Interleukin-12-Mediated Resistance to *Trypanosoma cruzi* Is Dependent on Tumor Necrosis Factor Alpha and Gamma Interferon

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Received 20 February 1996/Accepted 2 April 1996

The aim of this study was to determine if interleukin-12 (IL-12) has a role in the immune response to *Trypanosoma cruzi*. Infection of BALB/c mice with the virulent Tulahuen strain of *T. cruzi* is characterized by a high-level parasitemia, pathology in the heart associated with the presence of amastigotes, and death during the acute phase of the disease. Administration of IL-12 to BALB/c mice infected with *T. cruzi* resulted in a reduced parasitemia and a significant delay in the time to death compared with those for infected controls. This protective effect was correlated with increased levels of gamma interferon (IFN- γ) and tumor necrosis factor alpha (TNF- α) in serum. To determine if these cytokines were involved in the protective effects of IL-12, we treated infected mice with IL-12 alone or in combination with monoclonal antibodies specific for IFN- γ or TNF- α . These antibodies antagonized the protective effect of exogenous IL-12. Treatment of infected mice with a polyclonal antibody specific for IL-12 resulted in a significant increase in parasitemia but did not affect the time to death. These latter studies demonstrate a role for endogenous IL-12 in resistance to *T. cruzi*. Together, our data identify an IL-12-mediated mechanism of resistance to *T. cruzi*, which is dependent on IFN- γ and TNF- α .

Interleukin-12 is a recently described cytokine, produced by B cells and macrophages, which is a potent stimulator of cell-mediated immune responses (7, 38). IL-12 can stimulate the production of gamma interferon (IFN- γ) by T lymphocytes and NK cells, stimulate the proliferation of these cells, and enhance the cytotoxic activity of lymphocytes (7). Endogenous IL-12 has an important role in resistance to several infections, including *Leishmania major* (15, 33), *Plasmodium chabaudi* (28, 32), *Toxoplasma gondii* (13, 19), and *Listeria monocytogenes* (39) infections. Moreover, administration of IL-12 has been shown to be useful in the treatment of these infections in murine models (reviewed in reference 4). In these models of infection, the ability of IL-12 to stimulate the production of IFN- γ is critical to the protective effect of IL-12.

Chagas' disease is caused by infection with the parasitic protozoan *Trypanosoma cruzi*. This disease is characterized by acute and chronic phases (34). In the acute phase there are large numbers of trypomastigotes in the blood, whereas in the chronic phase there is development of pathology in the heart and other tissues, which is possibly caused by multiplication of parasites in these sites. The use of murine models to study the immune response to *T. cruzi* has identified an important role for T lymphocytes in resistance to this infection (1, 16, 22, 26, 35, 36). It is likely that the protective effect of T cells against Chagas' disease is through their production of IFN- γ (30). This cytokine, in combination with tumor necrosis factor alpha (TNF- α), can activate macrophages to kill intracellular parasites (10, 30).

Since cell-mediated immunity is important for resistance to *T. cruzi*, we hypothesized that IL-12 may be involved in such a response. To address this hypothesis, we infected mice with a virulent strain of *T. cruzi* and assessed the effect of administration of IL-12 or a neutralizing antiserum specific for IL-12 on the course of infection. Our results demonstrate that IL-12 has a protective role in the regulation of parasitemia and that this effect is dependent on IFN- γ and TNF- α .

MATERIALS AND METHODS

Mice and parasites. Adult BALB/c female mice (Simonsen Labs, Gilroy, Calif.) 6 to 8 weeks of age were used for all experiments. Mice were maintained with access to food and water ad libitum. The Tulahuen strain of *T. cruzi* was used for these studies (5). Infection was routinely maintained by transfer of blood from infected to uninfected mice. For experiments, the blood of infected animals was collected by using phosphate-buffered saline (PBS) containing 3.8% sodium citrate, and the numbers of trypomastigotes were counted (5). Mice were infected by intraperitoneal (i.p.) injection of 10^3 or 10^4 parasites in a volume of 0.2 ml. Parasitemia was determined as previously described (1) with blood diluted 1:10 in PBS with 3.8% sodium citrate.

Histopathology. In experiments to determine the effect of treatment with IL-12 on the parasite burden in tissue, we chose the heart as a representative tissue. Tissue was collected and processed for histopathology as described previously (17). Paraffin-embedded sections were stained with hematoxylin-eosin. Two sections per heart were stained with hematoxylin-eosin, and the numbers of foci of parasites per section were counted in a blinded fashion at a magnification of $\times 400$. Figure 3 illustrates typical foci of parasite replication.

Cytokines and antibodies. Endotoxin-free IL-12 (5.6×10^6 U/mg) was supplied by the Immunology Department of the Genetics Institute, Inc. (Cambridge, Mass.). A rat anti-mouse IFN- γ neutralizing monoclonal antibody (XMG1.2) or rat anti-mouse TNF- α (MP6-XT22) was administered i.p. to mice at 1 mg per mouse at 24 h preinfection and on days 3 and 7 postinfection. Rat monoclonal antibodies specific for β -galactosidase were used as isotype controls. All monoclonal antibodies employed in this study were purified by fast protein liquid chromatography anion-exchange chromatography under low-endotoxin conditions and were provided by John Abrams of DNAX (Palo Alto, Calif.). Polyclonal sheep anti-murine IL-12 was supplied by the Immunology Department of the Genetics Institute, Inc., and was administered i.p. at a dose of 200 μ g per mouse at 24 h prior to infection and every 2 days thereafter (20). Sheep immunoglobulin G (Sigma Chemical Co., St. Louis, Mo.) was used as a control for the IL-12-specific antiserum.

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FIG. 1. Administration of IL-12 to BALB/c mice infected with *T. cruzi* delays time to death. (A) Groups of four to six BALB/c mice were treated i.p. with IL-12 (10, 50, or 100 ng per mouse) at 24 h prior to infection and every day thereafter for 6 days. All three treatments resulted in a significant delay in the time to death ($P = 0.02$, 0.002 , and 0.002 , respectively). (B) Mice were treated with IL-12 (100 ng per mouse) beginning 3 days after infection and for a further 6 days. This treatment resulted in a significant delay in the time to death ($P = 0.008$). Mice were infected with *T. cruzi* as described in Materials and Methods. Mortality was monitored on a daily basis. Similar results were observed in two or three other experiments.

Assays for IFN- γ and TNF- α . A two-site enzyme-linked immunosorbent assay (ELISA) was employed to assay levels of IFN- γ with reagents supplied by DNAX, as previously described (20). The sensitivity of this assay was 19 to 38 pg/ml. TNF- α was assayed with a two site ELISA (Pharmingen, San Diego, Calif.). The sensitivity of this assay was 39 to 78 pg/ml.

Statistics. Statistical analysis (unpaired t test and Mann-Whitney test) was performed by using INSTAT software (GraphPad, San Diego, Calif.). A P value of less than 0.05 was considered significant. Kaplan-Meier survival analysis was performed with Stat-View software (Abacus Concepts, Inc, Berkeley, Calif.), and a P value of less than 0.05 was considered significant.

RESULTS

Administration of IL-12 delays time to death of BALB/c mice infected with *T. cruzi* and reduces parasitemia. Preliminary experiments revealed that daily administration of 0.5 to 1 μ g of IL-12 for 6 to 8 days to mice infected with *T. cruzi* did not result in a significant delay in the time to death (data not shown). In these experiments, infected mice treated with IL-12 became cachectic in appearance prior to controls and in some cases died before infected controls. Of note is that uninfected mice given the same treatment regimen became cachectic in appearance but did not die and recovered 3 to 4 days after IL-12 treatment was stopped. Since there have been recent reports that high doses of IL-12 are toxic to mice (11, 23) we decided to use doses of IL-12 that were lower than 0.5 μ g. In repeat experiments, administration of either 10, 50, or 100 ng of IL-12 to mice, beginning 24 h prior to infection and for 6 days thereafter, resulted in a significant delay in the time to

death (Fig. 1A). Surviving mice were alive at 50 days postinfection and showed no outward signs of disease.

In other models of parasitic infection, the protective effect of IL-12 is dependent on administration of the cytokine prior to infection and treatment after infection does not result in protection (21, 28). To determine if this was the case for mice infected with *T. cruzi*, we administered IL-12 (100 ng per mouse) to mice for 6 days beginning on day 3 of infection. This treatment regimen resulted in a significant delay in the time to death compared with that for sham-treated controls (Fig. 1B).

To further characterize the effect of treatment with IL-12 on the course of infection, we measured the parasitemia in sham-treated control mice or mice treated with IL-12. Administration of IL-12 (10, 50, or 100 ng) beginning 24 h prior to infection and for 6 days thereafter resulted in a significant decrease in the parasitemia compared with that for controls (Fig. 2). Similar results were observed with mice which were infected 3 days prior to treatment with IL-12 for 6 days (data not shown).

Since administration of low doses of IL-12 resulted in reduced parasitemia, a delay in the time to death, and survival of a low percentage of mice, we wished to assess whether the high doses of IL-12 used in our initial studies had an effect on parasitemia. In a typical experiment we observed that daily administration of IL-12 (500 ng per mouse) beginning 24 h prior to infection and for 5 days thereafter resulted in a significant reduction in parasitemia on day 10 postinfection, from $8.85 \times 10^5 \pm 97,601$ parasites per ml (mean $\pm$ standard deviation [SD]) ($n = 4$) to $1.85 \times 10^5 \pm 50,272$ parasites per ml ($n = 4$).

Examination of hematoxylin-eosin-stained tissue sections from infected mice sacrificed on day 10 postinfection revealed the presence of mild myocarditis and endocarditis. To assess the effect of treatment with IL-12 on the parasite burden in tissues, we counted the numbers of foci of parasite replication in the heart. Infected mice treated with 50 or 100 ng of IL-12 per day had a significant reduction in the numbers of foci of parasite replication in the heart (Table 1). This effect was not observed in mice treated with 10 ng of IL-12 per day (Table 1). Interestingly administration of IL-12 resulted in an increased cellularity associated with many of the foci of parasite replica-

FIG. 2. Administration of IL-12 to BALB/c mice infected with *T. cruzi* decreases parasitemia. Groups of four BALB/c mice were treated with IL-12 (10, 50, or 100 ng) at 24 h prior to infection and every day thereafter for 6 days. Mice were infected with *T. cruzi* as described in Materials and Methods. Parasitemia was monitored on days 10 and 14 after infection. Results presented are the means $\pm$ SDs for groups of four mice. Similar results were observed in three other experiments.

TABLE 1. Administration of IL-12 reduces numbers of foci of parasite replication in the heart^a

IL-12 treatment (ng)	No. of foci of parasite replication
0.....	20.1 (7.4)
10.....	21.7 (8.9)
50.....	11.2* (5.8)
100.....	5.1* (3.8)

^a Mice were infected with *T. cruzi* as described in Materials and Methods and treated with either IL-12 or diluent for 6 days starting 24 h prior to infection. Mice were sacrificed on day 14 after infection, and tissues were processed for histopathology. Parasite foci were counted as described in Materials and Methods. Results are from a single experiment with *n* = five or six mice per group. Similar results were observed in two repeat experiments. SDs are given in parentheses. *, *P* < 0.005 compared with sham-treated controls.

tion in the hearts of treated mice compared with that for untreated infected controls (Fig. 3).

Administration of IL-12 results in increased serum IFN- γ and TNF- α levels in infected mice. IFN- γ has previously been shown to be important in resistance to *T. cruzi* (30). Therefore, since IL-12 is a potent stimulus for the production of IFN- γ by T cells and NK cells, we assessed the effect of administration of IL-12 to uninfected or infected mice on levels of IFN- γ in serum. IFN- γ was not detected in the sera of uninfected mice, but administration of IL-12 or infection of mice resulted in elevated levels of IFN- γ in sera (Table 2). The levels of IFN- γ in the sera of infected mice treated with IL-12 were significantly higher than the levels found in sera from uninfected mice treated with IL-12 or from infected controls (Table 2). The results presented in Table 2 are for mice infected for 7 days. In repeat experiments we observed that by day 10 postin-

FIG. 3. Effect of administration of IL-12 on the pathology associated with *T. cruzi* infection in the heart. Mice were infected with *T. cruzi* as described in Materials and Methods and sacrificed on day 10 postinfection. Tissues were processed for histopathology and stained with haematoxylin and eosin. (A) Typical focus of infection observed in the heart. (B) Focus of infection (parasites [arrowhead]) in the heart of a mouse treated with IL-12 (100 ng per day for 6 days). Note the increased cellularity in response to the parasite. Magnification, $\times 400$.

TABLE 2. Administration of IL-12 to infected mice enhances levels of IFN- γ and TNF- α in serum^a

Treatment	Level (pg/ml) in serum			
	IFN- γ		TNF- α	
	Uninfected mice	Infected mice	Uninfected mice	Infected mice
Sham	0	1,998 (190)	109 (39)	102 (31)
IL-12	282 (106)	3,006 (489)	42 (17)	238 (69)

^a Mice were infected with *T. cruzi* as described in Materials and Methods and treated with IL-12 (100 ng per mouse) or diluent beginning 24 h postinfection and for a further 6 days. Uninfected control mice were given an identical treatment regimen. Mice were sacrificed on day 7 after infection, and levels of IFN- γ or TNF- α were measured by ELISA. Administration of IL-12 to infected mice resulted in a significant increase in levels of IFN- γ and TNF- α in serum compared with those for infected controls ($P = 0.0085$ and 0.0046 , respectively). For IFN- γ , results are from a single experiment with $n =$ four mice per group. Similar results were observed in three repeat experiments. For TNF- α , results are the pooled data from three experiments with $n = 9$ to 13 mice per group. SDs are given in parentheses.

fection there were even greater differences in the levels of IFN- γ in serum (infected mice, $6,296 \pm 2,015$ pg/ml [mean $\pm$ SD]; infected and IL-12-treated mice, $17,383 \pm 4,483$ pg/ml; $n = 4$).

Since TNF- α is associated with protection against *T. cruzi* (5), as well as with the protective effect of IL-12 against other intracellular pathogens (19, 29, 40), we analyzed the effect of treatment with IL-12 on levels of TNF- α in serum. Administration of IL-12 to uninfected mice or infection of mice with *T. cruzi* did not result in a significant change in the levels of TNF- α in serum compared with those for sham-treated uninfected controls. However, administration of IL-12 to infected mice did result in a significant increase in levels of TNF- α in serum at day 7 postinfection (Table 2).

IL-12-mediated reduction in parasitemia is dependent on IFN- γ and TNF- α . The results presented above associate the antiparasite effect of IL-12 treatment with increased levels of IFN- γ and TNF- α in serum. Thus, the ability of exogenous IL-12 to reduce parasitemia may be a consequence of an IL-12-induced increase in the levels of IFN- γ and/or TNF- α . To test this hypothesis, we treated mice with IL-12 alone or in combination with antibodies specific for IFN- γ or TNF- α . Treatment with anti-IFN- γ alone resulted in a remarkable increase in parasitemia, whereas anti-TNF- α had a significant but more marginal effect (Fig. 4). Analysis of the hearts of these animals revealed that anti-IFN- γ or anti-TNF- α alone did not affect the number of parasite foci present in the hearts. Coadministration of anti-IFN- γ with IL-12 antagonized the ability of treatment with IL-12 to reduce parasitemia and the numbers of foci of parasite replication (Fig. 4; Table 3). Coadministration of anti-TNF- α with IL-12 antagonized the ability of IL-12 treatment to reduce parasitemia (Fig. 4) but did not have a significant effect on the numbers of parasite foci in the heart at 7 days postinfection (Table 3).

Endogenous IL-12 is important in resistance to *T. cruzi*. To determine the role of endogenous IL-12 in resistance to *T. cruzi*, we administered a polyclonal antibody specific for IL-12 to infected mice beginning 24 h prior to infection, with two further treatments every 48 h. This regimen did not significantly affect the time to death of infected animals (data not shown). However, in three experiments this treatment regimen resulted in a significant increase in the numbers of parasites detected in the blood on day 7 postinfection (control, $3.22 \times 10^5 \pm 8,519$ parasites per ml [mean $\pm$ SD]; anti-IL-12, $1.19 \times$

$10^6 \pm 158,570$ parasites per ml; $n = 4$). Further analysis of these mice revealed that administration of anti-IL-12 resulted in a significant reduction in the levels of IFN- γ in serum (control, $2,677 \pm 433$ pg/ml [mean $\pm$ SD]; anti-IL-12, 446 ± 247 pg/ml; $n = 4$).

DISCUSSION

The data presented demonstrate that administration of IL-12 to mice infected with *T. cruzi* results in a reduced parasitemia and a delay in the time to death. Moreover, neutralization of endogenous IL-12 results in an increase in parasitemia as well as decreased levels of IFN- γ in serum. Together these results identify the presence of an IL-12-mediated mechanism of resistance to *T. cruzi*. An insight into this mechanism of resistance is provided by our studies which correlated administration of IL-12 to infected mice with increased levels of IFN- γ and TNF- α in serum. Furthermore, neutralizing antibodies specific for IFN- γ or TNF- α antagonized the protective effect of IL-12 treatment. These latter results indicate that the protective effect of IL-12 is dependent on IFN- γ and TNF- α . A similar mechanism of IL-12-induced resistance to other intracellular parasites has been described previously (12, 40).

It should be noted that although administration of IL-12 reduced the numbers of foci of intracellular parasites in the heart, there was also a remarkable reduction in the numbers of extracellular parasites present in the blood. It seems unlikely that IL-12 can kill parasites directly, since the protective effect of IL-12 treatment was inhibited by antibodies specific for IFN- γ or TNF- α . It may be that treatment with IL-12 results in nonspecific activation of macrophages which are able to actively phagocytose and kill the extracellular trypomastigotes. However, IL-12 has other properties that may be relevant to the protective effect that we have observed. These properties include a mitogenic effect on activated T lymphocytes and the ability to enhance the cytolytic activity of CD8⁺ T cells as well as the development of type 1 CD4⁺ T-helper-cell responses

FIG. 4. Antibodies specific for IFN- γ or TNF- α antagonize the IL-12-mediated decrease in parasitemia. Groups of four BALB/c mice were treated with IL-12 (100 ng) at 24 h prior to infection and every day thereafter for 6 days. Sham-treated controls and IL-12-treated controls received an isotype control monoclonal antibody. Cytokine-neutralizing antibodies were administered 24 h preinfection and on days 3 and 7 after infection. Mice were infected with *T. cruzi* as described in Materials and Methods. Parasitemia was monitored on day 10 after infection. Results presented are the means $\pm$ SDs. Treatment with anti-IFN- γ (anti-IFN-g) or anti-TNF- α (anti-TNF-a) resulted in a significant increase in the parasitemia of mice treated with IL-12 ($P = 0.0002$ and 0.0059 , respectively). Similar results were observed in three other experiments.

TABLE 3. Effect of neutralizing antibodies specific for IFN- γ or TNF- α on numbers of foci of parasite replication in the heart^a

Treatment	No. of foci of parasite replication
Control.....	11.0 (3.2)
Anti-IFN- γ	11.6 (7.7)
Anti-TNF- α	8.2 (4.0)
IL-12 alone.....	2.0 (1.4)
IL-12 + anti-IFN- γ	9.6 (5.5)
IL-12 + anti-TNF- α	2.8 (2.2)

^a Mice were infected with *T. cruzi* as described in Materials and Methods and treated with either IL-12 or diluent for 6 days starting 24 h prior to infection. Mice were sacrificed on day 10 after infection, and tissues were processed for histopathology. Parasite foci were counted as described in Materials and Methods. Administration of anti-IFN- γ resulted in a significant increase in the numbers of foci of parasite replication in mice treated with IL-12 ($P = 0.017$). Results are from a single experiment ($n = 5$ mice per group). Similar results were observed in three other experiments. SDs are given in parentheses.

which can affect the production of antibody (7, 38). Further studies are required to determine the role of IL-12 in the development of these T-cell responses during Chagas' disease.

Previous researchers have demonstrated a role for CD4⁺ and CD8⁺ lymphocytes in resistance to *T. cruzi* by using different murine models (1, 22, 26, 35–37). In addition, the elevated NK cell activity observed during acute infection indicates a role for these cells in the immune response to *T. cruzi* (14). Since IL-12 is a potent stimulator of the production of IFN- γ by CD4⁺ and CD8⁺ T lymphocytes as well as NK cells (38), it is likely that these cell types are involved in the IFN- γ -mediated resistance to this infection. Indeed, recent studies by Cardillo and colleagues suggest that NK cells are the source of most of the IFN- γ produced during the early stages of infection with *T. cruzi* (8). Those authors have also proposed that IL-12 may play a role in the modulation of IFN- γ production during infection with *T. cruzi*. Although our studies have demonstrated that IL-12 is involved in regulation of IFN- γ production during infection with *T. cruzi*, our studies have not addressed which of these cell types is involved in the IL-12-mediated resistance to *T. cruzi*. However, preliminary experiments using specific antibodies to deplete either CD4⁺ or CD8⁺ T cells or NK cells have failed to reverse the ability of treatment with IL-12 to reduce parasite numbers (unpublished data).

Studies with *T. gondii*, *L. major*, and *L. monocytogenes* have shown that treatment of infected mice with IL-12 can remarkably delay the time to death or can change susceptible mice to mice with a resistant phenotype (13, 15, 19, 21, 33, 39). In contrast, we observed that administration of IL-12 to mice infected with *T. cruzi* resulted in the survival of only a small percentage of mice and in only a modest delay in the time to death. It is of interest that our results with *T. cruzi* are more similar in many ways to results with *Candida albicans* and *P. chabaudi* than to those observed with *L. monocytogenes*, *T. gondii*, or *L. major*. Stevenson and colleagues (32) demonstrated that administration of anti-IL-12 to mice infected with *P. chabaudi* resulted in a more severe course of infection but did not affect mortality. Moreover, treatment with different doses of IL-12 (25 to 300 ng per mouse) resulted in decreased parasitemia (reduced from 60 to 40%), although only one dose (100 ng per mouse) resulted in increased survival. In studies with murine candidiasis, Romani and colleagues observed that endogenous IL-12 is important for acquired resistance to this infection (24). However, administration of IL-12 to mice with progressive systemic candidiasis did not result in a discernible protective effect (25). Nevertheless, our data raise the question

of why administration of IL-12 to mice infected with *T. cruzi* does not prevent infection-induced mortality, since it does have such a significant effect on parasite numbers. One possible explanation is that the death of these mice may not be simply due to parasite burden but may also be a consequence of infection-induced hyperreactivity and the overproduction of inflammatory cytokines such as IL-12 and IFN- γ . Thus, treatment with IL-12 may reduce parasite numbers but not affect the immune hyperresponsiveness. Indeed, treatment with IL-12 may result in a more vigorous immune response. This could explain our preliminary results in which we observed that treatment of infected mice with high doses of IL-12 resulted in earlier mortality compared with that of infected controls. Support for this hypothesis is provided by our studies in which mice lacking the IL-10 gene and infected with *T. cruzi* have a remarkable reduction in parasitemia but die before infected wild-type controls. The earlier mortality in these mice is associated with elevated levels of serum IFN- γ and IL-12 compared with those in wild-type controls (unpublished data).

A caveat for the studies we have presented is the large variation in virulence for different strains of *T. cruzi* and in the types of disease they cause in different strains of mice (6). It is possible that the ability of IL-12 to mediate resistance to *T. cruzi* may vary with the strain of the parasite. It has been proposed that parasite-induced production of cytokines such as IL-10 and transforming growth factor β can act as virulence factors (2, 30, 31). These cytokines can inhibit the immune response to *T. cruzi* and thereby result in parasite persistence. Both IL-10 and transforming growth factor β are potent inhibitors of IL-12-mediated effects (3, 9, 18, 20, 27, 40), and future experiments are required to characterize their role in the regulation of IL-12-mediated resistance to *T. cruzi*. Nevertheless, our data identify an IL-12-mediated mechanism of resistance to *T. cruzi* and highlight the important role of IFN- γ and TNF- α in this mechanism of resistance.

ACKNOWLEDGMENTS

This work is supported by Public Health Service grants AI04717 and AI03020 from the National Institutes of Health and by a MacArthur Foundation Grant in Molecular Parasitology. C. A. Hunter is a Fellow of the Irvington Institute for Immunological Research.

We thank Anis Khan for help with statistical analysis, Victor Van Cleave and Joe Sypek (Genetics Institute) for help during the course of these studies, and Jack Remington for support and critical commentary.

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