A. Louis.** 2000. An immunomodulatory function for neutrophils during the induction of a CD4+ Th2 response in BALB/c mice infected with *Leishmania major*. J. Immunol. **165:**2628–2636.
47. **Teixeira, M. J., J. D. Fernandes, C. R. Teixeira, B. B. Andrade, M. L. Pompeu, J. Santana da Silva, C. I. Brodskyn, M. Barral-Netto, and A. Barral.** 2005. Distinct *Leishmania braziliensis* isolates induce different paces of chemokine expression patterns. Infect. Immun. **73:**1191–1195.
48. **Titus, R. G., M. Marchand, T. Boon, and J. A. Louis.** 1985. A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. Parasite Immunol. **7:**545–555.
49. **Titus, R. G., G. Milon, G. Marchal, P. Vassalli, J. C. Cerottini, and J. A. Louis.** 1987. Involvement of specific Lyt-2+ T cells in the immunological control of experimentally induced murine cutaneous leishmaniasis. Eur. J. Immunol. **17:**1429–1433.
50. **Venuprasad, K., S. Chattopadhyay, and B. Saha.** 2003. CD28 signaling in neutrophil induces T-cell chemotactic factor(s) modulating T-cell response. Hum. Immunol. **64:**38–43.

Editor: W. A. Petri, Jr.