Brief Definitive Report

Resolution of Cutaneous Leishmaniasis: Interleukin 12 Initiates a Protective T Helper Type 1 Immune Response

By Joseph P. Sypek,* Charles L. Chung,* Sharon E. H. Mayor,* Janaki M. Subramanyam,* Samuel J. Goldman,* Derek S. Sieburth,‡ Stanley F. Wolf,‡ and Robert G. Schaub*

*From the Departments of *Preclinical Biology and ‡Cellular Immunology, Genetics Institute, Inc., Cambridge, Massachusetts 02140*

Summary

Resistance to *Leishmania major* in mice is associated with the appearance of distinct T helper type 1 (Th1) and Th2 subsets. T cells from lymph nodes draining cutaneous lesions of resistant mice are primarily interferon γ (IFN-γ)-producing Th1 cells. In contrast, T cells from susceptible mice are principally Th2 cells that generate interleukin 4 (IL-4). Although existing evidence is supportive of a role for IFN-γ in the generation of Th1 cells, additional factors may be required for a protective response to be maintained. A potential candidate is IL-12, a heterodimeric cytokine produced by monocytes and B cells that has multiple effects on T and natural killer cell function, including inducing IFN-γ production. Using an experimental leishmanial model we have observed that daily intraperitoneal administration at the time of parasite challenge of either 0.33 μg IL-12 (a consecutive 5 d/wk for 5 wk) or 1.0 μg IL-12 per mouse (only a consecutive 5 d) caused a >75% reduction in parasite burden at the site of infection, in highly susceptible BALB/c mice. Delay of treatment by 1 wk had less of a protective effect. Concomitant with these protective effects was an increase in IFN-γ and a decrease in IL-4 production, as measured by enzyme-linked immunosorbent assay of supernatants generated from popliteal lymph node cells stimulated with leishmanial antigen in vitro. The reduction in parasite numbers induced by IL-12 therapy was still apparent at 10 wk postinfection. In addition, we observed that the administration of a rabbit anti-recombinant murine IL-12 polyclonal antibody (200 μg i.p. every other day for 25 d) at the time of infection to resistant C57Bl/6 mice exacerbated disease. These effects were accompanied by a shift in IFN-γ production in vitro by antigen-stimulated lymph node cells indicative of a Th2-like response. These findings suggest that IL-12 has an important role in initiating a Th1 response and protective immunity.

The outcome of experimental infection in inbred strains of mice infected with *Leishmania major* is profoundly influenced by the nature of the $CD4^+$ T cell response to the disease. Disease resistance and susceptibility appear to depend on the cytokine profile secreted by particular subsets of $CD4^+$ lymphocytes. In mouse strains that develop small localized lesions that ultimately resolve, exemplified by the C57Bl/6 mouse, resistance is preferentially associated with the presence of IFN-γ-producing $CD4^+$ cells of the Th1 subset (1). In mice that develop a progressive and ultimately fatal infection, characterized by the BALB/c mouse, susceptibility is preferentially associated with the presence of IL-4-producing T cells of the Th2 subset (1). It has been directly demonstrated by adoptive transfer of specific Th1 and Th2 cell lines that these T cell subsets mediate disease resistance or susceptibility (2). A causal relationship between cytokine production and disease outcome is further suggested by the observation that anti-IL-4 treatment attenuates disease in susceptible mice (3), while anti-IFN-γ antibody treatment induces susceptibility in otherwise resistant mice (4). The observation, however, that administration of IFN-γ to BALB/c mice after the initiation of infection does not modify the progression of disease (3, 5) suggests that factors other than IFN-γ alone are necessary for the development of a durable Th1 response.

IL-12, also known as natural killer cell stimulating factor (NKSF) and cytotoxic lymphocyte maturation factor (CLMF), is a heterodimeric cytokine produced by monocytes and B cells (6–8). It displays a potent array of biological activities affecting NK and T cells. These biological activities include the ability to enhance proliferation of NK and T cells, to induce production of IFN-γ, and to enhance NK and T cell

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/06/1797/06 $2.00
Volume 177 June 1993 1797–1802

cytolytic activity (6–10). Of particular interest to the leishmanial model is the observation that IL-12 can promote the development of Th1 cells (11a).

In this study, we examine the effects of recombinant murine (rm)IL-12 administration on the immune response to cutaneous leishmaniasis in highly susceptible BALB/c mice. Conversely, we also examine the effects of anti-rmIL-12 antibody therapy in resistant C57Bl/6 mice. We demonstrate that IL-12 plays a critical role in the development of a protective Th1 immune response and in controlling the progression of chronic disseminating leishmaniasis in these animal models.

Materials and Methods

Leishmania. *L. major*, National Institutes of Health Seidman strain (WHOM/SN/74 Seidman), was used in these investigations (12). Amastigotes were propagated in mice by serial infection and obtained from infected footpad tissue as described (13).

Animals and Infection. Female BALB/cJ (H-2^d; amastigote and lymphocyte donors) and C57Bl/6 (H-2^b; lymphocyte donors) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were infected subcutaneously into each hind footpad with a 0.05-ml suspension of 2×10^5 tissue-derived amastigotes (13) in 0.15 M NaCl.

NKSF/IL-12. Serum-free culture supernatant was collected and purified from Chinese hamster ovary cells stably transfected with amplified cDNAs for the p40 and p35 subunits of mIL-12. SDS-PAGE analysis revealed this preparation to consist predominantly of mIL-12 heterodimeric (95%) with a small amount of monomeric p40 subunit (5%). rmIL-12 was stored in 500-μg aliquots of calcium- and magnesium-free modified Dulbecco's PBS at −80°C until used. The specific activity was $\sim 7.8 \times 10^6$ U/mg. 1 U is defined relative to a human IL-12 standard RB 012892 in a PHA blast proliferation assay. 1 U is approximately equal to the amount that yields half-maximal stimulation. Endotoxin levels as measured by the Limulus amebocyte lysate assay did not exceed 0.5–0.9 EU/mg.

In Vivo IL-12 Treatment. In the initial study BALB/c mice (n = 5 per treatment group) were given rmIL-12 (0.11 μg/d or 0.33 μg/d) or 0.9% NaCl (Abbott Laboratories, N. Chicago, IL) by single intraperitoneal administration for a consecutive 5 d/wk for 5 wk after a challenge with amastigotes in both hind footpads. At 1-wk time intervals during the 5 wk, lesion development was monitored by measuring with a venier caliper footpad thickness of infected footpads.

In the second study BALB/c mice (n = 10 per treatment group) were administered rmIL-12 intraperitoneally in the following manner. Immediately after a challenge with amastigotes in the hind footpads, one group received 0.33 μg/d rmIL-12 for a consecutive 5 d/wk for 5 wk, or alternatively another group received saline alone in a similar manner. An additional group received 1.0 μg/d rmIL-12 for a consecutive 5 d for the first week of infection only, while with another group treatment with 1.0 μg/d rmIL-12 was delayed until the second week of infection, where it was given for a consecutive 5 d/wk and continued for a total of 5 wk. Representative animals from each group at selected time intervals were killed and footpad parasite burdens were quantified by published methods (13).

Neutralizing Rabbit Anti-rmIL-12 Antibody. Female New Zealand white rabbits were immunized subcutaneously with 50 μg of rmIL-12 in adjuvant bimonthly. An immune Ig fraction from antisera pooled from one rabbit (03B02, anti-IL-12) was obtained by binding to protein A–agarose column (Repligen Corp., Cambridge, MA) according to established procedures (14, 15) using a FPLC chromatography system (Pharmacia Fine Chemicals, Piscataway, NJ). Purified antibody was stored at −80°C until needed. This anti-IL-12 preparation is not known to react with any other cytokines other than IL-12 (our unpublished observations).

In Vivo Anti-IL-12 Treatment. After a challenge with amastigotes in the hind footpads, C57Bl/6 mice (n = 8 per treatment group) were treated with either anti-IL-12 antibody or normal rabbit Ig (I-5006; Sigma Chemical Co., St. Louis, MO) by intraperitoneal injection of 200 μg of either material commencing on the day of challenge and continued every other day for 25 d. At selected time intervals parasite burdens were quantified as described above.

Lymph Node Lymphocytes and Lymphokine-containing Culture Supernatants. Lymph node lymphocytes were obtained from draining popliteal lymph nodes of *Leishmania*-infected or normal mice as described (13). Lymphokine-containing culture supernatants were prepared by published methods (16). Briefly, cultures of lymph node cells (10^6/ml) in RPMI supplemented with 2.5% FCS serum (Hyclone Laboratories, Logan, UT) were incubated at 37°C with either culture-derived promastigotes, tissue-derived amastigotes, anti-CD3 mAb (145-2C11, hamster IgG, 10 μg/ml; PharMingen, San Diego, CA), or left untreated. The cell-free conditioned medium from lymph node cell cocultures was harvested after 48 h and stored at −80°C until analyzed for activity.

Immunoassays for IFN-γ and IL-4. ELISAs using mAbs specific for mIFN-γ (Genzyme Corp., Cambridge, MA) or mIL-4 (Endogen Inc., Boston, MA) that have been previously described (17, 18) were used to quantify the amount of IFN-γ or IL-4 present in culture supernatants of lymph node cells cultured in vitro. A standard curve for each assay was generated with known concentrations of mIFN-γ or mIL-4 in protein-stabilized supplemented media.

Results

Treatment of BALB/c Mice with rmIL-2 Affects Progression of Lesion Development in Footpads Infected with Leishmania. To assess whether administration of rmIL-12 could influence leishmanial parasite growth in BALB/c mice (susceptible to cutaneous leishmaniasis), footpad thickness was monitored in infected mice treated by intraperitoneal injection with saline, 0.11 or 0.33 μg rmIL-12 once daily (qd), for a consecutive 5 d/wk for 5 wk. Footpad thickness steadily increased in saline-treated animals during the 5-wk course. In contrast, at both doses of rmIL-12, the increase in footpad thickness was reduced. By the fifth week of infection, animals treated with 0.11 or 0.33 μg rmIL-12 had footpad thicknesses of 4.23 ± 0.25 and 3.07 ± 0.21 mm (n = 5, mean ± SD), respectively. Footpad thickness in saline-treated animals was 5.60 ± 0.10 mm. These observations suggested that rmIL-12 induced a reduction in parasite burden. Histological examination of infected footpad tissue confirmed this conclusion (data not shown).

Administration of rmIL-12 to BALB/c Mice during the First Week of Infection Is Sufficient to Induce Resistance to Leishmania. To determine the temporal sequence of when rmIL-12 therapy was necessary to prevent lesion development, mice were infected by footpad injection and then treated with 1.0 μg rmIL-12 qd for a consecutive 5 d immediately after infection, or treated with 1.0 μg qd beginning 1 wk after infection. Mice given delayed therapy were administered rmIL-12

for a consecutive 5 d/wk for a total of 5 wk. As controls, additional groups of infected mice or uninfected mice received 0.33 μg rmIL-12 as described above, and an infected group received saline.

Parasite burdens in infected mice were determined by microscopic examination of stained amastigote suspensions derived from infected footpad tissue (Table 1). At 5 wk postinfection, parasite burden was reduced fivefold in mice treated continuously with 0.33 μg/d rmIL-12 when compared with saline-treated mice. More significantly, parasite reduction in mice treated with 1.0 μg rmIL-12 for the first week of infection was 15-fold compared with saline controls. In contrast, parasite reduction was less than twofold in mice where treatment began 1 wk after infection.

Treatment of mice for a consecutive 5 d postinfection was sufficient to prevent the development of footpad lesions. This protective effect of therapy was still apparent at 10 wk postinfection (Table 1), suggesting that the induced antileishmanial effects were sustained. Treatment starting 1 wk after parasite challenge reduced the rate of lesion progression compared with saline-treated animals. These mice, however, remained susceptible so that by 8 wk lesion size approached that observed in saline-treated controls at 4–5 wk of infection, necessitating that the mice be killed. Administration of rmIL-12 to uninfected mice did not induce an increase in footpad swelling.

Table 1. *Footpad Parasite Burdens from rmIL-12-treated BALB/c Mice Infected with Cutaneous Leishmaniasis for 5 and 10 wk*

Treatment	Amastigotes/mg tissue
5th week of infection	
Saline	$8.1 \times 10^4 \pm 2.4 \times 10^4$
0.33 μg IL-12 5 d/wk for 5 wk	$1.5 \times 10^4 \pm 5.1 \times 10^3$
1.0 μg IL-12 5 d/wk, 1st week only	$5.4 \times 10^3 \pm 2.1 \times 10^3$
1.0 μg IL-12 5 d/wk, 2nd to 5th week	$6.8 \times 10^4 \pm 2.3 \times 10^4$
10th week of infection	
Saline	Dissemination animals died
0.33 μg IL-12 5 d/wk for 5 wk	$2.8 \times 10^3 \pm 5.5 \times 10^2$
1.0 μg IL-12 5 d/wk, 1st week only	$9.5 \times 10^2 \pm 9.3 \times 10^2$
1.0 μg IL-12 5 d/wk, 2nd to 5th week	Dissemination animals died

BALB/c mice were infected with *L. major* for 5–10 wk by injection of 2×10^5 amastigotes/hind footpad. Mice were then treated with an intraperitoneal injection of saline or rmIL-12 qd starting on the day of infection for a consecutive 5 d/wk for 5 wk. In addition, one group was given rmIL-12 treatment for the first 5 d of infection, while in another group rmIL-12 was administered 1 wk after infection for a consecutive 5 d for 5 wk. After 5 or 10 wk representative animals from each group were killed and parasite density was quantified by enumerating the number of parasites/mg of footpad tissue. Each value represents the mean ± SD of three to five animals.

Administration of rmIL-12 at the Time of Leishmania Infection in BALB/c Mice Alters Cytokine Production. To determine whether resistance affected by rmIL-12 was due to the generation of a Th1 response and the production of IFN-γ, the cytokine profile produced in vitro by draining popliteal lymph node cells of infected BALB/c mice was measured. At 5 wk postinfection, cells from mice treated with 0.33 μg/d rmIL-12 or saline were stimulated in vitro with *L. major* amastigotes. IFN-γ production by lymph node cells of mice treated with rmIL-12 was 10-fold greater than that produced by saline-treated mice (Table 2). In contrast, cells from rmIL-12-treated mice produced fivefold less IL-4 than cells from saline controls. Semiquantitative PCR analysis of RNA from popliteal lymph node cells demonstrated that in vivo IFN-γ RNA levels in infected animals were increased by rmIL-12 treatment (10-fold increase compared with untreated controls; data not shown).

In addition, we observed that rmIL-12 treatment for 5 d postinfection appeared sufficient to initiate a protective Th1 response early in infection (Table 1). To assess the cytokine profile produced under these conditions, popliteal lymph node cells were collected 5 wk postinfection from these treated mice and assayed as described above. In vitro IFN-γ produc-

Table 2. *IFN-γ and IL-4 Levels Present in Cell-free Supernatants from Cultures of Lymph Node Cells Derived from rmIL-12- or Saline-treated BALB/c Mice Infected with Cutaneous Leishmaniasis for 5 wk under Various Culture Conditions*

In vivo treatment*	In vitro treatment‡	IFN-γ	IL-4
		pg/ml	*pg/ml*
Saline	Untreated	1,500	150
	Amastigotes	1,500	>750
0.33 μg/d	Untreated	8,000	25
rmIL-12, 5 wk	Amastigotes	>10,000	225
1.0 μg/d	Untreated	8,000	0
rmIL-12, 1 wk	Amastigotes	>10,000	75

ELISAs using mAbs specific for mIFNγ or IL-4 were used to quantify the amount of IFN-γ and IL-4 present in culture supernatants of lymph node cells. Cells used were derived from popliteal lymph nodes of BALB/c mice infected with *L. major* for 5 wk by injection of 2×10^5 amastigotes/hind footpad.

* Mice were treated intraperitoneally on the day of infection with saline, or 0.33 μg/d rmIL-12 qd for a consecutive 5 d for 5 wk, or 1.0 μg/d rmIL-12 qd for a consecutive 5 d for the first week of infection only.

‡ 10^6/ml lymph node cells were stimulated with 10^5 amastigotes/ml or left untreated for 48 h in supplemented media with 2.5% FCS. Cell-free supernatants were then collected and stored at −80°C before being assayed.

tion from mice treated with 1.0 μg/d rmIL-12 for a consecutive 5 d immediately after infection was 10-fold greater than that of cells from saline-treated mice. Concomitantly, there was a 10-fold reduction in the level of IL-4 produced by these cells compared with saline controls (Table 2). Accompanying these changes we also observed a decrease in the relative percentage of $CD4^+$ T cells of the Th2 subset in the draining popliteal lymph nodes of treated mice as determined by immunophenotyping using anti-CD45R isotype antibody MB23G2/15C11 (19, data not shown).

Treatment of C57Bl/6 Mice with Rabbit anti-rmIL-12 antibody Abrogates Natural Resistance to L. major. The observation above that rmIL-12 induces resistance in susceptible BALB/c mice suggested that host production of IL-12 might be responsible for protection in a resistant mouse strain. To test this hypothesis, mice from a resistant mouse strain, C57Bl/6, were administered polyclonal rabbit anti-rmIL-12 antiserum beginning at the time of infection with *L. major*. Infected footpad tissues of C57Bl/6 mice administered normal rabbit Ig had minimal swelling by 3 wk postinfection that returned to nearly baseline by 6 wk. In contrast, mice treated with anti-rmIL-12 antiserum developed large nonhealing lesions. By 4 wk postinfection, these footpads were twice as large as that of control animals and nearly as large as those of susceptible BALB/c mice. Examination of parasite suspensions derived from footpad tissue at 5 wk postinfection confirmed that footpad swelling was associated with increased parasite burden (>70% increase compared with untreated controls; $4.9 \times 10^4 \pm 1.9 \times 10^3$ amastigotes/mg tissue and $1.3 \times 10^3 \pm 3.6 \times 10^3$ amastigotes/mg tissue [$n = 3$, mean ± SD], respectively).

The increased disease susceptibility suggested that administration of anti-rmIL-12 antisera to C57Bl/6 mice resulted in the inhibition of their normal Th1 response to infection. To test this hypothesis, we measured in vitro the capacity of popliteal lymph node cells to produce IFN-γ. At 1–2 wk postinfection, there was a >50% decrease in IFN-γ production by lymph node cells from anti-rmIL-12-treated mice when compared with cells from control antisera-treated animals (Table 3). These observations taken together with those above demonstrate that the presence of IL-12 at the time of infection is crucial in determining whether a Th1 or Th2 response develops, and in the control of progressive disease.

Table 3. *IFN-γ Levels Present Cell-free Supernatants from Cultures of Lymph Node Cells Derived from Anti-rmIL-12- or Normal Sera-treated C57Bl/6 Mice Infected with Cutaneous Leishmaniasis for 1–2 wk under Various Culture Conditions*

In vivo treatment*	In vitro treatments‡	IFN-γ
		pg/ml
Normal rabbit Ig	Untreated	250
	Amastigotes	500
	Promastigotes	8,000
	Anti-CD3	500
Rabbit anti-rmIL-12	Untreated	250
	Amastigotes	250
	Promastigotes	3,000
	Anti-CD3	175

ELISAs using mAbs specific for mIFNγ were used. Cells were derived from popliteal lymph nodes of C57Bl/6 mice infected with *L. major* amastigotes for 1–2 wk.

* Mice were treated intraperitoneally on the day of infection with 200 μg normal rabbit Ig or rabbit anti-rmIL-12 qd and every other day thereafter for 25 d.

‡ Lymph node cells were stimulated with amastigotes, promastigotes, anti-CD3 mAb, or left untreated for 48 h in supplemented media. Supernatants were collected and stored at −80°C before analysis.

Discussion

$CD4^+$ T cells can be delineated on the basis of their cytokine profile into two subsets, Th1 and Th2. Th1 cells produce IL-2 and IFN-γ in response to stimulation, while Th2 cells produce IL-4, IL-5, and IL-10 (20). Additional cytokines are also produced by both these subsets. These subsets are thought to arise from a common precursor after stimulation via their TCRs (21). The mechanisms favoring the development of Th1 and Th2 cells and which factors or cytokines determine this differentiation are not well defined.

In mouse models of cutaneous leishmaniasis, a spectrum of disease ranging from self-healing cutaneous ulceration to progressive visceral dissemination occurs in association with distinct lymphokine responses. In particular, resolution of cutaneous disease requires the expansion of specific Th1 cells that produce IFN-γ, while exacerbation of infection requires the expansion of specific Th2 cells that produce IL-4 (1, 2). In this report, we provide evidence that IL-12 has a major role in influencing the development of a protective Th1 immune response in *L. major* infection. We show that treatment with rmIL-12 in BALB/c mice reverses their susceptibility to infection (Table 1). This alteration in susceptibility is due to a shift in the T cell response from a predominant Th2- to a Th1-type response (Table 2). Accompanying changes in IFN-γ and IL-4 levels produced by draining lymph node cells upon in vitro restimulation from these mice confirm that the IL-12-treated mice produce a more Th1-like response.

Two sets of data pinpoint the time most critical for the presence of IL-12 in the resolution of *L. major* disease. When IL-12 is administered only during the first week of infection, we observe resolution of infection. These data suggest that clearance of parasites from cutaneous tissues of BALB/c mice early after infection is influenced by IL-12. When treatment was delayed until the second week of infection, the animals were not able to heal the infection although disease progression was delayed. Thus, the presence of IL-12 during the initial parasite host–cell interactions in the first week of infection is clearly critical for resolution of disease. Further evidence in support of the role of IL-12 in the generation of a protective Th1 response is the demonstration that the administra-

tion of anti-IL-12 antibody in vivo exacerbates disease in normally resistant C57Bl/6 mice. Depletion of IL-12 profoundly affects the ability of these mice to clear the parasite inoculum from cutaneous tissues and is accompanied by a shift in IFN-γ and IL-4 production, rendering a more Th2-like response than in normal rabbit Ig–treated mice (Table 3). Combined with the ability of IL-12 to augment Th1 responses in BALB/c mice, these data in C57Bl/6 mice indicate that IL-12 is critical for the development of a Th1 response.

Earlier studies examining the role of Th1 and Th2 cells in the immune response to *Leishmania* provided evidence demonstrating the key role that IFN-γ plays in modulating the Th1 response to leishmanial infections. In particular, neutralizing antibodies to IFN-γ were shown to be capable of abrogating protective immunity in genetically resistant mice 4, 5). In addition, Th1 cells mediating immunity to infection produce IFN-γ, whose expression and secretion directly correlates with the reduction in parasite burdens in infected resistant mice (1, 2).

Paradoxically, however, the administration of IFN-γ itself after the initiation of infection, although leading to a delay in the generation of lesions, ultimately provides no long-term control of infection (3, 5). These observations suggest that factors in addition to IFN-γ were necessary for the development of a sustained Th1 response. We believe the evidence provided here strongly suggests that IL-12 is a critical factor in initially determining a Th1 response. Moreover, the ability of IL-12 to directly induce IFN-γ production in both NK and T cells (6, 7, 22), in addition to synergizing with other physiological IFN-γ inducers, is consistent with IL-12 having a key role as a immunopotentiating agent in generating a Th1 response. Our preliminary observations have also shown that the administration of anti-IFN-γ antibody to infected BALB/c mice treated with rmIL-12 abrogates their ability to initiate a protective immune response (data not shown). These data suggest that IL-12 may be influencing the early clearance of parasites by initiating a protective Th1 response, as well as by the induction of IFN-γ.

The murine model of cutaneous leishmaniasis provides an excellent system in which to examine the role $CD4^+$ T cells and their respective cytokines play in the resolution of pathogenesis of infectious diseases. We have shown that IL-12 plays a crucial role in initiating a Th1 cell response and is essential for inducing a protective host immune response. As $CD4^+$ subsets become better defined in other disease models, these observations will be useful in designing strategies for the development of vaccines and immunotherapy against other infectious pathogens.

We thank Brian Hubbard and Elliot Nickbarg for the preparation and purification of recombinant cytokine material; Kristin Murray, Scott Leppanen, Mary Lynn Kolowsky, and Victor Van Cleave for the preparation and purification of anticytokine antisera; Lori Hayes and Lawrence Mason for their excellent technical assistance; Steven Herrmann and John Leonard for their helpful advice and criticism during the course of this work and the preparation of this manuscript; and Lori Creilson for typing the manuscript.

Address correspondence to Joseph P. Sypek, Department of Preclinical Biology, Genetics Institute, Inc., 87 CambridgePark Drive, Cambridge, MA 02140.

Received for publication 9 February 1993 and in revised form 25 March 1993.

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