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Interleukin-12 Mediates Resistance to *Trypanosoma cruzi* in Mice and Is Produced by Murine Macrophages in Response to Live Trypomastigotes

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Host resistance to infection by *Trypanosoma cruzi* is dependent on both natural and acquired immune responses. During the first week of infection in mice, NK cell-derived gamma interferon (IFN-γ) is involved in controlling intracellular parasite replication, mainly through the induction of NO biosynthesis by activated macrophages. Interleukin-12 (IL-12) has been shown to be a powerful cytokine in inducing IFN-γ synthesis by NK cells, as well as in mediating resistance to different intracellular protozoa. We have therefore studied the ability of *T. cruzi* to elicit IL-12 synthesis by macrophages and the role of this cytokine in controlling parasite replication during acute infection in mice. Our results show that macrophages cultured in the presence of live trypomastigote forms (but not epimastigotes) release IL-12 that can induce IFN-γ production by normal spleen cells. IL-12 was detected in as little as 12 h after the addition of the trypomastigotes, and the level of IL-12 peaked at 48 h after the initial macrophage-parasite incubation. The addition of anti-IL-12 monoclonal antibody to macrophage-trypomastigote supernatants dose-dependently inhibited IFN-γ production by naive splenocytes. Finally, the in vivo role of IL-12 in resistance to infection by *T. cruzi* was analyzed. Mice treated with anti-IL-12 monoclonal antibody had significantly increased parasitemia and mortality in comparison with those of control infected mice treated with control antibody. Together, these results suggest that macrophage-derived IL-12 plays a major role in controlling the parasitemia in *T. cruzi*-infected mice and that the animal's resistance during the acute phase of infection may, at least in part, be a consequence of postinfection levels of IL-12.

Trypanosoma cruzi, a hemoflagellate protozoan parasite, is the causative agent of human Chagas' disease, a widely distributed debilitating infection which constitutes a major health problem in many Latin American countries. Following infection, the parasites are able to survive and replicate in a variety of nucleated cells, including nonactivated macrophages. Cytokines that enhance or inhibit parasite replication in macrophages seem to influence the outcome of infection, as well as the pathology of the disease.

The host's resistance during experimental Chagas' disease is dependent on both innate and acquired immunity, requiring the combined efforts of a number of cells, including NK cells (20) and $CD4^+$ (21) and $CD8^+$ (27) T cells, as well as antibody production by B cells (11). Cytokines play key roles in regulating both parasite replication and immune responses in infected animals. Gamma interferon (IFN-γ) has been most closely associated with host resistance during the acute phase of infection. This cytokine inhibits both in vivo and in vitro parasite replication through the induction of nitric oxide (NO) synthesis by IFN-γ-activated macrophages (5, 33). IFN-γ is produced by NK cells and activated T cells during infection and is involved in the control of parasite load, since the treatment of *T. cruzi*-infected mice with a monoclonal antibody (MAb) to IFN-γ results in exacerbation of the parasitemia and mortality (2, 18, 29). In contrast, the downregulatory cytokines interleukin-10 (IL-10) and transforming growth factor β (TGF-β) have been associated with susceptibility to *T. cruzi* infection (23, 24) by inhibiting IFN-γ-mediated macrophage activation (32).

A previous study demonstrated that supernatants from euthymic or athymic mouse macrophages cultured with live trypomastigotes induced IFN-γ production by spleen NK cells (2). Treatment with anti-IFN-γ MAb exacerbated the parasitemia only if administered before or soon after infection. Taken together, these results suggest that NK cells are the major source of IFN-γ in the acute phase of infection (2).

Recently, IL-12 has been described as a potent inducer of IFN-γ production by NK cells and different subsets of T cells (4). IL-12 is required for the T-cell-independent production of IFN-γ by NK cells that leads to the resistance of severe combined immunodeficiency (SCID) mice to *Toxoplasma gondii* (6) and *Listeria monocytogenes* (30). In addition, IL-12 is also required for the establishment of T-cell-dependent protective immunity in immunocompetent mice infected with a variety of microorganisms (reviewed in reference 1).

Since IFN-γ produced by NK cells in the early acute phase of *T. cruzi* infection is important in mediating resistance, we analyzed the ability of trypomastigotes to trigger IL-12 production by macrophages. In addition, we tested the effects of in vivo neutralization of IL-12 on resistance to *T. cruzi* infection in susceptible and resistant mice. Our findings support the hypothesis that live *T. cruzi* trypomastigotes induce IL-12 synthesis by normal macrophages and that this cytokine is responsible for the early IFN-γ production observed during infection and for the subsequent control of parasite proliferation in vivo.

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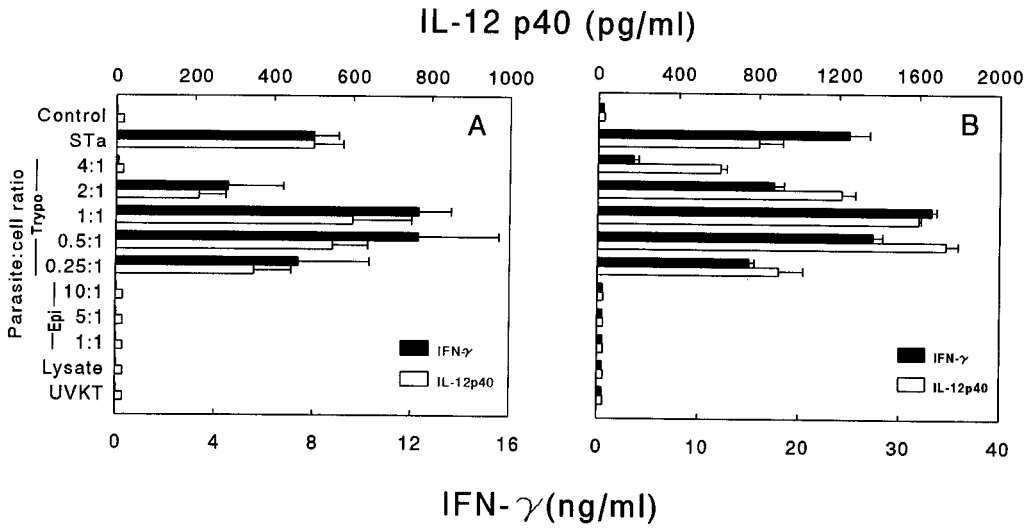

FIG. 1. Live *T. cruzi* trypomastigotes induce IL-12 p40 production by mouse splenocytes. Spleen cells were obtained from C57BL/6 (A) or BALB/c *nu/nu*$^+$ (B) mice and cultivated in medium alone (Control) or with freeze-thawed *T. gondii* tachyzoites (STa) or with live (Trypo), UV-killed (UVKT), or freeze-thawed (Lysate, 10 μg/ml) trypomastigotes or live epimastigotes (Epi). The parasite-to-cell ratios are indicated. The supernatants were harvested, and IFN-γ and IL-12 p40 were quantified by ELISA. Bars represent the means ± SD for six mice. Similar results were obtained in three additional experiments.

MATERIALS AND METHODS

Experimental animals. Five- to 6-week-old female C57BL/6 and BALB/c *nu/nu* mice were bred and maintained under standard conditions in the animal house of the Department of Immunology, University of São Paulo, Ribeirão Preto, Brazil.

Parasites and experimental infections. The Y and the Colombian strains of *T. cruzi* were used in in vivo experiments. C57BL/6 mice were infected intraperitoneally with 10^4 or 10^3 blood-derived trypomastigotes of the Y or Colombian strain of *T. cruzi*, respectively. For in vitro experiments, trypomastigotes of the Y strain were grown and purified from a monkey fibroblast cell line (LLC-MK_2). Epimastigotes were grown in Schneider's medium (Sigma Chemical Co., St. Louis, Mo.) supplemented with 20% fetal calf serum. UV-killed trypomastigotes (UVKT) were obtained by exposing a suspension of the purified forms to UV radiation for at least 1 h. UVKT were unable to grow in Schneider's medium. The parasite lysate preparation was obtained from freeze-thawed trypomastigotes or *T. gondii* tachyzoites (ME-49 strain), centrifuged at 10,000 × *g* for 30 min, and filtered through a 0.22-μm-pore-size membrane filter. The levels of parasitemia were evaluated by using 5 μl of blood obtained from the tail vein of infected mice as previously described (13).

Reagents and antibodies. Recombinant murine IL-12 (rMuIL-12) and recombinant murine IFN-γ (rMuIFN-γ) were provided by Genetic Institute Inc. (Boston, Mass.) and Genentech Inc. (San Francisco, Calif.), respectively. Anti-IL-12 was obtained from ascites collected from nude mice inoculated with rat hybridomas C17.8.20, C17.15.10, and C15.6.76 (kindly provided by G. Trinchieri, Wistar Institute, Philadelphia, Pa.). The MAbs were partially purified by ammonium sulfate precipitation, dialyzed against phosphate-buffered saline, assayed for their protein content with the Pierce (Rockford, Ill.) assay system, and then stored at −70°C until required.

In vivo IL-12 neutralization. Two hours before infection and again on days 2 and 4 after infection, C57BL/6 mice were injected intraperitoneally with 100 μg of anti-IL-12 MAb C17.8.20). Control mice received 100 μg of normal rat immunoglobulin G (IgG).

Spleen cell and macrophage cultures. Single-cell suspensions of splenocytes from C57BL/6 mice were washed in Hanks' medium and treated for 4 min with lysing buffer (9 parts 0.16 M ammonium chloride and 1 part 0.17 M Tris-HCl, pH 7.5). The erythrocyte-free cells were then washed three times and adjusted to 10^7 cells per ml in RPMI 1640 (Flow Laboratories, Inc., McLean, Va.) supplemented with 5% fetal calf serum (HyClone Laboratories, Inc., Logan, Utah), 5×10^{-5} M β-mercaptoethanol, 2 mM L-glutamine, and antibiotics. The cell suspension was distributed (100 μl) in 96-well tissue culture plates (Corning Glass Works, Corning, N.Y.) and cocultured with live trypomastigotes or UVKT, epimastigotes, or a parasite lysate for 48 h at 37°C in a humidified atmosphere containing 5% CO_2, with or without different concentrations of anti-IL-12 MAb. Parasite-to-cell ratios varied from 0.25:1 to 10:1. The supernatants were subsequently harvested, centrifuged, filtered through a 0.22-μm-pore-size membrane filter, and stored at 4°C until tested.

Mouse inflammatory macrophages were harvested from peritoneal cavities 3 days after the injection of 1 ml of 3% sodium thioglycolate (Difco). Macrophages were also harvested from normal bone marrow and spleens. The adherent cells were obtained after a 2- to 4-h incubation of single-cell suspensions in 96-well tissue culture plates at 37°C. The nonadherent cells were removed by exhaustive washing with Hanks' medium. Parasites were added in a 1:1 parasite-to-cell ratio and incubated for 48 h at 37°C as described above. The supernatants were then harvested, filtered through a 0.22-μm-pore-size membrane filter, and stored at −20°C until assayed. According to the manufacturers' specifications, all the media and reagents were endotoxin-free. In the absence of trypomastigotes, all of these cell populations were unable to produce detectable levels of IL-12 p40 or to induce IFN-γ production.

Measurement of IL-12 activity and IL-12 p40 levels. IL-12 activity was evaluated on the basis of its capacity to induce IFN-γ production by normal splenocytes. Culture supernatants or different rMuIL-12 concentrations (2 pg to 200 ng/ml) were added to spleen cell suspensions for 48 h at 37°C at a final concentration of 5×10^6 cells per ml. The culture supernatants were later harvested, and the IFN-γ concentration was determined. IL-12 p40 levels were determined by an enzyme-linked immunosorbent assay (ELISA) using 5 μg of anti-p40 MAb (C17.15.10) per ml as the capture antibody and biotinylated anti-IL-12 (C15.6.76), diluted 750-fold, as the detecting antibody. The IL-12 p40 concentration was calculated by reference to a standard curve for rMuIL-12.

IFN-γ assay. IFN-γ levels were determined by a two-site sandwich ELISA using XMG 1.2 (5 μg/ml) and biotinylated AN-18 anti-IFN-γ as the capture and detecting MAbs, respectively. The IFN-γ concentration was calculated by reference to a standard curve for rMuIFN-γ.

Statistical analysis. The results are expressed as means ± standard deviation (SD) for the indicated number of animals or experiments. Mann-Whitney and Kruskal-Wallis tests were used to determine the statistical significance of the intergroup comparisons. A P value of <0.05 was considered significant.

RESULTS

Live *T. cruzi* trypomastigotes induce IL-12 production in normal mouse splenocytes. It has previously been shown that live *T. cruzi* trypomastigotes induce IFN-γ production in normal mouse splenocytes (2). Here, we investigated whether this IFN-γ production correlated with the production of IL-12 by assaying p40 in the supernatants of splenocyte cultures stimulated with *T. cruzi* trypomastigotes. Since several studies have shown that the pathogen-to-cell ratio is crucial for optimum stimulation of IL-12 production (3, 6, 22), we used several such ratios. Trypomastigotes induced IFN-γ and p40 production by spleen cells at parasite-to-cell ratios of 2:1, 1:1, 0.5:1, and 0.25:1 (Fig. 1A). Interestingly, parasite-to-cell ratios higher than 2:1 did not induce the production of either IFN-γ or p40. The noninfective epimastigotes also did not induce IFN-γ or p40 production in these cultures; neither did the UVKT or parasite lysate at a 1:1 ratio (Fig. 1A). Thus, p40 was detected

TABLE 1. *T. cruzi* trypomastigotes induce IL-12 production by adherent splenocytes and inflammatory or bone marrow macrophages

Cell type[a]	IL-12 p40 (pg/ml)	IFN-γ (ng/ml)[b]
Spleen cells		
Total[c]	1,939 ± 49	23.5 ± 0.7
Adherent	1,742 ± 125	18.7 ± 0.3
Nonadherent	<50	2.6 ± 1.5
Macrophages		
Inflammatory peritoneal	3,212 ± 125	24.1 ± 0.2
Bone marrow	5,508 ± 150	24.6 ± 0.3

[a] The different cell populations were obtained and incubated with live trypomastigote forms for 48 h, and the IL-12 p40 was then assayed by ELISA. In the absence of parasite, the levels of IL-12 p40 were <50 pg/ml. The results are expressed as means ± SD of triplicate analyses performed with cells pooled from four mice.

[b] The supernatants of the different cell populations (except for total splenocytes) were added (50% [vol/vol]) to normal splenocytes in culture, and the IFN-γ was assayed 48 h later.

[c] The IL-12 p40 and IFN-γ concentrations were assayed with the same supernatant.

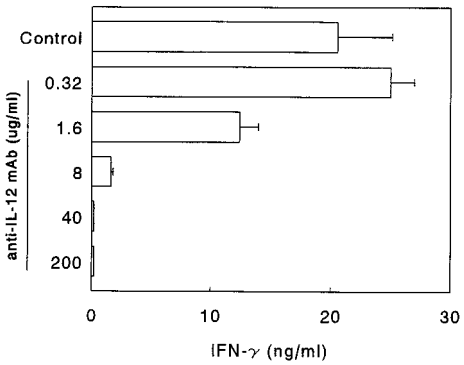

FIG. 2. The ability of adherent spleen cell supernatants incubated with trypomastigotes to induce IFN-γ production is abolished by anti-IL-12 MAb. Adherent spleen cells were obtained after splenocytes were cultured for 2 to 4 h in 96-well microplates at 37°C. The nonadherent cells were removed by extensive washing. Adherent spleen cells were stimulated with live *T. cruzi* trypomastigotes at a 1:1 parasite-to-cell ratio for 48 h, and the supernatants (50% [vol/vol]) were added to normal spleen cell cultures in the presence of different concentrations of neutralizing anti-IL-12 MAb or 200 μg of normal rat IgG per ml (control). The supernatants were harvested, and the IFN-γ concentration was determined by ELISA. Bars represent the means ± SD for six mice. Similar results were obtained in four additional experiments.

in all cultures in which IFN-γ was present and was undetectable in cultures in which no IFN-γ was found. Although we had no reason to suspect the presence of lipopolysaccharide as a possible inducer of IL-12 in these cultures, the fact that the epimastigotes, UVKT, and trypomastigote lysates did not induce p40 provides evidence to exclude the possibility that the p40 and IFN-γ production by splenocytes was triggered by contaminating endotoxin. Similar results were obtained when splenocytes from euthymic C57BL/6 (Fig. 1A) or athymic BALB/c (Fig. 1B) mice were used.

***T. cruzi* trypomastigotes induce p40 IL-12 production by adherent splenocytes and peritoneal or bone marrow macrophages.** IL-12 can be produced by macrophages, monocytes, and B cells (3). Of these cells, *T. cruzi* parasitizes mainly macrophages. We therefore investigated whether trypomastigotes could trigger IL-12 production by macrophages obtained from different sources. After 48 h in culture, adherent spleen cells produced levels of IL-12 p40 similar to those of whole spleen cells. Inflammatory and bone marrow macrophages also produced high levels of IL-12 p40 when stimulated with live *T. cruzi* trypomastigotes. In contrast, nonadherent spleen cells were unable to produce p40 IL-12 in response to stimulation by the parasite in vitro. Moreover, supernatants from adherent splenocyte cultures and peritoneal inflammatory and bone marrow macrophages stimulated with live *T. cruzi* trypomastigotes triggered IFN-γ production by normal spleen cells (Table 1). Thioglycolate-elicited macrophages incubated with *Corynebacterium parvum* (10 μg/ml) or with *Staphylococcus aureus* Cowan 1 (0.001%) secreted mean levels (± SD) of 22 ± 10 and 20 ± 7.5 ng of IL-12 p40 per ml, respectively.

The ability of macrophage supernatants incubated with live trypomastigotes to induce IFN-γ production is abolished by anti-IL-12 MAb. We next investigated whether IL-12 was the cytokine responsible for the induction of IFN-γ production by splenocytes stimulated with *T. cruzi* trypomastigotes. Adherent spleen cells were stimulated with live *T. cruzi* trypomastigotes for 48 h, and the supernatants (50% [vol/vol]) were added to normal splenocyte cultures in the presence of anti-IL-12 MAb (C17.8.20) or normal rat IgG. After a 48-h incubation, the IFN-γ concentration in the supernatants was assayed. The addition of anti-IL-12 MAb was found to inhibit IFN-γ production in a dose-dependent manner (Fig. 2). The addition of as little as 1.6 μg of anti-IL-12 MAb per ml inhibited around 50% of the IFN-γ production by splenocytes. The addition of 40 μg of anti-IL-12 MAb per ml totally abolished the IFN-γ production. Control rat IgG had no effect on IFN-γ levels (Fig. 2). Similar levels of IFN-γ were produced by normal splenocytes from BALB/c athymic nude mice. These results, together with the data in Fig. 1 and Table 1, indicate that IL-12 is produced by the macrophages of normal mice in response to stimulation with live *T. cruzi* trypomastigotes. In addition, these data provide evidence that IL-12 is produced at sufficient levels and is responsible for the induction of IFN-γ production by spleen cells in vitro.

Biological activity and kinetics of trypomastigote-induced IL-12 production by adherent splenocytes. Once the presence of IL-12 had been demonstrated by abolishing its effects with a specific MAb, its concentration in the parasite-stimulated macrophage supernatants was assayed. For this purpose, the activity of IL-12 was evaluated in a biological assay based on its ability to induce IFN-γ production by spleen cells (T and NK cells). The resulting IFN-γ levels were quantified by ELISA. The IL-12 produced by splenic macrophages was calculated from an rMuIL-12 standard curve. The results showed that the IL-12 produced by spleen macrophages (5×10^6 cells per ml) incubated with trypomastigotes had a biological activity corresponding to 2 ng of rMuIL-12 per ml (Fig. 3A). The p40 concentration in the same supernatants determined by ELISA was 1.7 ng/ml. Thus, these particular supernatants contained 42.5 pmol of IL-12 p40 and 30.6 pmol of IL-12 p70, which represents a 48.6% excess of p40. The dose-response curve for IFN-γ production by splenocytes in response to rMuIL-12 is shown in Fig. 3A.

It has been suggested previously that the timing of cytokine production after *T. cruzi* infection could be important for determining resistance or susceptibility to infection (23). Since IL-12 has been implicated as the major factor in the induction of IFN-γ biosynthesis by NK cells during the early acute phase of infection, and since IFN-γ is regarded as the key cytokine that mediates resistance to *T. cruzi* infection, the kinetics of *T. cruzi*-induced IL-12 production were determined. The supernatants of adherent splenocytes incubated with live trypomas-

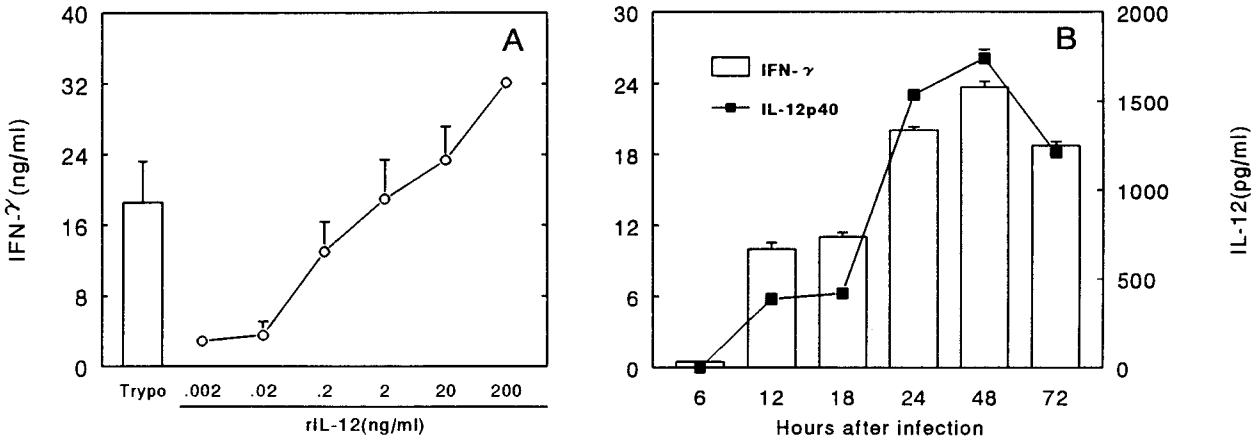

FIG. 3. Biological activity of *T. cruzi*-induced IL-12 produced by mouse splenic macrophages. IL-12 activity was determined by stimulating normal splenocytes with rMuIL-12 (2 pg to 200 ng/ml) or with supernatants (50% [vol/vol]) obtained from splenic macrophages cultured for 48 h with live *T. cruzi* trypomastigotes at a 1:1 parasite-to-cell ratio (Trypo), after which IFN-γ was assayed (A). Mouse splenic macrophages (obtained as described for Fig. 2) were stimulated with live trypomastigotes at a 1:1 parasite-to-cell ratio for 6, 12, 18, 24, 48, and 72 h, and the IL-12 p40 concentration in the culture supernatants was then determined by ELISA. The supernatants (50% [vol/vol]) were added to normal spleen cell cultures, which were then incubated for 48 h, and the IFN-γ concentrations in the supernatants of these cultures were determined (B). The results shown are the means ± SD for six mice. Similar values were obtained in three additional experiments.

tigotes were harvested at different times after culture and used to induce IFN-γ production by spleen cells. IL-12 p40 concentrations in these supernatants were also determined by ELISA. We observed that IL-12 p40 could be detected in these supernatants after as little as 12 h in culture, with a peak of production at 48 h that declined at 72 h. The levels of IFN-γ induced by these supernatants were directly correlated with the presence of IL-12 p40 (Fig. 3B).

IL-12 mediates resistance to *T. cruzi* infection in vivo. Since we have shown that IL-12 is produced by normal macrophages after stimulation with *T. cruzi* trypomastigotes in vitro, we evaluated whether this cytokine had any effect on parasitemia and mortality in infected mice. We used the Y and Colombian strains of *T. cruzi*, which induce different patterns of parasitemia and mortality in C57BL/6 mice. The Y strain is rapidly cleared and triggers a response that results in a relative resistance to infection, whereas the Colombian strain replicates progressively, leading to the death of 100% of the infected animals. The mice were each treated with 100 μg of anti-IL-12 MAb and infected 2 h later. The anti-IL-12 treatment was repeated on days 2 and 4 after infection, and parasitemia was determined. Although C57BL/6 mice are normally relatively resistant to infection by the *T. cruzi* Y strain, the anti-IL-12 treatment resulted in a significantly increased parasitemia 7 to 12 days postinfection (Fig. 4A). The parasitemia in mice infected with the Colombian strain was also significantly increased between days 12 and 16 in the animals treated with the anti-IL-12 MAb (Fig. 4B). More importantly, treatment with anti-IL-12 MAb resulted in increased and earlier mortality in mice infected with the Y and Colombian strains (Fig. 5A and B, respectively). These experiments establish the importance of endogenous IL-12 in controlling acute infection and suggest that the resistance mediated by IFN-γ is dependent on the presence of IL-12.

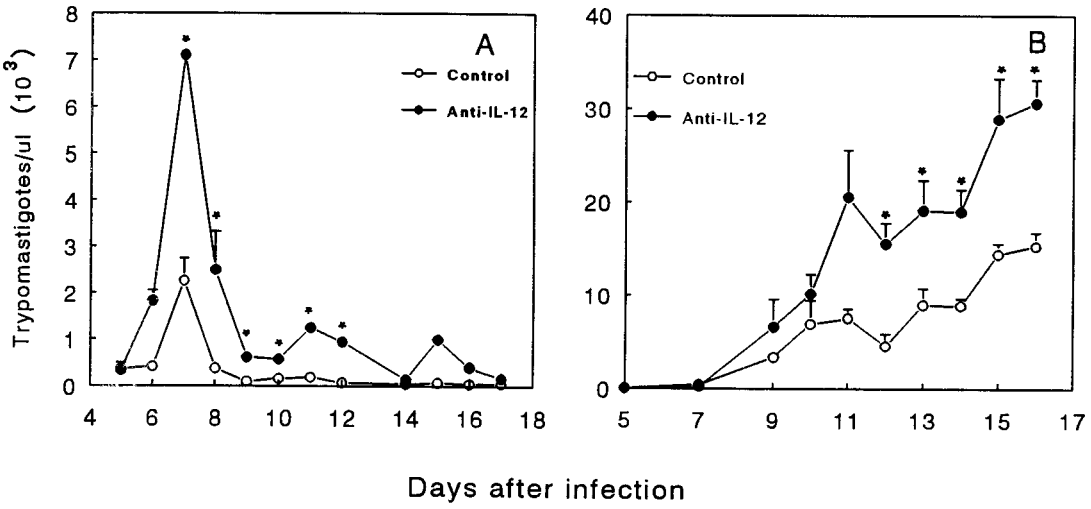

FIG. 4. Parasitemia in C57BL/6 mice infected with the Y (A) or Colombian (B) strain of *T. cruzi* in the presence or absence of anti-IL-12 MAb. The mice were infected with 10^4 (Y strain) or 10^3 (Colombian strain) *T. cruzi* blood trypomastigotes and treated with anti-IL-12 MAb or with normal rat IgG (control) 2 h before and again on days 2 and 4 after infection. Parasitemia was determined on the days indicated. Each point (mean ± SD) corresponds to five mice in an experiment representative of three. *, $P < 0.05$ compared with mice treated with normal rat IgG.

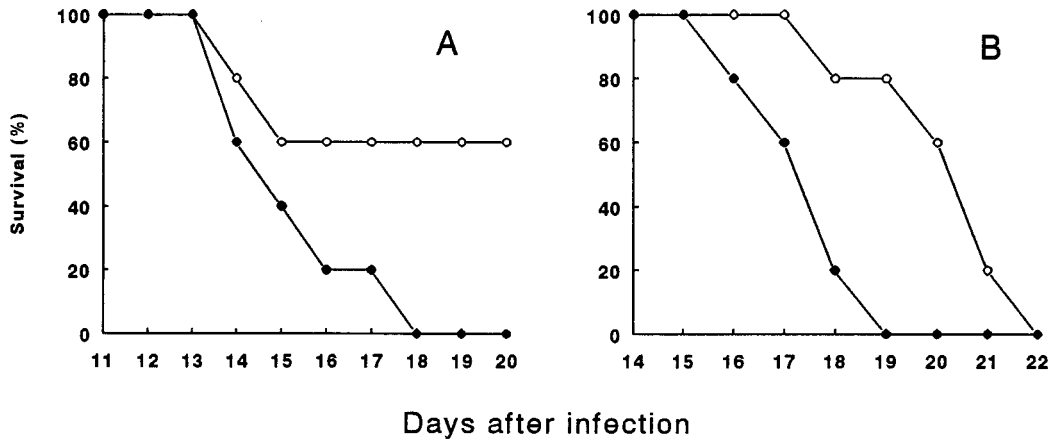

FIG. 5. Anti-IL-12 MAb increases the mortality of mice infected with *T. cruzi*. C57BL/6 mice were infected with 10^4 *T. cruzi* Y strain blood trypomastigotes (A) or 10^3 *T. cruzi* Colombian strain blood trypomastigotes (B) and treated with anti-IL-12 MAb (C17.8.20) (●) or normal rat IgG (○) 2 h before infection and again on days 2 and 4 after infection. The percent survival for each group ($n = 10$) was determined daily. The results are representative of two independent experiments.

DISCUSSION

Multiple components of the cellular compartment of both the innate and the adaptive immune systems are simultaneously required for resistance to acute infection by *T. cruzi*. Treatment with agents such as colloidal thorium dioxide and silica, which alter macrophage functions (7), or the elimination of NK cells (2) leads to increased susceptibility in the early stages of acute experimental Chagas' disease. Different studies have suggested that NK cells (2), $CD4^+$ T cells (21), and $CD8^+$ T lymphocytes (28) are all major sources of IFN-γ during acute infection with *T. cruzi*. In the early inflammatory response to such an infection, regulatory interactions mediated by cytokines could occur among these cell types. Thus, IFN-γ has been shown to be an important mediator of resistance to *T. cruzi* (9, 18, 23, 29). Whereas treatment with IFN-γ is protective, the neutralization of endogenously produced IFN-γ results in increased susceptibility during the acute stage of infection with *T. cruzi*. Together, these findings suggest that upon stimulation by this parasite, NK cells and different T-cell subsets produce IFN-γ, which in turn activates the parasiticidal functions of macrophages responsible for inhibiting parasite growth in the vertebrate host (5, 14, 16, 18, 23, 24, 29, 33). In the murine model, IFN-γ in combination with tumor necrosis factor alpha (TNF-α) (produced by macrophages, NK cells, and/or T lymphocytes) induces the expression of the nitric oxide synthase (15) and hence the synthesis of nitric oxide by macrophages. Nitric oxide derived from L-arginine degradation is the major effector molecule responsible for the trypanocidal effects displayed by macrophages both in vitro (5) and in vivo (33).

The major factor produced by infected phagocytic cells and responsible for the induction of IFN-γ production is IL-12, a heterodimeric cytokine that is a potent inducer of the biosynthesis of cytokines, particularly of IFN-γ by T and NK cells, and an enhancer of cytotoxic activity in both $CD8^+$ T and NK cells (10, 26). IL-12 has recently been shown to be essential in different infectious disease models in which resistance is mediated by IFN-γ (reviewed in reference 1). In this work, we examined the involvement of the IL-12-dependent pathway of IFN-γ production in resistance to an acute infection with *T. cruzi*. The results demonstrated that live *T. cruzi* trypomastigotes, but not epimastigotes or parasite lysates, were able to induce IL-12 secretion by mouse macrophages. This production was closely correlated with the ability of macrophage supernatants to induce IFN-γ secretion by normal murine splenocytes (Fig. 1) and was completely inhibited in the presence of anti-IL-12 MAb (Fig. 2). These results suggest that IL-12 is produced as the active p70 heterodimer. However, the proportion of IL-12 p40 found was higher than that previously reported for several other stimuli (3, 26, 34). In our experience, on a molar basis, *C. parvum* stimulates about 500 times more p40 chain than the whole IL-12 molecule in vitro, as measured by the same assays described here (34). However, in vivo infection of macrophages with *Leishmania major* rendered only 12 times more p40 than IL-12, again on a molar basis (34). Although for the *Leishmania* infections the results were obtained after in vivo infection, since *L. major* promastigotes do not induce IL-12 production by macrophages in vitro (19, 34), it is tempting to speculate that these intracellular parasites may induce a smaller p40 excess in relation to the biologically active IL-12 produced (12:1 for *L. major* infections in vivo and 2:1 for *T. cruzi* infections in vitro). The assay of IL-12 is complicated by the fact that recombinant p40 has been shown to inhibit IL-12 (12) and the fact that recombinant standards used for both p40 and IL-12 assays may contain inactive IL-12. Also, in our assay, the activity of the natural IL-12 could be higher than that of the recombinant IL-12 used as a standard, mainly because trypomastigotes also induce TNF-α production (25) that may potentiate the IL-12 activity (31).

Interestingly, parasite-to-cell ratios higher than 2:1 did not induce the production of either IFN-γ or IL-12 p40, possibly as a consequence of high levels of cellular infection or, alternatively, because of an active inhibition. Also striking is the absence of a response when epimastigotes, parasite lysate, or UVKT were used. These results might suggest that for induction of IL-12 production, active penetration or secretory-excretory products produced intracellularly by the parasite are necessary. Since the trypomastigote-induced IFN-γ production by normal splenocytes was inhibited by NK cell depletion (2) and since the addition of supernatants harvested from cultured macrophages incubated with trypomastigotes to splenocytes from athymic mice resulted in high levels of IFN-γ (Fig. 1B), we suggest that NK cells are the main source of this cytokine. IL-12 production was associated with the adherent spleen cell population. Moreover, inflammatory macrophages (98% of macrophages as determined by visual inspection) and bone

marrow-derived macrophages also produced p40 and induced IFN-γ production in response to infection with *T. cruzi*. Therefore, it seems that the macrophage is capable of producing IL-12 when faced with infection by *T. cruzi*. This is of utmost importance, since IL-12 elicits IFN-γ production by both T and NK cells (10, 26), and IFN-γ is essential for resistance to this parasite (18, 33). Macrophages are probably the first cells *T. cruzi* encounters and infects, and the fact that these cells can respond with a protective cytokine favors the host, leading to a chronic, benign infection. The IL-12 p40 chain could be detected as soon as 12 h after macrophage infection in vitro, as could the secretion of IFN-γ by splenocytes incubated with these supernatants, thus revealing a typical early effect of this cytokine (Fig. 3B). These results indicate that infected phagocytic cells could begin to produce IL-12 soon after *T. cruzi* infection in vivo and that this could in turn induce IFN-γ production by NK cells.

In an effort to understand the role of IL-12 in the control of *T. cruzi* infection in vivo, we treated C57BL/6 mice with an anti-IL-12 MAb and examined both the parasitemia curve and the mortality during the acute phase of infection. We found that during experimental acute Chagas' disease, the mice treated with the anti-IL-12 MAb had greater parasitemia and accelerated mortality in comparison with those of control mice infected with *T. cruzi* and treated with normal rat IgG. On the basis of these observations and a previous study (2), we postulate that early IL-12 secretion would induce IFN-γ synthesis by NK cells and that the latter cytokine could in turn activate macrophages to increase parasite killing during the early acute phase of infection. Although $CD4^+$ and $CD8^+$ T cells are also major targets for IL-12 and important sources of IFN-γ, our in vitro experiments suggest that NK cells are in fact the major source of IFN-γ during early infection with *T. cruzi*. However, we cannot exclude the possibility that both $CD4^+$ T cells and $CD8^+$ T cells also contribute to IFN-γ synthesis during this phase. In this regard, a recent study with another intracellular parasite (*T. gondii*) has demonstrated that by 5 days postinfection, $CD4^+$ T lymphocytes activated in vivo by this species produce high levels of IFN-γ in an IL-12-dependent manner (6).

Other cytokines have been shown to strongly influence the synthesis of and/or effects mediated by IL-12 in vitro and in vivo. For instance, both IL-10 (23) and TGF-β (8) are important regulators of IL-12-induced IFN-γ synthesis by NK cells. Whereas IL-10 appears to be a potent inhibitor of IL-12 synthesis by macrophages exposed to microbial products, the mechanism by which TGF-β inhibits IFN-γ synthesis by NK cells is unknown. Nevertheless, it is clear that both IL-10 and TGF-β are potent modulators of resistance during acute Chagas' disease (23, 24). In contrast to the downregulatory cytokines (IL-10 and TGF-β), other macrophage-derived (i.e., IL-1β and TNF-α) and T-cell-derived (IL-2) cytokines potentiate the effects of IL-12. We are now studying how these distinct cytokines regulate or potentiate the induction and/or effects of the IL-12 pathway during infection by *T. cruzi.*

In this study, we have also demonstrated that *T. cruzi* belongs to a large group of microorganisms which elicit the synthesis of IFN-γ through the induction of IL-12 (reviewed in reference 1). In addition, an IL-12-dependent pathway for IFN-γ production during resistance to this parasite in the acute phase of infection appears to be involved. It is noteworthy that in other experiments, we observed a more dramatic effect of treatment with anti-IFN-γ (the effector cytokine) MAb than with anti-IL-12 (the initiator cytokine) MAb on the parasitemia levels and mortality in *T. cruzi*-infected mice (data not shown). Thus, it is possible that during *T. cruzi* infection the parasite may trigger both IL-12-dependent and IL-12-independent pathways for IFN-γ synthesis and that they may have an additive effect on resistance to the parasite. Recent studies performed by Orange et al. (17) suggest the existence of both IL-12-dependent and IL-12-independent pathways for IFN-γ synthesis during viral infections. Further experiments are being performed in our laboratory to elucidate this question.

In terms of the parasite-vertebrate host interaction, several questions were raised by this study, including, for instance, whether the virulence and avirulence observed with different strains of *T. cruzi* are related to the parasite's ability to elicit or evade the induction of IL-12 synthesis by macrophages. On the basis of the present study, we believe that *T. cruzi* induces IL-12 to promote its own survival by protecting the host against a lethal infection. However, the cardiac lesions observed during infection with *T. cruzi* are typically of a delayed hypersensitivity type (i.e., mediated by Th1 lymphocytes), and therefore it is possible that the induction of IL-12 may also mediate the resulting immunopathology during chronic Chagas' disease by favoring parasite-specific $CD4^+$ T-cell differentiation in the direction of Th1 lymphocytes. We believe that elucidation of the role of IL-12 in resistance as well as in the pathogenesis of Chagas' disease may have important implications for the development of a vaccine and of therapy designed to protect the host against the infection and immunopathology induced by *T. cruzi.*

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