

Recombinant Interleukin 12 Cures Mice Infected with *Leishmania major*

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Summary

Resistant C57BL/6 mice infected with *Leishmania major* are self-healing, whereas susceptible BALB/c mice fail to contain cutaneous infection and subsequently undergo fatal visceral dissemination. These disparate outcomes are mediated by dissimilar expansions of T helper type 1 (Th1) and Th2 CD4⁺ T lymphocyte subsets in vivo during cure and progression of disease. Because interleukin 12 (IL-12) has potent T cell growth and interferon γ (IFN- γ) stimulatory effects, we studied its effect on CD4⁺ T cell differentiation during murine leishmaniasis. Treatment with recombinant murine (rMu)IL-12 during the first week of infection cured 89% of normally susceptible BALB/c mice, as defined by decreased size of infected footpads and 1,000–10,000-fold reduced parasite burdens, and provided durable resistance against reinfection. Cure was associated with markedly depressed production of IL-4 by lymph node cells cultured with antigen or mitogen, but preserved or increased production of IFN- γ relative to untreated mice. IL-4 and IFN- γ mRNA associated with CD4⁺ T lymphocytes isolated from infected lymph nodes showed similar reciprocal changes in response to rMuIL-12 therapy. A single injection of anti-IFN- γ monoclonal antibody abrogated the protective effect of rMuIL-12 therapy and restored Th2 cytokine responses. We conclude that rMuIL-12 prevents deleterious Th2 T cell responses and promotes curative Th1 responses in an IFN- γ -dependent fashion during murine leishmaniasis. Since BALB/c leishmaniasis cannot be cured with rMuIFN- γ alone, additional direct effects of IL-12 during T cell subset selection are suggested. Because rMuIL-12 is uniquely protective in this well-characterized model of chronic parasitism, differences in IL-12 production may underlie heterogeneous host responses to *L. major* and other intracellular pathogens.

Leishmania infects vertebrate hosts to cause a spectrum of disease ranging from self-limited cutaneous ulceration to fatal progressive disease. Healing and progression of murine leishmaniasis are causally linked to the dissimilar expansion of functionally distinct Th1 and Th2 CD4⁺ T lymphocyte subsets during infection (1, 2). Th1 cells producing IFN- γ necessary for control of infection are present in healing tissues, whereas progression of disease is mediated by Th2 CD4⁺ T lymphocytes that produce IL-4 (3–5). The reasons for such host-specific differences in CD4⁺ T cell differentiation are not well understood. Although IFN- γ and IL-4 are each necessary for the respective expansion of Th1 and Th2 cells in vivo (4, 6), a puzzling aspect of BALB/c susceptibility is that it is not reversed by treatment with rIFN- γ during infection (7). Because IL-12 has both T cell growth and IFN- γ stimulatory properties (8, 9), we hypothesized that this factor might contribute to the evolution of protective T cell immunity during murine leishmaniasis.

Materials and Methods

Mice. Female BALB/c and C57BL/6 mice (6 wk old) were purchased from Charles River Labs. (Wilmington, MA) and kept in the Cleveland Veterans Administration Medical Center animal care facility.

Cytokine and Antibody Reagents. The rat mAb XMG1.2 (anti-murine IFN- γ , IgG1) was a gift from Dr. T. Mosmann (University of Alberta, Edmonton, Canada) and was grown and purified as described (4). Mouse rIL-12 (sp act, 7.8×10^6 U/mg; endotoxin, 0.5–0.9 EU/mg) was a gift from Dr. S. Wolf (Genetics Institute, Cambridge, MA).

Parasite Cultivation and Antigen Preparation. *Leishmania major* (WHO strain WHOM/IR/-/173) were grown in M199 containing antibiotics, supplemental glutamine, and 30% FCS (Whittaker Bioproducts, Walkersville, MD) as described (10). Stationary-phase promastigotes were injected into the hind feet of recipient mice at a dose of 2×10^6 organisms/footpad to initiate infection. The course of infection was monitored by measuring the thickness of footpad swelling weekly using a dial gauge caliper.

Quantitative Parasite Cultures. Approximately 0.1–0.3 g of footpad tissue was minced in 10 ml of M199/30 FCS medium supplemented with 20 mM HEPES (pH 7.4), crushed through a no. 200 stainless steel screen, and disrupted using a Tenbroeck homogenizer (Fisher Scientific, Pittsburgh, PA). 50- μ l aliquots of footpad or lymph node suspension were sequentially diluted fivefold in promastigote growth medium, placed into flat-bottomed 96-well plates, and incubated at 26°C in humidified room air. Individual wells were examined using an inverted microscope at 200 \times at 2-d intervals for the presence of motile promastigotes. Data represent the geometric mean and standard error of the last positive reciprocal dilution for each experimental group.

RNA Hybridization. RNA was purified from lymph node tissue and lymphocyte subsets as described (1). After electrophoresis in a 1% agarose/1.9% formaldehyde denaturing gel, the RNA was transferred to nylon membranes and hybridized with ³²P-labeled antisense RNA probes specific for p35 and p40 IL-12, IL-4, and IFN- γ (4, 9).

Generation and Measurement of Lymphokines from Lymph Node Culture. Popliteal lymph nodes from mice infected for 4 wk with *L. major* and spleens from uninfected mice were disrupted into cellular suspension at 5 \times 10⁶ cells/ml in DMEM containing 10% FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine. Each specimen was cultured in media alone or in media with added Con A (4 μ g/ml), stationary phase promastigotes (10⁵/ml), or freeze-thaw promastigote antigen (equivalent of 2 \times 10⁶ promastigotes/ml). Conditioned media were removed after 48 h, centrifuged to remove cellular debris, and stored at 4°C for assay within 1 wk and/or frozen for later assay. ELISA techniques and reagents for the assay of murine IFN- γ and IL-4 are as described (5).

Magnetic Selection of CD4⁺ and CD8⁺ Lymph Node Cells. Popliteal lymph node cells were suspended in Mg²⁺/Ca²⁺-free HBSS with 1% FCS added, incubated 30 min with 10 μ g/ml of biotinylated anti-CD4 or anti-CD8 mAb, washed, and gently mixed with avidin-coated magnetic beads on a rotary wheel at 4°C (Dynabeads; Robbins Scientific, Mountain View, CA). Bead-bound lymphocytes were isolated using a magnetic separator (Robbins Scientific), washed once, and resuspended in 6 M guanidine HCl for RNA extraction. The purity of T cells obtained by this method has been consistently 90–95% for CD4⁺ and 85–90% for CD8⁺ T cells (4).

Bioassay for IL-12. BALB/c and C57BL/6 mice infected for 3 wk received 100 μ g of *Salmonella enteritidis* LPS (Sigma Chemical Co., St. Louis, MO) by intraperitoneal injection. Serially diluted aliquots of serum were incubated for 48 h with naive BALB/c splenocytes suspended at 10⁷ cells/ml in DMEM/10% FCS, and the conditioned media were assayed for IFN- γ by ELISA. Units of IL-12 activity were defined as the reciprocal dilution of serum at which half-maximal stimulation of IFN- γ release occurred; recombinant murine (rMu)IL-12 was used as an internal standard and positive control. Control serum with or without added LPS did not stimulate IFN- γ . Activity in serum peaked at 4 h after endotoxin injection. The presence of IL-12 was confirmed by incubation of serum samples in 96-well culture plates previously coated with non-neutralizing anti-MuIL-12 mAb 5C3 (Richard Chizzonite, Hoffmann-La Roche, Inc.) and subsequent measurement of lymphoblast proliferation induced by the captured cytokine as described (11).

Flow Cytometry. Suspensions of lymph node cells were labeled with rat anti-mouse CD4 (GK1.5; American Type Culture Collection, Rockville, MD) and anti-mouse CD8 (53-6.72; American Type Culture Collection) mAbs and then with FITC-conjugated anti-rat IgG antisera (mouse serum adsorbed; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) as previously described (4),

and analyzed using a FACScan[®] flow cytometer (Becton Dickinson & Co., Mountain View, CA).

Results and Discussion

To demonstrate a functional role for IL-12 in antileishmanial immunity, we treated BALB/c mice with mouse rIL-12 given by intraperitoneal injection for the first 7 d of infection. Therapy was limited to the first week of infection because discrete Th1 and Th2 responses first evolve during this time (12) and because other therapeutic interventions that act to alter CD4⁺ differentiation, such as treatment with anti-IL-4 and anti-CD4 mAbs, are only effective if given with 1 wk of infection (7, 12, 13). Treatment of BALB/c mice with either 0.2 and 0.5 μ g of rMuIL-12 markedly reduced footpad swelling after cutaneous inoculation with the parasite at that site (Fig. 1). This correlated with 1,000–10,000-fold decreases in numbers of parasites cultured from homogenized footpad and lymph node tissue (Table 1). As defined by decreasing footpad size or parasitic burden at 4–5 wk of infection, 16 of 18 rMuIL-12-treated mice healed in comparison with 1 of 18 untreated mice in three separate experiments (89% vs. 5.6%; *p* < 0.05, Fisher exact test). Approximately two-thirds of cured mice observed for 4 mo remained free of obvious disease and were resistant to reinfection with 2 \times 10⁶ stationary-phase promastigotes.

Lymphocytes obtained from the draining lymph nodes of treated mice produced 10–50-fold less IL-4 in response to Con A mitogen and *L. major* antigen compared with untreated

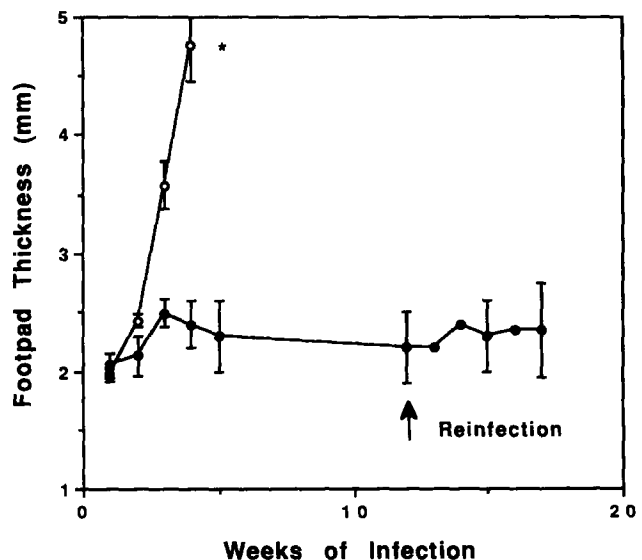


Figure 1. The effect of rMuIL-12 on progression of leishmaniasis in BALB/c mice. Footpad measurements represent the mean \pm SEM for four mice given either no treatment (O) or seven daily intraperitoneal injections of 0.2 μ g of rMuIL-12 commencing with the day of infection (●). Mice cured using rMuIL-12 were reinfected at 12 wk of infection with 2 \times 10⁶ promastigotes (arrow) and not further treated with rMuIL-12. Footpad necrosis necessitated euthanasia of control mice at the time indicated by the asterisk.

Table 1. Tissue Content of *Leishmania major* during Infection

Exp.	Mice	Titer of viable <i>Leishmania</i>	
		Footpad*	Lymph node*
		<i>log</i> ₁₀ ± SEM	
1	BALB/c	11.4 ± 0.0	9.7 ± 1.7
	BALB/c + rMuIL-12 [‡]	8.2 ± 1.3	7.6 ± 0.9
2	BALB/c	6.9 ± 0.8	7.2 ± 0.4
	BALB/c + rMuIL-12 [‡]	2.8 ± 0.4	5.9 ± 0.0

* Geometric mean and SEM of the last positive dilution obtained from 0.1 g of footpad tissue or 2.5 × 10⁶ cells of lymph node suspension obtained after 4 wk of infection.

[‡] Mice treated with 0.5 µg of rMuIL-12 daily for the first 7 d of infection.

mice (Table 2). Indeed, the Con A response was suppressed below that obtained from uninfected BALB/c spleen cells. IFN-γ production by rMuIL-12-treated mice was variably increased, with the mean values similar or greater than that of control-infected mice (Table 2). These data are consistent with a profound suppression of Th2 development in the treated mice, with concomitant sparing or promotion of Th1 activity.

Because IFN-γ production was inconsistently enhanced at 4 wk of infection in the rMuIL-12-treated mice, we sought to determine if the protective effects of rMuIL-12 were IFN-γ dependent. A single 1.0-mg dose of anti-IFN-γ mAb XMG1.2

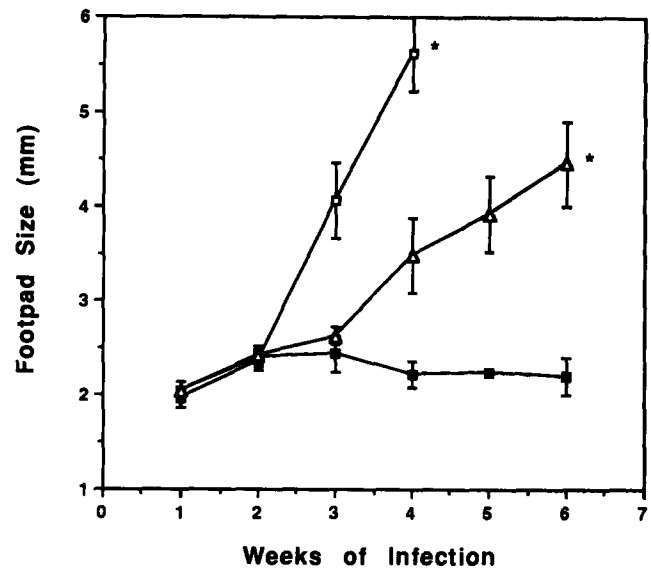


Figure 2. The effect of a single 1.0-mg injection of anti-IFN-γ mAb XMG1.2 on the healing of leishmaniasis induced by rMuIL-12 in BALB/c mice. Footpad measurements represent the mean ± SEM for four mice receiving either no therapy (□), seven daily injections of 0.5 µg of rMuIL-12 commencing on the day of infection (■), or 1.0 mg of XMG1.2 given by intraperitoneal injection on the day of infection in combination with 7 d of rMuIL-12 therapy (△). Asterisks denote footpad necrosis.

exacerbates leishmaniasis in normally resistant C57BL/6 and C3H mice (12). Cotreatment with 1.0 mg of XMG1.2 on the day of infection abrogated the protective effect of rMuIL-12 in BALB/c mice, although this exacerbation was delayed by

Table 2. Production of IFN-γ and IL-4 by Draining Lymph Node Cells

Exp.		Cytokine concentration			
		Con A		<i>L. major</i> antigen	
		IFN-γ	IL-4	IFN-γ	IL-4
		<i>ng/ml</i> ± SEM			
1*	Control	15.4 ± 4.1	36.1 ± 8.8	24.3 ± 2.6	30.5 ± 14.0
	rMuIL-12	8.0 ± 2.2	3.6 ± 2.0	18.8 ± 16.4	0.6 ± 0.2
2 [‡]	Uninfected BALB/c spleen	12.8 ± 2.1	41.0 ± 16.9	<0.05	<0.1
	Infected BALB/c	61.5 ± 20.8	85.6 ± 25.1	14.13 ± 3.6	54.0 ± 20.9
	rMuIL-12	17.8 ± 6.3	3.5 ± 1.2	32.1 ± 16.8	2.2 ± 1.9
	rMuIL-12 plus Anti-IFN-γ mAb	49.8 ± 13.1	72.0 ± 18.0	24.49 ± 9.8	62.9 ± 25.8

Suspensions of lymph node cells from mice infected for 4 wk and spleen cells from uninfected BALB/c mice were cultured for 48 h in the presence of 4 µg/ml of Con A, 10⁵/ml of viable promastigotes (Exp. 1), or freeze-thawed antigen equivalent to 2 × 10⁶ promastigotes/ml (Exp. 2). Conditioned media were assayed for IL-4 and IFN-γ by ELISA. Cells cultured in media alone yielded cytokine levels ≤20% of that produced in response to antigen. Designated BALB/c mice were treated with 0.5 µg of rMuIL-12 for the first 7 d of infection. Where specified, 1.0 mg of anti-IFN-γ mAb (XMG1.2) was given as a single dose on the day of infection.

* n = 4 mice for control and treated mice.

[‡] n = 4 for control, 3 for rMuIL-12, and 4 for rMuIL-12/anti-IFN-γ-treated mice.

1 wk relative to control BALB/c mice (Fig. 2). This confirmed that the final curative effect of IL-12 is dependent on IFN- γ . Draining lymph node cells from mice cotreated with anti-IFN- γ showed restored IL-4 production relative to mice treated with rMuIL-12 alone (Table 2).

IL-12 is a growth factor for several lymphocyte populations, including NK cells, γ/δ^+ , and CD4 $^+$ and CD8 $^+$ α/β^+ T lymphocytes (14). Although the proportions of lymph node cells that were CD4 $^+$, CD8 $^+$, and surface (s)Ig $^+$ (B cells) were not significantly altered in rMuIL-12-treated mice compared with untreated mice during infection (34.8 ± 12.4 vs. 32.5 ± 4.9 , 12.4 ± 1.0 vs. 14.9 ± 2.6 , and 47.5 ± 7.9 vs. $50.6 \pm 4.1\%$ respectively, for CD4 $^+$, CD8 $^+$, and sIg $^+$ lymphocytes), IFN- γ generated by the lymph node cells of rMuIL-12-treated mice may have been derived from CD8 $^+$ T lymphocytes or NK cells and not from CD4 $^+$ Th1 cells. Therefore, RNA was harvested after 4 wk of infection from draining lymph node populations separately enriched for CD4 $^+$, CD8 $^+$, and sIg $^+$ lymphocytes or depleted of these subsets (*Other*) for hybridization with IFN- γ and IL-4-specific RNA probes (Fig. 3). Only

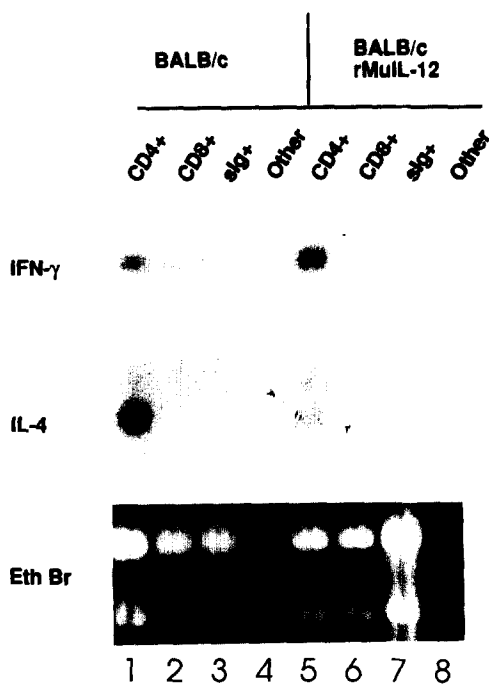


Figure 3. The relative abundance of IFN- γ and IL-4 mRNA in CD4 $^+$ and CD8 $^+$ T cells in control and rMuIL-12-treated BALB/c mice during infection. After 4 wk of infection, populations enriched for CD4 $^+$, CD8 $^+$, and sIg $^+$ cells by magnetic bead selection were obtained from the popliteal lymph nodes of BALB/c mice with progressive leishmaniasis (lanes 1–4) or from BALB/c mice cured after treatment with 0.5 μ g of rMuIL-12 for the first 7 d of infection (lanes 5–8). RNA obtained from lymph node cells relatively depleted of CD4 $^+$, CD8 $^+$, and sIg $^+$ lymphocytes (<6% of each) is also included (*Other*). 15- μ g of RNA from each cellular subpopulation was loaded per lane, except in the fractions labeled *Other*, which received 5 μ g. RNA loading was confirmed by ethidium bromide labeling of the agarose gel after electrophoresis (*Eth Br*).

CD4 $^+$ T cells expressed these lymphokine messages during infection. IFN- γ expression was increased twofold in rMuIL-12-treated mice, whereas IL-4 production was diminished fourfold as measured by densitometry. Combined with parallel changes observed in the production of cytokines by antigen-stimulated lymph node cultures (Table 2), these results confirm a reversal of Th2 and Th1 activities after rMuIL-12 therapy.

The protective effect of rMuIL-12 in susceptible BALB/c mice suggests that increased production of IL-12 may mediate the innate resistance of C57BL/6 mice against progressive leishmaniasis. Indeed, challenge of 3-wk infected mice with LPS resulted in the greater appearance of circulating IL-12 in C57BL/6 mice (400 U/ml) compared with BALB/c mice (≤ 10 U/ml), as measured by bioassay and confirmed by capture of 256 U/ml (or 1.4 ng/ml) of the C57BL/6 activity by specific anti-mouse IL-12 mAb combined with bioassay (11). IL-12 was not detected in the absence of a LPS challenge or in the serum of uninfected mice receiving LPS (≤ 10 U/ml). These results are consistent with a greater capacity for IL-12 release as a result of leishmaniasis in resistant mice and were prompted by parallel observations in C57BL/6 and BALB/c mice infected with *Bacillus-Calmette-Guerin* (15). Because strongly neutralizing antibodies against murine IL-12 are currently unavailable, we could not confirm if endogenous IL-12 plays a role in C57BL/6 resistance. Furthermore, although IL-12 p35 and p40 mRNA expression was increased in C57BL/6 mice during infection, the p40 and p35 transcripts were associated with different cell populations in the draining lymph nodes (data not shown). Because synthesis of functional IL-12 requires that both subunits be expressed in the same cell, these Northern blot data did not provide easily interpretable conclusions about the *in vivo* synthesis of IL-12 in response to infection. Further studies are necessary to determine the cellular origin and kinetics of IL-12 release during leishmaniasis.

Our data show that rMuIL-12 durably alters CD4 $^+$ subset differentiation when given in the critical first week of infection. Although cure was undoubtedly assisted by the increased Th1 activity in the draining lymph nodes, the relatively greater negative effect on IL-4 production suggests that considerable benefit may have been provided through removal of a known antagonist of IFN- γ -activated macrophage leishmanial killing (16, 17). The dependence of CD4 $^+$ subset outcomes, particularly that of Th2 cells, on IFN- γ after rMuIL-12 therapy is consistent with studies showing that IFN- γ is required for both cure and unipolar Th1 cell responses in infected C57BL/6 mice (12, 18). However, infected BALB/c mice cannot be cured by treatment with rMuIFN- γ alone (7), and this suggests that IL-12 may provide separate inductive signals necessary for the appearance of curative T cell responses.

The ability of rMuIL-12 to cure leishmaniasis in normally susceptible BALB/c mice strongly supports a unique role for this cytokine in the induction of protective immunity against intracellular parasitic infection. This is the first instance where any recombinant cytokine has been curative for infection with *L. major* (6). Further studies will be required to link differ-

ences in the production of IL-12 during leishmaniasis to the heterogeneity of disease outcomes observed in humans and inbred mice (2, 19), and to define the role of IL-12 in the

pathogenesis of inflammatory and allergic disorders similarly mediated by disparate CD4⁺ T cell responses.

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