Interleukin 10 and Interferon γ Regulation of Experimental *Trypanosoma cruzi* Infection

By João S. Silva,*‡ Philip J. Morrissey,§ Kenneth H. Grabstein,§ Kendall M. Mohler,§ Dirk Anderson,*§ and Steven G. Reed*‖

*From the *Seattle Biomedical Research Institute, Seattle, Washington 98109; the ‡University of São Paulo, Ribeirão Preto 14049, Brazil; §Immunex Corp., Seattle, Washington 98101; and ‖Cornell University Medical College, New York 10021*

Summary

Studies were undertaken to determine whether interleukin 10, (IL-10) a cytokine shown to inhibit interferon γ (IFN-γ) production, was involved in *Trypanosoma cruzi* infections in mice. Exogenous IFN-γ protects mice from fatal infection with *T. cruzi*. Furthermore, resistant B6D2 mice developed fatal *T. cruzi* infections when treated with neutralizing anti-IFN-γ monoclonal antibody (mAb). Thus, endogenous as well as exogenous IFN-γ is important in mediating resistance to this parasite. Because both *T. cruzi*–susceptible (B6) and –resistant (B6D2) mouse strains produced IFN-γ during acute infection, we looked for the concomitant production of mediators that could interfere with IFN-γ–mediated resistance to *T. cruzi*. We found that IL-10–specific mRNA was produced in the spleens of mice with acute *T. cruzi* infections. In addition, spleen cell culture supernatants from infected B6 mice, and to a lesser extent B6D2 mice, elaborated an inhibitor(s) of IFN-γ production. This inhibitor(s) was neutralized by anti–IL-10 mAb. These experiments demonstrated the production of biologically active IL-10 during *T. cruzi* infection. In further studies in vitro, it was shown that IL-10 blocked the ability of IFN-γ to inhibit the intracellular replication of *T. cruzi* in mouse peritoneal macrophages. Thus, in addition to its known ability to inhibit the production of IFN-γ, IL-10 (cytokine synthesis inhibitory factor), may also inhibit the effects of IFN-γ. These experiments demonstrate that IL-10 is produced during infection with a protozoan parasite and suggest a regulatory role for this cytokine in the mediation of susceptibility to acute disease.

The intracellular protozoan parasite *Trypanosoma cruzi* causes Chagas' disease in humans. Infection with this hemoflagellate has widely variable outcomes, determined by factors unknown. Human infections may be subclinical, often characterized by a prolonged indeterminate phase with only a positive serology to indicate the presence of parasites. Infection may also lead to acute disease with fatal outcome or, more commonly, the development of chronic disease with severe associated pathologies. In mice, different inbred strains may develop fatal or very mild infections when inoculated with the same *T. cruzi* isolate (1, 2). Similarly, different *T. cruzi* isolates produce variable courses of infection within a given mouse strain (3).

Two interconnected aspects of the immune response during experimental *T. cruzi* infection have been the subjects of considerable study. One is the nature of the protective host response to the parasite and the other is analysis of the profound immune depression that accompanies both acute and chronic infections. The latter is associated with defective macrophage (4) and T cell function (5, 6), including a lack of IL-2 production and utilization (7–11). Immune responses can be effectively restored in infected mice with exogenous cytokines, including IL-1 (12), IL-2 (7–9), and GM-CSF (4). It is not understood whether the immune depression provokes or results from infection-associated pathology.

The roles of cytokines in resistance and susceptibility to *T. cruzi* are only superficially understood. Macrophage activation by GM-CSF (13), IFN-γ (14, 15), or TNF (16) leads to inhibition of the replication of this parasite in vitro. Of these cytokines, IFN-γ has been most closely associated with host resistance. Treatment of infected animals with IFN-γ prevents acute disease, immune depression, and death induced by *T. cruzi* infection (17). Conversely, TGF-β, a potent macrophage deactivator (18), may counteract the effects of IFN-γ during *T. cruzi* infection (19). In this report, we document the necessity for endogenous IFN-γ in controlling *T. cruzi* infection in resistant mice. In addition, we provide evidence that another cytokine, IL-10 (cytokine synthesis inhibitory factor [CSIF][1]), may, by interfering with IFN-γ production

[1] *Abbreviation used in this paper:* CSIF, cytokine synthesis inhibitory factor.

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and/or utilization, play a role in regulating infection. The results of this study implicate this recently described cytokine (20, 21) in the regulation of an infectious disease.

Materials and Methods

Mice. Female C57Bl/6J (B6), (C57Bl/6J × DBA)F_1 (B6D2), and BALB/c mice, 8 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME).

Parasites. A clone of the Tulahuen strain of *T. cruzi*, designated in accordance with the World Health Organization classification as MHOM/CH/00/Tulahuen C2 (13), was used in all experiments. Mice were infected i.p. with 10^3 trypomastigotes grown in and purified from rat myoblast cells (L6E9).

Immune Reagents. Neutralizing anti-MuIFN-γ and IL-10 mAbs were obtained from ascites collected from nude mice inoculated with rat hybridomas XMG 1.2 (22, provided by DNAX Corp., Palo Alto, CA) or SXC1 (23, provided by Dr. Tim Mosmann, University of Alberta, Edmonton, Alberta, Canada). The mAbs were concentrated from nude mouse ascites by ammonium sulfate precipitation and dialyzed against PBS. Recombinant murine (rMu)-IFN-γ (5.2×10^6 U/mg protein) was provided by Genentech, Inc. (San Francisco, CA) as protein purified to homogeneity from *Escherichia coli*.

rIL-10. IL-10 cDNA was cloned from RNA from the 7B9 murine T cell clone (24) by PCR, using oligonucleotide primers designed from the IL-10 gene sequence (21). IL-10 was expressed in COS cells using the pDC302 vector previously described (24). COS cell supernatant containing rIL-10 (200 U/ml) and control COS cell supernatant were used in these studies. Units of rIL-10 were determined using the IFN-γ inhibition assay described below.

Northern Blot. Total RNA was isolated from mouse spleen cell lysate using a guanidine thiocyanate method (25). Samples were analyzed on 1.5% formaldehyde–denaturing agarose gels and transferred by capillary blotting onto Zeta Probe membrane (Bio-Rad Laboratories, Richmond, CA) using 50 mM NaOH (26). The membrane was rinsed in 2× SSC, air-dried, and ultraviolet cross-linked. Filters were prehybridized at 60°C as previously described, (27) except that the salt used was 6× SSC. Labeled IL-10 probe was prepared by the random priming method, as described (28), and added directly to the prehybridization mix at 2×10^6 cpm/ml of solution, and incubation continued for 16 h at 60°C. Posthybridization washes were twice at 65°C for 20 min with each of 2× and 0.5× SSC containing 0.1% SDS.

IFN-γ Inhibition (CSIF) Assay. Spleen cells from normal or infected animals at different days after infection were aseptically removed, washed, and adjusted to 5×10^6 leukocytes/ml in RPMI 1640 (Gibco Laboratories, Grand Island, NY), supplemented with 5% FCS serum (Cellect Gold; Flow Labs, McLean, VA), 100 U/ml of penicillin, 100 μg/ml streptomycin, and 5 mM of 2-ME (all from Sigma Chemical Co., St. Louis, MO). The cells were incubated for the time indicated in 24-well plates (Costar Data Packaging Corp., Cambridge, MA). Supernatants from triplicate cultures were tested for CSIF activity after depletion of IFN-γ with XMG 1.2 antibody covalently bound to AFFI-Gel (Bio-Rad Laboratories). Verification of IFN-γ depletion was determined using a sandwich ELISA for mouse IFN-γ.

The CSIF assay was performed using the BD7.7 cell line, maintained by stimulation with irradiated allogeneic mouse (DBA/2 or BALB/c) spleen cells and IL-2. Resting (7 d poststimulation) cells were added to dilutions of test supernatants at 2×10^5 cells/well with irradiated allogeneic stimulator cells (5×10^6/well) in 1 ml complete medium (dMEM supplemented with antibiotics, Na pyruvate, 2-ME, glutamine, and 5% FCS). After a 24-h incubation, supernatants were collected for determination of IFN-γ by ELISA.

Macrophage Microbicidal Activity. Peritoneal macrophages were harvested from BALB/c mice previously injected with 1 ml of 6% sodium caseinate in saline i.p. (Eastman Kodak Company, Rochester, NY). The cells were infected with *T. cruzi* for the determination of intracellular growth and inhibition thereof as described (13).

Results

The Importance of Endogenous IFN-γ in Control of T. cruzi Infection. To address the role of cytokines in resistance or susceptibility to *T. cruzi* infection, we developed a model using two inbred mouse strains. B6 mice are highly susceptible to the Tulahuen strain, while B6D2 mice are highly resistant (Fig. 1). Both mouse strains developed parasitemia, splenomegaly, and splenic parasite burdens. Using this system, we examined the role of IFN-γ and IL-10 during acute infection. Although we have established the importance of IFN-γ in protection from acute *T. cruzi* infection (17), it has not been demonstrated that endogenously produced IFN-γ is essential in mediating protection from fatal disease in a genetically resistant host. We therefore treated both susceptible B6 and resistant B6D2 mice with anti–IFN-γ mAb and looked for effects on the course of acute *T. cruzi* infection. Treatment with anti–IFN-γ mAb altered the course of infection by significantly increasing parasitemia in both groups (Fig. 1 *A*). Most dramatically, resistant B6D2 mice were rendered susceptible to acute *T. cruzi* infection, with 100% mortality, after anti–IFN-γ treatment (Fig. 1 *B*). These experiments established the importance of endogenous IFN-γ in controlling acute infection, and suggested that genetic resistance could the be mediated at least in part by IFN-γ.

IFN-γ Production by Resistant and Susceptible Mice during T. cruzi Infection. Based on these findings, and supported by our previous observations that the administration of IFN-γ could prevent fatal infections in susceptible mice, we examined IFN-γ production during the course of *T. cruzi* infection in B6 and B6D2 mice. To test for secreted IFN-γ, spleen cells were cultured from mice during progressive days after infection and stimulated in vitro with Con A. Mitogen stimulation led to increased IFN-γ production in both B6 and B6D2 mice, which peaked during the acute phase (days 10–30) of the infection (not shown). Spleen cells from susceptible B6 mice, but not resistant B6D2 mice, produced detectable IFN-γ during the acute phase of infection without stimulation (not shown).

Production of IL-10 mRNA during T. cruzi Infection. Endogenously produced IFN-γ failed to protect susceptible B6 mice, even though we and others (29) have shown that the IFN-γ produced by susceptible mice is biologically active in vitro. One reason for this may be that susceptible mice fail to produce IFN-γ during the very early stage of infection. Another is that molecules that inhibit the effects of IFN-γ are produced during infection. Among the cytokines, IL-10 may inhibit the production of IFN-γ (21), as well as the effects of cytokines on macrophage activation (30).

To evaluate production of IL-10 mRNA, spleen cell lysates

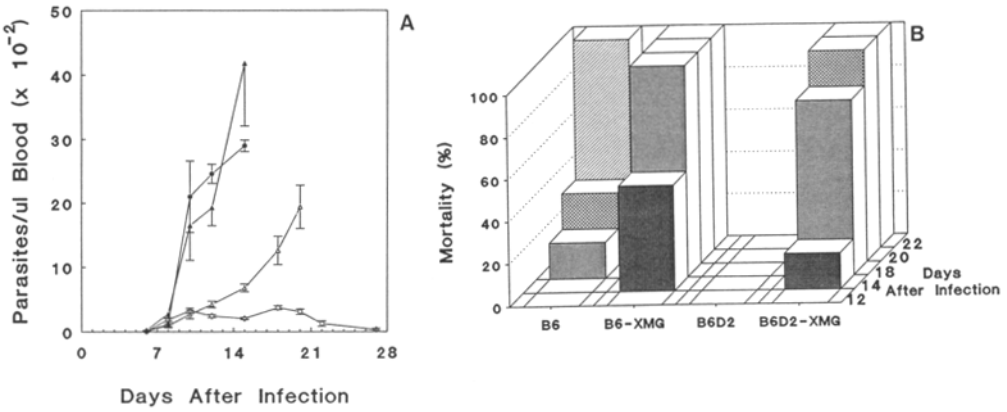

Figure 1. Effects of anti-IFN-γ treatment in resistant B6D2 (*circles*) and susceptible B6 (*triangles*) mice infected with *T. cruzi*. Mice were treated with 1 mg XMG 1.2 (*closed symbols*) or saline (*open symbols*) 1 d before and 3 d after intraperitoneal infection with 10^3 trypomastigotes. Parasitemias (*A*) and mortality (*B*) were determined at indicated days after infection. Each point (mean ± SEM) represents six mice. Mice treated with XMG 1.2 had parasitemias significantly higher ($p < 0.05$) than control mice at day 10 and beyond. In *B*, no mortality was seen in saline-treated B6D2 mice, as compared with 100% mortality in XMG-treated (B6D2 XMG) mice.

were prepared from B6 mice at different days during *T. cruzi* infection, and analyzed by Northern blot (Fig. 2). IL-10-specific mRNA was not detected in normal mouse spleens or in spleens from mice infected for 2 or 4 d. By day 8 after infection, and continuing through day 15 after infection, a period corresponding to the initiation and logarithmic increase of parasitemia (Fig. 1 *A*), spleen cell IL-10 mRNA was present. This demonstrated that an increase in IL-10 mRNA occurred in susceptible mice during the acute phase of *T. cruzi* infection.

Production of IL-10 in Mice with T. cruzi Infection. An IFN-γ inhibition assay was used to evaluate IL-10 activity in supernatants of cultured spleen cells from infected B6 and B6D2 mice. Spleen cells from mice infected for 6, 10, 14, or 17 d were cultured in vitro for 48 h and the supernatants tested for CSIF activity, the inhibition of IFN-γ production in response to recall antigenic stimulation (Fig. 3). The pattern of production of biologically active CSIF correlated directly with that seen for IL-10 mRNA production. In addition, it was found that spleen cells from infected B6D2 mice did not produce detectable levels of CSIF. Similar results were obtained when spleen cells from either of the mouse groups were stimulated in vitro with *T. cruzi* trypomastigote lysate (data not shown). Thus, the production of biologically active CSIF was a distinguishing feature of acute, fatal infections in this model. A neutralizing mAb (SXC1) was used for verification that the CSIF activity produced by spleen cells of infected B6 mice was due to IL-10. The IFN-γ-inhibiting activity produced by spleen cells of acutely infected mice, as well as that of rIL-10, was neutralized by anti-IL-10 mAb (Fig. 4).

Effects of IL-10 on the Intracellular Inhibition of T. cruzi. Because the course of acute *T. cruzi* infection depends largely on the degree of intracellular replication of the parasite in host cells, including macrophages, the ability of IFN-γ to inhibit this replication is of fundamental importance in the mediation of resistance by this cytokine. Interference with

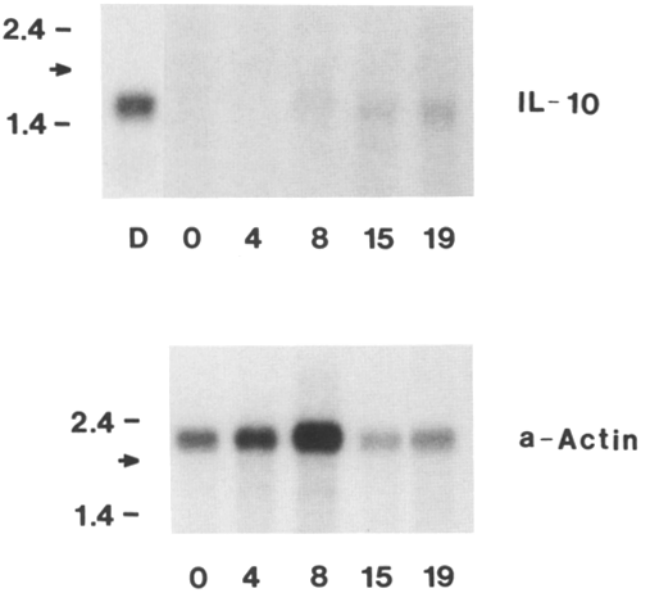

Figure 2. Northern blot for IL-10 in B6 mice during *T. cruzi* infection. 15 μg total RNA extracted from spleen cells of uninfected (day 0) or infected (days 4, 8, 15, 19) per lane. Shown is a 7-d exposure.

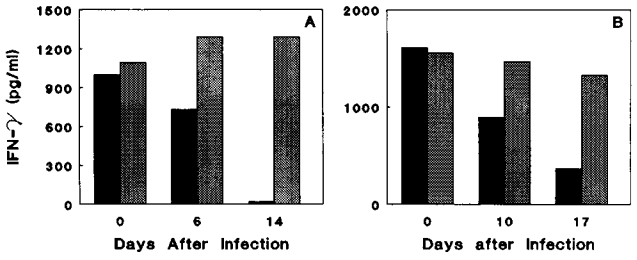

Figure 3. IL-10 activity in spleen cell supernatants from B6 (*filled bar*) and B6D2 (*stippled bar*) mice infected with *T. cruzi*. 48-h supernatants of 5 × 10^6 unstimulated spleen cells from uninfected and from mice infected for 6–17 d with *T. cruzi* were assayed for CSIF activity. Representative data from two experiments are shown. Each point represents pooled spleen cells from three mice.

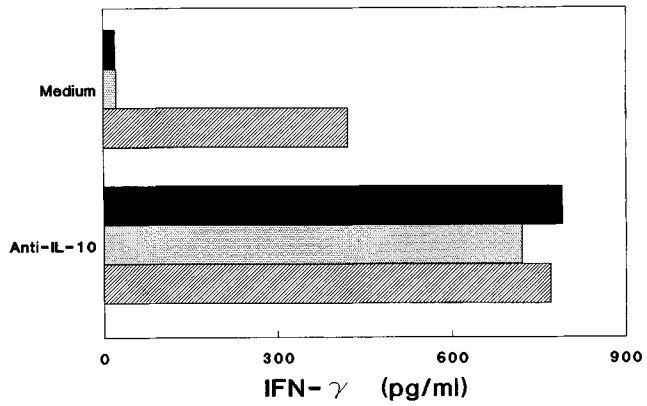

Figure 4. Anti-IL-10 blocks the IFN-γ-inhibiting activity of supernatants from spleen cells of B6 mice infected with *T. cruzi*. CSIF activity was determined using 10 U rIL-10 (*filled bar*), a 1/20 dilution of spleen cell supernatant from mice infected 17 d (*stippled bar*), and mock cos cell supernatant (*hatched bar*) in the absence or presence of SXC1 ascites (50 μg protein/ml).

this process could be an important mechanism in pathogenesis. We examined the ability of macrophages treated with rIL-10 to inhibit the intracellular replication of *T. cruzi* after activation with IFN-γ. Preincubation of mouse peritoneal macrophages with rIL-10–containing COS supernatant, but not with mock COS supernatant, significantly reduced the inhibitory effects of IFN-γ on the intracellular replication of *T. cruzi* (Table 1). Thus, in addition to the ability of IL-10 to interfere with the antigen presentation by macrophages (31), this cytokine can also block macrophage activation to inhibit intracellular parasites. This observation adds another important dimension to the regulatory functions of this cytokine.

Discussion

This study presents two findings relevant to mechanisms of host resistance to acute *T. cruzi* infection. The first is documentation of the importance of IFN-γ in controlling acute infection by using genetically resistant mice. The second is that IL-10, an inhibitory cytokine, is produced during *T. cruzi* infection. The latter observation may be an important step in understanding how this parasite escapes immune-mediated destruction.

IFN-γ is an effective inhibitor of the in vitro replication of *T. cruzi* in macrophages (13–15, 17). IFN-γ can also reverse the course of fatal infection in vivo in highly susceptible mice (17). More recently, it has been shown that treatment of susceptible mice with anti–IFN-γ can exacerbate infection (29). Using genetically resistant B6D2 mice, we have shown in this study that in vivo inhibition of a single cytokine is sufficient to eliminate that resistance, which establishes the importance of endogenous IFN-γ in mediating host resistance to *T. cruzi* infection. The question remains as to why IFN-γ produced during *T. cruzi* infection fails to protect mice from disease. One possibility is the kinetics of the cytokine response.

The importance of the early response to infection is illustrated by in vivo experiments with IFN-γ and anti–IFN-γ. To consistently prevent the development of acute disease in mice, it is necessary to administer IFN-γ before day 4 of infection (17). Similarly, to produce fatal infections in resistant B6D2 mice, it was necessary to administer anti–IFN-γ no later than 4 d after infection. These experiments may indicate the necessity of limiting the early replication of *T. cruzi* in host cells to obtain a healing response. They may also indicate the potential of IFN-γ to drive the development of a Th1 type T cell response.

Another possible way in which cytokines may exacerbate infection is to inhibit the effects of protective cytokines, such

Table 1. *rIL-10 Blocks the IFN-γ–mediated Inhibition of Trypanosoma cruzi in Mouse Peritoneal Macrophages*

		Exp. 1		Exp. 2	
	IFN-γ	Amastigotes /100 macrophages	Percent inhibition	Amastigotes /100 macrophages	Percent inhibition
		$\bar{X} \pm SD$		$\bar{X} \pm SD$	
Medium	–	33.1 ± 3.9		137.9 ± 13.5	
	+	5.6 ± 0.6	83.1	41.1 ± 8.7	70.2
COS-control	–	36.6 ± 6.3		130.0 ± 2.8	
	+	3.5 ± 1.6	90.4	32.6 ± 6.9	74.9
rIL-10	–	30.5 ± 1.7		166.8 ± 20.6	
	+	14.6 ± 2.6	52.1	120.9 ± 2.1	27.5

BALB/c peritoneal macrophages were treated with rMu-IFN-γ and rIL-10 or COS-supernatant control for 48 h, washed, and infected with *T. cruzi* trypomastigotes. The cells were washed thoroughly 2 h later and incubated for 48 h (Exp. 1) or 72 h (Exp. 2) and intracellular amastigotes enumerated. IFN-γ and COS-control + IFN-γ groups vs. rIL-10 + IFN-γ, $p < 0.05$.

as IFN-γ. We have recently shown that TGF-β production is increased during acute *T. cruzi* infections, and that this molecule can block the inhibitory effects of IFN-γ, both in vitro and in vivo (19). The present study demonstrates that IL-10 has a similar deactivating function. Thus, IL-10 may act to downregulate early IFN-γ production during *T. cruzi* infection, as well as to inhibit its effects. These results provide further information on the macrophage as a key target for the action of IL-10 (31, 31).

The concomitant occurrence or balance of Th1 and Th2 responses must be considered in determining disease outcome. A predominantly Th1 type response, characterized by the production of IFN-γ and IL-2, is associated with a healing response in experimental leishmania infections, while a Th2 response, characterized by the cytokines IL-4, IL-5, IL-6, and IL-10, is associated with disease exacerbation (32, 33). The situation appears to be different in *T. cruzi* infection, where both a Th1 response (IFN-γ) and a Th2 response (IL-10) occur during infection in susceptible mice. Thus, the infection appears to be characterized by the production of cytokines which can counteract the beneficial Th1 type response. The production of cytokines such as TGF-β and IL-10 may be the deciding factor in the outcome of *T. cruzi* infection. In support of this concept, production of IL-10 activity by spleen cells of infected B6 mice was elevated over that seen in identically infected B6D2 mice. Furthermore, it is possible that resistance or susceptibility to acute infection is determined by early production of protective or exacerbative cytokines.

T. cruzi infections in mice have provided an excellent model for the study of immune responses in acute and chronic infections. Studies on the regulation of cytokines during the course of infection as well the role of cytokines in host resistance and susceptibility have been performed with this model. It is of particular interest because *T. cruzi* survives and replicates in host macrophages, and evasion of killing mechanism by these cells must be considered. Although these parasites have unique ways of escaping from the phagocytic vacuole which may contribute to their intracellular survival (34), macrophages activated by cytokines, such as IFN-γ, can effectively eliminate intracellular infection. Thus, the inhibition of the production and/or effects of cytokines may be important parasite survival mechanisms which could be targets for therapeutic approaches. A recent study by Heinzel et al. (33) has demonstrated the production of IL-10 mRNA during murine infections with leishmania. We report herein a similar finding in murine *T. cruzi* infections. In addition, we document the production of biologically active IL-10 by genetically susceptible, but not resistant mice, and demonstrate that IL-10 may inhibit the antiparasite function of IFN-γ.

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Address correspondence to Dr. Steven G. Reed, Seattle Biomedical Research Institute, 4 Nickerson St., Seattle, WA 98109.

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