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Tumor Necrosis Factor Alpha Mediates Resistance to *Trypanosoma cruzi* Infection in Mice by Inducing Nitric Oxide Production in Infected Gamma Interferon-Activated Macrophages

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Cell invasion by *Trypanosoma cruzi* and its intracellular replication are essential for continuation of the parasite life cycle and for production of Chagas' disease. *T. cruzi* is able to replicate in nucleated cells and can be killed by activated macrophages. Gamma interferon (IFN-γ) is one of the major stimuli for the activation of macrophages and has been shown to be a key activation factor for the killing of intracellular parasites through a mechanism dependent upon nitric oxide (NO) biosynthesis. We show that although the addition of exogenous tumor necrosis factor alpha (TNF-α) does not potentiate the trypanocidal activity of IFN-γ in vitro, treatment of resistant C57BI/6 mice with an anti-TNF-α monoclonal antibody increased parasitemia and mortality. In addition, the anti-TNF-α-treated animals had decreased NO production, both in vivo and in vitro, suggesting an important role for TNF-α in controlling infection. In order to better understand the role of TNF-α in the macrophage-mediating killing of parasites, cultures of *T. cruzi*-infected macrophages were treated with an anti-TNF-α monoclonal antibody. IFN-γ-activated macrophages failed to kill intracellular parasites following treatment with 100 μg of anti-TNF-α. In these cultures, the number of parasites released at various time points after infection was significantly increased while NO production was significantly reduced. We conclude that IFN-γ-activated macrophages produce TNF-α after infection by *T. cruzi* and suggest that this cytokine plays a role in amplifying NO production and parasite killing.

The infection of inbred mice with trypomastigotes of *Trypanosoma cruzi*, the etiological agent of Chagas' disease, leads to an acute infection characterized by the presence of parasites in the blood and a severe immune depression mediated by T cells and macrophages which results in compromised T-cell functions, including help for antibody production (27) and cytotoxicity (24). The host resistance is dependent on a specific immune response that requires the combined efforts of a number of mechanisms including $CD4^+$ and $CD8^+$ T cells, antibody production, and NK cell activity (16, 28, 29, 34). The mechanism that controls parasite replication during the acute and chronic phases of infection in resistant mice and that maintains low but persistent numbers of circulating trypomastigotes during the chronic phase is not well understood. The parasite multiplies in many different mammalian cell types including primary resident macrophages. The outcome of the interaction of *T. cruzi* with macrophages may depend upon the level of activation of these cells.

Resistance to *T. cruzi* infection has been associated with the capacity of lymphocytes to generate gamma interferon (IFN-γ) which can in turn activate macrophages to kill the obligate intracellular amastigote form of the parasite in vivo (26) and in vitro (25, 37). The trypanocidal activity of IFN-γ-activated macrophages is mediated by nitric oxide (NO) generated from the guanidino nitrogen atom of L-arginine by an inducible NADPH-dependent enzyme, NO synthase (NOS). On the other hand, susceptibility to infection is associated with the production of interleukin 10 (10, 32) and transforming growth factor beta (33). The abilities of interleukin 10 and transforming growth factor beta to suppress microbicidal function have been positively correlated with the inhibition of nitrite generation by macrophages incubated with IFN-γ (9). In a previous report, we showed that the resistance to *T. cruzi* infection observed in C57BI/6 mice may result from their ability to produce adequate levels of NO. This conclusion was supported by the following observations: (i) the splenocytes from *T. cruzi*-infected C57BI/6 mice in the early phase of the infection released more NO than splenocytes from BALB/c mice, a strain which does not control the infection; (ii) the levels of nitrate plus nitrite in plasma in infected C57BI/6 mice were higher than those detected in BALB/c mice; and (iii) the infected C57BI/6 mice treated with NOS inhibitors became susceptible to infection. Additionally, the trypanocidal activity of IFN-γ-activated macrophages was inhibited by NOS inhibitors. Moreover, *S*-nitroso-acetyl-penicillamine, a NO donor, killed the trypanosomes in a dose-dependent manner (35).

Another cytokine that has been associated with macrophage microbicidal activity is tumor necrosis factor alpha (TNF-α) which provides a second signal to induce microbicidal activity in IFN-γ-activated macrophages by stimulating NO production (14, 17). However, the role of TNF-α in *T. cruzi* infections is controversial. Thus, the ability of recombinant TNF-α (rTNF-α) to induce macrophage trypanocidal activity (6) has not been confirmed by other investigators (37). Similarly, while Golden and Tarleton (11) have reported that TNF-α does not synergize with IFN-γ to enhance mac-

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rophage trypanocidal activity, a recent study has shown that TNF-α and IFN-γ do act synergistically to activate mouse macrophages to kill intracellular *T. cruzi* (21).

The purpose of this investigation was to examine the roles of TNF-α and IFN-γ in regulating NO production in *T. cruzi*-infected mice. Our results show that TNF-α and IFN-γ synergize to induce increased NO production in noninfected macrophages but not in *T. cruzi*-infected macrophages. We also report that IFN-γ-activated and infected macrophages produce TNF-α which affects their trypanocidal function via an autocrine pathway.

MATERIALS AND METHODS

Mice. Female C57BI/6 and BALB/c mice, 6 to 8 weeks old, were bred and maintained under standard conditions in the animal house of the Department of Immunology, University of São Paulo, Ribeirão Preto, Brazil.

Reagents. Recombinant murine IFN-γ (rMuIFN-γ) and rMuTNF-α were provided by Genentech Inc. (San Francisco, Calif.). Neutralizing anti-IFN-γ and anti-TNF-α were obtained from ascitic fluid samples collected from nude mice inoculated with rat hybridoma XMG 1.2 (provided by DNAX Corp., Palo Alto, Calif.) or XT 22.11 (provided by F. Y. Liew, Glasgow University, Glasgow, United Kingdom). The monoclonal antibodies (MAbs) were concentrated from ascitic fluid by ammonium sulfate precipitation, purified by chromatography on a Sepharose 4B-protein G column (Pharmacia Fine Chemicals, Uppsala, Sweden), dialyzed against phosphate-buffered saline, and stored at −70°C until use. The reagents and parasites utilized contained <0.05 ng of bacterial endotoxin per ml, as determined by the *Limulus* amoebocyte assay.

Infection and treatment with MAbs. The Y strain of *T. cruzi* was used in all experiments. For experiments in vitro, trypomastigotes were grown in and purified from rat myoblast cells (L6E9). C57BI/6 mice were infected intraperitoneally with 10^4 blood-derived trypomastigote forms. Parasitemia levels in 5-μl blood samples obtained from the tail vein were evaluated as previously described (19). Two hours before infection and again on days 3 and 5 of infection, C57BI/6 mice were injected intraperitoneally with 200 μg of an anti-IFN-γ or anti-TNF-α MAb. Control mice received 200 μg of normal rat immunoglobulin G (IgG). Parasitemia and mortality were determined during the acute phase of infection.

Spleen cell cultures. Spleen cells from noninfected or *T. cruzi*-infected C57BI/6 mice were washed in Hanks' medium and incubated for 4 min with lysing buffer (9 parts of 0.16 M ammonium chloride and 1 part of 0.17 M Tris [pH 7.65]). The erythrocyte-free cells were then washed three times and suspended to a final concentration of 5×10^6 cells per ml in RPMI 1640 (Flow Laboratories, Inc., McLean, Va.) supplemented with 5% fetal calf serum (Hyclone, Logan, Utah), 5×10^{-5} M 2-mercaptoethanol, 2 mM L-glutamine, and antibiotics. One milliliter of the cell suspension was distributed in each well of 24-well tissue culture plates (Corning, Corning, N.Y.) and incubated for 48 h at 37°C in a humidified 5% CO_2 incubator in the presence or absence of 20 μg of an anti-IFN-γ or anti-TNF-α MAb per well. Subsequently, the supernatants were harvested, filtered through 0.22-μm-pore-size membranes, and stored at 4°C until used for the nitrite assay.

Quantification of NO. Plasma samples from infected, resistant C57BL/6 mice were collected from the retro-orbital plexus 8 days after *T. cruzi* infection. Nitrate was reduced to nitrite with nitrate reductase as previously described (31), and the concentration of nitrite was determined by the Griess method. The nitrite concentration in the culture supernatants or in plasma was assayed in a microplate by mixing 0.1 ml of culture supernatant or plasma with 0.1 ml of Griess reagent (13). The A_{550} was read 10 min later, and the NO_2^- concentration was determined by reference to a standard curve of 1 to 100 μM $NaNO_2$.

NOS assay. Spleens from C57BI/6 mice which had or had not been treated with an anti-IFN-γ or anti-TNF-α MAb were collected on the eighth day after infection with *T. cruzi* and were immediately frozen at −70°C until processed for the NOS assay. NOS activity was determined by measuring the formation of L-[U-^{14}C]citrulline as previously described (30). The protein concentration in the samples was determined by the Coomassie blue binding method according to the manufacturer's recommendations (Pierce Chemical, Rockford, Ill.). NOS activity was expressed as picomoles of citrulline per milligram of protein per hour.

Microbicidal activity. Peritoneal macrophages were harvested from mice injected 3 days previously with 1 ml of 3% (wt/vol) sodium thioglycolate (Difco). The cells (10^6/ml) were plated onto 96- or 24-well plates and incubated overnight with rMuIFN-γ and/or rMuTNF-α (1 to 100 U/ml) in the presence or absence of 20 μg of an anti-TNF-α MAb per ml. Adherent cells were infected at a parasite/cell ratio of 4:1 for 90 min. Extracellular parasites were removed with six washes of RPMI 1640, and the cells were incubated at 37°C in 5% CO_2 with or without an anti-TNF-α MAb. The supernatants were harvested and assayed for their nitrate and TNF-α concentrations. The growth of parasites in the macrophages was evaluated by counting the trypomastigotes released at various times after the infection and by counting the intracellular amastigote forms as previously described (35).

TNF-α assay. Purified anti-MuTNF-α MAb and rabbit anti-MuTNF-α polyclonal sera were used to measure the TNF-α levels in culture supernatants by a two-site sandwich enzyme-linked immunosorbent assay performed as previously described (5). Briefly, microtiter plates were coated overnight at 4°C with an anti-TNF-α MAb (1 μg per well). The samples and standard rMuTNF-α were added in duplicate and incubated at room temperature for 2 h and then for 1 h with a 1/1000 dilution of a rabbit anti-MuTNF-α antibody. Alkaline phosphatase-conjugated goat anti-rabbit IgG antibody, diluted 1,000 times, was added and incubated for 1 h at room temperature.

Finally, *p*-nitrophenyl phosphate substrate was added, and the plates were read at 405 nm. The results were expressed as units of TNF-α per milliliter by comparing the optical density with a standard curve prepared using rMuTNF-α.

Statistical analysis. The results are expressed as the means ± standard errors of the mean. The 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 to indicate statistical significance.

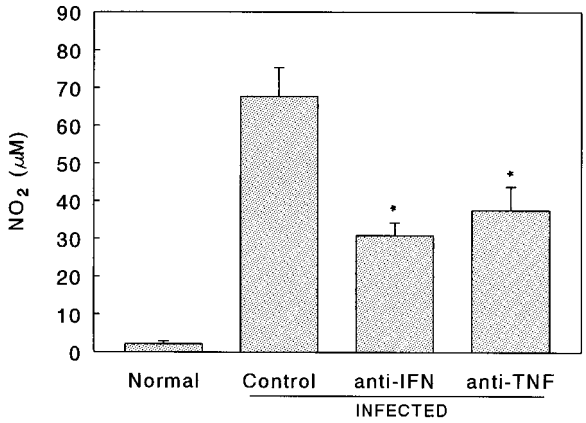

FIG. 1. Anti-TNF-α and anti-IFN-γ inhibit nitrite release by splenocytes from *T. cruzi*-infected mice. Splenocytes (5×10^6 cells per ml) from noninfected (normal) or infected C57BI/6 mice (8 days after infection) were harvested and cultured for 48 h in the presence or absence of 20 μg of an anti-TNF-α or anti-IFN-γ MAb per ml. The NO_2 released into the supernatants was determined by the Griess method. Each column represents the mean ± standard error of the mean of triplicate cultures obtained from three different mice and is representative of three independent experiments. Values that are significantly different ($P < 0.05$ by the Kruskal-Wallis test) from the value for the control mice (medium alone) are indicated by an asterisk.

RESULTS

IFN-γ and TNF-α modulate NO_2^- production by spleen cells from *T. cruzi*-infected mice. We have previously reported (35) that splenocytes from *T. cruzi*-infected C57BI/6 mice produce elevated amounts of NO in vitro, peaking on the eighth day of infection. In the present experiments, we examined whether NO production by splenocytes obtained from mice on day 8 after *T. cruzi* infection could be modulated by TNF-α and IFN-γ. We found that the addition of anti-TNF-α and anti-IFN-γ MAbs to splenocyte cultures resulted in significant inhibition by 45 and 55%, respectively, of the NO_2^- produced (Fig. 1).

IFN-γ and TNF-α mediate resistance in *T. cruzi*-infected mice. In order to investigate the role of TNF-α in mediating resistance to *T. cruzi*, we determined the parasitemia and mortality in infected resistant C57BI/6 mice treated with an anti-TNF-α or anti-IFN-γ MAb. Treatment with either MAb significantly increased the parasitemia levels on days 8 and 9 after infection (Fig. 2A). More dramatically, resistant mice were rendered susceptible to acute *T. cruzi* infection after treatment with both MAbs. While the control resistant mice did not die during the infection, the anti-TNF-α- and anti-IFN-γ-treated groups showed 100 and 80% mortality, respectively, by day 17 after infection (Fig. 2B).

We also found that the increased parasitemia and mortality

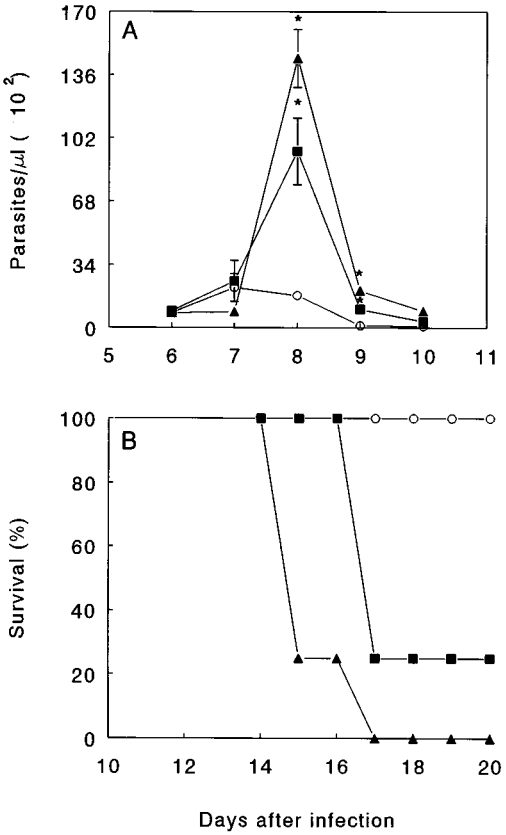

FIG. 2. TNF-α and IFN-γ confer resistance to *T. cruzi* infection in mice. C56BI/6 mice were treated with normal rat IgG (○) or anti-TNF-α (▲), or anti-IFN-γ (■) MAb on days 0, 3, 5, and 7 after *T. cruzi* infection, and the parasitemia (A) and survival (B) were determined. Values that are significantly different ($P < 0.05$ by the Kruskal-Wallis test) from the value for infected mice treated with normal rat IgG are indicated by an asterisk. The results shown (means ± standard errors of the means) are representative of two independent experiments, using five mice each.

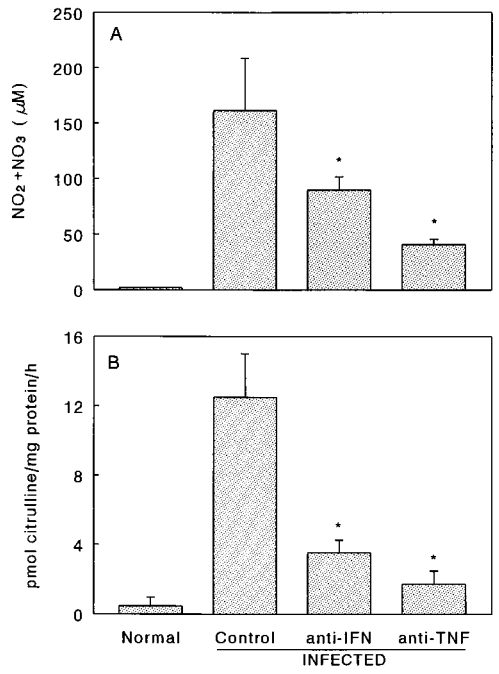

FIG. 3. TNF-α and IFN-γ modulate NO production in *T. cruzi*-infected mice. C57BI/6 mice were infected and treated with MAbs or normal rat IgG (control) as described in the legend to Fig. 2. The levels of NO_2^- plus NO_3^- (A) and the activity of NOS (B) were determined in the plasma and spleens, respectively, 8 days after infection. Each column is the mean ± standard error of the mean obtained from five mice per group. Values that are significantly different ($P < 0.05$ by the Kruskal-Wallis test) from the value for infected control mice are indicated by an asterisk.

induced by treatment of infected mice with an anti-IFN-γ or anti-TNF-α MAb were accompanied by significant reductions in the concentrations of the end metabolites of NO (nitrite plus nitrate) in the plasma of these animals (Fig. 3A). In addition, the expression of inducible NOS was increased in the spleens obtained from mice on the eighth day of infection, whereas treatment with anti-TNF-α and anti-IFN-γ MAbs markedly reduced NOS activity (Fig. 3B).

The trypanocidal effect of IFN-γ-activated macrophages is dependent on TNF-α. In an effort to understand the mechanisms by which TNF-α and IFN-γ modulate the intracellular replication of *T. cruzi*, we tested the abilities of these cytokines to induce trypanocidal activity in mouse peritoneal macrophages and correlated this activity with the amount of NO produced. As previously described, IFN-γ-activated macrophages significantly inhibit the intracellular replication of the parasite and this inhibition is correlated with the levels of NO produced. On the other hand, TNF-α alone does not activate macrophages to kill the intracellular parasites nor does it induce NO biosynthesis. However, TNF-α potentiates IFN-γ-induced NO production in normal macrophages. Interestingly, although IFN-γ alone did not induce TNF-α production, the addition of trypomastigotes to IFN-γ-stimulated macrophages resulted in marked increases in the amounts of both NO and TNF-α produced by these cells (Table 1). In contrast, even in a full titration experiment, the addition of exogenous TNF-α to IFN-γ-activated and infected macrophages did not potentiate the IFN-γ-induced trypanocidal effects or NO production (Fig. 4).

In order to determine whether the trypanocidal activity of IFN-γ was due to an autocrine effect involving the release of TNF-α from *T. cruzi*-infected macrophages that subsequently served as a trigger to stimulate NO byosynthesis, we neutral-

TABLE 1. Endogenous TNF-α is produced by IFN-γ-activated and infected macrophages and exogenous TNF-α potentiates the IFN-γ effects only in noninfected macrophages[a]

Macrophage treatment	No. of parasites/μl[b]	TNF-α concn (U/ml)	Amt (μM) of NO_2
No parasites added			
None		<0.5	3.3 ± 0.6
IFN-γ		1.0 ± 0.1	13.8 ± 1.2
TNF-α		NA[c]	1.0 ± 0.2
IFN-γ + TNF-α		NA	21.6 ± 1.6
Parasites added			
None	3,520 ± 120	0.9 ± 0.1	7.0 ± 2.0
IFN-γ	53 ± 10	10.2 ± 0.4	43.8 ± 1.1
TNF-α	3,850 ± 60	NA	15.3 ± 1.3
IFN-γ + TNF-α	40 ± 8.1	NA	61.7 ± 3.5

[a] Peritoneal macrophages (10^6/well) were treated with IFN-γ (10 U/ml) and/or TNF-α (10 U/ml) and were or were not infected with *T. cruzi*. The results are expressed as the means ± standard errors of the means for three replicate cultures and are representative of two independent experiments.
[b] Extracellular parasites were counted 4 days after infection.
[c] NA, not applicable.

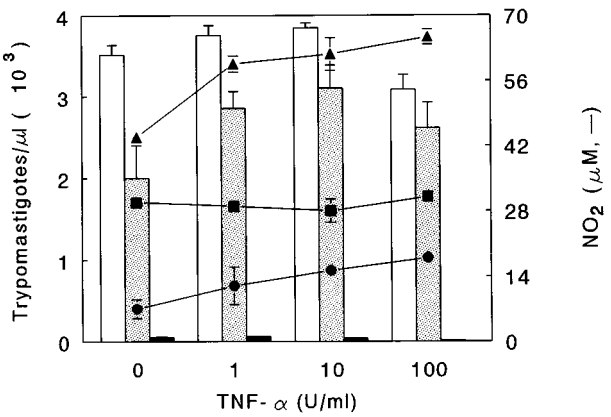

FIG. 4. Exogenous TNF-α does not potentiate the release of NO or the trypanocidal activity of IFN-γ-activated and infected macrophages. Thioglycolate-elicited macrophages were plated onto 96-well plates and incubated for 24 h with TNF-α (0 to 100 U/ml) alone (●, white bars) or with 1 (■, stippled bars) and 10 (▲, black bars) U of rMuIFN-γ per ml followed by the addition of *T. cruzi*. The extracellular parasites were removed, and the supernatants were harvested 48 h later for determination of NO_2 (lines). The trypomastigotes (bars) were counted in the supernatants 5 days after infection. Points and bars represent the means ± standard errors of the means and are representative of two independent experiments.

ized the TNF-α activity with an anti-TNF-α MAb. The presence of anti-TNF-α MAb in the cultures markedly inhibited both the IFN-γ-induced trypanocidal activity and NO production of the macrophages (Fig. 4). The results were similar when either the number of amastigotes in the macrophages (Fig. 5A) or the number of parasites released into the culture medium (Fig. 5B) was counted.

DISCUSSION

It is now generally accepted that NO or related nitrogen oxides produced by activated macrophages have an important role in the killing of different pathogens. In in vitro experiments, it has been shown that NO derived from activated macrophages is cytostatic or cytotoxic for a variety of pathogens including *Leishmania major* (2), *Mycobacterium bovis* (8), *Toxoplasma gondii* (1), *Schistosoma mansoni* (15), *Cryptococcus neoformans* (12), *Trypanosoma musculi* (36), and *T. cruzi* (9). In infected macrophages, NOS is inducible by cytokines such as IFN-γ (7), whereas TNF-α acts in an autocrine fashion to amplify the actual synthesis and release of NO by IFN-γ-primed macrophages (14, 17). Although it is well-known that IFN-γ-induced macrophage trypanocidal activity is mediated by NO (9, 35), the role of TNF-α in this activity is controversial (3, 6, 11, 21, 38). On the basis of the results of the present study, we suggest that TNF-α plays an essential role in such IFN-γ-induced trypanocidal activity of mouse macrophages by regulating the production of reactive nitrogen intermediate by these cells. First, we have shown in in vitro experiments that the spontaneous synthesis of NO by splenocytes from *T. cruzi*-infected mice is dramatically inhibited following the neutralization of TNF-α or IFN-γ, suggesting that these cytokines are involved in the modulation of NO production. These observations agree with the previously reported ability of spleen cells from C57BI/6 mice to produce detectable IFN-γ during the acute phase of infection in the absence of any stimulation (23, 32). Live parasites by themselves stimulated the production of TNF-α and low levels of NO which, however, had no protective action (Table 1). Second, the treatment of infected mice with an anti-TNF-α or anti-IFN-γ MAb increased the parasitemia and mortality of the animals (Fig. 2). These responses were correlated with significant reductions in the concentrations of NO_3^- in the plasma and of NOS activity in the spleens of *T. cruzi*-infected mice. A reduction in the NOS activity of the spleens of endotoxemic mice treated with anti-TNF-α or anti-IFN-γ has previously been reported (4). Probably as a result of stimulation by IFN-γ (23, 32) and endogenously produced TNF-α, splenocytes obtained from mice during the acute phase of *T. cruzi* infection are known to express inducible NOS mRNA (23) that, as we showed in Fig. 3, is subsequently translated into protein. This is important since the major control point in the regulation of inducible NOS is the stability of its mRNA that may be destabilized by TGF-β (22), which is increased during infection (33). Furthermore, the levels of NOS may be regulated at a posttranslational step via a mechanism that is dependent on the cytokine-induced synthesis of tetrahydrobiopterin (22).

Previous work has shown that treating macrophages with TNF-α alone inhibits the in vitro replication of *T. cruzi* in these cells (6). However, in our experiments (Table 1), TNF-α alone was unable to clear the parasites from macrophages and accordingly did not induce NO production. Also, exogenous rMuTNF-α did not synergize with IFN-γ to increase macrophage killing of *T. cruzi*. These results differ from our data obtained in in vivo experiments which showed that TNF-α is important in the resistance of mice to *T. cruzi* infections, since the treatment of infected animals with anti-TNF-α reduced

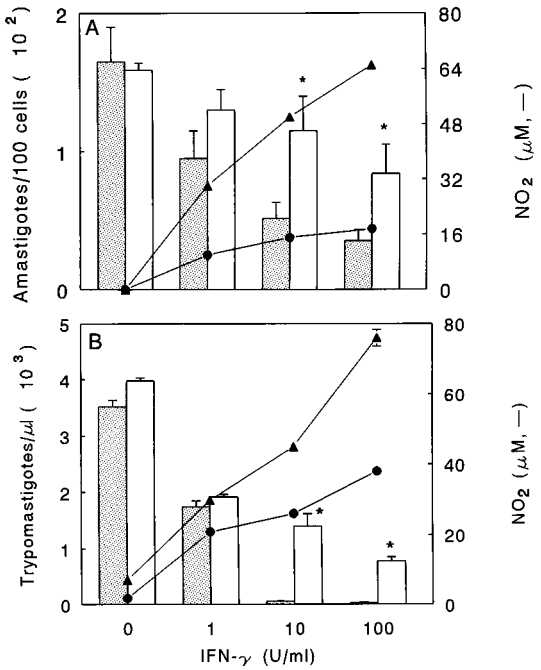

FIG. 5. IFN-γ-induced trypanosomal activity is dependent on TNF-α. Thioglycolate-elicited macrophages were plated onto 13-mm^2 round glass coverslips (A) or 96-well plates (B) and incubated for 24 h with rMuIFN-γ, with or without 100 μg of anti-TNF-α MAb per ml followed by the addition of *T. cruzi*. The extracellular parasites were removed, and the cells were incubated with (●, white bars) or without (▲, stippled bars) an anti-TNF-α MAb, and the supernatants were harvested 48 h later for determination of NO_2 (lines). The amastigotes (A) and trypomastigotes (B) were counted on coverslips fixed 48 h after incubation or in the supernatants 5 days after infection, respectively (bars). Points and bars represent the means ± standard errors of the means and are representative of three independent experiments. Values that are significantly different ($P < 0.05$ by the Mann-Whitney test) from the value for cells given IFN-γ and no anti-TNF-α MAb are indicated by an asterisk.

NO production and increased parasitemia and mortality (Fig. 2). To clarify this discrepancy and to further establish the possible role of endogenous macrophage-derived TNF-α in the control of intracellular parasite multiplication, the endogenous TNF-α produced in vitro by these cells was neutralized. These experiments revealed that both NO_2^- production and intracellular killing by IFN-γ-activated macrophages were significantly decreased when TNF-α was neutralized (Fig. 5). In a similar manner, an anti-TNF-α MAb attenuated the microbicidal activity of macrophages infected with *L. major* (14) and *T. gondii* (17). Otherwise, the administration of rTNF-α to mice infected with *T. cruzi* resulted in a significantly more rapid mortality (3). The exacerbating effect of such an administration during infection may involve a mechanism of TNF-α-induced acute endotoxic shock, particularly since *T. cruzi* possesses a lipopolysaccaride-like molecule (18) that could potentiate the TNF-α effects.

The infection of mouse peritoneal macrophages with *T. cruzi* in vitro resulted in the production of small amounts of TNF-α that had no significant effect on the induction of NO production or intracellular parasite killing. The addition of exogenous TNF-α also has no effect on the intracellular parasite multiplication, apparently because this cytokine does not activate macrophages or induce production of NO (11, 21), which has been reported to be the major effector molecule in the inhibition of intracellular *T. cruzi* multiplication (9, 35). In our experiments, the TNF-α and NO levels increased dramatically when IFN-γ-activated macrophages were infected with *T. cruzi* (Table 1), suggesting that the parasite acts as a second signal to induce TNF-α production. These data may explain why *T. cruzi* induces NO production in IFN-γ-activated and infected macrophages (Table 1) (20). It is likely that exogenous TNF-α did not potentiate IFN-γ-induced parasite killing, because the endogenous TNF-α concentrations were already sufficient to induce complete macrophage activation. Once again, the addition of exogenous TNF-α to these cells did not significantly increase the trypanocidal activity or the level of NO biosynthesis.

Taken together, these data suggest that TNF-α synthesized by macrophages acts in an autocrine fashion to initiate L-arginine metabolism in the presence of IFN-γ and that endogenous TNF-α has an important role in the killing of *T. cruzi* by IFN-γ-activated macrophages.

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