Trypanosoma cruzi: Tc52 Released Protein-Induced Increased Expression of Nitric Oxide Synthase and Nitric Oxide Production by Macrophages

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Trypanosoma cruzi: Tc52 Released Protein-Induced Increased Expression of Nitric Oxide Synthase and Nitric Oxide Production by Macrophages

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Trypanosoma cruzi target molecules that might regulate the host immune responses have not yet been fully identified. In the present study, we demonstrate that the parasite-released molecule (Tc52) was able to synergize with IFN-γ to stimulate nitric oxide production by macrophages. This synergistic effect was also observed at the level of inducible nitric oxide synthase gene expression. Furthermore, Tc52 was also shown to induce gene expression for IL-1α, IL-12, and IL-10. Moreover, the combination of Tc52 and IFN-γ down-regulates IL-10 gene expression, but not IL-12. Isotype profiles and Tc52 or anti-CD3-induced T cell proliferation were also analyzed, indicating that active immunization with Tc52 partially relieves the immunosuppression observed during the acute phase of the disease. Moreover, under conditions of experimental infection, the Tc52 appears immunologically silent, failing to elicit Ab response and lymphocyte proliferation during the initial acute phase infection. Following active immunization, Tc52 was capable of stimulating T cell proliferation and Ab production with a predominance of IgG1, IgG2a, IgG2b, IgG3, and to a lesser extent IgA. Taken together, these results demonstrate that T. cruzi Tc52-released Ag could be involved in the immunoregulatory processes. The immune response against Tc52 that appears late in the T. cruzi infection may play a role in the modulation of its biological function(s). The Journal of Immunology, 1998, 160: 3471–3479.
murine T cell proliferation in vitro (13, 14). The inhibition could be partially reversed by the addition of exogenous glutathione or cysteine. Moreover, these two components could partially restore the responsiveness of lymphocytes from T. cruzi-infected mice. These results prompt us to suggest that Tc52 may be a scavenger of free and macrophage-released cysteine as well as glutathione in vitro and in vivo. However, recent data demonstrated that exogenous NO diffusing into the extracellular environment may interact with cysteine or glutathione to form nitrosothiol groups, and this could be another mechanism involved in glutathione and cysteine depletion (15).

Inasmuch as NO synthesis is well defined in the murine macrophage system, we decided to determine whether Tc52 could modulate NO production by macrophages and if such activity could account for Tc52-mediated immunosuppression. In the present study, we have combined molecular and biologic approaches to demonstrate that, indeed, Tc52 synergized with IFN-γ for increased expression of iNOS mRNA and production of NO by macrophages. An additional unexpected effect of Tc52 was the induction of genes encoding the following cytokines: IL-1α, IL-12, and IL-10. Furthermore, the combination of Tc52 and IFN-γ had a negative effect on the IL-10 gene, but not the IL-12, suggesting therefore that Tc52 exerts a regulatory influence on inflammatory and immune responses and, thus, may play a role in T. cruzi-associated immunosuppression.

Materials and Methods

Reagents

RPMI 1640 and FCS were purchased from Life Technologies (New York, NY). The N°4-monomethyl-L-arginine (L-NMA), LPS from Escherichia coli (0.111:B4), and o-phenylenediamine dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO). The mouse rIFN-γ was obtained from Genzyme Diagnostic (Cambridge, MA). Fluorescein-conjugated goat anti-rabbit IgG was purchased from Diagnostics Pasteur (Marnes la Coquette, France). Nonlabeled and peroxidase-labeled Abs to goat IgG subclass determination and in vivo. However, recent data demonstrated that exogenous NO diffusing into the extracellular environment may interact with cysteine or glutathione to form nitrosothiol groups, and this could be another mechanism involved in glutathione and cysteine depletion (15).

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Animals

Male BALB/c, C57BL/6, and OF1 mice (5 to 6 wk old) were bred and maintained in a clean conventional colony at Pasteur Institute (Lille, France).

Macrophage cultures

Peritoneal macrophages were obtained from BALB/c mice 3 days after i.p. injection of 1.5 ml of 4% thioglycollate. Briefly, peritoneal cells were collected by sterile lavage with RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 2 mM sodium pyruvate, 100 U/ml penicillin, and 1% preservative-free heparin. The cells were washed, re-suspended in culture medium consisting of RPMI 1640 supplemented with heat-inactivated FCS and antibiotics, and plated in 24-well flat-bottom plates at 5 × 10^5/well. After 2-h incubation at 37°C, the cells were washed to remove nonadherent cells, filled with 600 μl of culture medium, and incubated for 48 h at 37°C in humidified 5% CO2 incubator. Before starting the experimental protocol, the wells were washed twice with medium to remove the remaining nonadherent cells. The adherent cells comprised 65% of the plated cells and were >92% macrophages by Wright and non-specific esterase staining or phagocytosis of opsonized SRBC.

Mouse macrophage cell line J774 obtained from American Culture Collection (Rockville, MD) was cultured in RPMI 1640 complete medium and incubated in 24-well tissue culture plates at 2.5 × 10^5 cells/well in 600 μl culture medium for 48 h at 37°C. Cells were cultured with or without the following reagents: Tc52 (5 μg/ml), LPS (10 ng/ml), or IFN-γ (100 U/ml). These concentrations were determined to result in significantly detectable levels of NO production by macrophages. In some experiments, Tc52 (5 μg/ml) or LPS (10 ng/ml) was added to the cells in the presence of IFN-γ (100 U/ml). Cells were also treated with either a combination of different doses of Tc52 (5 to 50 μg/ml) and a constant amount of IFN-γ (100 U/ml), or different concentrations of IFN-γ (1 to 100 U/ml) and a defined quantity of Tc52 (5 μg/ml). In control tests, cells were treated with the above reagents in the presence of 0.25 mM 1-NMA. Additional controls consisted of macrophages treated with either a T. cruzi protein named Tc24 (11) or BSA at a concentration of 50 μg/ml in the presence or absence of IFN-γ (100 U/ml).

NO measurement

Nitrite production was determined in culture supernatants using the Griess reaction with NaNO2 as standard (16). The lower limit of detection of the assay was 2 μM.

RNA isolation and reverse transcription

Total RNA was isolated using RNA Plus system from BALB/c macrophage cultures submitted to different treatments over 8 h. One microgram of RNA was reverse transcribed to cDNA with an oligonucleotide (poly(dT)16) using the SuperScript II reverse transcriptase, and the cDNA used as a template for PCR. As an internal control, a housekeeping gene, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript, was amplified.

PCR amplifications

PCR amplifications were performed using 1 μl of reverse-transcription products and 20 pmol of each primer, using Gold tag. Each PCR cycle consisted of a denaturation step (94°C, 1 min), an annealing step (55°C, 1 min), and an elongation step (72°C, 1 min). For the last cycle, the elongation step was extended to 7 min at 72°C. DNA was amplified for 35 cycles in a Perkin-Elmer Cetus thermocycler. Specific amplification of iNOS and the different cytokine cDNA was achieved using the following primers: iNOS sense, 5'-CACTCATCCTGGTACGGTCT-3'; iNOS antisense, 5'-GGCTAACACATCTGGGAAAGT-3'; IL-1 sense, 5'-CTCTAGACCCATGTACGAC-3'; IL-1 antisense, 5'-TGGAATCCGGGAAAACACGT-3'; TNF-α sense, 5'-AGCCCACTTGCGTACAAACACAC-3'; TNF-α antisense, 5'-ACACATGTCCTCCTCAGAAG-3'; IL-10 sense, 5'-GCTCTGACCTGCTATGCTGC-3'; IL-10 antisense, 5'-GGAGGATACACCTGGCACAACAGG-3'; IL-12p40 sense, 5'-GGGGAGTACCACCTGCCACAAAGGAGG-3'; IL-12p40 antisense, 5'-CCTCATTGCGACCGTCAAGGCAAC-3'. GAPDH was used as internal control: GAPDH sense, 5'-GTCTGTACCACTCATGAG-3'; GAPDH antisense, 5'-CCAAAGTTGTACTGATGAGCAAC-3'. PCR products were analyzed on 1.5% agarose gel and visualized with ethidium bromide.

Tc52-binding studies

Suspensions of 1 × 10^6 of J774 macrophage cell line or splenic macrophages, prepared from normal BALB/c mice as previously (11), were washed three times in RPMI 1640 at 4°C and incubated 30 min at 4°C with normal mouse serum diluted 1/50 to block FcR. After washing, the cells were incubated for 1 h at 4°C with Tc52 (5 μg/ml) in RPMI 1640. After three washes in HBSS, the cells were incubated with rabbit anti-Tc52 Abs diluted in HBSS for 1 h at 4°C, washed three times with HBSS, and treated with fluorescein-conjugated goat anti-rabbit IgG diluted 1/100 in HBSS for 30 min at 4°C. After two washes, labeled cells were fixed with 1% paraformaldehyde and analyzed for surface fluorescence using an Orthocyt-offuorograph 50 M (OrthoDiagnostic Systems, Westwood, MA).

Immunization and infection

Five-week-old male BALB/c mice (n = 24) were inoculated three times i.p. at 2-wk intervals with three doses of 50 μg of Tc52 in association with 30 μl of Bordetella pertussis and Alum (BpAl) as adjuvant (VAXICOQ, Institut Merieux, Lyon, France; 4 IU B. pertussis and 1.25 mg aluminum hydroxide/500 μl). Control groups corresponded to untreated mice (n = 24) and mice immunized with BpAl alone (n = 24). Two weeks after the last immunization, mice were infected i.p. with 10^7 bloodstream trypomastigotes (Y strain), as described in previous reports (11, 17).

IgG subclass determination

This was performed as reported in previous studies (11, 17). Briefly, flexible polystyrene microtitration plate (Dynatech Labs., Alexandria, VA) wells were coated with Tc52 at concentration of 5 μg/ml in carbonate
buffer, pH 9.6, and incubated overnight at 4°C. The unbound Ag was discarded and plates were blocked with 200 μl of PBS, containing 1% BSA and 0.05% Tween-20 for 2 h at 37°C. Further washing steps were conducted, and the following peroxidase-labeled rabbit Abs to mouse Ig subclasses were added: anti-IgG1 (1:2000), anti-IgG2a (1:500), anti-IgG2b (1:1000), and anti-IgG3 (1:500). All of the Abs were diluted in PBS/0.05% Tween-20 and incubated for 1 h at 37°C. After five washes, 100 μl o-phenylenediamine dihydrochloride was added as substrate and the reaction was allowed to proceed for 20 min at 37°C before being stopped with 1 N HCl. The absorbance was read at 492 nm by an ELISA reader.

**Detection of specific IgE Abs**

The specific IgE Abs were detected using an RIA technique. Tubes (NUNC, Roskilde, Denmark) were coated overnight at 4°C with 5 μg/ml Tc52 Ag in 0.015 M Na2CO3/0.035 M NaHCO3 buffer, pH 9.6. Tubes were then saturated for 2 h at room temperature with PBS containing 2% BSA, and incubated overnight at 4°C with 250 μl of diluted (1/5) mouse anti-Tc52 immune serum. After washing with 0.1% PBS/Tween-20, tubes were reacted with 20 ng of 125I-labeled monoclonal anti-mouse IgE (sp. act. 10^6 cpm/μg) overnight at 4°C. The tubes were then washed three times with PBS/Tween and counted in a Packard (Downers Grove, IL) gamma counter.

**T cell proliferation assay**

Spleens were removed aseptically from anesthetized BALB/c mice, and spleen cell suspensions were prepared as described (14). Cultures of spleen cells were set up in triplicate in microculture plates (Nuclon, Roskilde, Denmark), as follows: cell suspension (1 × 10^5 in RPMI 1640 medium, containing 10% heat-inactivated FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 U/ml gentamicin) was cultured with or without 10 μl (0.1 μg/ml) rat mAb to mouse CD3 and with or without Tc52 native protein (an optimal dose of 5 μg/ml was used throughout the experiments) in a total volume of 200 μl culture medium. After culture for 3 days at 37°C under humidified atmosphere of 5% CO2 in air, 0.5 μCi [methyl-3H]TdR per well was added. Sixteen hours later, cells were harvested using a multiple automated cell harvester onto filters, dried, and placed in scintillation fluid and counted in a scintillation counter.

**Statistical analysis**

Statistical significance was analyzed by paired Student’s t test.

**Results**

**Tc52 synergize with IFN-γ to induce increased production of NO by macrophages**

Previous studies have shown that the degree of resistance to T. cruzi varied among mice strains and also depends on the parasite genotype (18). Therefore, we have compared the effect of Tc52 on macrophages from different origins. As shown in Figure 1, Tc52 alone had no effect on NO production by macrophages from BALB/c, OF1, or BALB/c mice. In contrast, the levels of NO produced by mice macrophages activated with Tc52 protein (5 μg/ml) in the presence of IFN-γ (100 U/ml) were either
IFN-γ (100 U/ml) plus LPS (10 ng/ml). Under our experimental conditions, neither IFN-γ (100 U/ml) nor LPS (10 ng/ml) alone had the capacity to activate the production of NO by macrophages. It is known that IFN-γ on its own is a relatively poor stimulator of NO production by macrophages, and the addition of a second signal can significantly enhance NO production (16). Moreover, the timing and quantity of LPS stimulation are important for effective NO production (19–21). Furthermore, a variety of mouse strains required LPS triggering of IFN-γ-primed macrophages to render them tumoricidal (22). Taken together, these observations would account for the poor activity of IFN-γ or LPS alone in our experiments. As expected, in all cases, LPS plus IFN-γ induced increased production of NO by macrophages from different origins, although macrophages from BALB/c mice were found to produce significantly lower levels of NO compared with those from C57BL/6 mice. Interestingly, the addition of 250 µM L-NMMA to 500 g constant amount of IFN-γ increased production of L-NMMA to 30 to 50% reduction of the NO production compared with that observed in the absence of L-NMMA. Increasing the concentrations of L-NMMA to 500 µM resulted in more than 70% decrease of NO production (data not shown). It is noteworthy that all Tc52 preparations used in our studies contained less than 0.01 ng/ml of endotoxin, as determined by the Limulus amebocyte assay.

In the following experiments, NO production was measured in cell culture supernatants of macrophages from different origins treated with different doses of Tc52 (5 µg to 50 µg/ml) and a constant amount of IFN-γ (100 U/ml) or different concentrations of IFN-γ (1 to 100 U/ml) and a defined quantity of Tc52 (5 µg/ml). The results obtained showed that Tc52 alone had no significant effect on macrophage NO production, but synergized in a dose-dependent fashion with IFN-γ to mediate NO production. This synergistic effect was evident in the case of macrophages from BALB/c and OF1 mice as well as the J774 cell line (data not shown). When increasing concentrations of IFN-γ (1 to 100 U/ml) in the presence of a constant amount of Tc52 (5 µg/ml) were used, NO production reached a plateau with 10 U/ml IFN-γ. Thus, although different profiles of NO production by macrophages could be observed, a synergistic effect of Tc52 and IFN-γ for NO production in a dose-dependent manner was always evidenced. Furthermore, we examined the effect of two control proteins (BSA and T. cruzi Tc24 protein) in the presence or absence of 100 U/ml IFN-γ on NO production by macrophages. The results obtained showed no significant increase of NO production (the mean values obtained varied between 5±2 and 10±4 µM NO), and were comparable with those obtained with IFN-γ, LPS, or Tc52 alone, p > 0.5). Therefore, these results clearly indicated that Tc52 can synergize with IFN-γ to induce NO synthesis.

**Tc52 induces macrophage iNOS gene expression**

Having found the up-regulation of macrophage NO secretion upon incubation with Tc52 and IFN-γ, it was of interest to investigate whether the macrophage iNOS gene expression is also affected by Tc52 and IFN-γ treatment. As shown in Figure 2, a strong positive iNOS signal was seen in the case of macrophages treated with Tc52 and IFN-γ. Weak positive signals were observed in the case of macrophages treated with LPS alone or LPS together with IFN-γ. In addition, IFN-γ alone had no effect on iNOS gene expression. Control tests showing similar signals for GAPDH gene transcript in all treated and untreated macrophage cultures confirmed that mRNA isolated from cells was intact.

**Complementary experiments**

To further examine the effect of Tc52 and IFN-γ on iNOS expression, RT-PCR analysis of iNOS and cytokine mRNA in macrophages was performed as described in Materials and Methods. One microgram of total RNA from control (non-treated) or from macrophages treated with either of the following reagents, Tc52 (5 µg/ml), LPS (10 ng/ml), IFN-γ (100 U/ml), Tc52 plus IFN-γ (5 µg/ml, 100 U/ml, respectively), or LPS plus IFN-γ (10 ng/ml, 100 U/ml, respectively), was reverse transcribed to cDNA. The cDNA sample was used as a template for 35 cycles of PCR using primers for iNOS, IL-1α, IL-10, TNF-α, and IL-12. GAPDH was used as control housekeeping gene to check the homogeneity of different samples.

**Tc52 induces cytokine gene expression**

Complementary experiments were performed to examine whether Tc52 could modulate the level of macrophage cytokine gene expression. Surprisingly, Tc52 (5 µg/ml) induced the expression of genes encoding IL-1α, IL-10, and IL-12, whereas LPS alone had no effect, indicating therefore that the Tc52 activity was not due to LPS contaminants. Furthermore, IFN-γ alone could stimulate IL-1α and IL-12 gene expression. Interestingly, while Tc52 alone induced increased IL-10 gene expression, the combination of Tc52 and IFN-γ had the opposite effect (Fig. 2). The signal intensities obtained for TNF-α gene transcripts were of the same order for treated and nontreated cells.

**Binding of Tc52 to macrophages**

We attempted to determine whether Tc52 could be bound to the macrophage surface. As shown in Figure 3, the FACS analysis revealed that the percentage of Tc52-positive cells was about 78% after incubation with Tc52 for 1 h at 4°C and multiple washings. Control tests in which cells were treated with medium alone showed minimal fluorescence (12% positive cells). These observations suggested the presence of Tc52 binding sites at the macrophage surface. Experiments done with J774 cells gave similar results (data not shown). Further studies will be needed to determine whether the Tc52 binding to macrophages involved a specific receptor-ligand interaction.

**Active immunization with Tc52 partially relieved immunosuppression**

To further define the relationship between Tc52 and immunosuppression of T cell function, we conducted immunization experiments in BALB/c mice and followed the capacity of T cells to respond to anti-CD3 stimulation. Mice were immunized with three doses of purified native Tc52, starting 6 wk before T. cruzi infection. Three mice were sacrificed at different times and their spleen cells were pooled for in vitro experiments. Figure 4 illustrates the effects of Tc52 on anti-CD3-induced proliferation indexes of T
cells from immunized and infected mice compared with those observed in the case of T cells from the following groups: 1) non-immunized and infected mice; 2) adjuvant alone immunized and infected mice, and 3) normal mice. On day 18, both mice that had received adjuvant alone and the nonimmunized mice showed suppressed anti-CD3 mAb-induced T cell proliferation, whereas the group that had received three doses of Tc52 showed enhanced responses, although lower than the normal mice. Similar results were obtained when using the mitogen Con A in the proliferation assay (data not shown). These observations indicated that immunization with Tc52 markedly prevented the immunosuppression observed during T. cruzi acute phase infection.

Production of anti-Tc52 Abs during experimental infection and after active immunization and subsequent challenge infection

These studies were conducted to determine the antigenicity of Tc52 during experimental T. cruzi infection as well as after immunization followed by challenge infection. Sera collected from mice on various days before and after infection were examined for anti-Tc52 Abs by ELISA to determine the nature and the levels of anti-Tc52 isotype production. As shown in Figure 5, sera from adjuvant-immunized and nonimmunized and infected mice exhibited low levels of IgM, IgA, and IgE Abs recognizing Tc52. Moderate levels of IgG2a, IgG2b, and IgG3 were produced during the early acute phase of T. cruzi infection. Moreover, the production of IgG1 Abs to Tc52 was observed only after 21 days postinfection. In contrast, Tc52-immune mice showed higher levels of total anti-Tc52 Abs than adjuvant-immunized or nonimmunized mice, and this is true at later time points as well. Isotype and IgG subclass analysis showed the production of IgG1, IgG2a, IgG2b, IgG3, IgA, and only moderate levels of IgM. The IgE-specific Ab response was also examined. The results showed that immunization with Tc52 Ag induced a weak IgE response. However, a transient enhancement of IgE levels in sera from T52-immunized mice was observed upon infection with T. cruzi when compared with the control groups. Thus, immunization with Tc52 Ag leads to very high levels of Ab with a relative prominence of the IgG subclasses: IgG1, IgG2a, IgG2b, and to a lesser extent IgG3.

**FIGURE 3.** The panel shows the fluorescence intensity vs relative cell number, obtained using BALB/c mouse peritoneal macrophages incubated at 4°C with Tc52 Ag and treated with rabbit anti-Tc52 Abs, followed by fluorescein-conjugated goat anti-rabbit Ig (right peak, 78% fluorescent cells). The left peak represents cells that were incubated in the absence of Tc52 Ag and treated with the Abs as above (12% fluorescent cells).

**FIGURE 4.** Kinetic of splenic cell proliferation upon stimulation with anti-CD3 mAb. Three groups of mice were used: 1) mice immunized with Tc52 plus adjuvant; 2) mice immunized with adjuvant alone; and 3) nonimmunized mice. The immunization scheme was as described in Materials and Methods. Two weeks after the last immunization, mice were infected i.p. with 10^7 bloodstream trypanosomes. At each time point, three mice were sacrificed, and their spleen cells were pooled and stimulated with anti-CD3 mAb. Control tests consisted of spleen cells from normal mice (NSC) stimulated with anti-CD3 mAb or kept only in culture medium.
**FIGURE 5.** Kinetics of specific isotype profiles of Abs induced by immunization with Tc52 and after challenge infection, in sera from nonimmunized mice (control), sera from mice immunized with BpAl alone (BpAl), and sera from mice immunized with Tc52 plus BpAl (Tc52-BpAl). The levels of anti-Tc52 Abs in sera from Tc52-immune mice were compared with those observed in the case of control mice (nonimmunized or adjuvant-immunized mice). Asterisks indicate time points at which differences are statistically significant ($p < 0.05$ (*)) or $p < 0.001$ (**) by Student’s $t$ test.
or nonimmunized and infected mice. Chronic phase. The proliferative responses of Tc52-immunized and nonimmunized mice. The immunization scheme was as described in Materials and Methods. Two weeks after the last immunization, mice were infected i.p. with 10^7 bloodstream trypanosomes. At each time point, three mice were sacrificed, and their spleen cells were pooled and stimulated with 5 \mug/ml Tc52 Ag. Spleen cells from normal mice were used as control.

**Study of cellular response against Tc52**

Complementary experiments were performed to test the capacity of Tc52 to stimulate the proliferation of T cells from acute and chronically T. cruzi-infected mice that had been previously immunized or nonimmunized with Tc52 Ag. As shown in Figure 6, Tc52 induced proliferation of splenic cells from T. cruzi-infected mice in a time-dependent manner. Thus, the proliferation started to become evident at 40 days postinfection and was reinforced in the chronic phase. The proliferative responses of Tc52-immunized and infected mice remained higher than of adjuvant-immunized or nonimmunized and infected mice.

**Discussion**

The impaired immune response in Chagas’ disease has been attributed to a wide range of mechanisms, including IL-2 production; increased suppressor activity by splenic T cells and macrophages; down-regulation of CD3, CD4, CD8, and TCRs; and inhibition of IL-2R expression in the case of human PBMC (2). Furthermore, recent observations have suggested that increased production of NO by macrophages would account for the impairment of T cell response in T. cruzi-infected mice (9). Other investigators have shown that splenic CD4^+ T cells from T. cruzi-infected mice died from apoptosis upon stimulation with T cell mitogen Con A and anti-TCR-\alpha\beta mAb, a phenomenon that might play a role in immunosuppression observed in infected hosts (23). Although all of these mechanisms might participate separately or in combination in the immunosuppression observed during the acute phase of Chagas’ disease, it is of interest to search for host- and/or parasite-derived molecules that might trigger the alteration of the immune response.

In view of the fact that increased NO production by splenic macrophages has been involved in the suppression of lymphocyte proliferation in mice infected with *Trypanosoma brucei* (24), *P. chabaudi* (25), and *T. cruzi* (9), we decided to investigate whether Tc52 modifies macrophage NO production and cytokine gene expression. In the present study, we have shown that Tc52, when incubated with macrophages in the presence of IFN-\gamma, induced the production of high amounts of NO comparable with those observed when using a mixture of LPS and IFN-\gamma. Furthermore, the synergistic effect of Tc52 and IFN-\gamma was shown to be dose dependent. LPS alone did induce different levels of NO production according to the macrophage type used in the assay, whereas Tc52 alone had no significant effect on NO production. This observation indicates that a possible contamination of Tc52 by LPS, which might account for the activity of Tc52 on NO production by macrophages, is unlikely. Furthermore, no increase of NO production by macrophages was observed when using other proteins (*T. cruzi* Tc24 or BSA) as stimulatory factors. Additional experiments showed that Tc52 could be bound to macrophage surface, suggesting therefore the presence of Tc52 binding sites at the cell surface. However, further studies will permit detailed observation of how Tc52 modify the intracellular molecular events leading to macrophage activation.

To further characterize the signal pathway leading to the activation of NO production, we followed the expression by reverse transcription (PCR) of the iNOS-encoding gene upon Tc52-macrophage interaction. The results obtained showed that indeed, the levels of iNOS transcripts had increased markedly in samples from macrophages treated with Tc52 and IFN-\gamma or LPS and IFN-\gamma compared with control tests (medium alone, Tc52, LPS, or IFN-\gamma). However, further studies will have to investigate in detail whether the increased iNOS gene expression and NO production depend on the sequence of Tc52/IFN-\gamma signaling, and whether other cytokine gene expressions could have been affected by Tc52 Ag.

It is noteworthy that a colocalization of *T. cruzi* intracellular amastigotes and macrophage iNOS has been documented recently (26). Therefore, our study provides the first identification of a parasite-released molecule that induced macrophage iNOS gene expression and thus might participate in the accumulation of the gene product around intracellular amastigotes.

A number of reports have shown that IFN-\gamma and nonoxidative molecules (TNF-\alpha and NO) could play a role in the control of *T. cruzi* infection in mice (4). Furthermore, a recent study has reported a series of experiments supporting the notion that IFN-\gamma and TNF-\alpha-mediated activation of macrophages leads to increased production of NO, which in turn suppresses T cell activation (9). Moreover, the participation of NO in the suppression of T cell activation has been reported in a number of other biologic systems (4). Its involvement in apoptosis of thymocytes and macrophages has also been documented (27, 28). Furthermore, NO markedly inhibited the induction of IL-2 promoter, which could account for most of the reduction in IL-2 production, and weakly increased the activation of IL-4 promoter (29). This mechanism could be involved in the down-regulation of IL-2 gene expression observed during *T. cruzi* infection (30). Therefore, it is likely that NO production during the initial phase of acute *T. cruzi* infection might participate in the clearance of parasites by macrophages, whereas its overproduction during the late phase of acute infection would account for the immunosuppression observed. Recent studies have shown that lipophosphoglycan glycoconjugates from *Leishmania* and soluble proteins from *Entamoeba histolytica* were capable of modulating the iNOS gene and NO production (31, 32). Moreover, it has been reported recently that NO-mediated suppression of T cells during *B. brucei* infection could result from a synergistic effect of soluble trypanosome products and IFN-\gamma on iNOS expression (33). *T. gondii* uses this kind of mechanism to generate a
transient immunosuppression that probably helps in the establishment of the chronic infection in animals and humans (6). Although *T. gondii*-derived factor(s) has been shown to down-regulate the immune response, its possible involvement in NO production was not explored (34).

Previous studies have shown that *T. cruzi* releases factor(s) that exerted potent immunosuppressive properties (*Tryptosoma* immunosuppressive factor(s)) (35). The parasite-immunosuppressive material has been shown to comprise polypeptides ranging from 30 to 100 kDa molecular mass. Interestingly, the immunosuppressive activity could be removed from *T. cruzi*-released Ags by treatment with chronic chagasic sera, but not by sera from acute phase infection (36). Therefore, our present work is the first demonstration that a parasite-released Ag (Tc52) could modulate macrophage iNOS gene expression and NO production, and this may represent a mechanism participating in the depression of immune function in the late acute phase of Chagas’ disease. Indeed, in preliminary experiments, we found that the addition of 500 μM L-NMMA to Tc52/IFN-γ-treated spleen cells could partially reverse the inhibition of T cell proliferation induced by anti-CD3 mAb, suggesting therefore that at least part of the observed inhibition results from an effect of NO on responding lymphocytes (data not shown).

In a previous report, we have shown that Tc52 circulating in the blood of infected mice could be detected from day 5 postinfection (14). In contrast, under conditions of experimental infections, the Tc52 appears immunologically relatively silent during the early acute phase, failing to elicit significant levels of Abs and lymphocyte proliferation. In contrast, the immunization with Tc52 partially abrogated the immunosuppression and stimulated both arms of the immune system. This observation contrasts with other parasite Ags such as the shed acute phase Ag (SAPA, 37) and the Tc24 protein (38) among others, against which an Ab response could be detected earlier during T. cruzi infection. It is thus reasonable to assume that during the chronic phase, Abs to parasite immunosuppressive factors present in the sera of infected hosts bound to their respective Ag(s) inhibited their biologic immunosuppressive properties.

Another interesting observation is that Tc52 acts directly on macrophages to modulate IL-1α, IL-10, and IL-12 mRNA expression, and this may have implications as to whether Th1 or Th2 immune response will predominate. Indeed, IL-12 is able to facilitate proliferation and activation of Th1 cells. Moreover, it could synergize TNF-α and IL-1α to induce production of IFN-γ by T and NK cells, which in turn has a positive feedback effect by enhancing production of IL-12 by macrophages (39). The up-regulation of macrophage IL-10 gene expression by Tc52 might act as a negative feedback regulatory mechanism on IL-12 production. However, it is noteworthy that Tc52-induced up-regulation of IL-10 gene expression was abrogated by addition of IFN-γ. Furthermore, it is interesting to remind that high levels of IFN-γ and low amounts of IL-10 have been recorded in mice during *T. cruzi* infection. Other parasite molecules may also participate in the modulation of macrophage activation. Indeed, a recent study has shown that glycosylphosphatidylinositol-anchored mucin-like glycoproteins isolated from *T. cruzi* trypomastigotes induce IL-12 and TNF-α synthesis by macrophages (40). Therefore, it is reasonable to assume that Tc52, among other parasite molecules, through its direct or indirect action toward IL-10, IL-12, and iNOS gene expression, could participate in the mechanisms that may play a role in the balance of Th1 and Th2 immune response.

One of the consequences of Chagas’ disease is inflammatory damage of myocardium and skeletal muscles (1). Multiple mechanisms of myocardioocyte alterations, including cytotoxic T cells, Abs to cardiac cell Ags, TNF-α cytotoxicity, and overproduction of NO, might also operate in pathogenesis of Chagas’ disease cardiopathy (1, 9). Therefore, we cannot rule out the possibility that during the chronic phase, in situ production of low amounts of Tc52 (not neutralized by Abs) by amastigotes inside *T. cruzi*-infected cardiac endothelial cells and myocytes that have a transcriptionally iNOS, induces local production of NO and nitrotyrosine formation that might participate in myocardial dysfunction and heart failure.

Parasitic infections lead to the development of a complex network of T and non-T cells and cross-regulatory cytokines (41). Although our study does not completely support the triggering of Th1-like or Th2-like response, it provides, to our knowledge, the first evidence that a *T. cruzi*-defined protein could contribute to the immune dysfunction observed during Chagas’ disease through its capacity to modulate macrophage cytokine and iNOS gene expression. The elucidation of the molecular mechanisms that contribute to the pathophysiological processes and disease progression might allow rational design of tools to control *T. cruzi* infections.

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References


