

Regulation of *Trypanosoma cruzi* Infections In Vitro and In Vivo by Transforming Growth Factor β (TGF- β)

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Summary

The effects of transforming growth factor β (TGF- β) on interferon γ -mediated killing of the intracellular protozoan parasite *Trypanosoma cruzi* and on the course of *T. cruzi* infection in mice were investigated. Spleen cells from mice with acute *T. cruzi* infections were found to produce elevated levels of biologically active TGF- β in vitro, and the possibility that TGF- β may mediate certain aspects of *T. cruzi* infection was then addressed. When mouse peritoneal macrophages were treated with TGF- β in vitro, the ability of IFN- γ to activate intracellular inhibition of the parasite was blocked. This occurred whether cells were treated with TGF- β either before or after IFN- γ treatment. TGF- β treatment also blocked the *T. cruzi*-inhibiting effects of IFN- γ on human macrophages. Additionally, treatment of human macrophages with TGF- β alone led to increased parasite replication in these cells. The effects of TGF- β on *T. cruzi* infection in vivo were then investigated. Susceptible C57BL/6 mice developed higher parasitemias and died earlier when treated with TGF- β during the course of infection. Resistant C57BL/6 \times DBA/2 F1 mice treated with TGF- β also had increased parasitemias, and 50% mortality, compared with no mortality in infected, saline-treated controls. A single dose of TGF- β , given at the time of infection, was sufficient to significantly decrease resistance to infection in F1 mice and to exacerbate infection in susceptible C57BL/6 mice. Furthermore, a single injection of TGF- β was sufficient to counter the in vivo protective effects of IFN- γ . We conclude that TGF- β , produced during acute *T. cruzi* infection in mice, is a potent inhibitor of the effects of macrophage activating cytokines in vivo and in vitro and may play a role in regulating infection.

The hemoflagellate protozoan *Trypanosoma cruzi* causes Chagas' disease, a major public health problem in many countries of Latin America. Infection with this parasite may be acute or chronic, and frequently involves the development of progressive pathology in tissues of the heart, esophagus and colon. In vitro and in vivo, the parasites infect a variety of nucleated cells, including macrophages. In both humans and laboratory animals, *T. cruzi* infection is accompanied by a nonspecific immune depression mediated by T cells and macrophages which results in reduced T cell function, including help for antibody production (1) and cytotoxicity (2). T cells from infected mice may have reduced capacity to produce IL-2 and display IL-2R (3). Several cytokines have been shown to reverse depressed immune function in infected mice, including IL-2 (4-6), IL-1 (7), and granulocyte macrophage colony-stimulating factor (GM-CSF)¹ (8). Evidence has been pre-

sented that IL-1 and GM-CSF may function at least in part by restoring or increasing IL-2 production in infected animals (7, 8).

The mechanisms which control parasite replication during the acute and chronic phases and which maintain low but persistent numbers of circulating parasites during the chronic phase are not well understood, but both T cell cytokines and lytic antibodies appear to be involved. Among cytokines, IFN- γ has a remarkable capacity to activate macrophages and inhibit the replication of *T. cruzi* both in vitro (9, 10) and in vivo (11). IFN- γ appears to have an important role in the very early stage of *T. cruzi* infection in determining disease outcome (11). Endogenous IFN- γ is important in controlling *T. cruzi* infection since treatment with anti-IFN- γ mAb rendered resistant mice susceptible to acute disease (Silva, J.S., P.J. Morrissey, K.H. Grabstein, K.M. Mohler, and S.G. Reed, manuscript submitted for publication).

Thus cytokines play key roles in regulating both parasite replication and immune responses in infected animals. One cytokine which is associated with both immune regulation

¹ Abbreviation used in this paper: GM-CSF, granulocyte macrophage colony-stimulating factor.

and control of macrophage activation is TGF- β . This 24-kD protein is produced by many cells including B, T, and activated macrophages (12–14) and has been implicated as a mediator of immunosuppression, inhibiting IL-2 receptor induction (14), IL-1 induced thymocyte proliferation (15), B cell proliferation and differentiation (12), IFN- γ induced class II antigen expression (16), cytotoxic lymphocyte generation, and lymphokine activated killer cells (17). In addition, TGF- β has the ability to inhibit cytokine-induced macrophage activation (18), suppressing the production of reactive oxygen and nitrogen intermediates (18, 19). The potent effects of TGF- β on cellular immune responses resemble in many ways those accompanying acute *T. cruzi* infection. In the present report, we have evaluated the effects of TGF- β on experimental *T. cruzi* infection, both in vitro and in vivo, and present evidence for the involvement of TGF- β in parasite survival and the production of fatal infections.

Materials and Methods

Mice. 6–8-wk-old female BALB/c, C57BL/6, (B6), and (C57BL/6 \times DBA)F1 (B6D2) mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Parasite. A clone of the Tulahuen strain of *T. cruzi*, designated as MHOM/CH/00/Tulahuen C2, was used in all experiments. Trypomastigotes were grown in the L6E9 rat myoblast cell line and used to infect mice by i.p. injection of 10^3 parasites in 0.2 ml of PBS, or s.c. injection in the footpad in 0.02 ml. *T. cruzi* trypomastigote lysate was used at 10 μ g protein/ml and consisted of freeze-thawed trypomastigotes centrifuged at 10,000 *g* for 30 min and filtered through a 0.22 μ m filter.

Cytokines. rMu-IFN- γ (5.2×10^6 U per mg protein) and rHu-IFN- γ (10^7 U per mg protein) was provided by Genentech, Inc. (San Francisco, CA) as protein purified to homogeneity from *Escherichia coli*. Human TGF- β was purchased from Calbiochem Corp. (La Jolla, CA) and rMu-TGF- β was a gift from Genentech, Inc.

Macrophages. Mouse macrophages were harvested from peritoneal cavities 3–5 d after injection of 1 ml of 6% sodium caseinate (Eastman Kodak Company, Rochester, NY). The cells were harvested with cold RPMI 1640 (Gibco Laboratories, Grand Island, NY) washed, and plated on the 12 mm round coverslips in 24-well plates (5×10^5 cells/well) or directly into 48-well plates (2.5×10^5 cells/well). After 2 h at 37°C in 5% CO₂, adhered cells were washed with warm PBS and cultured in RPMI/5% endotoxin-free FCS (Collect Gold, Flow Labs, McLean, VA) plus antibiotics. Human macrophages were obtained from normal donor PBMC (American Red Cross, Portland, OR) purified on Percoll gradients and cultured for 5 d in RPMI/5% human AB serum.

Microbicidal Activity. Mouse or human macrophages were incubated with or without TGF- β and/or IFN- γ for 48 h. Cells were washed and infected at a parasite:cell ratio of 1:1 for 2 h at 37°C. Extracellular parasites were removed by three washes with RPMI and the cells incubated for the indicated times in the presence or absence of cytokine. The growth of parasites in macrophages was evaluated by counting trypomastigotes released at various times after infection and by counting intracellular amastigotes as described (20).

Transforming Growth Factor β Bioassay. Production of active TGF- β was determined as described (21). The mink lung epithelial cell line CCL64 was plated in 96-well tissue culture plates at 3.5×10^3 cells per 50 μ l of DMEM containing 10% FCS. Supernatant

from spleen cell cultures from uninfected or *T. cruzi* infected mice were incubated at 5×10^6 cells/ml in RPMI + 5% FCS (both endotoxin free) for 48 h in the presence or absence of *T. cruzi* lysate at 37°C and assayed for TGF- β activity. Samples were added to triplicate wells 5 h after plating of cells. After incubation at 37°C for 72 h ¹²⁵I-IodoUdr (M 355; Amersham, Arlington Heights, IL; 1 μ Ci/ml) was added and the cells were incubated an additional 24 h. Inhibition of growth was expressed in the percent decrease of ¹²⁵I-IodoUdr incorporation in treated cells when compared to incorporation in untreated cells. A standard curve with human platelet TGF- β 1 (0.05–10 ng/ml) was run in each assay. The specificity of this bioassay has been demonstrated, including inhibition of the assay by monoclonal anti-TGF- β antibody (22).

Statistics. Student's *t* test was used to analyze data.

Results

Production of Transforming Growth Factor β by Spleen Cells from Mice Infected with *T. cruzi*. Experimental *T. cruzi* infection is associated with a profound and lasting depression of spleen cell immune responsiveness (1, 4–8). Because of the association of TGF- β with immune suppression, we looked for production of this cytokine by spleens of infected mice. The production of active TGF- β by mouse spleen cells was determined using a sensitive growth inhibition assay. Fig. 1 shows TGF- β production by spleen cells from uninfected mice and from mice at different time points after infection. Increasing amounts of active TGF- β were produced and released by cells taken during the course of acute infection. Levels of TGF- β were significantly elevated by 8 d after infection. Cells

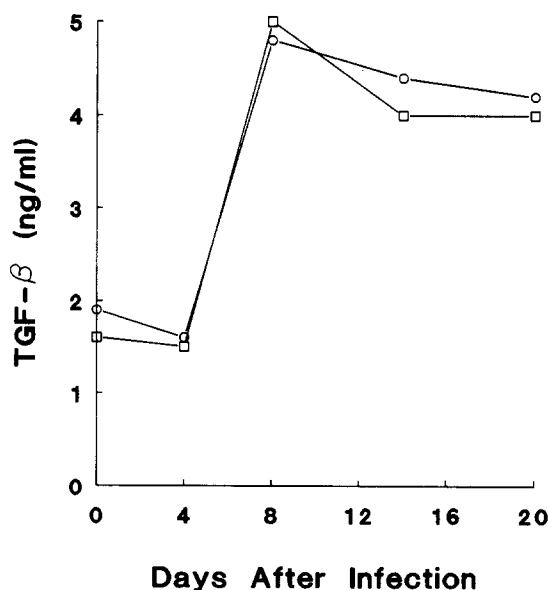


Figure 1. Production of active TGF- β by spleen cells from mice infected with *T. cruzi*. Spleen cells from B6 mice obtained at different days after infection with 10^3 trypomastigotes of *T. cruzi* were incubated for 48 h in the presence (□) or absence (O) of trypomastigote lysate, and supernatants were tested for biologically active TGF- β . The first points (0 d) indicate uninfected control mice.

incubated in the presence of parasite lysate produced the same amounts of TGF- β as those incubated in medium alone. Of particular interest was that detection of active TGF- β did not require prior acidification. This indicated that the cells were producing the active form of TGF- β , and suggested that activation occurred within the spleen cells.

Effects of TGF- β on IFN- γ -Induced Inhibition of *T. cruzi* in Macrophages. We and others have associated cytokine-induced inhibition of *T. cruzi* in macrophages with enhanced oxidative metabolism (20, 23). One of the molecules shown to decrease macrophage ability to respond to cytokine with increased oxidative metabolism is TGF- β (18). Therefore we first examined the in vitro effects of TGF- β on the ability of mouse or human macrophages to inhibit intracellular replication of *T. cruzi* following activation with IFN- γ . Mouse peritoneal macrophages were treated with TGF- β or IFN- γ 48 h before or 2 h after infection. Numbers of extracellular trypomastigotes released from the cells were determined 5 d later. Pre- or post-infection treatment with 10 U of murine rIFN- γ significantly inhibited *T. cruzi* replication. The degree of inhibition was less than we have previously reported (11) due to the lower amount of IFN- γ used in the present study, but either pre- or post-treatment of macrophages led to 70–75% fewer parasites released from the cells (Table 1). However, treatment of the macrophages with TGF- β 48 h before infection prevented the inhibition of parasite replication by IFN- γ . Whereas IFN- γ treatment 2 h after infection led to an approximately 70% reduction in parasite liberation (Table 1, group 4), prior incubation with TGF- β prevented this inhibition (group 7). Similarly, whereas a 48 h preincubation of macrophages in IFN- γ led to a 76% inhibition of parasites (group 2), post-infection treatment of the IFN- γ -treated cells with TGF- β eliminated the parasite inhibition (group 6). Pre- or post-treatment with TGF- β alone had little effect on parasite numbers in this system. In another

series of experiments, mouse peritoneal macrophages were treated with different doses of IFN- γ before infection with *T. cruzi*, in the presence or absence of TGF- β (Fig. 2). Concentrations of 10 and 100 U IFN- γ /ml inhibited both intracellular amastigotes (Fig. 2 A) and the liberation of extracellular trypomastigotes (Fig. 2 B). Post- and or pre-infection treatment with TGF- β significantly inhibited the effects of IFN- γ at all doses tested. Together, these experiments (Table 1, Fig. 2) demonstrate that the IFN- γ -mediated inhibition of *T. cruzi* in mouse peritoneal macrophages is blocked by TGF- β .

Further experiments examined the effect of TGF- β on the inhibition of *T. cruzi* by human macrophages. Peripheral blood

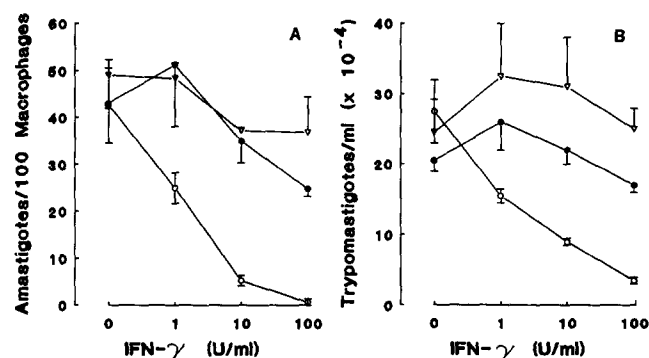


Figure 2. Effects of TGF- β on inhibition of *T. cruzi* development in mouse peritoneal macrophages in vitro. The cells were plated on 13 mm² round glass coverslips (A) or 48 well plates (B) and incubated for 24 h with the indicated concentration of rMuIFN- γ without (O), or with 1 ng/ml rMuTGF- β before (●), or before and after (Δ) infection with *T. cruzi*. (A) Coverslips were fixed 48 h after infection, and numbers of amastigotes per 100 macrophages determined. (B) Extracellular trypomastigotes were counted 5 d after infection. Points represent $\bar{x} \pm SD$.

Table 1. Transforming Growth Factor β Blocks the Interferon γ -mediated Inhibition of *T. cruzi* in Cultured Mouse Macrophages*

Group	Cytokines added		No. trypomastigotes/ μ l	% Inhibition
	48 h before infection	2 h after infection		
			$\bar{x} \pm SD$	
1	-	-	356 \pm 32	
2	IFN- γ	-	87 \pm 15	76
3	TNF- β	-	400 \pm 28	-12
4	-	IFN- γ	113 \pm 12	68
5	-	TGF- β	293 \pm 15	18
6	IFN- γ	TGF- β	307 \pm 25	14
7	TGF- β	IFN- γ	380 \pm 28	-7

* Peritoneal macrophages were treated with rIFN- γ (10 U/ml) or TGF- β (1 ng/ml) as indicated. Cells were infected with *T. cruzi* trypomastigotes, washed thoroughly after 2 h to remove extracellular parasites, and incubated with the indicated cytokines. After 5 d, extracellular trypomastigotes were counted. Groups 2 and 4 vs. groups 1, 3, 5, 6, 7, $p < 0.05$.

monocytes were adhered to 12 mm round glass coverslips for 5 d before treatment with TGF- β and/or IFN- γ . 48 h later, the macrophages were infected with *T. cruzi*, and numbers of intracellular amastigotes were determined after 72 h. Data from four individual donors are shown in Table 2. As was seen with mouse macrophages, treatment with TGF- β blocked the IFN- γ -mediated inhibition of parasite replication. Pre- or post-infection treatment with IFN- γ significantly reduced

the replication of *T. cruzi* in human macrophages as previously described (20). Treatment of the macrophages with TGF- β either before or after the addition of IFN- γ inhibited this effect. In addition, treatment with TGF- β alone, either before or after infection of the cells, led to a significant increase in intracellular parasite numbers. This demonstrated that TGF- β treatment exacerbated *T. cruzi* infection in normal human macrophages.

Table 2. Transforming Growth Factor β Exacerbates In Vitro *T. cruzi* Infection in Human Macrophages and Blocks the Effects of Interferon γ *

Group	TGF- β		IFN- γ		Amastigotes/100 cells	% Inhibition
	Before	After	Before	After		
Donor 1					$\bar{x} \pm SD$	
1	-	-	-	-	246 \pm 16	
2	-	-	+	-	110 \pm 25	55
3	+	-	-	-	365 \pm 76	-48
4	-	-	-	+	55 \pm 6	78
5	-	+	-	-	411 \pm 27	-67
6	+	-	-	+	264 \pm 38	-7
7	-	+	+	-	220 \pm 23	11
Donor 2						
1	-	-	-	-	265 \pm 3	-
2	-	-	+	-	76 \pm 23	71
3	+	-	-	-	295 \pm 19	-11
4	-	-	-	+	116 \pm 6	56
5	-	+	-	-	434 \pm 94	-64
6	+	-	-	+	263 \pm 46	1
7	-	+	+	-	274 \pm 90	-3
Donor 3						
1	-	-	-	-	247 \pm 29	-
2	-	-	+	-	124 \pm 13	50
3	+	-	-	-	384 \pm 47	-55
4	-	-	-	+	195 \pm 29	21
5	-	+	-	-	385 \pm 59	-56
6	+	-	-	+	279 \pm 31	-13
7	-	+	+	-	276 \pm 74	-12
Donor 4						
1	-	-	-	-	297 \pm 103	-
2	-	-	+	-	139 \pm 33	53
3	+	-	-	-	508 \pm 88	-71
4	-	-	-	+	131 \pm 55	56
5	-	+	-	-	549 \pm 57	-85
6	+	-	-	+	303 \pm 24	-2
7	-	+	+	-	231 \pm 46	22

* PBMC-derived macrophages were treated with TGF- β (1 ng/ml) or rIFN- γ (100 U/ml) 48 h before or 2 h after infection with *T. cruzi* trypomastigotes. Number of intracellular amastigotes determined 72 h after infection. In all donors, groups 4 vs. 6, and 2 vs. 7, $p \leq 0.05$.

Effects of Transforming Growth Factor- β on *T. cruzi* Infection In Vivo. In vitro, TGF- β treatment blocked inhibition of *T. cruzi* mediated by IFN- γ and, in some cases, exacerbated intramacrophage replication of the parasite. We therefore examined the effects of treatment of mice with TGF- β on *T. cruzi* infection. We have developed a model to examine parameters of resistance and susceptibility to *T. cruzi*. B(B6)6 mice are extremely susceptible to the Tulahuen strain of *T. cruzi*, resulting in 100% mortality following infection with as few as 50 blood forms. B6D2 mice, however, are highly resistant to this organism, with 100% survival resulting from infection with as many as 10^5 parasites. To examine the effects of TGF- β on the course of infection in resistant and susceptible mice, B6 and B6D2 were injected with TGF- β , 1 d before infection, and at multiple d after infection with 10^3 *T. cruzi*. The results are shown in Fig. 3. In B6 mice, TGF- β treatment led to parasitemias which were significantly higher than in saline-treated mice, with an accompanying decrease in survival time. Similarly, a striking increase in parasitemia and mortality was seen in B6D2 mice treated with TGF- β . Whereas none of the saline treated mice died, 50% of the TGF- β -treated mice died by 20 d after infection.

The in vivo effects of TGF- β administration on infection with *T. cruzi* were confirmed in experiments in which susceptible B6 mice were infected s.c. in the footpad. This infection route produced a lower parasitemia and mortality rate than i.p. infection. In these experiments, illustrated in Fig. 4, mice were given a single s.c. injection of 1 μ g of rMuTGF- β (or saline) at the time of infection. As was observed with i.p. infection experiments, treatment with TGF- β led to significantly increased parasitemias and mortalities in B6 mice. TGF- β -treatment also eliminated the in vivo protective effect of IFN- γ on *T. cruzi* infection (11). Thus, in vivo results cor-

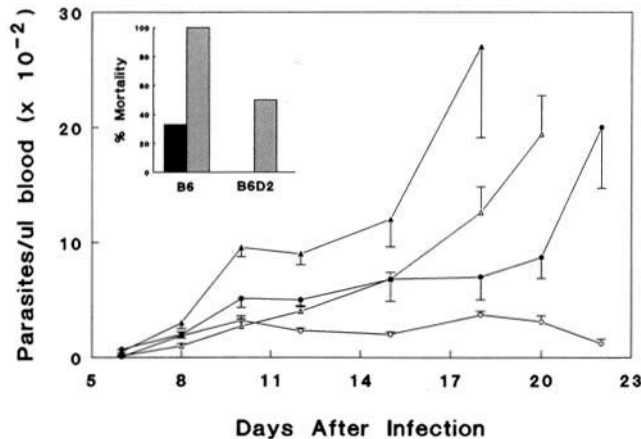


Figure 3. Effects of TGF- β on the course of *T. cruzi* infection in resistant (B6D2) and susceptible (B6) mice. The B6D2 (O) or B6 mice (Δ) were treated i.p. with PBS (opened symbols) or 10 μ g of rMuTGF- β (closed symbols) on -1, 0, 2, 5, and 9 d after i.p. infection with 10^3 trypomastigotes. Points represent the mean \pm SEM of 6 mice. At 10, 12, 15, 18, 20 and 22 d, TGF- β treated vs. control, $p < 0.05$ for both mouse strains. Inset shows cumulative mortality on d 20 after infection in PBS (black bar) or TGF- β (grey bar) treated mice.

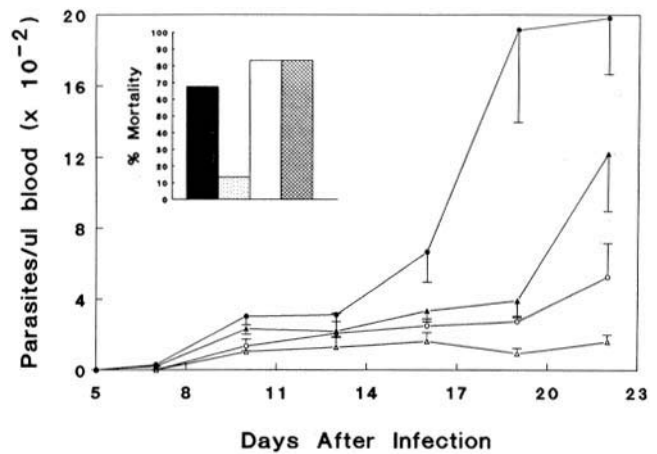


Figure 4. TGF- β inhibits the effects of IFN- γ in vivo. Susceptible (B6) mice were infected s.c. in the footpad with 10^3 trypomastigotes in PBS only (O), or PBS containing 1 μ g of rMuTGF- β (\bullet), 2 μ g of rMuIFN- γ (Δ), or both 1 μ g of rMuTGF- β and 2 μ g of rMuIFN- γ (\blacktriangle). Points represent mean \pm SEM of 6 mice. At 16, 18, and 22 d, mice treated with IFN- γ (Δ) vs. mice treated with IFN- γ + TGF- β (\blacktriangle), $p < 0.05$. Inset shows cumulative mortality on d 28 after infection in mice treated with saline (black bar), IFN- γ (stippled bar), TGF- β (white bar) or both TGF- β and IFN- γ (cross-hatched bar). Mice treated with IFN- γ vs. other groups, $p < 0.05$.

roborated those obtained in vitro. Mice treated with TGF- β had exacerbated infections as well as an inhibition of the therapeutic effect of IFN- γ treatment.

Discussion

The macrophage is a pivotal cell in the host immune response to *T. cruzi*. During the very early stages of infection the parasites are found within macrophages, which may inhibit their replication or provide a favorable environment in which *T. cruzi* can multiply and be disseminated. Evidence for the importance of macrophage activity in determining the outcome of infection comes largely from experiments done in vivo which demonstrated that early treatment with rIFN- γ can reverse the course of fatal infection (11). Thus mice treated with rIFN- γ beginning within 24 h after infection had 100% survival, vs. 100% mortality in saline-treated mice or in mice treated with IFN- γ 7 d after infection. Further, a single dose of neutralizing antibody to IFN- γ , given within 24 h after infection, is sufficient to produce fatal infections in resistant B6D2 mice, as well as significantly exacerbate infections in susceptible B6 mice (Silva, J.S., P.J. Morrissey, K.H. Grabstein, K.M. Mohler, and S.G. Reed, manuscript submitted for publication), providing evidence for the importance of physiological levels of endogenous IFN- γ . The macrophage is the most likely target for the dramatic effects of IFN- γ , suggesting that severity of infection with *T. cruzi* depends significantly upon how effectively these phagocytic host cells limit early parasite replication.

In vitro, mouse macrophages activated by IFN- γ are very effective at limiting *T. cruzi* replication (20). This activity

has been associated, at least in part, with increased oxidative metabolism resulting from cytokine stimulation (20, 23). One of the described effects of TGF- β on macrophage function is the down regulation of oxidative metabolism (18). Results in the present study indicated that TGF- β could both increase intra-macrophage replication of *T. cruzi* as well as block the inhibitory effect of IFN- γ . These findings support the importance of reactive oxygen intermediates in the intramacrophage inhibition of *T. cruzi*.

It is apparent from this and other studies that cytokines play a variety of crucial regulatory roles in the host response to parasites. They are also essential in regulating host responses not directed at the parasite. Several cytokines, including IL-2 (5, 6), IL-1 (7), and GM-CSF (8), have been shown to have the capacity of restoring immunological competence to spleen cells from infected mice. At least three cytokines, IFN- γ , GM-CSF, and TNF (20, 24) have been shown to have the capacity to limit replication of *T. cruzi* in macrophages. The present report demonstrates that another cytokine, TGF- β , is produced during *T. cruzi* infection, and has the potential of altering the course of that infection. At least one of the ways

in which this may be effected is via the inhibition of the activity of IFN- γ . Such a mechanism has been suggested in recent in vitro studies using mouse macrophages infected with *Leishmania major* (25). Thus susceptibility to the parasite may be manifested in part by molecules which counteract host effector responses.

Another way in which TGF- β may mediate immune depression is by inhibiting cytokine production (26). However, we have no evidence for the inhibition of IFN- γ production by *T. cruzi* infected mice, even in highly susceptible B6 animals. A more likely cause for the failure of endogenous IFN- γ to limit parasite replication may be inhibition of its effectiveness, such as we have demonstrated herein. Thus, TGF- β produced in vivo may be important in mediating susceptibility to *T. cruzi* infection. Of interest is that biologically active TGF- β requiring no acidification was produced by spleen cells from infected mice. Together the in vitro and in vivo results of this study provide evidence for the importance of TGF- β as a mediator of acute *T. cruzi* infection, and provide insight into molecular mechanisms of susceptibility in infectious disease.

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