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Intracellular Growth of *Trypanosoma cruzi* in Cardiac Myocytes Is Inhibited by Cytokine-Induced Nitric Oxide Release

Laura Edith Fichera, Maria Cecilia Albareda, Susana Adriana Laucella, and Miriam Postan*

Instituto Nacional de Parasitología "Dr. Mario Fatala Chaben"/ANLIS/Malbrán and Consejo Nacional de Investigaciones Cientificas y Tecnicas, Buenos Aires, Argentina

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The effect of interleukin-1β (IL-1β), tumor necrosis factor alpha (TNF-α), and gamma interferon (IFN-γ) on *Trypanosoma cruzi* multiplication and nitric oxide (NO) production in cardiac myocytes was investigated. Cardiac myocyte cultures were obtained from neonatal Wistar rat hearts, infected with *T. cruzi*, and treated with IL-1β, TNF-α, IFN-γ, or *N*-monomethyl-L-arginine (L-NAME) for 72 h. Parasite growth was calculated from the number of infected cells in Giemsa-stained smears. Nitric oxide production was determined with the Griess reagent. Inducible nitric oxide synthase (iNOS) expression by cardiac myocytes was detected by Western blot. The results showed that the percentages of cardiac myocytes containing *T. cruzi* amastigotes in cytokine-treated cultures were significantly lower than in nontreated cultures. The addition of L-NAME reversed the inhibitory effect on parasite growth of IL-1β and TNF-α but not of IFN-γ. Nitrite levels released by *T. cruzi*-infected and noninfected cardiac myocyte cultures after 72 h of stimulation with IL-1β were significantly higher than those produced upon treatment with TNF-α, IFN-γ, or medium alone, regardless of the infection status. Nitrite levels in TNF-α-stimulated infected cultures were significantly higher than in untreated infected cultures and TNF-α-treated noninfected cultures. L-NAME inhibited IL-1β- but not TNF-α-induced NO production, indicating the presence of iNOS-dependent and iNOS-independent mechanisms for NO formation in this experimental system. iNOS expression was detected in infected and noninfected cardiac myocytes stimulated with IL-1 β and TNF-α but not with IFN-γ. These results suggest an important role for cardiac myocytes and locally secreted cytokines in the control of parasite multiplication in *T. cruzi*-induced myocarditis.

One of the characteristics of Chagas' disease is the cardiomyopathy which develops following chronic infection with *Trypanosoma cruzi*. Various arms of the host immune response have been demonstrated to participate in the control of *T. cruzi* infection, including T and B lymphocytes, NK cells, and macrophages. *T. cruzi* invades and multiplies within the cytoplasm of a wide variety of mammalian cells, including macrophages and cardiac myocytes. Macrophages activated by gamma interferon (IFN-γ), and by tumor necrosis factor alpha (TNF-α) to a lesser extent, have the ability to kill intracellular parasites in vitro, exerting their trypanocidal activity through a nitric oxide (NO) mechanism (15).

Treatment of mice with IFN-γ for the acute phase of infection results in lower parasitemias and prevents death from acute disease (20). The administration of NO inhibitors increases parasitemia and mortality rate in infected mice, further demonstrating the relevant role of NO in the resistance to *T. cruzi* (16). NO produced by activated macrophages is also known to be cytotoxic for other intracellular pathogens, including *Leishmania major, Toxoplasma gondii, Schistosoma mansoni,* and *Plasmodium falciparum* (2, 11). In the heart, NO is an essential mediator of different biological functions, influencing the heart rate and modulating myocardial contractile responses. Cytokines released locally in several inflammatory conditions of the heart induce inducible nitric oxide synthase (iNOS) expression and NO release, implicated in the myocardial dysfunction associated with cardiomyopathies of different etiologies, such as Coxsackie virus-induced myocarditis, cardiac allograft rejection, and myocardial infarction (5, 7, 9, 12, 23).

We have demonstrated previously that the adaptive response of ventricular myocytes from *T. cruzi*-infected rats depends on the presence of inflammatory cells in the surroundings; whereas myocyte nuclear division and DNA repair prevail at inflammation sites, myocyte hypertrophy is the hallmark of the noninflamed myocardium (1). Other authors have reported enhanced expression of iNOS, TNF-α, IL-1β, transforming growth factor beta, IL-6, and, at a relatively lower level, IFN-γ in the myocardium of *T. cruzi*-infected mice, supporting the hypothesis that inflammatory cells and their products might play a role in the pathogenesis of chagasic cardiomyopathy (6, 10, 14, 24).

In order to determine if cardiac myocytes play a role in the control of *T. cruzi* infection under the influence of locally released cytokines, we investigated the effect of IL-1β, TNF-α, and IFN-γ on parasite growth in Wistar rat cardiac myocytes infected with *T. cruzi* in vitro.

MATERIALS AND METHODS

Preparation of ventricular myocytes. Two- to three-day-old Wistar rats were sacrificed by cervical dislocation under light ether anesthesia, and the heart was removed under sterile conditions. Ventricular myocytes were isolated as described by Ellingsen et al. (8). Briefly, the hearts were cut into small fragments and dissociated with Hanks' balanced salt solution (HBSS, Gibco, Rockville, Md.) containing 0.02% trypsin at 37°C for four cycles of 15 min each. The cell

* Corresponding author. Mailing address: Av. Paseo Colón 568 (1063), Buenos Aires, Argentina. Phone: (5411) 4331 7732. Fax: (5411) 4331 7142. E-mail: mpostan@mail.retina.ar.

suspensions were pooled and resuspended in HBSS supplemented with 10% fetal bovine serum (FBS, Gibco, Rockville, Md.). For selective enrichment of cardiac myocytes, the dissociated cells were preplated for 1 h, during which nonmyocytes readily attached to the bottom of the flask (22). The cells remaining in the supernatant were resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco, Rockville, Md.) supplemented with 10% FBS and 10% horse serum (complete medium) and transferred to 12-mm coverslips contained in 24-well culture plates (Nunc, Naperville, Ill.). The cultures were kept in a 5% CO_2 incubator at 37°C to allow the cells to adhere firmly to the coverslip and start beating before use in the specific experiments (usually within 36 to 72 h of initiated the cultures).

Infection of cardiac myocytes and cytokine treatment. *T. cruzi* clone Silvio-X10/4 trypomastigotes derived from bovine embryo skin and muscle (BESM) cell cultures were used in this study (18). Cardiac myocyte cultures were infected with trypomastigotes at a 5:1 parasite-to-cell ratio, followed by incubation for a 3-h period at 37°C in 5% CO_2. Afterwards, the cultures were washed thoroughly with medium, and the cells were incubated with complete medium in the presence or absence of additives for 72 h according to the experimental protocol. Cardiac myocytes (5×10^4/well) were treated with 10 ng of recombinant human IL-1β (PharMingen, San Diego, Calif.), 100 ng of recombinant rat TNF-α (PharMingen, San Diego, Calif.), or 500 U of recombinant rat IFN-γ (BD PharMingen, San Diego, Calif.) per ml. *N*-Monomethyl-L-arginine (L-NAME, Sigma, St. Louis, Mo.), a competitive antagonist of NOS activity, was added to some cultures.

The optimal concentrations of the cytokines were selected in preliminary assays based on their capacity to inhibit *T. cruzi* growth. Different concentrations of L-NAME were tested for the capacity to inhibit cytokine-induced NO release and/or parasite growth. Noninfected and nontreated cultures were used as controls. The supernatants were collected daily and stored at −70°C for nitrite measurement at the end of each experiment, and the wells were replenished with the appropriate fresh medium. Cultures of cardiac myocytes were set up in triplicate wells for each treatment, and three independent experiments including all treatments were performed.

Measurement of nitrite release. Nitrite (NO_2^-) content was used as an index of nitric oxide production. Nitrite levels were determined in supernatant of cell cultures by spectrophotometry, with Griess reagent (Promega, Madison, Wis.). Fifty microliters of the samples was added to 50 μl of freshly prepared Griess reagent in a 96-well plate, and the absorbance was read at 550 nm. The nitrite concentration was calculated with a sodium nitrite standard.

Assessment of infection rates. To determine the infection rate, the number of cardiac myocytes containing amastigotes in the cytoplasm was evaluated with a light microscope. Covers with cardiac myocytes were removed from the culture plates after 72 h of treatment, gently rinsed with phosphate-buffered saline, air dried, and fixed in absolute methanol for 10 min prior to staining with Giemsa. In each experiment, a total of 300 cardiac myocytes (100 cells/cover) were evaluated for each treatment in randomly selected fields. Results are expressed as the mean percentages of infected cells. The percent growth inhibition was calculated as [(experimental infected cells − control infected cells)/(control infected cells)] × 100.

Effect of cytokines on parasite growth. Epimastigotes from *T. cruzi* clone Sylvio-X10/4 were grown in BHT medium containing brain heart infusion (Difco, Detroit, Mich.), 0.3% tryptose (Difco, Detroit, Mich.), 0.002% bovine hemin (Sigma, Saint Louis, Mo.), and 10% heat-inactivated fetal calf serum (Bioser, Buenos Aires, Argentina). Axenic culture epimastigotes were centrifuged at 2,300 rpm, resuspended in 1 ml of BHT supplemented with 10 ng of IL-1β, 100 ng of TNF-α, or 500 U of IFN-γ per ml at a final concentration of 5×10^5 epimastigotes in 15-ml polystyrene disposable tubes (Falcon, Oxnard, Calif.), and kept at 26°C. The parasite concentration was calculated in samples obtained at 48, 96, and 144 h of incubation with a Neubauer chamber.

Inducible nitric oxide synthase detection by Western blot. iNOS protein production was evaluated by immunoblotting with a rabbit anti-iNOS/NOS type II polyclonal antibody (Transduction Laboratories, Lexington, Ky.). The cells were resuspended in phosphate-buffered saline and sonicated three times for 10 s each. Equal amounts (90 μg) of the denatured proteins were loaded per lane, separated on a sodium dodecyl sulfate–7.5% polyacrylamide gel (Sigma Aldrich, St. Louis, Mo.), and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat milk for 40 min and incubated with the rabbit anti-mouse iNOS primary antibody for 3 h, followed by incubation with peroxidase-labeled goat anti-rabbit immunoglobulin G (Vector Laboratories, Burlingame, Calif.) for 1 h. The reaction was visualized with 3,3′-diaminobenzidine and H_2O_2. Thioglycolate-activated mouse peritoneal macrophages stimulated with 500 U of IFN-γ per ml in vitro for 72 h were used as controls.

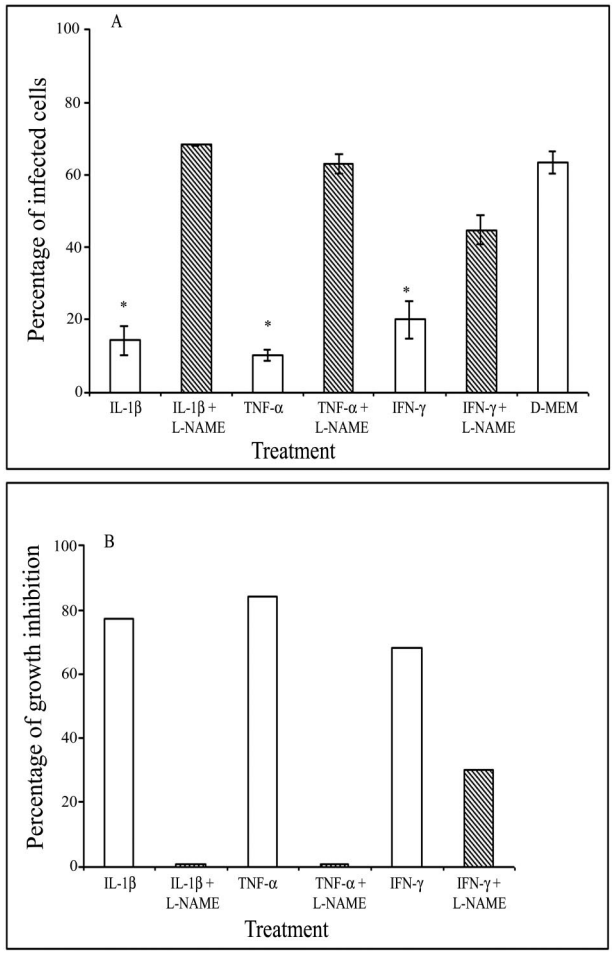

FIG. 1. Effect of cytokines on *T. cruzi* infection of cardiac myocytes. Infected cardiac myocytes were cultured in the presence of 10 ng of IL-1β per ml, 10 ng of IL-1β per ml + 1 mM L-NAME, 100 ng of TNF-α per ml, 100 ng of TNF-α per ml + 2 mM L-NAME, 500 U of IFN-γ per ml, 500 U of IFN-γ per ml + 6 mM L-NAME, or DMEM. (A) Percentage of infected cardiac myocytes after 72 h of treatment. (B) Percentage of parasite growth inhibition (see text). *, $P < 0.05$ compared with controls.

Statistical analysis. Comparison of nitrite levels and percentages of infected cardiac myocytes among the different treatments were performed with analysis of variance followed by the Bonferroni (SAS software) *t* test. The correlation between parasite burden and NO production was studied by Spearman' s rank correlation coefficient. $P < 0.05$ was regarded as statistically significant.

RESULTS

Effect of cytokines on the number of *T. cruzi*-infected cardiac myocytes. The quantification of cardiac myocytes containing *T. cruzi* amastigotes showed that the number of infected cells in cultures treated with IL-1β, TNF-α, or IFN-γ for 72 h was significantly lower ($P < 0.05$) than in nontreated cultures (Fig. 1A). Parasite growth inhibition achieved by treatment with IL-1β, TNF-α, and IFN-γ with respect to the controls was 77, 84, and 68%, respectively (Fig. 1B). The addition of L-NAME reversed the inhibitory effect of IL-1β and TNF-α on parasite growth ($P < 0.05$), but did not significantly modify the inhibitory effect of IFN-γ.

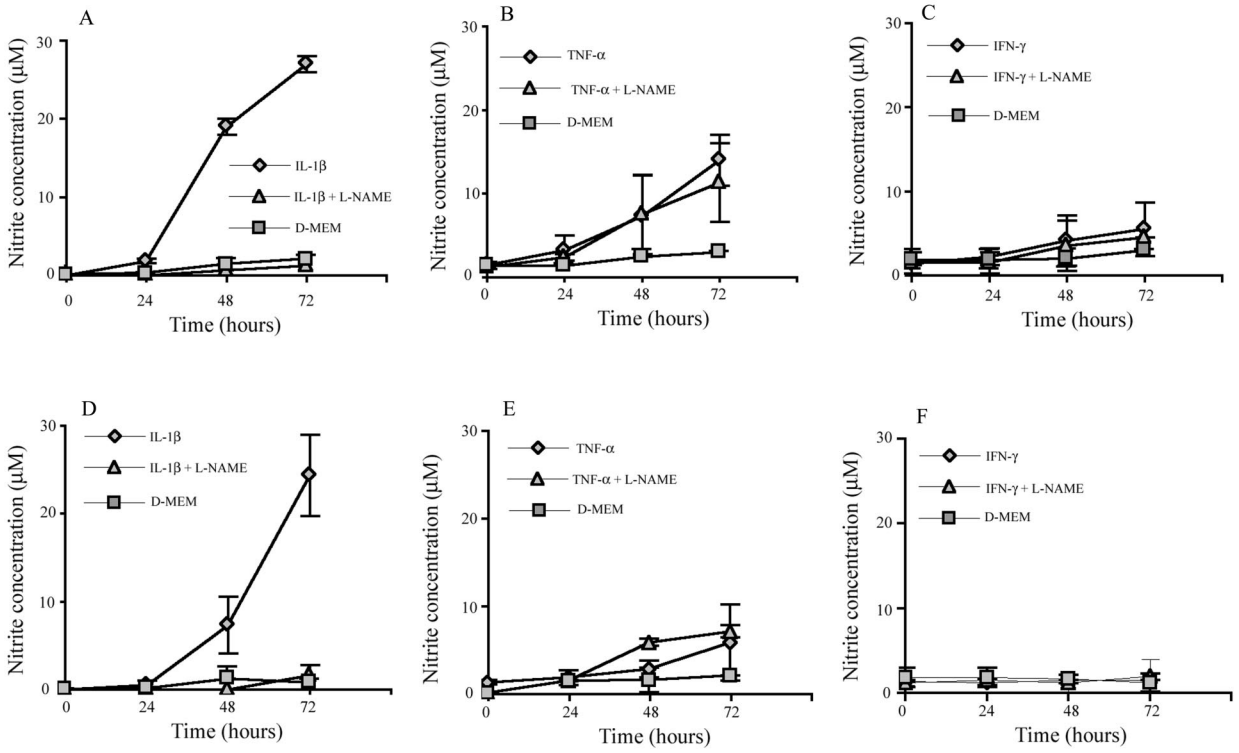

FIG. 2. Kinetics of NO production in cardiac myocyte cultures infected with *T. cruzi*. (A, B, and C) Infected cultures. (D, E, and F) Noninfected cultures. (A and D) Treatment with 10 ng of IL-1β per ml, 10 ng of IL-1β per ml + 1 mM L-NAME, or medium. (B and E) Treatment with 100 ng of TNF-α per ml, 100 ng of TNF-α per ml + 2 mM L-NAME, or medium. (C and F) Treatment with 500 U of IFN-γ per ml, 500 U of IFN-γ per ml + 6 mM L-NAME, or medium. Each point represents the mean ± standard deviation of triplicate samples. The data are representative of three independent experiments.

NO production by *T. cruzi*-infected rat cardiac myocyte cultures stimulated with cytokines. Nitrite release by *T. cruzi*-infected and noninfected rat cardiac myocyte cultures stimulated with IL-1β, TNF-α, and IFN-γ was variable, depending upon the cytokine and the time of stimulation (Fig. 2).

Treatment of cultures with IL-1β resulted in time-dependent increases of nitrites being released into the supernatants (Fig. 2A and 2D). Nitrite levels induced by the cytokine in infected and noninfected cultures were comparable, with 13- and 26-fold average increases after 48 and 72 h, respectively, of incubation in infected cultures with respect to basal levels ($P < 0.05$) and 11- and 27-fold average increases, respectively, in noninfected cultures ($P < 0.05$). The addition of 1 mM L-NAME to IL-1β-treated cultures inhibited the cytokine-induced nitrite release by more than 90%, regardless of the infection status.

Increasing amounts of nitrites were also observed following treatment with TNF-α for 48 and 72 h, with 6.8- and 9.3-fold increases, respectively, in infected cultures ($P < 0.05$) and 2.3- and 3.5-fold increases, respectively, in noninfected cultures compared to basal levels (Fig. 2B and 2E). The lower doses of TNF-α tested (25 and 50 ng/ml) failed to induce significant nitrite release. The addition of 1 to 2 mM L-NAME did not considerably modify the production of NO induced by TNF-α.

Average nitrite level increases of 2.9- and 3.9-fold with respect to basal values were observed in *T. cruzi*-infected cultures after 48 and 72 h of incubation with IFN-γ, respectively (Fig. 2C and 2F). Nitrite levels in nonstimulated wells remained low, regardless of the infection status.

In order to determine the relative efficacy of the cytokines to induce NO production, we compared the levels of nitrites reached by *T. cruzi*-infected and noninfected myocyte cultures after 72 h of treatment. The results of this analysis showed that IL-1β induced significantly higher nitrite levels than TNF-α, IFN-γ, and medium alone ($P < 0.05$), regardless of the infection status. The nitrite levels reached by infected cultures after 72 h of stimulation with TNF-α were significantly higher ($P < 0.05$) than those produced by untreated infected cultures and noninfected cultures treated with TNF-α. No differences were found between the nitrite levels of IFN-γ-stimulated and nonstimulated cultures, nor were there statistically significant differences in nitrite release between TNF-α- and IFN-γ-stimulated cultures, irrespective of the infection status.

The content of nitrites in the supernatant of cytokine-treated cultures was inversely correlated with the number of infected cells at 72 h of incubation ($r = 0.768$), suggesting an NO-related mechanism in the control of intracellular parasite growth.

iNOS expression in cardiac myocytes. The expression of iNOS in cardiac myocyte cultures was analyzed after 72 h of exposure to IL-1β, TNF-α, and IFN-γ by immunoblotting. iNOS was found to be expressed by infected and noninfected cultures stimulated with IL-1 β and TNF-α but not in cultures stimulated with IFN-γ (Fig. 3). iNOS expression induced by

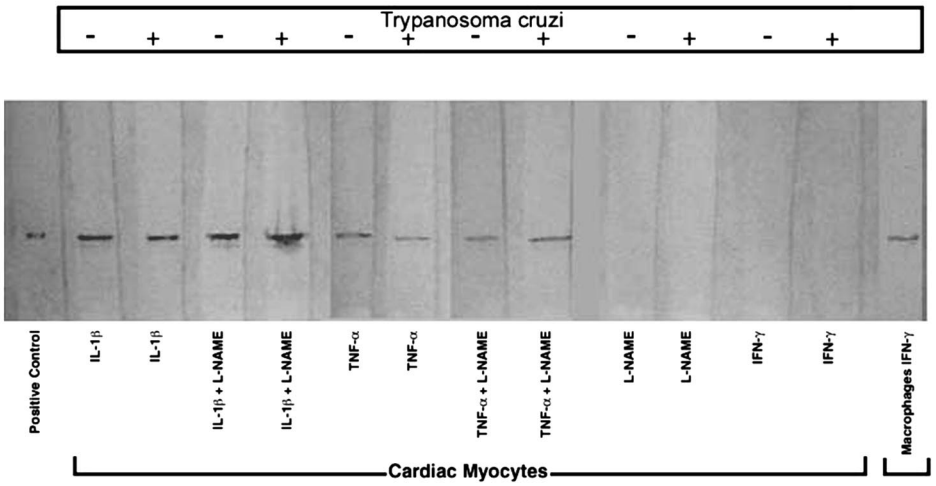

FIG. 3. Detection of iNOS protein in *T. cruzi*-infected cardiac myocytes after exposure to cytokines. iNOS protein production on *T. cruzi*-infected (+) and noninfected (−) cardiac myocytes was analyzed by immunoblotting after 72 h of exposure to 10 ng of IL-1β per ml, 10 ng of IL-1β per ml +1 mM L-NAME, 100 ng of TNF-α per ml, 100 ng of TNF-α per ml + 2 mM L-NAME, 500 U of IFN-γ per ml, 500 U of IFN-γ per ml + 6 mM L-NAME, 1 mM L-NAME, or medium alone. Mouse macrophages treated with 500 U of IFN-γ per ml were included as controls.

IL-1β and TNF-α remained detectable after the addition of L-NAME to cytokine-stimulated cultures. iNOS expression was not detected in nonstimulated cardiac myocyte cultures or cultures treated with L-NAME exclusively.

Effect of cytokines on the epimastigote proliferative stage of *T. cruzi*. We addressed the possibility that cytokines may exert a direct effect on the multiplication of *T. cruzi* and conducted growth kinetics studies of *T. cruzi* clone Sylvio-X10/4 epimastigotes in the presence of IL-1 β, TNF-α, or IFN-γ. The results showed significantly lower parasite densities at 96 h of culture with IL-1 β ($P < 0.05$), TNF-α ($P < 0.05$), and IFN-γ ($P < 0.05$) with respect to the controls, with growth inhibition of 45, 58, and 41%, respectively. This inhibitory action of the cytokines on epimastigote multiplication was not observed at 48 and 144 h of culture.

DISCUSSION

We previously demonstrated that neonatal cardiac myocytes exhibit a marked inhibitory effect on intracellular growth of *T. cruzi* amastigotes in vitro upon stimulation with a mixture of IL-1β, TNF-α, and IFN-γ (19). Moreover, we showed that the cytokine-induced trypanocidal action of cardiac myocytes was associated with the generation of NO. In the present study, we demonstrate that each cytokine separately has the capacity to stimulate cardiac myocytes to limit proliferation of intracellular *T. cruzi* amastigotes, further supporting our original hypothesis that myocardial cells do not remain passive at inflammation sites but actively collaborate with the immune response in the elimination of the parasite (19).

Because of the reported differences in the ability of individual cytokines to induce iNOS expression and NO release in cardiac myocyte cultures (3, 17) and in the levels of cytokine expression for *T. cruzi*-induced myocarditis (24), it was important to elucidate the role of individual cytokines in the outcome of our experimental system of infection. As described for other rat systems (3, 17, 22), we found that noninfected neonatal Wistar rat cardiac myocytes stimulated with IL-1β generate NO. The ability of cardiac myocytes to generate NO upon stimulation with IL-1β remained unaltered after infection with *T. cruzi* in vitro. Furthermore, we demonstrate by means of L-NAME inhibition that NO in IL-1β-stimulated cardiac myocyte cultures is produced through the iNOS pathway. Conversely, TNF-α induced significant levels of NO only in infected cultures, even though iNOS expression was detectable in both infected and noninfected cultures.

The unresponsiveness of TNF-α-stimulated cultures to the NOS inhibitor L-NAME indicates the involvement of an iNOS-independent pathway in NO formation. TNF-α was suggested to facilitate acidification in macrophages infected with *Mycobacterium bovis* via an oxidative mechanism (4), and acidic conditions are known to favor enzyme-independent NO formation (25). On the other hand, it was demonstrated that iNOS loses its activity under low oxygen tension and low pH (13). Thus, it is possible that one or more of these mechanisms might be acting in cardiac myocyte cultures stimulated with TNF-α, explaining in part the expression of iNOS without inhibition by L-NAME. NO production by *T. cruzi*-infected but not noninfected TNF-α-stimulated cultures suggests that the parasite itself might contribute to NO formation by cardiac myocytes. These results support the hypothesis of other authors about the ability of *T. cruzi*-infected murine cardiomyocytes to secrete mediators that induce the production of chemokines, proinflammatory cytokines, and iNOS expression (14).

IFN-γ failed to induce NO production and iNOS protein expression in our rat cardiac myocyte system. In this regard, conflicting results on the ability of cardiac myocyte cultures to produce NO under stimulation with IFN-γ were reported by other authors (3, 17).

The comparison of nitrite levels induced by single cytokine treatments revealed that IL-1β has the most potent effect on *T. cruzi*-infected rat cardiac myocytes to produce NO. This pattern of response contrasts with that described for *T. cruzi*-

infected embryonic mouse cardiac myocytes, which produce higher levels of NO when stimulated with IFN-γ than with IL-1β and TNF-α (14).

The inverse correlation between parasite load and nitrite levels released upon stimulation with the cytokines indicates that the parasiticidal action of cardiac myocytes is at least partially mediated by NO. The participation of the iNOS pathway in the control of parasite growth exerted by IL-1β and TNF-α was confirmed by the neutralizing effect of L-NAME, even though the iNOS pathway did not appear to be the main source of NO produced by TNF-α stimulation. IFN-γ also exerted a trypanocidal action, though neither iNOS expression nor significant NO production was recorded in our experimental system, indicating the involvement of iNOS- and NO-independent mechanisms of parasite growth inhibition. On the other hand, each cytokine exerted a direct but transient inhibitory effect on the multiplication of the epimastigote form of parasite development.

In summary, this study demonstrates the ability of cardiac myocytes to limit the growth of *T. cruzi* in vitro in response to cytokines known to be secreted by inflammatory cells for the in vivo infection, confirming an active role for these cells in resistance against the parasite. Although NO might be helpful for limiting parasite load and therefore the extent of inflammation, there are data in the literature suggesting its participation in the pathogenesis of experimental chagasic heart disease (10, 21). Clarifying the role of individual cytokines on *T. cruzi* persistence is crucial for further understanding of the immunological mechanisms involved in parasite clearance from target tissues.

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