INFECTION AND IMMUNITY, Aug. 2004, p. 4455–4463 Vol. 72, No. 8
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.8.4455–4463.2004

$CD4^+$ Th1 Cells Induced by Dendritic Cell-Based Immunotherapy in Mice Chronically Infected with *Leishmania amazonensis* Do Not Promote Healing

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Received 12 March 2004/Returned for modification 18 April 2004/Accepted 9 May 2004

The susceptibility of mice to *Leishmania amazonensis* infection is thought to result from an inability to develop a Th1 response. Our data show that the low levels of gamma interferon (IFN-γ) produced by the draining lymph node (DLN) cells of chronically infected mice could be enhanced in vitro and in vivo with *L. amazonensis* antigen-pulsed bone marrow-derived dendritic cells (BM-DC) and the Th1-promoting cytokine interleukin-12 (IL-12). Given intralesionally to chronically infected mice, this treatment induced the upregulation of mRNA levels for IFN-γ, the transcription factor T-box expressed in T cells, and IL-12 receptor β2 in $CD4^+$ T cells from the DLN and an increase in parasite-specific immunoglobulin G2a in the serum. However, this Th1 response was not associated with healing, and the antigen-specific enhancement of IFN-γ production remained impaired in the DLN. However, addition of IL-12 to the in vitro recall response was able to recover this defect, suggesting that antigen-presenting cell-derived IL-12 production may be limited in infected mice. This was supported by the fact that *L. amazonensis* amastigotes limited the production of IL-12p40 from BM-DC in vitro. Altogether, our data indicate that the immune response of mice chronically infected with *L. amazonensis* can be enhanced towards a Th1 phenotype but that the presence of Th1 $CD4^+$ T cells does not promote healing. This suggests that the phenotype of the $CD4^+$ T cells may not always be indicative of protection to *L. amazonensis* infection. Furthermore, our data support growing evidence that antigen-presenting cell function, such as IL-12 production, may limit the immune response in *L. amazonensis*-infected mice.

Leishmaniasis is a zoonotic disease caused by intracellular protozoa of the genus *Leishmania*. These parasites are transmitted to the host by a sandfly vector, and the resulting clinical disease can be categorized into three forms: cutaneous, mucocutaneous, and visceral. Infection of mice with various *Leishmania* species has led to an understanding of some of the immune mechanisms necessary to control these intracellular protozoa. As in humans, murine cutaneous leishmaniasis can have different outcomes depending on the parasite strain and species and the genetic background of the host (1, 4, 10, 11, 21, 37). For example, the outcome of murine cutaneous leishmaniasis caused by *L. major* is determined by the phenotype of the $CD4^+$ T-cell response: Th1 $CD4^+$ T cells producing gamma interferon (IFN-γ) promote resistance, while Th2 $CD4^+$ T cells producing interleukin (IL)-4 induce susceptibility (reviewed in references 36 and 38).

C3HeB/FeJ and C57BL/6 mice are able to control an *L. major* infection by mounting a Th1 immune response. This resistant phenotype requires a sustained source of IL-12 and the expression of IL-12 receptor β2 (IL-12Rβ2) and T-bet, a Th1-associated transcription factor (14, 15, 18, 31, 41). The associated cutaneous lesion subsequently resolves and contains very low parasite numbers (36, 38). In contrast, when infected with *L. amazonensis*, C3HeB/FeJ and C57BL/6 mice develop chronic cutaneous lesions containing from 10^5 to 10^8 parasites. This is associated with low levels of both IFN-γ and IL-4 produced in the in vitro recall responses from the draining lymph node (DLN) (1, 20, 39), which suggests that an antigen-specific memory/effector cell population may not develop and persist after infection. In addition, there is decreased IL-12Rβ2 expression in the $CD4^+$ T cells from the DLN, and this persists even in the absence of IL-4 (17, 20). Also, mice given the Th1-promoting cytokines IL-12 and IFN-γ and mice deficient for the Th2 cytokines IL-4 and IL-10 remain susceptible to *L. amazonensis* (5, 17, 19, 20). Therefore, in addition to the mouse models of visceral leishmaniasis caused by *L. donovani* and *L. chagasi*, the murine model of cutaneous leishmaniasis caused by *L. amazonensis* is an experimental system in which the Th1 response is limited without the expansion of Th2 cells, (23, 45).

Dendritic cells (DC) are the main antigen-presenting cells in vivo and in vitro (3). They play an important role in priming naïve T cells toward a Th1 and Th2 phenotype (reviewed in reference 30). Their potential as prophylactic vaccine adjuvants has been exploited to promote an efficient immune response before infection with *L. major* (8, 12, 44). However, DC-based immunotherapies have not been tried in mice with established cutaneous lesions. Protection to a *L. amazonensis* challenge has been achieved in mice by giving parasite antigen and DNA and by adoptively transferring an *L. amazonensis*-specific Th1 $CD4^+$ T-cell line before the infection (9, 17, 32, 34, 40). This was associated with an increase in IFN-γ production within the DLN, suggesting a Th1 phenotype of the local immune response. Furthermore, Ji et al. showed that $CD4^+$ T cells from the DLN of protected mice had increased expression

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of mRNA levels for IL-12Rβ2, further supporting that a Th1 response could provide protection to *L. amazonensis* infection (17).

Our data show that upon *L. amazonensis* infection, the phenotype of the $CD4^+$ T cells is not indicative of disease outcome, as the induction of Th1 $CD4^+$ T cells expressing T-bet and IL-12Rβ2 in chronically infected mice did not promote healing. This was associated with an impaired production of IFN-γ in the DLN, which could be restored by addition of IL-12 in the DLN recall response. This suggests that IL-12 may have been a limiting factor in the DLN and is further supported by the ability of *L. amazonensis* amastigotes to limit IL-12p40 production by bone marrow-derived DC (BM-DC).

MATERIALS AND METHODS

Mice. C3HeB/FeJ mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and bred and maintained in a specific-pathogen-free facility. Prkdc.Scid (C3H SCID) mice were purchased from the same source and maintained in our specific-pathogen-free facility. The Committee on Animal Care at Iowa State University approved all protocols involving animals. Five- to 8-week-old C3HeB/FeJ females were inoculated with 5×10^6 stationary-phase promastigotes in 50 μl of phosphate-buffered saline (PBS) in the left hind footpad. The lesion size was monitored weekly with a dial micrometer (L. S. Starrett Co., Athol, Mass.), and the results were expressed as the difference between the footpad thickness for the infected foot and the footpad thickness for the uninfected foot. C3H SCID mice were inoculated subcutaneously with 10×10^6 to 20×10^6 stationary-phase promastigotes in 50 μl of PBS in the left hind foot and later sacrificed to harvest tissue-derived amastigotes.

Parasites. Culture of *L. amazonensis* (MHOM/BR/00/LTB0016) and *L. major* (MHOM/IL/80/Friedlin) parasites and the preparation of parasite antigen were performed as previously described (19). For parasite quantification from cutaneous lesions, the infected feet were disinfected with 70% ethanol, and the skin was dissected away. The remaining subcutaneous lesion was homogenized with a Tenbrock tissue homogenizer, washed twice in PBS, and resuspended in Grace's insect cell culture medium (Life Technologies, Grand Island, N.Y.) supplemented with 20% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. A 10-fold serial dilution of the parasite suspension was then performed and incubated at 24°C for 8 days before parasite quantification was assessed. Amastigotes were directly recovered from the lesions of C3H SCID mice. The number of live amastigotes recovered from the footpad tissue was determined by fluorescence microscopy with fluorescein diacetate (Acros Organics, Morris Plains, N.J.) and propidium iodide (Sigma, St. Louis, Mo.).

Antigen challenge. At 10 weeks postinfection, mice were injected with 20 μg of antigen in 50 μl of PBS in the right hind footpad. Footpad swelling was measured daily for 72 h with a dial micrometer, and the results were expressed as the difference between the footpad thickness of the antigen-challenged foot and its thickness before the antigen challenge.

Histology. Feet from challenged mice were fixed in 10% buffered formalin and routinely processed for paraffin-embedded sectioning. Sections 5 μm thick were cut, applied to poly-L-lysine-coated slides and stained with hematoxylin and eosin. Toluidine blue stains were performed to assess basophilic infiltration.

Isolation and culture of BM-DC. BM-DC were cultured in vitro in the presence of 10 ng of murine granulocyte-macrophage colony-stimulating factor (PeproTech Inc., Rocky Hill, N.J.) according to the method of Lutz et al. (26). At day 10 of culture, approximately 90% of the BM-DC were positive for the DC marker CD11c. For in vitro studies, 10-day-old BM-DC were incubated for 24 h at 37°C with fresh, tissue-derived amastigotes at a cell-parasite ratio of 1:3.

BM-DC immunotherapy. Mice infected with *L. amazonensis* for 10 weeks and naïve control mice were injected every other day for 12 days in their left hind foot. Each injection consisted of 20 μg of *L. amazonensis* antigen and 0.2 μg of murine IL-12 (PeproTech Inc.) with 10^6 BM-DC resuspended in a total of 25 μl of PBS. Control groups were injected with 25 μl of PBS. The mice were sacrificed 3 days after the last injection. As a control for the in vivo efficacy of murine IL-12, BALB/c mice were infected with *L. major* and simultaneously injected with IL-12, as previously described (31).

Recall responses. Lymph node cell recall responses were performed as previously described (19). Spleen cells were incubated with a lysing buffer (0.15 M ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM ethylene diamine tetraacetic acid) to lyse the red blood cells and plated as lymph node cells. Supernatants were harvested after 72 and 96 h (in experiments in which BM-DC were added to the cultures), and IFN-γ and IL-4 levels were determined by enzyme-linked immunosorbent assay (ELISA). Recombinant IFN-γ (Pharmingen, San Diego, Calif.) and IL-4 (PeproTech) were used on each ELISA plate to set up a standard curve. The sensitivity of the IFN-γ ELISA ranged between 39 and 78 pg/ml; the sensitivity of the IL-4 ELISA was 78 pg/ml. When BM-DC were added to the cultures, a total of 1×10^5 and 2×10^5 BM-DC were cocultured with fourfold more LN cells in 200 μl of complete tissue culture medium plus *L. amazonensis* antigen (50 μg/ml). The concentrations of murine IL-12 (PeproTech) and anti-murine IL-12p70 (Pharmingen, San Diego, Calif.) used were 1 ng/ml and 10 μg/ml, respectively. Endotoxin levels in culture supernatants were undetectable, as determined by the *Limulus* amoebocyte assay (Sigma).

Purification of $CD4^+$ T cells. Lymph node cells were incubated with anti-CD4-coupled microbeads (Miltenyi Biotec, Auburn, Calif.) according to the manufacturer's protocol, followed by one passage through an AutoMACS. The purity of the cell preparation was higher than 95%.

Flow cytometry. Intracellular staining was performed as previously described (20). Live events were gated based on forward scatter versus side scatter.

Amastigote infection of BM-DC. *L. amazonensis* and *L. major* amastigotes were harvested from C3H SCID mice as indicated above. BM-DC (2×10^6) were incubated for 24 h with 6×10^6 live amastigotes (3:1 parasite-to-BM-DC ratio). The infection rate was determined by microscopic examination of Hema 3 (Fisher Scientific, Middletown, Va.)-stained cytospin preparations. Infection rates ranged from 56 to 65%. There were no significant differences in the infection rate between the two infections.

IL-12p40 ELISA. Supernatants from BM-DC 24-h post-amastigote infection were harvested, and the IL-12p40 ELISA was performed with commercially available antibodies (Pharmingen, San Diego, Calif.), peroxidase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, West Grove, Pa.) and ABTS microwell peroxidase substrate (Kirkegaard & Perry Laboratories, Gaithersburg, Md.).

IgG2a ELISA. ELISA plates were coated with 10 μg of *L. amazonensis* antigen per ml. The samples were serially diluted from 1:100 to 1:12,800, and the ELISA was performed as previously described (19).

RT-PCR. Total RNA was extracted from $CD4^+$ T cells with the Rapid Total RNA purification system (Marligen Biosciences, Ijamsville, Md.) according to the manufacturer's protocol. The RNA concentration was measured spectrophotometrically; RNA samples were then treated with 1.5 U of DNase I (Pierce, Milwaukee, Wis.) per μg of RNA for 45 min at 37°C, and 2 μg of each DNase I-treated RNA sample was reverse transcribed in 80-μl reaction volumes with 1X Moloney murine leukemia virus reaction buffer (Promega), 200 U of Moloney murine leukemia virus reverse transcriptase (Promega), 40 U of RNase inhibitor (Applied Biosystems, Foster City, Calif.), 2.5 μM random hexamers (Amersham Biosciences Corp., Piscataway, N.J.) and 0.5 mM each of the four deoxynucleoside triphosphates (USB Corporation, Cleveland, Ohio). RNA samples and random hexamers were incubated together at 70°C for 3 min and room temperature for 10 min. The reverse transcription reaction was performed at 42°C for 1 h; samples were then heated at 95°C for 5 min and incubated on ice for 10 min. Real-time PCR experiments were run on the ABI Prism 5700 sequence detection system (Applied Biosystems, Foster City, Calif.). Each 50-μl reaction contained 1 μl of cDNA and 1X ABsolute QPCR Mix (Abgene, Surrey, United Kingdom).

Forward and reverse primers were used at either 300 or 350 nM (IDT, Coralville, Iowa) and TaqMan probes (labeled with the reporter dye 6-carboxyfluorescein and the quencher dye 6-carboxytetramethylrhodamine; Applied Biosystems, Foster City, Calif.) were used at either 75 or 100 nM. Primer pairs and probes were designed with Primer Express 1.5 software (Applied Biosystems) and are listed in Table 1. For quantitative analysis of gene expression, normalized to an endogenous control, we used the standard curve method, which was detailed previously (13). Briefly, control cDNA samples were serially diluted to obtain standard curves for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA and each target cDNA. For each assay, the resultant cycle threshold (C_t) values were plotted against the input of RNA. For all experimental samples, the cDNA quantity for the target of interest was then determined from its standard curve and normalized to the GAPDH control.

Statistical procedure. Statistical analysis was performed with Statview (SAS, Cary, N.C.). When multiple treatment groups were compared, the data were entered into an analysis of variance and analyzed with Fisher's protected least significance difference post-hoc test. When a comparison between two groups was done, a *t* test was performed. Differences were considered significant when P was <0.05.

TABLE 1. Sequences of PCR forward and reverse primers and probes

Target gene	GenBank accession no.	Type[a]	Sequence (5′→3′)
T-bet	AF241242	F	GCCAGGGAACCGCTTATATG
		R	AACTTCCTGGCGCATCCA
		p	CCCAGACTCCCCCAACACCGG
GATA-3	NM_008091	F	CCTACCGGGTTCGGATGTAAG
		R	TTCACACACTCCCTGCCTTCT
		p	AGGCCCAAGGCACGATCCAGC
IL-12Rβ1	NM_008353	F	CCTCTGTACTTGACGATCTTTGGA
		R	GATGCAACAGTCAGGCCTCTT
		p	AGCCGGGACCGCCCACTTTG
IL-12Rβ2	U64199	F	CTTCACTTCCGCATACGTTCAC
		R	GCTCCCAGAAGCATTTAGAAAGTT
		p	TCCCTCTTCCTCCGTGGGACATCA
IFN-γ	XM_125899	F	ACAATGAACGCTACACACTGCAT
		R	TGGCAGTAACAGCCAGAAACA
		p	TTGGCTTTGCAGCTCTTCCTCATGG
GAPDH	M32599	F	TGTGTCCGTCGTGGATCTGA
		R	CCTGCTTCACCACCTTCTTGA
		p	CCGCCTGGAGAAACCTGCCAAGTATG

[a] F, forward primer; R, reverse primer; p, probe.

RESULTS

Low levels of IFN-γ production in the DLN and spleen from mice chronically infected with *L. amazonensis*. While C3HeB/FeJ mice are able to resolve a *L. major* infection, they develop chronic cutaneous lesions when infected with *L. amazonensis* (Fig. 1). The susceptibility to *L. amazonensis* is thought to result from an inability to develop a Th1 response. Within DLN and splenic recall responses from *L. amazonensis*-infected mice, the absolute amounts of IFN-γ detected were low (0.31 ± 0.11 and 1.75 ± 0.37 ng/ml, respectively), and typically 10 to 20 times lower than the ones detected in the recall responses from the DLN and spleen from *L. major*-infected animals (6.79 ± 2.59 ng/ml and 21.57 ± 4.45 ng/ml, respectively). No IL-4 was detected in the recall responses at 10 weeks postinfection. However, the low levels of IFN-γ detected in vitro, together with the absence of IL-4, do suggest that the immune response of mice chronically infected with *L. amazonensis* has some component of a Th1 response.

Antigen-specific footpad swelling upon subcutaneous antigen challenge indicates a functional memory response in vivo during chronic *L. amazonensis* infection. The low levels of effector cytokines within the recall response of mice chronically infected with *L. amazonensis* may reflect an absence of effector/memory response in vivo. To test this, mice infected for 10 weeks with *L. amazonensis* in their left hind foot were challenged with 20 μg of *L. amazonensis* antigen in the contralateral foot, and the associated footpad swelling was measured over a 3-day period. Naïve mice challenged with *Leishmania* antigen were used as negative controls. In parallel, as a positive control for the kinetics and histological changes of a delayed-type hypersensitivity response, similar experiments were done with *L. major*-infected mice challenged with 20 μg of *L. major* antigen.

As shown in Fig. 2A, mice chronically infected with *L. amazonensis* developed an antigen-specific footpad swelling, indicating the presence of a memory/effector response in vivo. The observed footpad swelling was not as pronounced as in *L. major*-infected mice challenged with *L. major* antigen, but it was significantly higher than in uninfected control mice challenged with *Leishmania* antigen (Fig. 2A). Histologically, the footpad swelling observed in the antigen-challenged feet of mice infected with *L. amazonensis* and *L. major* was similar and characterized by an infiltrate of mononuclear cells with scattered neutrophils and rare basophils (Fig. 2B and data not shown), which is consistent with a delayed-type hypersensitivity response. In mice infected with *L. amazonensis*, the cellular infiltrate was found multifocally throughout the dermis and subcutaneous tissue, while it was diffusely present in the dermis and subcutis of mice infected with *L. major*. A cellular infiltrate was not present in naïve mice challenged with *Leishmania* antigen (Fig. 2C). However, a focus of neutrophils and few mononuclear cells was occasionally observed in the feet of these mice, likely resulting from traumatic injury at the injection site (data not shown).

Impaired IFN-γ production in the lymph node draining the site of antigen challenge. Since antigen-specific memory cells were able to elicit a delayed-type hypersensitivity in vivo, we determined if this response was associated with increased effector cytokine production, i.e., IFN-γ and IL-4. *L. major*-infected mice demonstrated a typical memory response when challenged with *L. major* antigen in the contralateral footpad. The cells from the lymph node draining the site of antigen challenge produced two times more IFN-γ than their uninfected controls during the recall response (Fig. 3). In contrast, *L. amazonensis*-infected mice had an inhibition of effector cytokine production when challenged with *L. amazonensis* antigen in the contralateral footpad. The cells of the lymph node draining the site of antigen challenge had a 75% reduction in IFN-γ production in comparison to the uninfected mice challenged with *L. amazonensis* antigen (Fig. 3). The IFN-γ production was dependent on the presence of leishmanial antigen in all assays, and no IL-4 was detected in any of the recall responses (data not shown). This demonstrates that in mice

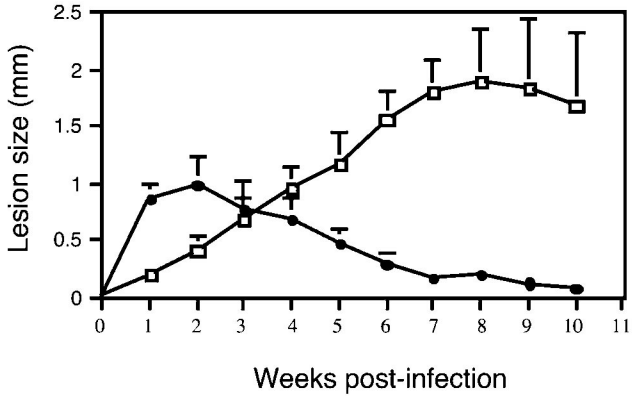

FIG. 1. Upon infection with *L. amazonensis*, C3HeB/FeJ mice develop a chronic cutaneous lesion. We infected 5- to 8-week-old C3HeB/FeJ mice in the left hind footpad with 5×10^6 stationary-phase *L. amazonensis* (open squares) or *L. major* (solid circles) promastigotes. Lesion size was monitored weekly as described in Materials and Methods. The values shown are the mean ± standard deviation (12 mice per group).

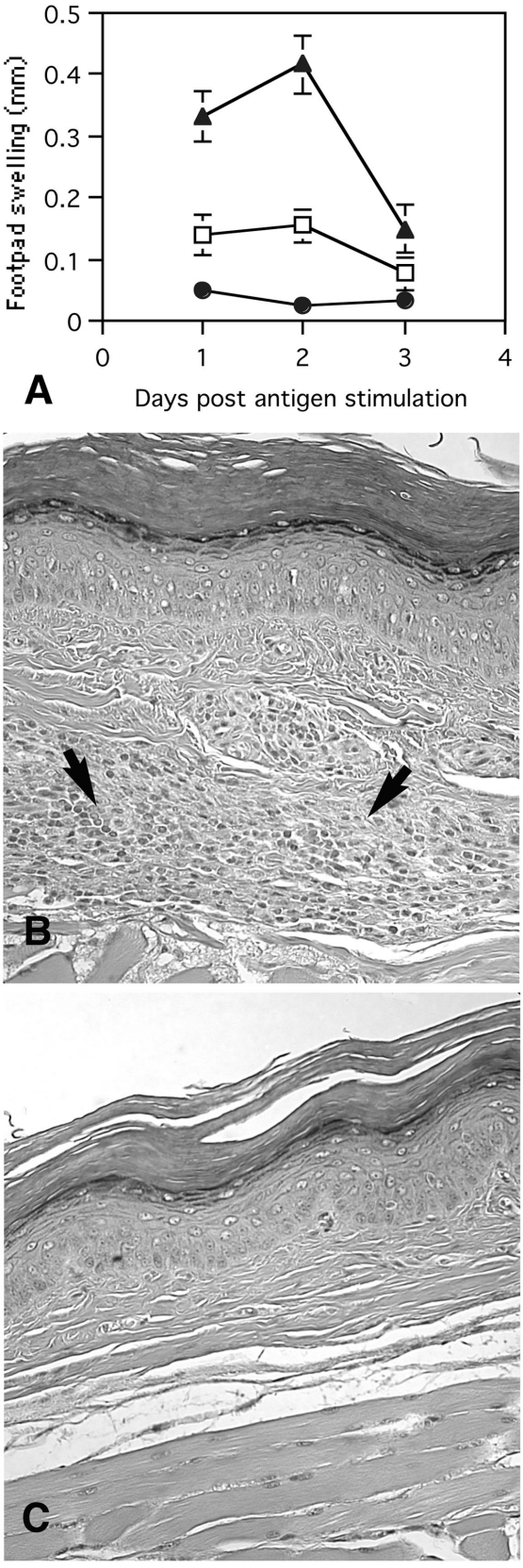

FIG. 2. Antigen-specific footpad swelling consistent with delayed-type hypersensitivity indicates the presence of a memory/effector response in vivo. (A) C3HeB/FeJ mice were infected in the left hind footpad with *L. amazonensis* or *L. major* as described for Fig. 1. Ten weeks postinfection, the *L. amazonensis*-infected mice were challenged subcutaneously with *L. amazonensis* antigen (open squares) and the *L. major*-infected mice were challenged subcutaneously with *L. major* antigen (solid triangles) in the right hind foot as described in Materials and Methods. Uninfected mice challenged subcutaneously with either *L. amazonensis* or *L. major* antigen (solid circles) were used as negative controls. The footpad thickness was monitored daily over 3 days and expressed as the difference between the footpad thickness of the challenged foot at the time of measurement and its thickness before the antigen challenge. The values shown are the mean ± standard error of the mean based on three separate experiments. (B and C) Two days post-antigen challenge, the mice were sacrificed, and the antigen-challenged feet were fixed and processed for histology (hematoxylin and eosin staining). (B) Foot from a mouse infected with *L. amazonensis* and challenged with *L. amazonensis* antigen; the cellular infiltrate (arrows) within the dermis is composed of mononuclear cells, scattered neutrophils, and rare basophils. (C) Foot from an uninfected mouse challenged with *L. amazonensis* antigen; there is no inflammatory infiltrate within the dermis.

chronically infected with *L. amazonensis*, the production of IFN-γ is also impaired in lymph nodes distant from the site of infection, i.e., the lymph node draining the site of a subcutaneous antigen challenge.

Antigen-pulsed BM-DC enhance IFN-γ production from DLN cells in an IL-12-independent manner. Dendritic cells have previously been shown to enhance the in vitro production of IFN-γ by T cells from the DLN of *Leishmania*-infected mice (6). Therefore, we wanted to test the hypothesis that the addition of BM-DC as antigen-presenting cells in the in vitro recall response would enhance the secretion of IFN-γ of DLN cells. Cells from the DLN of mice chronically infected with *L. amazonensis* were cocultured for 4 days with either BM-DC alone (nonpulsed) or *L. amazonensis* antigen-pulsed BM-DC (see Materials and Methods) at a 4:1 ratio. As shown in Fig. 4A, *L. amazonensis* antigen-pulsed BM-DC increased IFN-γ levels up to three to seven times the level of IFN-γ detected in the standard recall response. In addition, nonpulsed BM-DC were unable to promote IFN-γ production by the lymph node cells, demonstrating the antigen specificity of the response. In our system, IFN-γ was not detected in cultures containing antigen-pulsed BM-DC alone, and IL-4 was not detected in any culture conditions (data not shown).

We then hypothesized that this antigen-specific enhancement of the IFN-γ production could result from enhanced IL-12p70 production by the BM-DC, skewing the results towards a Th1 response. Blocking the bioactive IL-12p70 in the cocultures did not result in a significant decrease in the levels of IFN-γ recovered in the supernatants, consistent with a Th1 bias of the antigen-responsive cells (Fig. 4B). Addition of IL-12 in the cocultures significantly enhanced the levels of IFN-γ detected, further suggesting that the lymphocyte response is skewed towards a Th1 response (Fig. 4C).

Immunotherapy with antigen-pulsed BM-DC and IL-12 promotes the polarization of $CD4^+$ T cells from the DLN towards a Th1 phenotype. Since antigen-pulsed BM-DC and IL-12 were able to enhance the Th1 phenotype of the recall response in vitro, we tested if such treatment would promote a Th1 response and subsequent healing in vivo. In particular, we determined the phenotype of the $CD4^+$ T cells from the DLN.

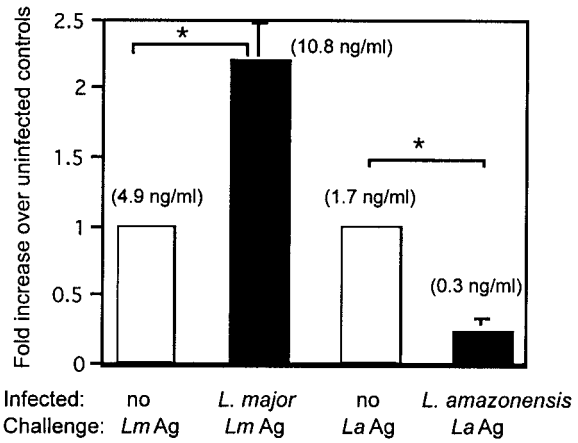

FIG. 3. Impaired IFN-γ production during the memory response of mice infected with *L. amazonensis*. Mice were infected and challenged with antigen as in Fig. 2. Three days postchallenge, the mice were sacrificed, and cells from the lymph node draining the antigen-challenged feet were plated in vitro for 3 days with *L. amazonensis* (*La* Ag) or *L. major* (*Lm* Ag) antigen. IFN-γ levels were detected by ELISA. Results are expressed as the increase ± standard error of the mean over the respective negative controls, that is, uninfected mice challenged with *L. amazonensis* or *L. major* antigen. In parentheses are shown the mean amounts of IFN-γ detected by ELISA for each group. The results shown are from three separate experiments with three mice per group. *, statistically significant difference ($P < 0.05$).

Th1 $CD4^+$ T cells have been shown to play a major role in resistance to *L. major* infection (36, 38) and to promote protection to an *L. amazonensis* challenge after adoptive transfer (17, 34). Therefore, mice chronically infected with *L. amazonensis* were injected in their infected foot every other day for 12 days with either PBS or *L. amazonensis* antigen-pulsed BM-DC plus IL-12 (see Material and Methods). Naïve mice were included in each treatment group as negative controls. Three days after the last injection, the $CD4^+$ T cells from the DLN were purified, and their mRNA levels for T-bet, GATA-3 (a Th2-associated transcription factor [46]), IFN-γ, IL-12Rβ1, and IL-12Rβ2 were determined by RT-PCR.

In comparison to the PBS control, the administration of antigen-pulsed BM-DC and IL-12 led to the upregulation of T-bet, IFN-γ, and IL-12Rβ2 mRNA levels in both naïve and infected mice (Fig. 5A and data not shown). Furthermore, mRNA levels for IL-12Rβ1 and GATA-3 remained similar between treated and control mice (Fig. 5A), indicating that the administration of antigen-pulsed BM-DC and IL-12 led to specific enhancement of a Th1 phenotype of the $CD4^+$ T-cell population from the DLN. Finally, as another indication that the BM-DC immunotherapy induced a Th1 response, treated mice had increased levels of *L. amazonensis*-specific IgG2a in their serum in comparison to PBS control mice (Fig. 5B).

Specific limitation of antigen-specific IFN-γ production in the DLN of mice chronically infected with *L. amazonensis*. As antigen-pulsed BM-DC and IL-12 induced a Th1 response in vivo, we hypothesized that it would be accompanied by an enhancement of the IFN-γ production in the in vitro recall response from the DLN. In the absence of antigen in the recall response, DLN cells from treated infected mice produced significantly more IFN-γ than DLN cells from PBS control mice (6.8 ± 1.6 ng/ml versus 0.5 ± 0.1 ng/ml, respectively). This enhanced IFN-γ production induced after immunotherapy recapitulated the one previously observed in vitro when antigen-pulsed BM-DC and IL-12 were added to the cell cultures (Fig. 4C). However, the increased levels of IFN-γ produced by DLN cells from chronically infected mice treated with antigen-pulsed BM-DC plus IL-12 were not further enhanced in the presence of antigen in vitro, in contrast to what is seen in treated naïve mice (Fig. 6A). In fact, the amount of IFN-γ recovered in the recall response of treated naïve mice was significantly greater than that of treated infected mice (Fig. 6A).

These discrepancies occurred despite the presence of similar percentages of IFN-γ-producing cells within the DLN of both groups of mice (Fig. 6B). However, the addition of IL-12 to the cell cultures led to similar levels of IFN-γ produced by the DLN cells from treated naïve and treated infected mice (59.35 ± 15.94 and 47.41 ± 10.96 ng/ml, respectively; Fig. 6A). This demonstrates that the defective antigen-specific IFN-γ production of infected mice could be overcome by IL-12 and supports a Th1 phenotype of the DLN cells. Finally, a limitation of IFN-γ production was not observed in the spleen, as after

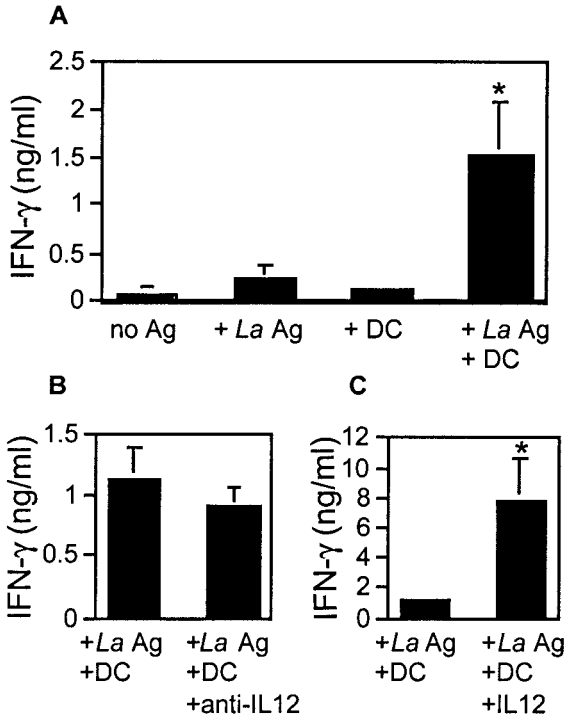

FIG. 4. Antigen-pulsed BM-DC enhance the production of IFN-γ by lymph node cells from mice chronically infected with *L. amazonensis*. Ten weeks postinfection with *L. amazonensis*, mice were sacrificed, and lymph node cells from the lymph node draining the infected foot were plated either (A) in medium alone (no antigen), with *L. amazonensis* antigen (+*La* Ag), with BM-DC (+DC), or with *L. amazonensis* antigen-pulsed BM-DC (+*La* Ag +DC); (B) with *L. amazonensis* antigen-pulsed BM-DC and an IL-12p70 blocking antibody (+*La* Ag +DC +anti-IL-12); or (C) with *L. amazonensis* antigen-pulsed BM-DC and recombinant murine IL-12 (+*La* Ag +DC +IL-12). The DLN cell-to-DC ratio was 4:1. After 4 days of in vitro culture, IFN-γ levels in the culture supernatants were detected by ELISA. The values shown are the mean ± standard error of the mean based on three (C) or four (A and B) separate experiments. *, statistically significant difference in comparison to the other group(s) ($P < 0.05$).

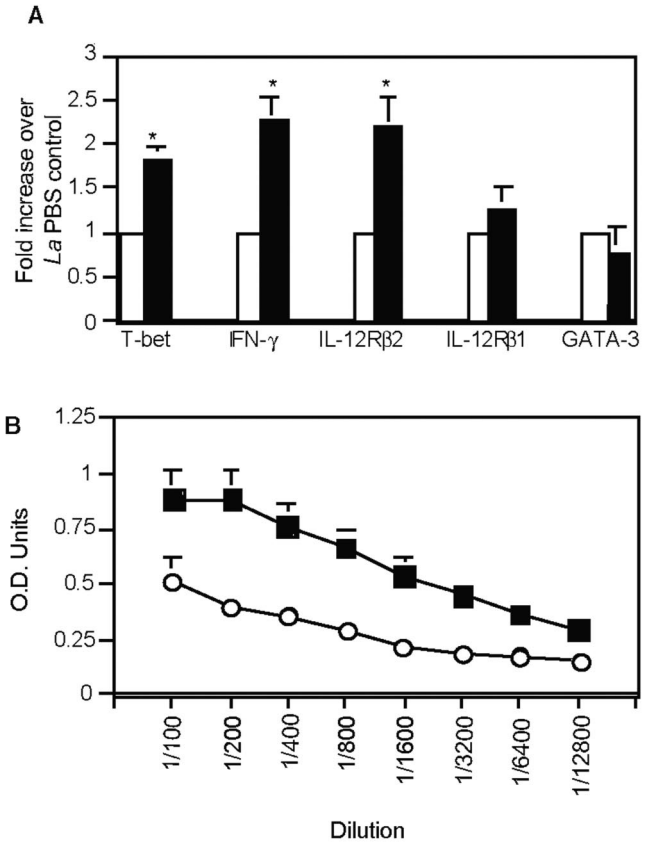

FIG. 5. Immunotherapy with antigen-pulsed BM-DC plus IL-12 induced a Th1 response in chronically infected mice. Mice were infected with *L. amazonensis* as in Fig. 1. Ten weeks postinfection, the mice were injected every other day for 12 days in their infected foot with *L. amazonensis* antigen-pulsed BM-DC and IL-12. Negative control mice were injected with PBS. Three days after the last injection, the mice were sacrificed. (A) $CD4^+$ T cells from the DLN were purified, and the expression of GAPDH, T-bet, IFN-γ, IL-12Rβ2, IL-12Rβ1, and GATA-3 was determined by RT-PCR as described in Materials and Methods. In each experiment, the amount of target cDNA in $CD4^+$ T cells from PBS control mice was given a value of 1 (open histogram) and the amount of target cDNA in $CD4^+$ T cells from treated mice was expressed as an increase over this value (solid histogram). The values shown are the mean increase ± standard error of the mean based on three separate experiments. *, statistically significant difference with the PBS control mice ($P < 0.05$). (B) Sera from treated (solid squares) and PBS control (open circles) mice were collected, and the levels of *L. amazonensis*-specific IgG2a were determined by serial dilution as described in Materials and Methods. The data shown are the mean optical density ± standard error of the mean based on four separate experiments.

immunotherapy with antigen-pulsed BM-DC and IL-12, splenocytes from both naïve and infected animals produced comparable levels of antigen-specific IFN-γ in the recall response (Fig. 6C). Altogether, this indicates that the limitation of antigen-specific IFN-γ production from the lymphocyte population is confined to the DLN but not the spleen of mice chronically infected with *L. amazonensis* and that it can be overcome by IL-12.

Consistent with the limitation of the lymphocyte response in the DLN, the immunotherapy did not induce a reduction in lesion size 2 weeks after the first BM-DC injection: the mean lesion size ± standard error of the mean of the PBS control and treated mice was 0.99 ± 0.11 and 0.95 ± 0.11 mm, respectively. This was further supported by the absence of a significant decrease in parasite numbers in the infected feet at this time point (Fig. 6D). Furthermore, to determine if a healing response would require more time, in one experiment lesion size and parasite loads were measured 5 weeks after the first BM-DC injection. At this time point, lesion size and parasite loads were still similar between treated mice and PBS controls (data not shown). Altogether, these data demonstrate that the administration of antigen-pulsed BM-DC plus IL-12 to mice chronically infected with *L. amazonensis* induced a Th1 response in vivo; however, this was not associated with lesion resolution.

***L. amazonensis* amastigotes limit IL-12p40 production by BM-DC.** As the impaired production of IFN-γ in the DLN of infected mice after treatment was restored by the addition of IL-12 in the in vitro recall response, it suggested that the infection with *L. amazonensis* may be limiting the production of IL-12 by antigen-presenting cells, i.e., dendritic cells. Therefore, we determined the ability of BM-DC to produce IL-12p40 in vitro after infection with *L. amazonensis* amastigotes. BM-DC were infected or not with *L. amazonensis* amastigotes at a 1:3 ratio. As a positive control, BM-DC were infected with *L. major* amastigotes at a similar cell-parasite ratio. The levels of IL-12p40 were determined 24 h postinfection by ELISA. As shown in Fig. 7, BM-DC infected with *L. amazonensis* amastigotes and noninfected BM-DC produced similar levels of IL-12p40. In contrast, BM-DC infected with *L. major* amastigotes produced significantly larger amounts of IL-12p40. Altogether, this demonstrates that *L. amazonensis* amastigotes can limit the production of IL-12p40 by BM-DC.

DISCUSSION

Our data demonstrate that the poor lymphocyte response of mice chronically infected with *L. amazonensis* is not the result of an absence of a memory cell population that can recognize parasite antigen, as these mice developed a delayed-type hypersensitivity response after a subcutaneous antigen challenge (Fig. 2A and B). However, the elicited delayed-type hypersensitivity reaction is attenuated in comparison to that of *L. major*-infected animals, which developed a strong Th1 response (Fig. 2A). In addition, the undetectable levels of IL-4 and the impaired IFN-γ production from the lymph node draining the site of antigen challenge indicate that the defective Th1 response of mice chronically infected with *L. amazonensis* is present at peripheral sites distant from the primary infection (Fig. 3).

It has recently been suggested that defective priming of T cells by DC may be responsible for the poor lymphocyte response to *L. amazonensis* antigen (17). Dendritic cells play a crucial role in priming T cells; in particular, IL-12 produced by DC promotes the polarization of the T cells towards a Th1 phenotype (reviewed in references 30 and 25). During both acute and chronic *L. major* infection, DC are the main antigen-presenting cells in the in vitro recall response (28, 29). Our data show that BM-DC could enhance the antigen-specific production of IFN-γ from DLN cells in vitro in an IL-12-independent manner (Fig. 4A and B). This enhanced lymphocyte response could result from a more efficient DC-to-T-cell

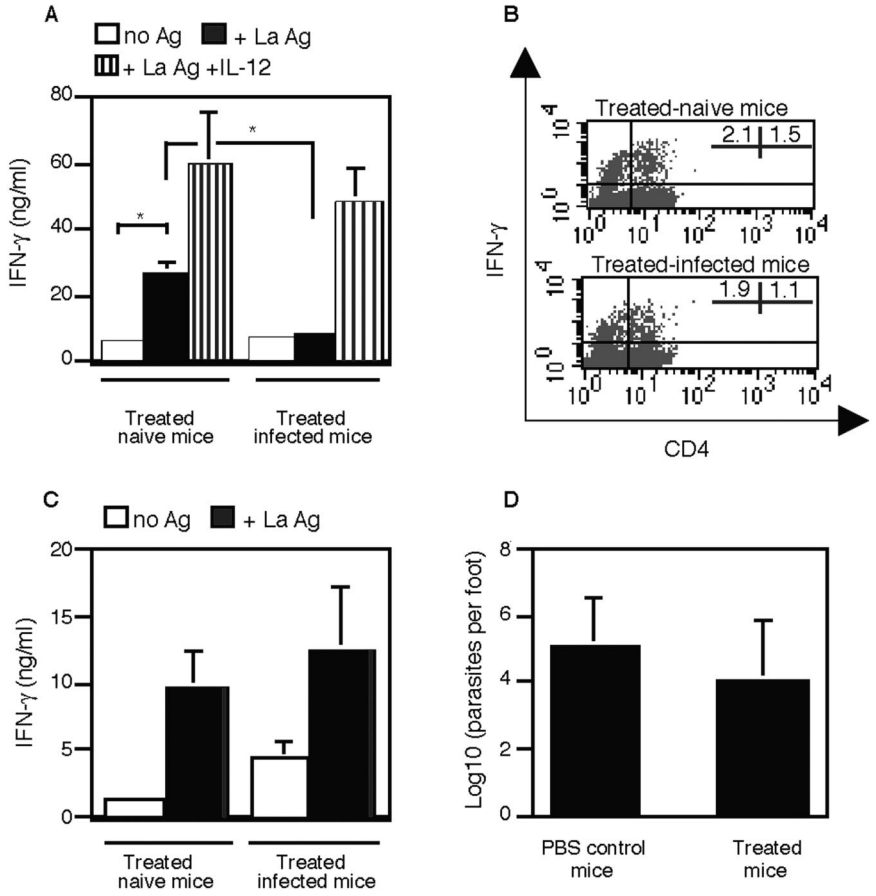

FIG. 6. BM-DC-based immunotherapy does not promote decreased parasite numbers and is still associated with impaired antigen-specific production of IFN-γ within the DLN. Naïve and *L. amazonensis*-infected mice were injected every other day with PBS (PBS control) or *L. amazonensis* antigen-pulsed BM-DC plus IL-12 (treated) as in Fig. 5. Three days after the last injection, the mice were sacrificed. (A) DLN cells from each treatment group were plated for 3 days in vitro in medium alone (no antigen) or in medium containing *L. amazonensis* antigen (*La* Ag) or *L. amazonensis* antigen and IL-12 (+*La* Ag +IL-12). The levels of IFN-γ were determined in the supernatants by ELISA. The values shown are the mean ± standard error of the mean based on five separate experiments (three experiments with exogenous IL-12 in the cell culture). *, statistically significant difference between the groups ($P < 0.05$). (B) DLN cells from naïve- and infected treated mice were plated in vitro with *L. amazonensis* antigen for 24 h, and the percentage of $CD4^+$ IFN-γ-producing cells was determined by flow cytometry. The data shown are representative of two separate experiments. (C) Splenocytes from each treatment group were plated for 3 days in vitro in medium alone (no antigen) or in medium containing *L. amazonensis* antigen (+*La* Ag). The levels of IFN-γ were determined in the supernatants by ELISA. The values shown are the mean ± standard error of the mean based on five separate experiments. *, statistically significant difference between the groups ($P < 0.05$). (D) Infected feet from PBS control and treated infected mice were collected, and parasites were quantified. The values shown are the mean ± standard deviation based on five separate experiments.

ratio, antigen presentation, and/or costimulation. In addition, the levels of IFN-γ detected in the DLN recall response were further increased when IL-12 was added to the cocultures (Fig. 4C). This and the fact that IFN-γ, but not IL-4, was detected in the DLN in vitro recall response indicate a Th1 bias of the lymphocyte response in mice infected with *L. amazonensis*. Furthermore, the enhanced production of IFN-γ in response to IL-12 suggests that cells fully capable of producing IFN-γ are present within the DLN.

Therefore, in addition to the $CD4^+$ T-cell defects present during *L. amazonensis* infection (17, 20), it is probable that antigen-presenting cells from the DLN are limited qualitatively and/and quantitatively, thereby contributing to low effector cytokine production in the recall response. This is supported by the small numbers of IL-12-producing cells associated with the DLN of *L. amazonensis*-infected mice (20) and by studies

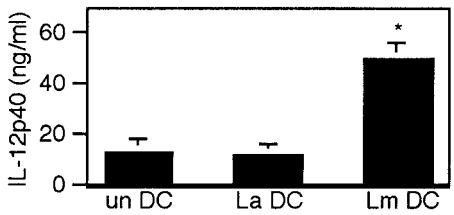

FIG. 7. *L. amazonensis* amastigotes limit IL-12p40 production from BM-DC. Ten-day-old BM-DC were infected or not with *L. amazonensis* or *L. major* amastigotes at a parasite-cell ratio of 3:1. Culture supernatants were harvested 24 h later, and the levels of IL-12p40 were determined by ELISA. The values shown are the mean ± standard error of the mean based on three separate experiments ($P < 0.05$).

showing that *Leishmania* parasites can modulate the phenotype and functions of antigen-presenting cells, i.e., macrophages and dendritic cells (2, 7, 22, 24, 27, 33, 35, 43).

Based on our in vitro results, we hypothesized that giving *L. amazonensis* antigen-pulsed BM-DC plus IL-12 as an immunotherapy to mice chronically infected with *L. amazonensis* would promote a Th1 response in vivo. Variable degrees of protection to *L. amazonensis* infection have been achieved in mice by prophylactic administration of antigen (32, 40), DNA (9), attenuated *L. major* promastigotes (16, 42), and an *L. amazonensis*-derived Th1 $CD4^+$ T-cell line (17, 34). DC-based vaccines have been used successfully prophylactically in experimental studies of cutaneous leishmaniasis caused by *L. major* (8, 12, 44), and protection has been linked to the ability of the DC to produce IL-12 (8).

However, to our knowledge, the present study is the first to use a therapeutic DC-based strategy in mice with established cutaneous lesions. The repeated administration of this treatment led to the development of a Th1 response in vivo, as measured by the upregulation of T-bet, IFN-γ, and IL-12Rβ2 mRNA levels on the $CD4^+$ T-cell population from the DLN (Fig. 5A). This is different from what was previously observed in BALB/c mice, where administration of IL-12 alone 48 h after infection with *L. major* did not induce a Th1 phenotype of the $CD4^+$ T cells from the DLN (14). In our study, the presence of a Th1 response was further demonstrated by the presence of increased levels of *L. amazonensis*-specific IgG2a in the serum (Fig. 5B). As adoptive transfer of Th1 $CD4^+$ T cells was recently shown to promote protection to a subsequent *L. amazonensis* infection, a correlation between healing and the vaccine-induced Th1 $CD4^+$ T-cell response was expected (17, 34).

This idea was also supported by a recent study of murine visceral leishmaniasis caused by *L. donovani*, in which the administration of BM-DC alone was associated with decreased parasite burden 7 days later (2). However, in the present study, despite the Th1 response, there was no significant decrease in parasite numbers in treated mice, as measured at 2 weeks (Fig. 6D) and 5 weeks (data not shown) after the first BM-DC injection. In addition, although the percentage of IFN-γ-producing cells was similar between treated infected and treated naïve mice, there was no enhancement of IFN-γ production when antigen was added to the DLN recall response of infected mice (Fig. 6A and B). This indicates that the impaired antigen-specific IFN-γ production in the DLN of mice chronically infected with *L. amazonensis* does not result from quantitative defects from the IFN-γ-producing cell population but rather from qualitative defects of these cells, i.e., ability to produce IFN-γ.

In addition, it is possible that the qualitative defect affects cells other than $CD4^+$ T cells, possibly antigen-presenting cells. This is supported by the fact that addition of IL-12 in the recall response from the DLN overcomes the defective production of IFN-γ by DLN cells (Fig. 6A). Furthermore, we tested the hypothesis that *L. amazonensis* amastigotes, the only parasite stage present during chronic infection, could limit IL-12p40 production by BM-DC in vitro. Our data provide evidence that *L. amazonensis* amastigotes-infected BMDC do not have enhanced production of IL-12p40 in comparison to noninfected BMDC (Fig. 7), supporting previous studies showing that *Leishmania* parasites can modulate the production of IL-12 by DC (7, 27, 35). Therefore, our data suggest that in chronically infected mice, and even in the presence of Th1 $CD4^+$ T cells, the production of IFN-γ may be limited by insufficient IL-12 production. This is consistent with studies showing that sustained production of IL-12 is critical to promote and maintain resistance to *L. major* (31). However, we cannot rule out that the IFN-γ production from the DLN cells is actively suppressed by immunomodulatory factors produced in the DLN.

Finally, our results show that the limitation of IFN-γ production in response to antigen was localized to the DLN, as it was not observed in the spleen, suggesting a compartmentalization of the immune response (Fig. 6C). As *L. amazonensis* parasites are present in the DLN but not the spleen of infected animals, it is tempting to suggest that such limitation could be specifically mediated by *L. amazonensis* amastigotes. This would be consistent with a very recent study showing that the transfer of a Th1 cell line prior to infection did confer resistance to a subsequent challenge with *L. amazonensis* promastigotes but not amastigotes (34).

Altogether, our data indicate a local impairment of antigen-specific IFN-γ production in the DLN of mice chronically infected with *L. amazonensis*. This defective response persists even after the induction of a Th1 $CD4^+$ T cells within the DLN, suggesting that the phenotype of the $CD4^+$ T cells may not always be indicative of protection to *L. amazonensis*, as is the case with *L. major*. Furthermore, our data support growing evidence that, in addition to $CD4^+$ T-cell defects, antigen-presenting cell functions such as IL-12 production may be impaired in *L. amazonensis*-infected mice (17).

ACKNOWLEDGMENTS

This work was supported by NIH grant AI48357 and the Biotechnology Council and College of Veterinary Medicine at Iowa State University. Y.V. was supported by a Van Roekel scholarship.

We thank Dennis Byrne for technical help.

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Editor: W. A. Petri, Jr.