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CTLA-4 Blockage Increases Resistance to Infection with the Intracellular Protozoan *Trypanosoma cruzi*1

Gislâine Aparecida Martins,* Carlos Eduardo Tadokoro,* Roberta Borges Silva,* João Santana Silva,† and Luiz Vicente Rizzo2*‡§

Recent studies have revealed an important role for CTLA-4 as a negative regulator of T cell activation. In the present study, we evaluated the importance of CTLA-4 to the immune response against the intracellular protozoan, *Trypanosoma cruzi*, the causative agent of Chagas’ disease. We observed that the expression of CTLA-4 in spleen cells from naïve mice cultured in the presence of live trypomastigote forms of *T. cruzi* increases over time of exposure. Furthermore, spleen cells harvested from recently infected mice showed a significant increase in the expression of CTLA-4 when compared with spleen cells from noninfected mice. Blockage of CTLA-4 in vitro and/or in vivo did not restore the lymphoproliferative response decreased during the acute phase of infection, but it resulted in a significant increase of NO production in vivo and in vitro. Moreover, the production of IFN-γ in response to parasite Ags was significantly increased in spleen cells from anti-CTLA-4-treated infected mice when compared with the production found in cells from IgG-treated infected mice. CTLA-4 blockade in vivo also resulted in increased resistance to infection with the Y and Colombian strains of *T. cruzi*. Taken together these results indicate that CTLA-4 engagement is implicated in the modulation of the immune response against *T. cruzi* by acting in the mechanisms that control IFN-γ and NO production during the acute phase of the infection. *The Journal of Immunology*, 2004, 172: 4893–4901.

*Trypanosoma cruzi* is an obligate intracellular parasite that causes American trypanosomiasis (Chagas’ disease), a chronic and debilitating syndrome that affects millions of people in Latin America (1). Infection of mice with this parasite reproduces many of the characteristics observed in human disease (1, 2).

Similarly to humans, during the acute phase of infection, mice present suppressed lymphoproliferative responses to parasite Ags and to mitogens (2–4). The decreased ability to proliferate observed in T cells from infected mice has been ascribed to many different mechanisms, including the excessive production of NO (3, 4), which is markedly increased during the acute phase of disease (5). The increase in NO synthesis results from the enhanced expression of inducible NO synthase (4–6) by cytokines such as IFN-γ and TNF-α, which are produced in high levels as a consequence of the infection (7–9). The resultant increase in NO contributes to parasite killing and host survival (5, 10, 11) but may also lead to myocardial dysfunction (12). It has been suggested that IFN-γ-induced NO production could be involved in down-regulating the immune response in mice acutely infected (4, 6) since the excess NO produced has also been implicated in lymphocyte apoptosis during the infection (4).

CTLA-4 (or CD152) plays a significant role in regulating the immune response. CTLA-4 is 76% homologous to CD28 and binds to the same costimulatory ligands, B7-1 (CD80) and B7-2 (CD86), with a 20-fold higher affinity than CD28 (13, 14). Nevertheless, unlike CD28, CTLA-4 expression is rarely detected on nonstimulated T cells, and peak expression occurs only after activation of CD4+ or CD8+ T cells (15–17). In addition, CTLA-4 and CD28 play opposite roles in regulating the immune response. CD28 has been shown to provide the critical second signal required for optimal T cell activation (17, 18), whereas CTLA-4 is implicated in the negative regulation of T cell activation (15–17, 19). It has been proposed that CTLA-4-mediated termination of T cell responses may facilitate the generation of memory T cells, which become ready to respond to Ag stimulation after the consequent decay of CTLA-4 expression (16, 20). CTLA-4 has also been implicated in the regulation of T cell anergy (21).

The importance of CTLA-4 as a regulator of lymphocyte homeostasis was confirmed by the generation of CTLA-4-deficient mice, whose phenotype includes a severe lymphoproliferative disorder that is usually fatal by 4–5 wk of age (22, 23). The involvement of CTLA-4 as a negative regulator of the immune response was additionally confirmed by reports showing that blocking of CTLA-4 in mice enhances antitumor immunity (24, 25), antagonizes the onset, and exacerbates the severity of experimental autoimmune encephalomyelitis (26, 27). CTLA-4 has also been implicated as the primary checkpoint for clonal expansion of uncommitted T cells, thus controlling the size of the T cell pool in the periphery and allowing the development and maintenance of memory cells (28).

It has also been demonstrated that CTLA-4 blockade in vivo enhances the immune response to *Mycobacterium* infection (29), leading to rapid and protective primary responses against *Nippostrongylus brasiliensis* (30), and enhances host resistance to the...
intracellular pathogens \textit{Leishmania donovani} and \textit{Leishmania major} (31, 32). The blockade of CTLA-4 in vitro also led to increased cytokine production and microbicidal activity in cells from \textit{Leishmania chagasi}-infected mice (33).

In the present study, we sought to evaluate whether signaling through CTLA-4 would be implicated in regulating the immune response in mice infected with \textit{T. cruzi}. Our data show that the blockade of CTLA-4 results in increased resistance to infection associated with increased production of IFN-\(\gamma\), TNF-\(\alpha\) and NO.

Materials and Methods

\textbf{Animals and Ab treatments}

Female 5- to 8-wk-old C57BL/6 mice were obtained from the animal facilities of the Department of Immunology, Institute of Biomedical Sciences, University of São Paulo (São Paulo, Brazil) and were maintained in specific pathogen-free conditions with food and food ad libitum. Blockage of CTLA-4 function in vivo was performed by i.p. injection of 100 \(\mu\)g/mice of purified mAb anti-CTLA-4 (clone UC10-4F10; BD PharMingen, San Diego, CA) diluted in PBS. Ab infusions were performed 4 h before or 24 h after the infection. Control mice received 100 \(\mu\)g of hamster IgG (BD PharMingen) diluted in PBS. All procedures performed in the studies described here were approved by the Ethics Committee on Animal Research of the University of São Paulo.

\textbf{Parasites and infection}

Mice were infected i.p. with \(1 \times 10^5\) or \(1 \times 10^2\) (Y strain) blood-derived \textit{T. cruzi} trypomastigote forms. Parasitemia was evaluated in 5 \(\mu\)l of blood drawn from the tail vein as previously described (4). For in vitro experiments, trypomastigote forms (Y strain) were grown and purified from the monkey kidney fibroblast cell line LLC-MK\(_2\). For preparation of soluble \textit{T. cruzi} Ags (sTcAg),\(^3\) trypomastigote forms were washed twice in cold PBS, submitted to 10 freeze-thaw cycles, and centrifuged (9000 \(\times\) g, 10 min, 4°C). The supernatant was filtered through a 0.22-\(\mu\)m filter and the protein concentration was assayed by using the Pierce (Rockford, IL) assay system.

\textbf{Spleen cell cultures}

Single-cell suspensions from the spleen were obtained by dissociating the organ in HBSS followed by treatment with an erythrocyte-lysing agent. The erythrocyte-free cells were then washed three times in HBSS and adjusted to \(3 \times 10^6\) cells/ml in RPMI 1640 (Flow Laboratories, McLean, VA) supplemented with 10% FCS (HyClone, Logan, UT), 2-ME (5 \(\times\) \(10^{-5}\) M), 1-glutamine (2 mm), and antibiotics (all from Sigma-Aldrich, St. Louis, MO). The cell suspension was distributed (1 ml/well) in 24-well tissue culture plates (Corning, Corning, NY) and cultured for 48 h at 37°C in a humidified 5\% CO\(_2\) atmosphere in the presence or absence of soluble parasite Ags (sTcAg; 10 \(\mu\)g/ml) or Con A (2 \(\mu\)g/ml). Cells were used to assay apoptosis, or surface expression of CTLA-4, and the supernatant was collected to evaluate NO and cytokine production.

\textbf{Quantification of nitrite and nitrate}

Blood was obtained from the retro-orbital plexus. Nitrate was reduced to nitrite with nitrite reductase as described previously (5), and the nitrite concentration was determined by the Griess method (34). In this assay, 0.1 ml of cell-free culture medium or nitrite reductase-treated serum was mixed with 0.1 ml of Griess reagent in a multiwell plate and the absorbance at 550 nm was then read 10 min later. The NO\(_2\) concentration was determined by reference to a standard curve of NaNO\(_3\) (1–200 \(\mu\)M).

\textbf{Cytokine quantification}

Total spleen cells were cultured as described above. Supernatants were harvested after 24 or 48 h and stored at \(-20°C\) until use. IFN-\(\gamma\), TNF-\(\alpha\), IL-2, and IL-4 levels were evaluated in the supernatants by a two-site sandwich ELISA, as previously described (6), and confirmed using the cytometric beads assay for murine cytokines from BD Immunocytometry Systems.

\textbf{Statistical analysis}

The results are expressed as the mean \(\pm\) SD of the indicated number of animals or as the mean \(\pm\) SEM of the data obtained in the indicated number of experiments. Statistical analysis was performed using repeated measures ANOVA (for parasitemia data) or one-way ANOVA (for nonpaired measures) followed by the Student-Newman-Keuls test or Mann-Whitney \(U\) test (INSTAT software; GraphPad, San Diego, CA) as indicated in the figure legends. Values of \(p<0.05\) were considered to be significant.

\textbf{Results}

\textit{T. cruzi} trypomastigote forms induce expression of CTLA-4 in spleen cells in vitro and in vivo

To investigate whether CTLA-4 expression could be induced by infection with \textit{T. cruzi}, we cultured total spleen cells from C57BL/6 naïve mice in the presence of live trypomastigote forms, sTcAg, or Con A and evaluated CTLA-4 expression in CD3\(^+\) T cells after 24, 48, and 72 h. The presence of live parasites in the cultures resulted in a time-dependent increase in CTLA-4 expression on T cells, similar in magnitude and timing to that induced by Con A stimulation. In comparison to cells cultured in medium only, cells cultured with live parasites showed a significant increase in CTLA-4 expression (\(p<0.02\), which was about four times higher at 24 h and six times higher at 48 h, returning to normal levels after 72 h of culture. Cells cultured in the presence of parasite Ags showed significantly increased (\(p<0.05\) expression of CTLA-4 only after 48-h culture (3-fold increase, as compared with cells cultured with medium only) (Fig. 1A).

Since exposure to live parasites in vitro had a significant effect on CTLA-4 expression, we next investigated whether \textit{T. cruzi} infection in vivo could also result in induction of CTLA-4 expression. C57BL/6 mice were infected with \textit{T. cruzi} and the expression of CTLA-4 was evaluated on spleen T cells by flow cytometry. Significantly increased expression of CTLA-4 was found on T cells from \textit{T. cruzi}-infected mice as early as 3 days after infection and remained elevated until 11–15 days after infection, returning from the analysis by setting the appropriate gate on a side scatter vs forward scatter dot plot. Live cells exclude 7-AAD completely and therefore are 7-AAD\(^-\), whereas apoptotic cells are 7-AAD\(^{int}\) and necrotic cells or cells that lost membrane integrity are 7-AAD\(^{bright}\). The 7-AAD\(^{bright}\) cells were excluded from the analysis gate. All measurements were made using the same instrument settings.

To evaluate the expression of CTLA-4, spleen cells from uninfected or infected mice were incubated for 30 min at 4°C with 0.5 \(\mu\)g of anti-CD16/CD32 mAb (Fc block), followed by the addition of 0.5 \(\mu\)g of FITC-labeled anti-murine CD3, CD4, or CD8 and 0.5 \(\mu\)g of PE-labeled anti-murine CTLA-4 (all from BD PharMingen). To determine the background staining, cells were incubated with 0.5 \(\mu\)g each of hamster IgG1 anti-trinitrophenol FITC and hamster IgG1 anti-trinitrophenol PE, for 30 min at 4°C, in the dark in 100 \(\mu\)l of PBS with 3% FCS. Subsequently, cells were washed twice and resuspended in 300 \(\mu\)l of PBS-BSA. Multivariate data analysis was performed using the CellQuest software (BD Biosciences).

\(\textup{3}\) Abbreviations used in this paper: sTcAg, soluble \textit{T. cruzi} Ag; 7-AAD, 7-aminoactinomycin; T reg, T regulatory cell.
to normal levels by day 20 after infection (Fig. 1B). The percentage of CTLA-4–expressing-cells was increased in both, CD4⁺ and CD8⁺ T cells from infected mice (Fig. 1C). At days 7–9, the expression of CTLA-4 is 8–10 times higher on cells from infected mice as compared with cells from uninfected mice (Fig. 1B). In vitro restimulation with Con A, but not with sTcAg, resulted in an additional increase of surface CTLA-4 expression in both CD4⁺ and CD8⁺ T cells from infected mice (Fig. 1D).

**Impaired lymphocyte proliferation in the acute phase of T. cruzi infection in mice is not restored by blocking CTLA-4**

Since CTLA-4 has been implicated in modulating T cell unresponsiveness (14,16, 17, 20) and taking into account that the expression of this molecule is significantly enhanced after T. cruzi infection, we next investigated whether CTLA-4 engagement could be involved in determining the T cell unresponsiveness observed during the acute phase of T. cruzi infection (3, 4, 6). Spleen cells harvested from infected or naive mice were cultured in the presence of anti-CTLA-4 (mAb clone UC104F10) or anti-CTLA-4 plus parasite Ags and the lymphoproliferative response was evaluated. The administration of mAb anti-CTLA-4 in vitro did not lead to any significant changes in the lymphoproliferative response to Ag or mitogen in cells from infected mice (data not shown). It is possible, however, that events occurring in vivo after infection would preclude us from seeing any effect of blocking CTLA-4 in vitro. To explore this possibility, we treated infected and control mice with mAb anti-CTLA-4 before analyzing the lymphoproliferative response to parasite Ags or parasite Ags plus anti-CTLA-4 in vitro. Treatment with anti-CTLA-4 in vivo (4 h before infection) did not alter significantly the proliferation profile in either naive or infected mice to any of the stimuli tested (Fig. 2A). Similar results were obtained when the anti-CTLA-4 mAb was administrated (at the same dosage) 24 h after infection (data not shown).

**CTLA-4 blockade does not inhibit apoptosis induced after T. cruzi infection**

It has been reported that CTLA-4 engagement might also induce apoptosis on activated T cells (36); therefore, we asked whether blocking CTLA-4 would decrease T cell death by apoptosis induced in vivo and in vitro by infection with T. cruzi. To answer this question, apoptosis induction was evaluated in spleen cells harvested from control or infected mice treated with either anti-CTLA-4 or control IgG Ab 4 h before infection. As previously demonstrated (4, 6), apoptosis levels were significantly enhanced in cells from T. cruzi–infected mice, when compared ex vivo to cells from uninfected mice (3.6 ± 1.2% and 13.2 ± 2.1%, respectively). Following the same pattern observed for the lymphoproliferative response, treatment with anti-CTLA-4 mAb in vivo did not cause any significant alteration in the amount of apoptotic cells, either in the control or in the infected mice (4.9 ± 1.3% and 14.2 ± 2.3%, respectively).

Induction of apoptosis after CTLA-4 engagement has been reported to occur in strict conditions, namely, only primed T cells in the presence of specific Ags (36). Thus, we studied whether CTLA-4 blockade could decrease the number of apoptotic cells observed after contact with T. cruzi Ags. The percentage of apoptotic cells was evaluated in spleens from anti-CTLA-4, IgG-treated and untreated infected mice. Flow cytometry was performed after culturing the cells for 48 h in the presence or absence of parasite Ags with or without additional anti-CTLA-4 mAb added to the cultures. The results presented on Fig. 2 indicate that the treatment with anti-CTLA-4 in vivo did not reduce apoptosis when cells were cultured in medium only or when they were stimulated with parasite Ags or parasite Ags plus anti-CTLA-4 (Fig. 2B). Indeed, as compared with cells from control IgG–treated mice, cells from mice treated with anti-CTLA-4 in vivo showed a
slightly increased percentage of apoptosis when cultured in the presence of sTcAg plus anti-CTLA-4 (Fig. 2B).

NO production after T. cruzi infection is enhanced by blocking CTLA-4 in vivo

In an effort to evaluate whether the increased CTL-4 expression observed after the infection could influence other aspects of the immune response against T. cruzi, we next evaluate NO production in anti-CTLA-4-treated mice. To determine whether or not the blockade of CTLA-4 could interfere with the induction of NO, we compared its production in vitro and in vivo in T. cruzi-infected and anti-CTLA-4 or IgG-treated mice. The evaluation of NO produced after culturing spleen cells for 48 h showed that in infected mice treated in vivo with anti-CTLA-4, NO synthesis was greatly enhanced in the presence of sTcAg or Con A when compared with IgG-treated mice. However, addition of anti-CTLA-4 in vitro did not result in any additional increase of NO production (Fig. 3A).

NO synthesis on noninfected mice was not altered by treatment with anti-CTLA-4 either in vivo or in vitro (Fig. 3A).

Evaluation of NO production in vivo by measuring nitrite and nitrate levels on the plasma showed that the blockage of CTLA-4 also led to a significant increase (p < 0.05) in NO production in vivo (3-fold increase as compared with control Ab-treated mice; Fig. 3B). Uninfected mice synthesized minimal amounts of NO, which were not affected by anti-CTLA-4 treatment (Fig. 3B). Cells from naive mice treated with anti-CTLA-4 did not increase their NO synthesis in response to parasite Ags. Treatment of T. cruzi-infected mice with anti-CTLA-4 mAb 24 h after infection instead of 4 h before infection resulted in a similar increase in NO production, assayed both in vivo and in vitro (data not shown).
Treatment with anti-CTLA-4 increases the production of IFN-γ and TNF-α induced by T. cruzi infection

It is known that IFN-γ and TNF-α are two of the major cytokine inducers of iNOS in T. cruzi-infected mice. Since blocking CTLA-4 substantially increased NO production in infected mice, we asked whether CTLA-4 could be involved in the regulation of the production of these cytokines in response to the infection. To investigate this possibility, spleen cells from T. cruzi-infected, anti-CTLA-4, or IgG-treated mice were harvested 11 days after infection and cultured in the presence or absence of parasite Ags, anti-CTLA-4 mAb, or parasite Ags plus anti-CTLA-4 mAb. The concentration of IFN-γ and TNF-α was measured in the culture supernatants after 48 h. As previously published (5, 6), T. cruzi infection leads to an increase in the production of IFN-γ by spleen cells in vitro when the cultures are stimulated with parasite Ags (data not shown). Treatment of infected animals in vivo with anti-CTLA-4 resulted in increased production of both IFN-γ (Fig. 4A) and TNF-α (Fig. 4B). Culture of spleen cells in the presence of additional anti-CTLA-4 in vitro minimally enhanced IFN-γ production in cells from infected mice treated with anti-CTLA-4 in vivo; however, the same in vitro treatment resulted in a further increase in the synthesis of TNF-α (Fig. 4B). Furthermore, IL-2 synthesis in response to parasite Ags was also increased by treatment with anti-CTLA-4 in vivo (Fig. 4C), suggesting that the treatment with anti-CTLA-4 was effective (14, 16, 20).

Blocking CTLA-4 in vivo decreases susceptibility to T. cruzi infection

Because IFN-γ, TNF-α, and NO have all been reported as playing major roles in resistance against infection with T. cruzi (3, 5–8, 10, 11), the changes induced by blocking CTLA-4 in the synthesis of these factors could enhance the ability to kill the parasite. To test this hypothesis, C57BL/6 mice were treated with mAb anti-CTLA-4 or control IgG and infected with increasing numbers of T. cruzi. Parasitemia and mortality were evaluated at different time points after the infection. When infection was performed with 10³ parasite forms, the treatment with anti-CTLA-4, but not with control hamster IgG, led to a significant reduction in parasitemia, most notably at days 9 (p < 0.01) and 10 (p < 0.05) postinfection (Fig. 5A).

Since 100% of the C57BL/6 mice infected with 10³ forms survive acute and chronic phases of infection (6, 10), we next infected C57BL/6 mice with 10⁴ forms (which leads to death from acute disease in all mice infected) and 24 h later treated them with anti-CTLA-4 or control IgG. This treatment schedule has been used before to efficiently block CTLA-4 in mice infected with other intracellular parasites (31), and it was preferred instead of the treatment schedule used in the previous experiment (Fig. 5A) in an attempt to maximize the efficacy of CTLA-4 blockage. We hypothesized that infection with either higher inoculums or more virulent strains could result in different kinetics of T cell activation and consequently of CTLA-4 expression, which would be presumably more efficiently blocked if the neutralizing Ab anti-CTLA-4 was administered early after the infection. In addition, since the kinetics of CTLA-4 expression induced by the infection in vivo is delayed compared to that observed in vitro, we assumed that the anti-CTLA-4 Ab effects would last longer if administered 24 h after infection instead of 4 h before the infection. Indeed, the treatment with anti-CTLA-4 24 h after the infection resulted in a significant decrease in parasitemia (Fig. 5B). Nine days after infection, anti-CTLA-4-treated mice had a 6-fold decrease in the number of parasites in the blood as compared with the IgG-treated group. At day 10 after infection, parasitemia was still significantly smaller in the anti-CTLA-4-treated group (~3-fold lower) in comparison to the parasitemia of the IgG-treated group. By day 11 postinfection, the differences between the two groups were no longer statistically significant. Evaluation of mortality rates showed that while 100% of the IgG-treated mice died by day 20 after infection, all of the anti-CTLA-4- and T. cruzi-infected mice survived the acute phase (Fig. 5C). A significant reduction in parasitemia levels and similar rescue of mortality were observed when
mice were treated with anti-CTLA-4 Ab 4 h before instead of 24 h after the infection with 10^4 parasite forms (data not shown).

Whereas infection with 10,000 trypomastigotes from the Y strain is a suitable model to study resistance to the acute phase of Chagas’ disease, there are other, more aggressive, strains of the parasite that can cause death at much smaller parasite inoculums. To study whether blocking CTLA-4 could modulate resistance to the infection with a more virulent parasite strain, C57BL/6 mice were infected with 100 μg of control hamster IgG (●) or hamster anti-CTLA-4 (▲) mAb, and 4 h later infected with 10^4 T. cruzi trypomastigote forms (Y strain, A). Alternatively, mice (five per group) were infected with 10^4 parasite forms and 24 h later treated with the anti-CTLA-4 Ab (B and C). Parasitemia (A and B) and survival rates (C) were evaluated at different time points. Asterisks indicate statistical significance, p ≤ 0.05 (+) or p ≤ 0.001 (++) (Mann-Whitney U test) compared with the values obtained from control IgG-treated mice at the same time point. Similar results were obtained in three (A) or two (B and C) other experiments performed independently.

Discussion

The acute phase of murine infection with T. cruzi spans from 7 to at least 20 days after parasite inoculation. This phase of disease is characterized by high parasitemia, suppression of lymphocyte proliferative responses, decreased IL-2 production, anergy, and apoptosis of parasite-specific and bystander lymphocytes (2–4, 37). The role of Fas-Fas ligand interaction in this process has been extensively studied (6, 38). Nevertheless, the participation of other T cell down-regulatory molecules such as CTLA-4 in suppression of lymphoproliferative response during the acute phase of this infection is not understood. CTLA-4 engagement has been broadly associated with the down-regulation of the immune system. Under the overwhelming activation of the cellular immune response induced by infection with T. cruzi, CTLA-4 may be prematurely expressed, resulting in an untimely termination of the process that leads to clonal expansion. This may constitute an escape mechanism used by the parasite to survive the initial attack of the immune response and/or part of a safety instrument used by the immune system to avoid the consequences of extreme activation of the immune response. The latter hypothesis is corroborated by the increase in the expression of CD28, CD69 CD45RB, and, to a lesser extent, CD40 ligand observed in infected mice during the

**FIGURE 5.** Blockage of CTLA-4 leads to increased resistance to T. cruzi (Y strain) infection in mice. C57BL/6 mice (10/group) were treated with 100 μg of control hamster IgG (●) or hamster anti-CTLA-4 (▲) mAb, and 4 h later infected with 10^4 T. cruzi trypomastigote forms (Y strain, A). Alternatively, mice (five per group) were infected with 10^4 parasite forms and 24 h later treated with the anti-CTLA-4 Ab (B and C). Parasitemia (A and B) and survival rates (C) were evaluated at different time points. Asterisks indicate statistical significance, p ≤ 0.05 (+) or p ≤ 0.001 (++) (Mann-Whitney U test) compared with the values obtained from control IgG-treated mice at the same time point. Similar results were obtained in three (A) or two (B and C) other experiments performed independently.

**FIGURE 6.** Blockage of CTLA-4 leads to increased resistance to T. cruzi (Colombian strain) infection in mice. C57BL/6 mice were infected with 10^2 trypomastigote forms (Colombian strain) and treated 24 h later with 100 μg of control hamster IgG (●) or anti-CTLA-4 (▲) mAb anti-CTLA-4. Parasitemia (A) and survival rates (B) were evaluated at different time points. Results shown (mean ± SD) are from one experiment performed with 10 mice/group. Asterisks denote statistical significance, p ≤ 0.05 (+) or p ≤ 0.005 (++) (Mann-Whitney U test) compared with the values obtained from control IgG-treated mice at the same time points. Similar results were obtained in another two experiment performed independently.
acute phase of disease preceding the decrease in the lymphoproliferative response (G. A. Martins, R. B. Silva, L. V. Rizzo, and J. S. Silva, unpublished observations). In this present study, we evaluate the possible implications of CTLA-4-B7 interaction in controlling the immune response during the infection with *T. cruzi*.

We first observed that exposure to live trypomastigote forms resulted in a significant increase in CTLA-4 expression in spleen T cells in vitro. We also observed that the infection of mice with *T. cruzi* resulted in increased CTLA-4 expression in both CD4+ and CD8+ T cells in vivo. Increased CTLA-4 expression in CD4+ T cells has been previously reported in mice and humans infected with different parasites; however, in many of these reports, CTLA-4 expression was selectively increased in CD4+ T cells as opposed to CD8+ T cells (39–41). It is noteworthy that *T. cruzi* infection resulted in increased CTLA-4 expression in both CD4+ and CD8+ T cells. Although we did not further explore the biological importance of this finding, it is likely that CTLA-4 expression could increase as a consequence of CD8+ T cell activation soon after the infection. It is also tempting to speculate that the increased CTLA-4 expression by CD8+ T cells after *T. cruzi* infection could be potentially implicated in modulating the recently described CD8+ T cell dysfunction in nonlymphoid tissues from chronically infected mice (42). Interestingly, a more recent report showed that CTLA-4-expressing CD8+ T cells play a decisive role in the outcome of *Plasmodium yoelii* infection in mice (43). It is also possible that the CTLA-4-expressing CD4+ and/or CD8+ T cells described in the present study could belong to regulatory T cells (T reg) subpopulations, perhaps expanded during the infection. This possibility remains to be investigated. Both CD4+ and CD8+ T reg have been described elsewhere (44, 45) and many examples of pathogen-specific regulatory cells have now been reported (reviewed in Ref. 46); however, regulatory T cells seem to be a heterogeneous population and not necessarily all of them function in a CTLA-4-dependent manner (47). In addition, CTLA-4 is also expressed by T cells out of the T reg pool (47, 48).

The mechanisms bearing the increased and sustained CTLA-4 expression on T cells induced by *T. cruzi* infection are not fully understood. The increased CTLA-4 expression could simply be a consequence of T cell activation caused by the infection. It could also result from the presence of soluble factors secreted by the infected cells, such as cytokines. The latter hypothesis is supported by the finding that naïve spleen T cells also up-regulate CTLA-4 expression when cultured with parasite-free supernatant from infected mice spleen cells cultures, or with supernatant from *T. cruzi*-infected peritoneal macrophages (G. A. Martins, R. B. Silva, L. V. Rizzo, and J. S. Silva, unpublished observations). The notion that CTLA-4 expression might be regulated in response to cytokines is emphasized in other infectious diseases such as tuberculosis, where administration of anti-IL-10 restored CTLA-4 expression in PBMC from *Mycobacterium tuberculosis*-infected patients (49). IL-10 is produced during *T. cruzi* infection in vivo and in vitro and it has been implicated in the sensitivity to infection by antagonizing IFN-γ effects (50, 51). Considering that IFN-γ is produced in high levels after infection with *T. cruzi* (3, 5, 51), one could speculate that IFN-γ induced by *T. cruzi* infection might somehow participate in the induction of CTLA-4 expression.

A previous report has shown that blockage of CTLA-4 in vitro is able to restore the proliferation of T lymphocytes from *L. chagasi*-infected mice (33). Since mice acutely infected with *T. cruzi* also show a profound suppression of immune response and we have found increased CTLA-4 expression in spleen T cells from the infected mice, we asked whether CTLA-4 could be implicated in modulating the decreased T cell proliferation in those animals. Unlike the results with *Leishmania*-infected mice, we demonstrated that CTLA-4 blockage in vitro does not modulate proliferation of T cells from *T. cruzi*-infected animals (Fig. 3A). The fact that similar results were obtained when CTLA-4 was blocked by treating mice with the specific Ab anti-CTLA-4 in vivo indicates that the inability to restore the lymphoproliferative response was not due to inefficient blockage of CTLA-4 functions in vitro but rather to differences in the regulation of the immune response after infection with these two protozoan parasites. Furthermore, the enhanced production of IL-2 following treatment with anti-CTLA-4 suggests that the blockage was effective in modulating immune function in these animals (14, 16, 20), although the increased production of this cytokine did not restore the T cell proliferation.

The blockage of CTLA-4 did not result in any reduction in apoptosis induction in cells from *T. cruzi*-infected mice. As a matter of fact, neutralization of CTLA-4 in vitro in spleen cells from anti-CTLA-4-treated infected mice resulted in increased apoptosis when cells were simultaneously stimulated with parasite Ags. Although we have not further investigated the mechanisms behind this effect, it is possible that the increase in apoptosis rates in this culture is due to the increase in NO production observed in these conditions (see Fig. 3A). As previously reported, NO induces apoptosis in cells from *T. cruzi*-infected mice (4, 6). Furthermore, since IL-2 production is increased by CTLA-4 blockage in these cultures, it is also possible that the neutralization of CTLA-4 resulted in an increase in IL-2-mediated activation-induced cell death.

In an effort to understand the reason why CTLA-4 blockage was unable to restore the lymphoproliferative response in *T. cruzi*-infected mice, we investigated whether CTLA-4 was involved in modulating other mechanisms implicated in induction of unresponsiveness after infection with *T. cruzi*. Since excessive NO synthesis has been associated with suppression of the proliferative response during infection with many different parasites, including *T. cruzi* (2, 3), we analyzed whether the blockage of CTLA-4 would affect the production of NO. Our results showed that neutralization of CTLA-4 in vivo led to an increased production of NO by cells from *T. cruzi*-infected mice. The fact that the blockage of CTLA-4 function also resulted in increased IFN-γ and TNF-α production indicated that CTLA-4 engagement would modulate NO production probably through an increased production of these cytokines. In support of this hypothesis, modulation of IFN-γ production by CTLA-4 has been demonstrated to occur in mice infected with other parasites, including *L. major* and *L. chagasi* (32, 33). In *L. chagasi*-infected mice, the increase in IFN-γ production resulting from the neutralization of CTLA-4 was linked to inhibition of TGF-β synthesis (52), which might be induced after CTLA-4 engagement in T cells (53). We cannot discard the possibility that the increase in IFN-γ production we observed when *T. cruzi*-infected mice were treated with anti-CTLA-4 could also be mediated by an inhibition of TGF-β. Indeed, TGF-β has been associated with inhibition of IFN-γ production and impairment of microbicidal activity in *T. cruzi*-infected mice (54, 55). Nevertheless, it is important to take into account that CTLA-4 function is not always dependent on induction of TGF-β synthesis (56).

Since the treatment with anti-CTLA-4 Ab was shown to enhance production of IFN-γ, TNF-α, and NO, all important factors involved in resistance to *T. cruzi* infection, we next evaluated whether the blockage of CTLA-4 function could result in resistance to infection with *T. cruzi*. Strikingly, the treatment with anti-CTLA-4 led to a significant decrease in parasitemia and mortality of mice infected with the Y strain of *T. cruzi*, indicating that engagement of CTLA-4 does modulate susceptibility to infection, probably through modulation of the production of IFN-γ, TNF-α, and consequently NO. When the CTLA-4 function was blocked in...
mice infected with the Colombian strain of *T. cruzi*, which is significantly more virulent than the Y strain (57), and normally results in elevated mortality rates in the C57BL/6 mouse, we could not detect any significant difference in the mortality rates, comparing anti-CTLA-4 and control Ab-treated mice. We speculated that the inability to rescue survival in these settings could be due to a less efficient blockage of CTLA-4. It is possible that the kinetics of CTLA-4 expression in cells from mice infected with the Colombian strain would somehow differ from that observed when mice were infected with the Y strain, and treatment with a single dose of anti-CTLA-4 mAb could be insufficient to fully prevent CTLA-4 signaling in this system. We evaluated the expression of CTLA-4 in cells from mice infected with Colombian strain, but it did not differ significantly from that observed in cells from Y strain-infected mice (data not shown). Even so, as we observed increased CTLA-4 expression until day 20 postinfection in infected mice, we did treat Colombian strain-infected mice with two doses of anti-CTLA-4 Ab by administrating anti-CTLA-4 mAb (100 μg/mice) 4 h before and 24 h after infection; however, this treatment did not result in any significant increase in survival rates (data not shown).

Since elevated expression of CTLA-4 is seen 3 days after infection and continues up to 11 days after infection, one could argue that a different treatment schedule (administrating the Ab anti-CTLA-4 at a later time point, rather than increasing the number of doses) would perhaps be more efficient in blocking CTLA-4 activity. Nonetheless, our decision to treat mice 24 h after infection was guided by previous data indicating that this treatment schedule is efficient to block factors induced soon after the infection in a similar mode to CTLA-4 (4, 5). In addition, reports in the literature (31, 32) suggest that these time points are most effective at influencing the outcome of infection in other parasitic diseases. When testing the consequences of CTLA-4 blockage after the infection with higher parasite inoculums or more virulent parasite strains, we took into account that a more virulent infection could result in a higher T cell activation, possibly resulting in increased or longer lasting CTLA-4 expression. Thus, we used the same treatment schedule used by others who efficiently blocked CTLA-4 in a different infection model. Regardless of the treatment schedule used, we always observed a consistent increase in IL-2 synthesis by cells from anti-CTLA-4-treated mice as compared with that observed in cells from control Ab-treated mice (Fig. 4C), indicating that CTLA-4 activity was efficiently blocked.

The finding that CTLA-4 blockage results in decreased parasitemia in C57BL/6 mice infected either with the Colombian or Y strain, but results in improved survival rates only in mice infected with the Y (less pathogenic) strain, could suggest that although CTLA-4 may play a role in modulating resistance to *T. cruzi* infection, its protective effect seems to be restricted to the less stringent circumstances. A similar finding was reported in mice infected with *Mycobacterium*, where the blockage of CTLA-4 led to increased immune response but did not improve parasite clearance (30).

Recent studies have suggested that CTLA-4 works as a negative signal for the development of Th2 cells by decreasing the strength of the TCR interaction and consequently favoring Th1 differentiation (58). It has also been proposed that engagement of CTLA-4 may control the generation of memory T cells by regulating the expression of activated rapidly proliferating uncommitted T cells in the periphery (16, 20, 28). We have not investigated the effect of blocking CTLA-4 in vivo on the development of a secondary response to *T. cruzi*. The temporary blockage of CTLA-4 by the Ab does not seem to skew the response against the parasite toward the Th2 phenotype. This is supported by the fact that IL-4 synthesis in response to parasite Ags is not increased in cells from anti-CTLA-4-treated mice (data not shown). On the contrary, we observed an increase in production of IFN-γ, which is consistent with data showing that Th1 responses are protective against infection with *T. cruzi*. Altogether, the data presented in this study reinforces the notion that CTLA-4 is an important molecule in the control of the immune response against parasites and that its induction by infectious agents may be part of the mechanisms they have developed to exploit normal regulatory pathways of the immune system to evade destruction.

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References


