Impaired Expression of Inflammatory Cytokines and Chemokines at Early Stages of Infection with *Leishmania amazonensis*

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Infection of mice with *Leishmania major* results in disease progression or resolution, largely depending on the genetic backgrounds of the mouse strains. Infection with *Leishmania amazonensis*, on the other hand, causes progressive cutaneous lesions in most inbred strains of mice. We hypothesized that deficient activation of early immune responses contributes to the pathogenesis in *L. amazonensis*-infected mice. To distinguish early molecular events that determine the outcome of *Leishmania* infections, we examined cytokine gene expression in C57BL/6 mice infected with either *L. amazonensis* or *L. major* (a healing model). After 2 to 4 weeks, *L. amazonensis*-infected mice had significantly delayed and depressed expression of inflammatory cytokines (interleukin-12 [IL-12], gamma interferon, IL-1 α , IL-1 β), CC chemokines (CC chemokine ligand 3 [CCL3]/macrophage inflammatory protein 1 α [MIP-1 α], CCL4/MIP-1 β , CCL5/RANTES, MIP-2), and chemokine receptors (CCR1, CCR2, CCR5) in foot tissues and draining lymph nodes compared to the expression in *L. major*-infected controls. These findings correlated with defective T-cell responsiveness to parasite stimulation in vivo and in vitro. Adoptive transfer of *L. amazonensis*-specific Th1 cells prior to infection overcame the immune defects of the animals, leading to complete control of the disease. Studies with gene knockout mice suggested that IL-10, but not IL-4, contributed partially to compromised immunity in *L. amazonensis*-infected hosts. The data suggest that there is impairment in multiple immune functions at early stages of infection with *L. amazonensis* parasites and provide a compelling rationale to explore immune augmentation as an intervention in American cutaneous leishmaniasis.

Leishmania infections can result in a wide spectrum of clinical manifestations, and the outcome of disease is determined by complex host-parasite interactions. *Leishmania amazonensis*, a member of the *Leishmania mexicana* complex, has been identified from patients with diverse clinical forms, including cutaneous leishmaniasis, diffuse cutaneous leishmaniasis (DCL), and visceral leishmaniasis in South American countries (2). Patients with DCL are often resistant to chemotherapy and defective for the leishmanin skin test or antigen-stimulated T-cell proliferation in vitro, but they remain responsive for other antigens, such as tuberculin and lepromin (23, 41). The mechanisms responsible for this specific suppression of cell-mediated immune responses remain obscure, although it has been suggested that epidermal deficiencies of cytokines and cytokine-mediated accessory signals may play a role in DCL (52).

The regulation of host immune responses to *Leishmania* parasites has been well defined in cutaneous *Leishmania major* infections in inbred strains of mice that are either genetically susceptible (BALB/c mice) or resistant (most mouse strains) to the parasite. In this model, dominant Th1-type responses lead to the control of infection in resistant mice, whereas an early burst of interleukin-4 (IL-4) determines disease progression in susceptible hosts (27, 35). High levels of intralésional IL-10, on the other hand, are associated with both parasite persistence,

even in mice genetically resistant to *L. major* infection (8), and unresponsiveness of patients to chemotherapy (11). However, most inbred strains of mice are susceptible to *L. amazonensis* infection, developing chronic nonhealing skin lesions that differ at the onset and during the disease. There is increasing evidence that the host immune responses to *L. amazonensis* infection are different from the host immune responses to *L. major* infection. For example, during the course of infection with *L. amazonensis*, BALB/c and C57BL/6 mice do not mount a vigorous Th2 response, but they do generate detectable levels of Th1-type responses (1, 29, 49). Moreover, targeted deletion of the IL-4 or IL-10 gene in mice has no effect or a minimal effect on *L. amazonensis*-induced lesion development and tissue parasite loads (30, 31), whereas deletion of genes involved in the development of cell- and antibody-mediated immune responses significantly increases susceptibility (33, 50). Jones et al. (31) have recently described decreased expression of the IL-12 receptor β_2 (IL-12 β_2) chain in CD4⁺ T cells of *L. amazonensis*-infected C3H mice, as well as in CD4⁺ T cells of IL-4^{-/-} C57BL/6 mice, suggesting that an IL-4-independent mechanism is responsible for reduced IL-12 responsiveness and consequently for impaired Th1 responses in *L. amazonensis*-infected hosts. However, treatment of mice with either gamma interferon (IFN- γ) (7) or IL-12 (31) had no significant effect on the course of *L. amazonensis* infection. Since these two cytokines are known to promote and induce IL-12R β_2 chain expression (47, 51), these data suggest that the mechanisms responsible for susceptibility of mice to this New World parasite species are complex and warrant further investigation.

The susceptibility of mammalian hosts to intracellular pathogens is largely determined during the transition from

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innate immunity to specific immunity (19, 37). The local immunological milieu created at the initial stage of the host-pathogen interaction shapes adaptive cell-mediated immunity in subsequent development of disease or protective immunity (22, 38, 54). Studies with human biopsy samples have shown that lesions of patients with localized cutaneous leishmaniasis contain high levels of CC chemokine ligand 2 (CCL2)/monocyte chemoattractant protein 1 (MCP-1), CXCL9/Mig, and CXCL10/IFN- γ -inducible protein 10 (IP-10), whereas samples from patients with chronic DCL predominantly express CCL3/MIP-1 α , suggesting that different chemokine patterns may be linked to unique phenotypes of leishmaniasis (44). In mice, in vitro and in vivo infections with *L. major* can induce diverse inflammatory mediators, including tumor necrosis factor alpha (TNF- α), IL-1 β , and CC and CXC chemokines (13, 16, 39, 40, 43). Little is known about the expression profiles of these mediators in *L. amazonensis*-infected patients and mice. Given the low levels of IL-12 and IFN- γ production in *L. amazonensis*-infected mice (1, 31, 49), we hypothesized that progressive disease in these mice is due to a failure of induction of early inflammatory cytokines and chemokines that are important for Th1-cell development and macrophage effector functions. To test this hypothesis, we compared side by side the early immune responses in C57BL/6 mice to infections with *L. amazonensis* and *L. major* parasites and focused on the IL-1 and CC chemokine families. The expression profiles of cytokines and chemokines in foot tissues and draining lymph nodes (LNs) were assessed by using a multiprobe RNase protection assay and enzyme-linked immunosorbent assay (ELISA). Our results revealed that there is profound impairment in multiple immune functions at early stages of infection with *L. amazonensis* parasites. The mechanisms underlying this immune down-regulation were examined further by using mice adoptively transferred with *L. amazonensis*-specific Th1 cells and with targeted deletion of the IL-10 or IL-4 gene.

MATERIALS AND METHODS

Mice. Female C57BL/6 and BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, Ind.). Female IL-10^{-/-} and IL-4^{-/-} mice generated with a C57BL/6 background and wild-type controls were purchased from Jackson Laboratory (Bar Harbor, Maine). All mice were maintained under specific-pathogen-free conditions and used for experiments when they were 6 to 8 weeks old with protocols approved by the Animal Care and Use Committee of the University of Texas Medical Branch (Galveston, Tex.). Age- and sex-matched mice (five to eight mice/group) were infected subcutaneously in the hind foot with 2×10^6 stationary-phase promastigotes of *L. amazonensis* or *L. major*. Lesion size was monitored with a digital micrometer (Control Company, Friendswood, Tex.) and was expressed as the difference between the thickness of infected feet and the thickness of uninfected feet. Tissue parasite burdens were measured at different times by using a limiting dilution assay as previously described (49).

Parasite culture and antigen preparation. The infectivity of *L. amazonensis* (MHOM/BR/77/LTB0016) and the infectivity of *L. major* (MRHO/SU/59/P/LV39; obtained from Richard Titus, Colorado State University, Fort Collins) were maintained by regular passage through BALB/c mice. Promastigotes were cultured at 23°C in Schneider's *Drosophila* medium (pH 7.0 to 7.3) (Life Technologies, Rockville, Md.) supplemented with 20% fetal bovine serum (Sigma, St. Louis, Mo.), 2 mM L-glutamine, and 50 μ g of gentamicin per ml. To prepare amastigote lysates for the generation of T-cell lines and other experiments, lesion-derived amastigotes were recovered from foot tissues of BALB/c mice and cultured at 32°C for 5 to 7 days in the same Schneider's *Drosophila* medium except that the pH was adjusted to 5.0 to 5.3 and the gentamicin concentration was reduced to 25 μ g/ml. Parasites (1×10^8 cells/ml in phosphate-buffered

saline) were subjected to three freeze-thaw cycles and sonication for 45 min in an ice bath and then were stored at -70°C.

Detection of cytokines and chemokines and their receptor mRNAs by RPA. Total RNAs were extracted from draining LNs, isolated CD4⁺ LN T cells, or soft tissue of the infected foot at different times during infection by using a Micro RNA isolation kit (Stratagene, La Jolla, Calif.). An RNase protection assay (RPA) was conducted by using RiboQuant multiprobe RPA kits together with the following four template sets (all obtained from BD Biosciences): (i) set mCK-2b for the cytokines IL-4, IL-12p40, IL-1 α , IL-1 β , IL-1 receptor antagonist (IL-1Ra), IL-18/IGIF, and IFN- γ ; (ii) set mCK-5b for the chemokines RANTES, eotaxin, MIP-1 β , MIP-1 α , MIP-2, and MCP-1; (iii) set mCR-3 for the cytokine receptors IL-12 β 1, IL-12 β 2, IL-6R, IFN- γ R α , and IFN- γ R β ; and (iv) set mCR-5 for the chemokine receptors CCR1, CCR3, CCR4, CCR5, and CCR2. Briefly, antisense cRNA probes were transcribed via T7 RNA polymerase in the presence of [α -³²P]UTP (3,000 Ci/mmol; ICN Biomedical, Inc., Irvine, Calif.). After heating the preparations briefly at 90°C to denature the RNA, we incubated the labeled probes with 5- μ g portions of total RNA samples in hybridization buffer at 45°C for 16 h. Annealed products were digested with an RNase A-RNase T₁ mixture for 1 h at 30°C. The protected fragments were precipitated, dried, resuspended in loading buffer, and then denatured for 3 min at 90°C before electrophoresis on QuickPoint gels (NOVEX, San Diego, Calif.). The gels were dried for 1 h at 80°C before exposure to Kodak X-AR film at -70°C. The identity of each protected fragment was established by analyzing its migration distance by using a standard curve of migration distance versus the log nucleotide length for each undigested probe. The quantity of a given mRNA species in the original RNA sample was determined based on the signal densities as determined with an AlphaImager 2200 optical densitometer (Alpha Innotech Corp., San Leandro, Calif.) for the appropriately sized, protected probe fragment bands. Sample loading was normalized to the amount of the housekeeping gene coding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Measurement of cytokines and chemokines by ELISA. LN cells (5×10^6 cells) were prepared at different times during infection and cultured in 24-well plates in the presence or absence of parasite lysates (5×10^6 parasite equivalents). Culture supernatants were collected at 72 h. Specific ELISAs were performed by using pairs of monoclonal antibodies and recombinant mouse IL-4, IL-10, and IFN- γ (BD Biosciences). To directly assess the levels of cytokines and chemokines at the inflamed sites, foot tissues were homogenized in lysis buffer (15 mM Tris-HCl, 0.5% Triton X-100, 150 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂). Homogenized tissue samples were kept on ice for 30 min before centrifugation (850 $\times$ g for 30 min). After filtration through 0.2- μ m-pore-size syringe filters, supernatants were tested by using ELISA kits specific for IL-1 β , MIP-1 α (R & D System, Minneapolis, Minn.), and MCP-1 (BD Biosciences).

Proliferation of draining LN cells and purified CD4⁺ LN T cells. CD4⁺ T cells were purified from draining LNs by positive selection by using magnetic beads (Dyna, Lake Success, N.Y.). Briefly, LN cells (pooled from five mice/group) were incubated with appropriate amounts of anti-CD4 monoclonal antibody-coated beads for 20 min at 4°C with gentle shaking. The rosetted cells were collected by using a magnetic separator, and bound cells were separated from beads by using DETACHaBEAD mouse CD4 (Dyna). Purified cell preparations usually contained >90% CD4⁺ T cells, as analyzed by fluorescence-activated cell sorting. CD4⁺ LN T cells (10^5 cells) were cultured with 5×10^5 irradiated syngeneic splenocytes of naive C57BL/6 mice together with promastigote lysates (equivalent to 0, 10^3 , 10^4 , 10^5 , and 10^6 parasites/well) for 4 days in 96-well plates. One microcurie of [³H]thymidine was added 18 h before harvest, and the radioactivity was counted. Proliferative responses of LN cells were similarly tested in the presence or absence of parasite lysates (29).

Generation of *L. amazonensis*-reactive Th1-cell line and adoptive cell transfer. The procedures used for generating CD4⁺ T-cell lines were similar to those of Holaday et al. (28). Briefly, splenocytes were recovered from *L. amazonensis*-infected C57BL/6 mice (age, 4 months) and depleted CD8⁺ cells by using anti-CD8-coated beads (Dyna). CD8-depleted cells (5×10^6 cells) were cultured with *L. amazonensis* amastigote lysates (equivalent to 2×10^6 parasites) in complete IMDM containing 5 ng of IFN- γ per ml and a 1:8 dilution of culture supernatant of an anti-IL-4 hybridoma (11B11; American Type Culture Collection) for 7 days in 24-well plates. After centrifugation with Lympholyte-M separation medium (Cedarlane, Westbury, N.Y.) at 2,000 rpm (model BR 4i centrifuge; Jouan, Inc., Winchester, Va.) for 20 min, viable cells enriched at the interphase were collected, the concentration was adjusted to 2×10^5 cells/ml, and the preparation was allowed to rest in the absence of antigen for 7 days. After this, 2×10^5 viable cells were restimulated every 12 to 14 days with 5×10^6 syngeneic splenocytes pulsed with amastigote lysates in the presence of IFN- γ and anti-IL-4. Viable cells harvested after a 7-day rest were injected intravenously into the tail vein at different concentrations (1×10^6 to 5×10^6 or 10^7

FIG. 1. Lesion development during the course of infection with *L. amazonensis* and *L. major*. C57BL/6 mice (five mice/group) were infected subcutaneously with 2×10^6 stationary-phase promastigotes of *L. amazonensis* (*La*) or *L. major*. Lesion sizes were measured with a micrometer. The parasite load in foot tissues was assessed after 10 weeks of infection, and the numbers in parentheses are means $\pm$ standard deviations for the groups (in log scale). Two asterisks indicate that there is a statistically significant difference ($P < 0.01$) between the two infection groups.

cells/mouse) 1 day prior to infection with 2×10^6 stationary-phase promastigotes of *L. amazonensis*.

Statistical analysis. The differences between experimental groups were examined by using the Student's *t* test. A difference in mean values was considered significant when the *P* value was <0.05 or very significant when the *P* value was <0.01 .

RESULTS

Delayed production of inflammatory cytokines and CC chemokines during *L. amazonensis* infection. Following infection with *L. amazonensis*, C57BL/6 mice developed progressive disease, with the lesion sizes increasing from 0.5 mm at 2 weeks to 2.1 mm at 8 weeks, while the lesions in *L. major*-infected mice reached the peak values (1.1 mm) at 4 weeks and then declined to baseline values (0.1 mm) by 8 weeks (Fig. 1). At 10 weeks, the parasite loads in foot tissues of *L. amazonensis*-infected mice were about 4 logs higher than the parasite loads in foot tissues of *L. major*-infected mice ($\log_{10} 6.2 \pm 0.49$ versus $\log_{10} 2.1 \pm 0.18$). However, it is unclear why *L. amazonensis*-infected mice failed to mount a strong Th1 response, despite the absence of Th2 dominance (29). To test the hypothesis that the failure of these mice to mount a Th1 response was due to a change in the production of proinflammatory cytokines and Th1-favored CC chemokines at an early stage of infection, we examined the expression profiles of cytokines and chemokines during the course of infection with these two parasite species. Total RNAs were isolated from draining LNs and foot tissues after 0, 1, 2, 4, or 8 weeks of infection (three to five mice/group), and the levels of specific transcripts were measured by the RPA. The quantity of each mRNA species was normalized and expressed relative to the level of the housekeeping GAPDH gene. Consistent with previous reports (30, 31), we found that LNs from *L. amazonensis*-infected mice expressed almost no IL-12 or IFN- γ and a low level of IL-4 mRNA during the first 2 weeks of infection (Fig. 2A and B). Compared to the 2-week data, a very similar profile of gene expression was observed in LNs of *L. amazonensis*-infected mice at 4 weeks (data not shown). Expression of IFN- γ and IL-1 β became detectable after 8 weeks of infection with *L. amazonensis*.

In sharp contrast, the transcripts for IL-12, IFN- γ , IL-1 α , and IL-1 β were readily detectable even after 1 week of infection with *L. major*. Expression of these genes reached their peak levels after 2 weeks, and the levels remained relatively high at 8 weeks. After 2 weeks, the levels of IFN- γ and IL-1 β in *L. major*-infected mice were approximately 20- and 6-fold higher, respectively, than the levels in *L. amazonensis*-infected mice.

A more striking difference was observed for the foot tissues of *L. amazonensis*- and *L. major*-infected mice (Fig. 2C). In this case, expression of IL-1 α and IL-1 β was barely detectable throughout the 4-week infection period with *L. amazonensis*. However, the levels of expression peaked after 2 weeks of infection with *L. major* and were approximately 35- to 70-fold greater than the levels in *L. amazonensis*-infected mice, and then they decreased by 4 weeks. These results indicate that there was prompt and regulated expression of IL-1-type cytokines in self-healing mice; however, expression of these genes in the nonhealing mice was delayed for about 2 to 4 weeks and significantly reduced after onset. Interestingly, expression of IL-1Ra, a specific IL-1 receptor antagonist that functions as an anti-inflammatory cytokine by competing with IL-1 α and IL-1 β for binding to the IL-1 receptor (3), was induced like expression of IL-1 α and IL-1 β was induced (Fig. 2), suggesting that there is a balance between IL-1 and IL-1Ra in local tissues of *L. major*-infected hosts.

Chemokines in the CC family, especially the ligands for CCR5 and CCR2, are involved in differentiation of T-cell subsets, recruitment of leukocytes to the site of infection (37, 38), and activation of the leishmanicidal activity of macrophages (9, 45). We next examined whether a deficient Th1-type response in *L. amazonensis*-infected mice correlated with alterations in expression of CC chemokines. Foot tissues of *L. amazonensis*-infected mice showed no sign of chemokine expression after 1 and 2 weeks but exhibited low levels of chemokine expression after 4 weeks (Fig. 3). In contrast, significantly higher levels of CCL5/RANTES, CCL3/MIP-1 α , CCL4/MIP-1 β , and MIP-2 were detected after 2 weeks of infection with *L. major*, and the levels were about 6- to 20-fold higher than those in *L. amazonensis*-infected mice. Except for expression of CCL5/RANTES and CCL2/MCP-1, chemokine expression declined after 4 weeks of infection with *L. major* (Fig. 3B). As in the foot tissues, deficient expression of these chemokines was also observed in draining LNs of *L. amazonensis*-infected mice (data not shown).

To determine whether the change in cytokines and chemokines at the mRNA level correlated with protein expression, we collected LN cells and foot tissue extracts 1, 2, and 4 weeks after infection. The levels of IL-4, IL-10, and IFN- γ in culture supernatants of in vitro restimulated LN cells, as well as the levels of IL-1 β , MIP-1 α , and MCP-1 in foot tissue extracts, were determined by specific ELISAs. Compared with infection in *L. major* controls, *L. amazonensis* infection resulted in significant reductions in protein levels for IFN- γ , IL-1 β , MIP-1, and MCP-1 (Fig. 4). Although expression of the IL-10 gene was not always easy to document in an RPA (data not shown), the levels of IL-4 and IL-10 secretion were consistently higher in LN cells derived from *L. amazonensis*-infected mice than in cells from *L. major*-infected mice (Fig. 4). Once again, differences between the two infection models were most apparent after 2 and 4 weeks. Consistent with the RPA data (Fig. 2),

FIG. 2. Deficient expression of proinflammatory cytokines during infection with *L. amazonensis*. C57BL/6 mice were infected subcutaneously with 2×10^6 stationary-phase promastigotes of *L. amazonensis* (*La*) or *L. major*. At 0, 1, 2, and 8 weeks after infection, draining LNs were harvested and pooled (three to five LNs/group) for total RNA extraction. RPA analyses were performed for the genes indicated, as described in Materials and Methods, and the results of a representative assay are shown (A). (B and C) Quantities of each mRNA species detected in LNs (B) and foot tissues (C), normalized and expressed relative to the amount of GAPDH. The data are means $\pm$ standard deviations for three separate experiments. wk, week(s).

IL-4 production was not detectable after 2 weeks of infection with *L. major*, while the levels in *L. amazonensis*-infected mice decreased from 1.6 ng/ml after 1 week to 0.05 ng/ml by 4 weeks. It should be mentioned that although the levels of IL-4 production and IL-10 production at early stages of *L. amazonensis* infection were significantly higher than those in *L. major*-infected C57BL/6 mice, they were much lower than those reported for *L. major*-infected BALB/c mice (26, 29).

Deficient expression of cytokine and chemokine receptors in *L. amazonensis*-infected mice. The functions of inflammatory and chemotactant cytokines are often regulated at the level of their corresponding receptors. To examine whether *L. amazonensis*-infected mice were also deficient in expression of cytokine receptors, CD4⁺ T cells were purified from draining LNs after 2 weeks of infection (five to eight mice/group). Total RNA was immediately extracted for RPA. Consistent with a

FIG. 3. Deficient expression of CC chemokines during infection with *L. amazonensis*. C57BL/6 mice (five mice/group) were infected with 2×10^6 promastigotes of *L. amazonensis* (*La*) or *L. major*. At 0, 1, 2, and 4 weeks after infection, total RNA was extracted from foot tissues. RPA analyses were conducted for the genes indicated, and the results of a representative assay are shown (A). (B) Quantity of each mRNA species normalized and expressed relative to the amount of GAPDH. The data are means $\pm$ standard deviations for three separate experiments. wk, week(s).

previous report (31), we found a five- to sevenfold reduction in expression for the IL-12 receptor $\beta 2$ subunit (IL-12R $\beta 2$) in *L. amazonensis*-infected mice compared to the expression in *L. major*-infected controls (Fig. 5A and B) ($P < 0.01$). Furthermore, *L. amazonensis*-infected mice had significantly lower levels of CCR1, CCR2, and CCR5 mRNAs than *L. major*-infected controls (Fig. 5C and D) ($P < 0.05$). No apparent

changes were detected in the levels of expression of IL-12R $\beta 1$, IL-6R, gp130, IFN- γ R α , CCR3, or CCR4, suggesting that there was selective, rather than global, impairment in expression of cytokine and chemokine receptors in CD4 $^+$ T cells of *L. amazonensis*-infected mice.

Reduced T-cell responses to *L. amazonensis* antigens. To test whether the decreased cytokine and cytokine receptor levels have a major effect on T-cell responsiveness to *L. amazonensis* antigens, we collected draining LN cells from two groups of mice after 2 weeks of infection and stimulated these cells (5×10^5 cells/well/200 μ l) with lysates of *L. amazonensis* or *L. major* promastigotes. At concentrations equivalent to 10^3 to 10^6 parasites/well, there was a significant reduction in thymidine incorporation in LN cells of *L. amazonensis*-infected mice (Fig. 6A) compared with the incorporation in *L. major*-infected controls ($P < 0.01$). The differences were not due to a reduction in

FIG. 4. Differential production of cytokines and chemokines following infection with *L. amazonensis* or *L. major*. C57BL/6 mice (six to eight mice/group) were infected with 2×10^6 promastigotes of *L. amazonensis* (open bars) or *L. major* (solid bars). LN cells were cultured in vitro in the presence of parasite lysates (equivalent to 5×10^6 parasites/well), and the supernatant was collected 3 days later. Foot tissue homogenates were prepared and pooled from three naive and infected mice as described in Materials and Methods. The levels of IL-4, IL-10, and IFN- γ in the supernatants of LN cell cultures and of IL-1 β , MIP-1 α , and MCP-1 in foot tissue homogenates were determined by ELISAs. The data are means $\pm$ standard deviations for three animals per group in three independent experiments. One asterisk ($P < 0.05$) and two asterisks ($P < 0.01$) indicate that there are statistically significant differences between the two infection groups.

FIG. 5. Reduced expression of cytokine and chemokine receptors in *L. amazonensis*-infected mice. C57BL/6 mice (five to eight mice/group) were not treated or were infected with 2×10^6 promastigotes of *L. amazonensis* (*La*) or *L. major*. At 2 weeks after infection, draining LNs were harvested and pooled for isolation of CD4⁺ T cells by positive selection. Total RNA was extracted from CD4⁺ T cells, and RPA analyses were conducted for cytokine receptors (A) and chemokine receptors (B). (C and D) Quantities of mRNA species for cytokine receptors (C) and chemokine receptors (D) normalized and expressed relative to the amount of GAPDH. The data are means $\pm$ standard deviations for three separate experiments. One asterisk ($P < 0.05$) and two asterisks ($P < 0.01$) indicate that there are statistically significant differences between the two infection groups.

the levels of CD4⁺ T cells in the LNs of *L. amazonensis*-infected mice because the percentages of CD4⁺ T cells per LN were $22.97\% \pm 0.82\%$ and $17.71\% \pm 0.52\%$ in *L. amazonensis*- and *L. major*-infected mice, respectively. Moreover, LN cells from both groups of mice responded similarly to concanavalin A (data not shown). These data suggest that there was selective impairment in parasite-specific immune responses during *L. amazonensis* infection. To further define the function of CD4⁺ cells, we isolated CD4⁺ LN T cells after 2 weeks of infection and stimulated the cells with the corresponding parasite lysates together with irradiated, syngeneic splenocytes of naive C57BL/6 mice. A reduction in the proliferation of CD4⁺ LN T cells of *L. amazonensis*-infected mice was still evident, but the reduction was less (Fig. 6B) ($P < 0.05$). Therefore, it appears that CD4⁺ T cells in *L. amazonensis*-infected mice are intrinsically deficient in responsiveness to *L. amazonensis* antigen, probably due to impaired priming and optimal activation through their interaction with dendritic cells, which are known to shape antigen-specific T-cell responses in *L. amazonensis*-infected mice (42). In agreement with this view, we found

significantly smaller LNs in *L. amazonensis*-infected mice than in *L. major*-infected controls after 5 and 9 days of infection (data not shown). After 2 weeks, the total numbers of cells in *L. amazonensis*- and *L. major*-infected mice were 7.6×10^6 and 21.0×10^6 cells per LN, respectively ($P < 0.01$). These in vitro and in vivo observations suggest that there was impairment in CD4⁺ T-cell priming and activation during *L. amazonensis* infection.

Reversal of *L. amazonensis*-specific immune deficiency via adoptive transfer of Th1 cells. Given that there is insufficient priming of antigen-specific CD4⁺ T cells in *L. amazonensis*-infected mice and that treatment with recombinant IFN- γ or IL-12 alone does not result in parasite resolution (7, 31), we next examined whether bypassing T-cell priming via adoptive transfer of fully primed Th1 cells would restore gene expression and reverse the outcome of infection. We generated a Th1 cell line, which contained about 40% TNF- α -producing cells and 95% IFN- γ -producing cells and expressed predominantly V β 4 (14%) and V β 6 (58%) T-cell receptors. C57BL/6 mice were transferred intravenously with increasing doses of Th1

FIG. 6. Parasite-specific proliferation of draining LN cells and purified CD4⁺ LN T cells. Draining LN cells were collected from C57BL/6 mice at 2 weeks after infection with *L. amazonensis* (□) or *L. major* (■), and CD4⁺ LN T cells were isolated by positive selection. Draining LN cells (5×10^5 cells) (A) and CD4⁺ LN T cells (10^5 cells) (B) were cultured without and with 5×10^5 irradiated syngeneic splenocytes in the presence of different concentrations of promastigote lysates for 4 days in 96-well plates. One microcurie of [³H]thymidine was added 18 h before harvest, and the radioactivity was counted. The data are means $\pm$ standard deviations for two separate experiments. One asterisk ($P < 0.05$) and two asterisks ($P < 0.01$) indicate that there are statistically significant differences between the two infection groups.

cells (2×10^5 , 1×10^6 , 5×10^6 , or 1×10^7 cells per mouse) 1 day prior to infection. As shown in Fig. 7A, there were dose-dependent reductions in lesion size and tissue parasite burdens in mice undergoing cell transfer. Mice receiving 5×10^6 or 1×10^7 cells were completely protected against promastigote challenge, with no sign of lesions after 12 weeks and a 3- to 4-log reduction in the parasite load. This protection correlated with an average fivefold increase in IFN- γ production in LN cells and a 10-fold increase in splenocytes (data not shown).

Mice that received 5×10^6 Th1 cells were selected for detailed gene expression analyses. Compared to control animals, cell-transferred mice had significantly higher levels of IFN- γ , IL-1 β , and IL-1Ra in LN cells after 2 weeks (Fig. 7B), higher levels of RANTES, MIP-1 α , MIP-1 β , and MIP-2 in foot tissues (Fig. 7C), and increased levels of IL-12R β 2, CCR2, and CCR5 in CD4⁺ LN T cells (Fig. 7D and E) ($P < 0.01$). Enhanced levels of IL-12, IL-1 α , IL-12 β 1, and CCR1 were also detected in mice that received cells ($P < 0.05$). Overall, the levels of expression of these genes in cell-transferred, *L. amazonensis*-infected mice closely resembled the levels observed in *L. major*-infected mice (compare Fig. 7 with Fig. 2 to 4). These

studies suggest that an early, vigorous Th1 response to the parasite contributes to the induction of inflammatory mediator production in the skin.

Partial role of IL-10 in immune down-regulation. It is known that IL-4 and IL-10 can down-regulate inflammatory responses in many infection models (25, 55) and that both cytokines play critical roles in disease progression and parasite persistence in *L. major* infection (32). Given the enhanced production of IL-4 and IL-10 in the first few weeks of *L. amazonensis* infection (Fig. 4), it was important to examine whether endogenous production of these two cytokines contributed to impaired expression of inflammatory mediators. IL-10^{-/-} and wild-type C57BL/6 mice were infected with *L. amazonensis* promastigotes, and total RNA was extracted from LNs or foot tissues at zero time and after 2 and 4 weeks for RPA analysis. As shown in Fig. 8B, approximately 3- to 10-fold increases in the levels of IL-12, IFN- γ , and IL-1 β were detected in LNs of IL-10^{-/-} mice compared with the levels in wild-type controls. Foot tissues of IL-10^{-/-} mice exhibited two- to threefold increases in the levels of CCL5/RANTES, CCL3/MIP-1 α , CCL4/MIP-1 β , and MIP-2 compared to the levels in wild-type controls. This enhancement was most evident after 4 weeks of infection with *L. amazonensis*; however, the overall levels of gene expression in *L. amazonensis*-infected mice remained much lower than those in resistant mice (compare the data for *L. amazonensis*-infected mice to the data for *L. major*-infected mice in Fig. 2 and 3). Consistent with a previous report (30), IL-10^{-/-} mice remained susceptible to *L. amazonensis* infection and developed slightly smaller, but progressive lesions that contained about 10-fold fewer parasites than the wild-type control lesions contained (Fig. 8A). Similar studies were conducted with IL-4^{-/-} mice; however, we did not observe a significant increase in gene expression (data not shown). As reported by Jones et al. (31), *L. amazonensis*-infected IL-4^{-/-} mice developed progressive lesions with a high number of parasites in foot tissue ($\log_{10} 4.55 \pm 0.37$ parasites at 2 weeks and $\log_{10} 5.32 \pm 0.51$ parasites at 4 weeks), compared to the numbers in the wild-type controls ($\log_{10} 4.32 \pm 0.28$ parasites at 2 weeks and $\log_{10} 5.37 \pm 0.45$ parasites at 4 weeks). There were no statistical differences between the two infection models at the 2- and 4-week time points ($P > 0.05$). Collectively, these data suggest that IL-10, but not IL-4, contributes in part to immune down-regulation in *L. amazonensis* infection.

DISCUSSION

To address how susceptible mice develop progressive *L. amazonensis* infection in the absence of a robust Th2 response, we examined inflammatory responses at the site of parasite inoculation and in the draining LNs during the course of infection with *L. amazonensis* and compared the resultant expression profiles with those of *L. major*-infected, self-healing mice. A major finding of this study is that during the first 2 to 4 weeks after infection with *L. amazonensis* parasites, there are profound delays and reductions in expression of multiple inflammatory mediators, including IL-1 α , IL-1 β , CCL3/MIP-1 α , CCL4/MIP-1 β , CCL5/RANTES, and MIP-2, as well as receptors, such as IL-12R β 2, CCR1, CCR2, and CCR5 (Fig. 2 to 5). These alterations are accompanied by reduced T-cell respon-

FIG. 7. Reversal of immunodeficiency and control of *L. amazonensis* infection following Th1-cell transfer. C57BL/6 mice (five to eight mice/group) were not treated or received different doses of Th1 cells and then were infected with 2×10^6 promastigotes of *L. amazonensis* 1 day later. (A) Lesion sizes, as determined biweekly with a micrometer. The parasite load in foot tissues was assessed after 14 weeks of infection, and the numbers in parentheses are means $\pm$ standard deviations for the groups (in log scale). The parasite loads in mice that received 5×10^6 or 1×10^7 Th1 cells were lower than those in nontransferred controls ($P < 0.01$). Mice that received 5×10^6 Th1 cells were selected for subsequent gene expression analyses. At zero time and 2 weeks after infection, draining LNs (B), foot tissues (C), and purified CD4⁺ LN T cells (D and E) were collected and used for total RNA extraction. RPA analyses were performed for different genes. The quantity of each mRNA species was normalized and expressed relative to the amount of GAPDH. The data are means $\pm$ standard deviations for three separate experiments. One asterisk ($P < 0.05$) and two asterisks ($P < 0.01$) indicate that there are statistically significant differences between the transferred and nontransferred groups.

siveness (Fig. 6), which can be overcome by direct transfer of antigen-specific Th1 cells (compare Fig. 7 and Fig. 2 to 5). Therefore, coordination of multiple immune effector functions leads to the control of *L. major* infections in resistant mice, whereas impairments in this coordination fail to activate monocytes and macrophages in inflamed skin, leading to progressive disease in *L. amazonensis*-infected hosts. To our knowledge, this is the first report of a detailed analysis of a

FIG. 8. Partial reversal of immunodeficiency in *L. amazonensis*-infected IL-10^{-/-} mice. IL-10^{-/-} and control (WT) C57BL/6 mice were infected with 2×10^6 promastigotes of *L. amazonensis*. (A) Lesion sizes, as determined with a micrometer. (Inset) Parasite loads in foot tissues were assessed (log₁₀ scale) after 4 and 10 weeks of infection. One asterisk indicates that there is a statistically significant difference ($P < 0.05$) between the two infection groups at 4 and 10 weeks after infection. (B) Total RNAs were extracted from LNs or infected foot tissues at zero time and 2 and 4 weeks after infection and used for RPA analyses of different genes. The quantity of each mRNA species detected in LNs and foot tissues was normalized and expressed relative to the amount of GAPDH. The data are means $\pm$ standard deviations for three separate experiments.

large set of immunoregulatory genes in which infections caused by two clinically important *Leishmania* species were compared. This study also provides a compelling rationale for exploring immune augmentation for the control of cutaneous leishmaniasis.

It has been shown that in vitro infection of murine macrophages with *L. major* promastigotes rapidly induces expression of several CC chemokines; however, this induction is transient, as it reaches a peak at 2 to 4 h and declines to basal levels by 8 h (39). *L. major* infection in mice can also trigger the production of inflammatory cytokines, including TNF- α by epidermal keratinocytes and IL-1 β by Langerhans cells in murine epidermis (5, 37). These cytokines, in turn, induce the production of additional inflammatory mediators, including chemokines (40). Thus, the production of early cytokines and chemokines is critical for developing effective immunological control of *L. major* infection (37, 52). It was evident in this study (Fig. 2 to 4) that expression of inflammatory and chemoattractant cytokines in *L. major*-infected mice reached peaks after 2 to 4 weeks of infection and then declined. Of note, we found that expression of IL-1Ra was regulated like expression of IL-1 α and IL-1 β was regulated (Fig. 2). It has been demonstrated in certain cases that induced expression of IL-1 is compensated for, to some degree, by increased production of endogenous IL-1Ra (20, 34). In several cases, an excess amount of IL-1Ra (10- to 100-fold) over IL-1 is necessary to functionally inhibit the biological effects of IL-1 because of the spare receptor effect (4, 21). Although we detected comparable levels and kinetics of gene expression for IL-1 and IL-1Ra (Fig. 2) in a self-healing model of cutaneous leishmaniasis, the role of IL-1Ra in this disease remains unclear at this stage. Nevertheless, it appears that a balance between IL-1 and IL-1Ra is efficacious in preventing tissue damage in a variety of experimental models of inflammatory disease (3) and that regulated expression of IL-1 and IL-1Ra also correlates with the control of disease progression following *L. major* infection in genetically resistant mice (Fig. 1 and 2). In *L. amazonensis*-infected mice, however, expression of several inflammatory mediators in draining LNs and foot tissues was nearly undetectable after 1 to 4 weeks. Besides a marked delay in the onset of gene expression, the overall levels of secretion of these mediators were much lower in *L. amazonensis*-infected mice than in the healing mice. These observations are consistent with clinical studies, in which the levels of IL-1 β , IL-6, TNF- α , and CCL2/MCP-1 mRNAs are high in patients with self-healing lesions but are not detectable or very low in DCL lesions (14, 18, 46).

It is known that CCR5 ligands (CCL3/MIP-1 α , CCL4/MIP-1 β , and CCL5/RANTES) are involved in the development of Th1 cells, whereas CCR2 ligands (CCL2/MCP-1 and MCP-2) promote the differentiation of Th2 cells (10, 24). Chemokines are also involved in effector functions of Th subsets and can act directly on macrophages (22). Of relevance to this study are the finding that CCL3/MIP-1 α and CCL2/MCP-1 orchestrate the induction of leishmanicidal activities in murine macrophages via the generation of nitric oxide (9) and the finding that CCL2/MCP-1 and IFN- γ synergistically activate human monocytes to clear intracellular *L. major* (45). Although in the present study we did not directly address whether the ligands for CCR5 or CCR2 act at the level of Th-cell development and/or macrophage effector functions, the data strongly sup-

port the view that deficiency in protective immunity to *L. amazonensis* parasites occurs at multiple levels, including impairment in innate mediators in inflamed skin and subsequently acquired cell-mediated immunity to parasite antigens.

Despite the profound delay and reduction in the expression of inflammatory mediators in *L. amazonensis*-infected mice, these mice showed levels of cellular infiltration and percentages of CD4⁺ and CD8⁺ T cells in foot tissues after 1 to 4 weeks of infection comparable to the levels of cellular infiltration and percentages of CD4⁺ and CD8⁺ T cells in foot tissues in self-healing mice (data not shown). The histopathological characteristics imply that in vivo trafficking of T cells into the inflamed skin of *L. amazonensis*-infected mice is not significantly altered, presumably due to the overlapping functions of chemokines in leukocyte migration. However, these T cells apparently are not activated properly in the cytokine and chemokine milieu in the draining LNs (Fig. 6). While CD4⁺ T cells fail to activate monocytes and macrophages to eliminate *L. amazonensis* parasites, they actually may facilitate further recruitment of immature macrophages for propagation of the parasites and contribute to disease pathogenesis (49, 50). Thus, *L. amazonensis* parasites may have unique strategies to delay the induction of host immune responses in favor of the establishment of infection at early stages and to take advantage of host immune responses for disease pathogenesis at later stages of infection.

Since parasite inoculation doses can influence the outcome of infection (12), it is reasonable to suspect that deficiency in mounting an early immune response in *L. amazonensis*-infected mice is the consequence of slow parasite replication and thus low levels of antigenic stimulation. However, our parasite burden data argue against this possibility. At 2 weeks, foot tissues of *L. amazonensis*- and *L. major*-infected mice contained $\log_{10} 4.38 \pm 0.49$ and $\log_{10} 4.52 \pm 0.55$ parasites, respectively ($P > 0.05$). Therefore, different host immune responses in these two infection models cannot be attributed to parasite replication rates in vivo but rather to the biological characteristics of the two parasite species. One possibility is that the *L. amazonensis* parasite is, by nature, a weaker inducer of host responses than *L. major* is. It has been suggested that these two parasite species utilize different virulence factors for invading macrophages (17, 53) and have different requirements for host-derived factors for parasite growth (15) and disease progression (33, 49). Our comparative proteomics studies suggest that there are marked differences in protein expression patterns between promastigotes of *L. amazonensis* and promastigotes of *L. major* (Ji and Soong, unpublished data). Further studies are needed to explore this possibility. Another possibility is that the *L. amazonensis* parasite may have evolved unique mechanisms to prevent or delay activation of the host immune system. Data from this and other studies (30) suggest that there is a role for IL-10, but not IL-4, in the deficient production of IL-12, IFN- γ , IL-1 β , CCL5, and CCL2 ligands in *L. amazonensis*-infected mice. Given that the levels of expression of these mediators in *L. amazonensis*-infected IL-10^{-/-} mice are only partially restored (Fig. 8), other negative regulators, such as transforming growth factor β (6), granulocyte-macrophage colony-stimulating factor (48), and prostaglandin E₂ (36), may also contribute to down-modulation of host immune responses to the *L. amazonensis* parasite.

We are currently examining whether IL-10- or transforming growth factor β -producing suppressive and regulatory T cells are involved in immune down-regulation in *L. amazonensis* infection. Regardless of the mechanism, it is clear from this study that adoptive transfer of parasite-specific Th1 cells prior to infection with *L. amazonensis* parasites can bypass a deficiency in early production of inflammatory mediators, leading to control of parasite growth in the foot tissue and prevention of pathological changes (Fig. 7). These results reemphasize the need to further define the parasite-derived mechanism responsible for the impaired immune responses in *L. amazonensis*-infected hosts.

In summary, the findings presented here indicate that *L. amazonensis* may evade host immune responses by preventing early production of inflammatory cytokines and chemokines and preventing the development of antigen-specific Th1 cells in the presence of low levels of Th2 cytokines. Although vigorous Th1 responses at the time of parasite invasion can overcome immune deficiency, leading to control of disease in *L. amazonensis*-infected mice, the interplay between the early immune events and antigen-specific T-cell responses remains unclear at this stage. Thus, it will be of interest to determine the parasite-derived factors that are involved in this modulation. Comparative studies of the gene and protein expression profiles of *L. amazonensis* and *L. major*, as well as the different responses in parasite-exposed Langerhans cells and dendritic cells, should provide new insight into the pathogenesis of cutaneous leishmaniasis and the biology of *Leishmania* parasites.

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