Chagas’ disease is the most important cause of acquired cardiomyopathy in Latin America and is one of the outcomes resulting from the interaction between the human immune system and the hemoflagellate prokaryote Trypanosoma cruzi. In the natural infection, the flagellated forms in the feces of infected hematophagous insects of the Triatominae subfamily invade the host through skin lesions or intact mucosa. The parasite then proliferates intracellularly and disseminates systemically from the site of inoculation, causing an inflammatory reaction of variable intensity, along with splenomegaly, cardiac damages due to the combined effects of parasite persistence, immune deregulation, autonomic denervation, and microvascular damages (21, 30).

The immunological mechanisms underlying this silent, relentless infection and heart pathology remain elusive despite several decades of research. It is known that T cell-mediated immune responses are essential to control the parasite replication during the acute phase of the infection (33). The cytokines gamma interferon (IFN-γ), interleukin-12 (IL-12), and tumor necrosis factor alpha (TNF-α) strengthen the activation of innate and adaptive effector immune responses, resulting in more efficient killing of the parasite and a strong inflammatory response in several tissues where parasites replicate, including the myocardium. On the other hand, the cytokines IL-10 and transforming growth factor β (TGF-β) counterregulate the inflammatory process, indirectly favoring parasite persistence within infected host cells because they are potent inhibitors of nitric oxide (NO) production and other IFN-γ- and IL-12-mediated cell activation processes that are important for the killing of the parasite (12, 32). The extent of this regulation seems to be crucial for the final outcome of the illness, since patients with the indeterminate (asymptomatic) form of the disease have a more controlled immune response (38) than patients with advanced stages of infection.
The intensity of a protective immune response can be determined by the balanced expression of costimulatory and coinhibitory molecules during priming of T cells by antigen-presenting cells (APC). CD28 and inducible T cell costimulator (ICOS) are costimulatory receptors, while cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death cell receptor 1 (PD-1) exert a negative function to prevent excessive T cell activation (8). PD-1 is a member of the CD28 family, expressed mainly on activated T, B, and myeloid lineage cells (1). It signals through the ligands PD-L1 (9) and PD-L2 (18), which are expressed by an increasing number of cell types, including myeloid, lymphoid, and nonlymphoid cells. Engagement of PD-1 with any of its two ligands inhibits the activation of T cells and the production of cytokines, especially IL-2 and TNF-α (8). Mice of the BALB/c strain which are deficient in PD-1 lost their immunological tolerance to cardiac autoantigens and can suffer spontaneous autoimmune dilated cardiomyopathy (25). In addition, the blockade of PD-1 engagement accelerates autoimmune disorders (3, 31) and graft-versus-host disease (7, 24). Induction of PD-L1 expression has also been demonstrated as a mechanism for immune evasion by intracellular pathogens (15). Recent evidence suggests that avoiding signaling through coinhibitory molecules could constitute promising immunotherapeutic strategies in antiviral and anti-tumor cellular immunity (14). For example, treatment with anti-CTLA-4 antibodies improves the cellular immune response against T. cruzi (23). It therefore seems reasonable to hypothesize that PD-1 may participate in the cell-mediated immune response and in the maintenance of cardiac tolerance during an infection with T. cruzi. Here we show that this infection induces increased expression of PD-1 by cells of the immune system and that this regulatory pathway is involved in the control of acute myocarditis, as its inhibition or gene deletion leads to increased cardiac inflammation.

**MATERIALS AND METHODS**

Mice, antibodies, and treatments. C57BL/6 mice aged 6 to 8 weeks, obtained from local animal facilities (FMRP-USP), were treated with anti-PD-1 (RPMI-14, anti-PD-L1 (MH5), anti-PD-L2 (TY25), or normal rat IgG starting 48 h before infection and lasting for 2 weeks. During this period, intraperitoneal (i.p.) injections containing 250 μg of antibody were administered to mice every 72 h. Antibodies against PD-1 (RPMI-14), PD-L1 (MH5), and PD-L2 (TY25) were produced in the labs of T. Honjo and M. Azuma. Four or five hearts were collected from mice at 14 and 20 days postinfection (p.i.) for histology, immunohistochemistry, PCR, and enzyme-linked immunosorbent assay (ELISA) studies. Noninfected age-matched mice were used as controls. For survival studies, two independent (anti-PD-1-treated and rat IgG-treated) groups of 8 animals were followed until 35 days postinfection. Mice were cared for according to the local guidelines on ethics in animal experiments.

**Parasites and experimental infection.** Mice were infected (i.p.) with 1,000 bloodstream forms of T. cruzi (Y strain) obtained from intermediary strain-matched mice. Parasitemia levels were evaluated in 5 μl of blood drawn from the tail. Before infection of intermediary mice, parasites were grown and purified from the monkey kidney fibroblast line LLC-MK2 (ATCC).

**Histological analysis.** Quantification of heart tissue inflammation was assessed by stereologically counting inflammatory cells in four representative nonconsecutive hematoxylin-eosin (H&E)-stained sections (thickness of 5 μm) per organ (per side). The day of death was determined as the time of last contact (adult mouse) or the day of birth (newborn mouse). Histological sections were stained with eosin and examined with a Zeiss microscope. The percentage of inflammatory cells was evaluated in 1.5% agarose gel.

**Synthesis of cDNA and real-time PCR.** cDNA was synthesized using 2 μg of RNA by a reverse transcriptase reaction, using ImProm-II reagents (Promega, Madison, WI) in a PTC 100 thermal cycler (MJ Research, Watertown, MA). The conditions used for the reaction were as follows: 5 min at 70°C and 1 h at 42°C, followed by refrigeration at 4°C. The total volume of the reaction was 25 μl, which was diluted by 8-fold, reaching a total volume of 200 μl. Real-time PCR was performed using the Platinum SYBR green qPCR SuperMix uracil-DNA glycosylase (UDG) with ROX reference dyes (Invitrogen, Carlsbad, CA). PCRs were performed using the Platinum SYBR green qPCR SuperMix uracil-DNA glycosylase (UDG) with ROX reference dyes (Invitrogen, Carlsbad, CA) using forward and reverse primers (sequences are listed in Table 1) that we designed with Primer Express software (Applied Biosystems, Foster City, CA), according to nucleotide sequences available in the GenBank database. Expression of each mRNA was normalized to a constitutive mRNA (β-actin) by the threshold cycle (ΔΔCT) method as previously described (27).

**Isolation of inflammatory cells from cardiac tissues and cytometry.** Hearts collected from 5 mice at day 20 p.i. were minced, pooled, and incubated for 1 h at 37°C with RPMI 1640, supplemented with NaHCO₃, penicillin-streptomycin-gentamicin, and 0.05 g/ml of liberase blendzyme CI (Roche, Basel, Switzerland). The organs were processed in a Medimachine (BD Biosciences, San Jose, CA) using forward and reverse primers (sequences are listed in Table 1) that we designed with Primer Express software (Applied Biosystems, Foster City, CA), according to nucleotide sequences available in the GenBank database. Expression of each mRNA was normalized to a constitutive mRNA (β-actin) by the threshold cycle (ΔΔCT) method as previously described (27).

**TABLE 1. Sequences of primers used in real-time PCR**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5'-AGC TGC GTT TTA CAC CCT TT-3'</td>
<td>5'-AAG CCA TGC CAA TGT TGT CT-3'</td>
</tr>
<tr>
<td>PD-1 mouse</td>
<td>5'-TTC AGG TTT ACC ACA AGC TGG-3'</td>
<td>5'-TGA CAA TAG GAA ACC GGG AA-3'</td>
</tr>
<tr>
<td>PD-L2 mouse</td>
<td>5'-TTG TCG GTG TGA TTG GCT TC-3'</td>
<td>5'-AAA AGG CAG CAC ACA GTT GC-3'</td>
</tr>
</tbody>
</table>

**Histopathological analysis.** Histopathological analysis was performed with a FACScan apparatus and a Coulter counter (Beckman Coulter, Miami, FL). Fluorescently labeled antibodies were used to evaluate the expression of various markers: CD3 (1:100 dilution of mouse anti-human CD3 antibody, BD Biosciences, San Jose, CA), CD4 (1:100 dilution of mouse anti-human CD4 antibody, BD Biosciences, San Jose, CA), CD8 (1:100 dilution of mouse anti-human CD8 antibody, BD Biosciences, San Jose, CA), CD11b (1:100 dilution of mouse anti-human CD11b antibody, BD Biosciences, San Jose, CA), and CD19 (1:100 dilution of mouse anti-human CD19 antibody, BD Biosciences, San Jose, CA). The stained cells were analyzed by flow cytometry using a FACScan apparatus and a Coulter counter (Beckman Coulter, Miami, FL). Flow cytometric analysis was performed with a FACScan apparatus and a Coulter counter (Beckman Coulter, Miami, FL).
results demonstrated for the first time that cells showed no correlation with the time of infection. These levels after day 20. Expression of PD-1 on CD8, NK, or NKT the case of CD4 T cells, the expression of PD-1 fell to normal along the time course of the infection (Fig. 1A, E, and F). In addition of complete culture medium, and the cells were centrifuged (5 min, 37°C, and protected from light). Staining was stopped by 300

padatime points after infection with T. cruzi, and the expression of PD-1 was determined in each cell subset by cytometry. (A to F) Gated on lymphocytes (A to D) and on monocytes (E, F). Data are representative values obtained from three independent experiments. Asterisks denote P values of <0.005 by ANOVA compared to uninfected values (I).

CellQuest software (both obtained from BD Biosciences) as well as FlowJo software (Tree Star, Ashland, OR).

**Lymphocyte proliferation assays.** Analysis of lymphocyte proliferation was performed by carboxyfluorescein diacetate succinimidyl ester (CFSE) staining. In brief, spleen-derived leukocytes (1 × 10⁶ cells/ml) were stained with 5 μmol/liter CFSE (5 min, 37°C, and protected from light). Staining was stopped by addition of complete culture medium, and the cells were centrifuged (5 min, 300 × g). The cell suspension was adjusted to 5 × 10⁶ cells/ml and plated in a 96-well culture plate (Nunc) at 200 μl cells/well, and then the cells treated with 1 μg of each antibody/well or left alone in medium for 72 h. Some wells were precoated with anti-CD3 (2.5 μg/ml). Data analysis was performed using a flow cytometer on a FACSCanto II apparatus (BD) using FACSDiva (BD) and FlowJo (Tree Star) softwares by setting a gate on the live cells to side-scatter versus forward-scatter dot plots and determining the expression of the CFSE.

**Statistical analysis.** Data are expressed as means ± standard errors of the means (SEM). Analysis of variance (ANOVA) followed by Student’s t test was used to determine the statistical significance of the observed differences between the treated and control groups. The Kaplan-Meier method was used to compare the survival times of the study groups. Differences were considered statistically significant at P values of <0.05. All analyses were performed using the PRISM 3.0 program (GraphPad Software, San Diego, CA).

**RESULTS**

**Modulation of PD-1 expression in immune cells by T. cruzi infection.** To evaluate if T. cruzi infection upregulates the expression of PD-1 in immune cells in vivo, flow cytometry was performed on spleen cells at several time points after infection, and the percentage of cells expressing PD-1 on the surface was quantified. The results showed that the infection leads to a gradual increase in the expression of PD-1 in spleen cells (Fig. 1). This was particularly seen within TCD4 cells and APC (CD11b⁺ CD11c⁺, CD11b⁺), where a curve was observed along the time course of the infection (Fig. 1A, E, and F). In the case of CD4 T cells, the expression of PD-1 fell to normal levels after day 20. Expression of PD-1 on CD8, NK, or NKT cells showed no correlation with the time of infection. These results demonstrated for the first time that T. cruzi is able to modulate the expression levels of the negative coreceptor PD-1, a molecule known to trigger a regulatory pathway that inhibits T cell activation.

**Increased levels of mRNA transcripts for PD-1 and its ligands in heart tissue from T. cruzi-infected mice.** To assess the levels of mRNA for PD-1 and its ligands, cardiac tissues were collected at days 14 and 20 p.i. and compared to normal hearts. The results showed significantly increased levels of mRNA transcripts for PD-1 on both of the examined time points (by 5- and ~3-fold, respectively) (Fig. 2A). Transcripts for PD-L1 were significantly increased on day 20 p.i. (Fig. 2B), while the PD-L2 transcripts remained unchanged (Fig. 2C). We further confirmed the presence of PD-1 and its ligands by immunofluorescence of the heart and spleen by comparing infected and normal tissues. The results revealed marked expression of PD-L1 (see Fig. S1E in the supplemental material) and low expression of PD-L2 (see Fig. S1F) in cardiac tissue obtained from infected mice at day 20 p.i., while no perceptible expression of PD-1 was detected by this methodology. Of note, no expression of the three molecules in normal hearts (see Fig. S1A to C in the supplemental material) was observed. Interestingly, reduced expression levels of PD-1 and its ligands in the spleen after infection were observed. From being expressed typically in the B-T cell region to agglomerating mostly within
T cell zones (see Fig. S1G and J in the supplemental material), these data show that *T. cruzi* infection modulates the expression of PD-1 and its ligands in the heart and spleen.

Heart-infiltrating CD4<sup>+</sup> and CD8<sup>+</sup> T cells express PD-1. The expression of PD-1 was determined by flow cytometry in CD4 and CD8 T cells extracted from heart tissues on day 20 p.i. (Fig. 3). The results showed that 88.03% and 99.34% of CD4 T cells express PD-1 and PD-L1 (Fig. 3C and D). The frequencies of CD8 T cells expressing PD-1 and PD-L1 was 98.62% and 98.62%, respectively, while the frequencies of being PD-L<sub>2</sub> among CD8 and CD4 T cells was 79.29% and 49.68%, respectively. These results clearly show that PD-1 and its ligands are highly expressed in lymphocytes found in the hearts of *T. cruzi*-infected mice.

PD-1 blockade results in increased acute myocarditis and reduced survival in mice infected with *T. cruzi*. Aiming to test if the PD-1-dependent regulatory pathway is indeed involved in the maintenance of cardiac tissue tolerance during *T. cruzi* infection in vivo, we treated *T. cruzi*-infected mice with blocking antibodies against each one of the PD-1-related molecules (PD-1, PD-L1, and PD-L2) and studied the heart histopathology as well as the local production of cytokines and chemokines. The results showed that the blockade of PD-1 and PD-L1 (but not of PD-L2) led to increased myocarditis (Fig. 4E and F). This was not observed in the skeletal muscle or in the hepatic tissue (Fig. 4I to Q). In addition, all treatments induced increased expression of NO synthase 2 (NOS2) in the cardiac tissue observed at the same time point (Fig. 4S). The treatment with anti-PD-1, anti-PD-L1 or anti-PD-L2 MAb did not induce any cell migration to the heart tissue of normal C57BL/6 mice (Fig. 4B to D). These results suggest a role for the PD-1 pathway in regulating the inflammatory response at the myocardium during *T. cruzi* infection. In fact, the analysis of inflammatory cells infiltrating the heart tissue of PD-1<sup>−/−</sup> and wild-type (WT) mice showed significantly increased numbers of macrophages and T cells in PD-1<sup>−/−</sup> mice (see Fig. S2G and C, respectively, in the supplemental material), and this was in association with increased expression of pStat5 (see Fig. S2M).

To further explore the effects of the PD1/PDL1/PDL2 blockade treatments in the induction of increased myocarditis, we assayed the levels of the proinflammatory chemokines CCL5, CCL3, and CCL2 and the cytokines IFN-γ and TNF-α in heart homogenates obtained from mice belonging to each experimental group (see Fig. S3 in the supplemental material). Increased levels of all analyzed markers were found in hearts obtained from anti-PD-1-treated mice at day 14 p.i. CCL3 and CCL2 were reduced at day 20 p.i. in mice receiving anti-PD-L1 or anti-PD-L2. In addition, the expression of mRNA for CCR5, which is a receptor for these chemokattractants involved in the Th-1-biased immune response, was higher in the group that received anti-PD-L1 treatment (not shown).

PD-1-dependent regulation is involved in the mechanism that mediates host protection during the acute phase of *T. cruzi* infection. The increased myocardial inflammation observed in *T. cruzi*-infected mice after the blockade of PD-1 signaling molecules suggests that it could be also involved in the resistance to *T. cruzi* infection. In agreement with the increased levels of proinflammatory factors observed in cardiac tissues after the blockade of PD-1, the results showed that mice treated with anti-PD-1 present higher serum levels of IFN-γ and TNF-α than the control mice (Fig. 5A and B) at day 14 p.i. Furthermore, this increased production of IFN-γ (but not of TNF-α) was maintained at day 20 p.i. These results point to a role for PD-1 in the control of the systemic inflammatory response during this infection. To determine the role of PD-1 in the control of parasite proliferation and resistance to the acute phase of the infection, we studied the parasitism and...
mortality of mice belonging to each experimental group. The results demonstrated that anti-PD-1-treated mice have a significant reduction in tissue parasitism (Fig. 5D), and the same results were obtained with PD-1/H11002 mice, which also exhibited reduced parasitemia (Fig. 5E) compared to that of the control group. However, the blockade of PD-1 also lead to a significantly decreased survival rate compared to that of the control group, as they started to die by day 15 p.i. By day 35 p.i., more than 90% of them had succumbed, while in the control group, the survival rate at this date was more than 55% (Fig. 5C). Additionally, PD-1/H11002 mice showed significantly reduced parasitemia compared to that of the WT mice (Fig. 5E). These results demonstrate that, in spite of mice becoming more resistant to infection, PD-1 blockade/deletion makes the host less tolerant to the inflammatory response.

PD-1 blockade induced the increased proliferative response by lymphocytes. To gain further insight into the mechanisms underlying the increased inflammation after PD-1 blockade/gene deletion, CFSE-stained naïve or primed spleen cells were analyzed for proliferation after being incubated with anti-PD-1, anti-PD-L1, or anti-PD-L2. Our results showed that the blockade of PD-1 and PD-L1 led to the increased proliferation of spleen cells from uninfected or infected mice (Fig. 6A). These data provide definitive evidence that the blockade of PD-1 and its ligand PD-L1 induces an increased proliferative response by lymphocytes.

PD-1 deficiency induced diminished apoptosis of T cells during T. cruzi infection. Aiming to study a possible mechanism behind the increased inflammatory response and lymphocyte proliferation observed in mice after PD-1 blockade, we
finally studied the frequency of apoptosis of lymphocytes in WT or PD-1−/− mice. The results showed that *T. cruzi*-infected PD-1−/− mice exhibited significantly reduced numbers of apoptotic CD4+ and CD8+ T cells (Fig. 6B).

In summary, our data demonstrate that PD-1 blockade/deficiency lead to a reduction in the regulation of the immune response, which is related to increased lymphocyte proliferation and reduced apoptosis.

**DISCUSSION**

This study demonstrates for the first time that *T. cruzi* modulates the expression of PD-1, which has been widely involved in T cell exhaustion and persistent infections (5), features that have been associated with *T. cruzi* infection. The expression of PD-1 in association with parasite persistence has been reported for filariasis (4). In addition, increased expression of the ligands PD-L1 and PD-L2 has been reported to be induced by *Taenia crassiceps* in macrophages, which is associated with the inhibition of T cell proliferation by this parasite (36).

*T. cruzi* infection is characterized by acute parasitemia that is usually cleared out raising an intense cellular immune response against the parasite that usually causes extensive tissue damage, leading to fibrosis and dysfunction of the myocardium and other organs. However, tissue parasitism can persist, being responsible for continued tissue destruction. In this study, we show that *T. cruzi* infection upregulates the expression of PD-1 by CD8 and CD4 T cells migrating to the myocardium during the acute phase of this systemic infection. The high expression levels of PD-1 and its ligands reported in effectors T cells can be responsible for such pathogen persistence. In fact, the blockade of PD-1 and PD-L1 or deletion of the PD-1 gene ameliorated the control of parasite burden both systemically and in cardiac tissue.

Similar to our current results, we previously reported that *T. cruzi* induces upregulated expression of another coinhibitor molecule, CTLA-4, in lymphocytes *in vivo* and *in vitro*, and the blockade of this inhibitory signaling pathway lead to increased inflammation and decreased tissue parasitism (23).

*T. cruzi* appears to have evolved to manage the expression of costimulatory molecules as a strategy to persistently survive within the mammalian host. A recent study showed that the parasite is also able to exert immune evasion by downregulating CD28 ligands and major histocompatibility complex (MHC) molecules in dendritic cells (28). In addition, it has been proposed that differential expression levels of these costimulator molecules induced by the parasite can be associated with the intensity of the inflammatory response, leading to different clinical forms of the disease in humans (28, 34). Whether differential expression of PD-1 and CTLA-4 would be a marker of clinical forms of the disease in humans is currently under investigation in our laboratory.

The modulation of expression of PD-1, PD-L1, and PD-L2 by lymphocytes in response to *T. cruzi* is
infection in vivo became clear by the time course cytometry study. A wave of increasing expression of PD-1 in T cells (both CD4 and CD8) obtained from the spleen was detected on days 10 and 15 p.i. By day 20 p.i., however, the expression of PD-1 fell. This may correspond to a migration effect from the spleen to the heart and to other tissues. It can be confirmed by an indirect fluorescent-antibody assay (IFA) of the spleen, as shown in Fig. S1 in the supplemental material, in which the expression of these molecules is lower in infected mice than in uninfected mice. These data clearly show that T. cruzi infection lead to modulation in the normal patterns of expression of PD-1 and its ligands by lymphocytes and monocytes. In all cases, PD-L1 exhibited the most upregulated expression, which is known to participate in immune evasion in other microorganisms (6). Previous reports showed that PD-1 and its ligands are induced in immune cells late after activation, and PD-1 is
now considered a marker of late activation and cell exhaustion during chronic infections and tumor immune evasion (6). The T cells present at the myocardium migrate in response to the parasite’s presence and are known to exhibit a phenotype of activated cells, producing massive amounts of cytokines, predominantly those of the Th-1 immune response (13, 35). In addition, we demonstrated that nearly 100% of these cells express high levels of PD-1. Such high expression of PD-1 in T cells has already been described during viral infections (16). The apparent discrepancy in the detection of PD-1 by IFA and flow cytometry is due to differential sensibility among the two methods. In addition, the effect of concentration is also evident, as flow cytometry takes into account cells found in a whole heart, while IFA focuses only on a small area of the heart. Our findings suggest the participation of a novel regulatory mechanism in the control of the inflammatory response in cardiac tissues during this parasitic infection.

We believe that the balanced expression of positive and negative coreceptors is what determines the final outcome of infection in terms of the intensity and clearance of the parasite. The pathogenic role of collateral destruction of cardiac tissue during this inflammatory reaction is also undeniable. It is mediated by cellular and soluble components of the immune response, which is poorly regulated by classic immune regulatory mechanisms. For instance, regulatory cells do not play a strong role in the modulation of this inflammatory response (17, 20). Our data demonstrate that PD-1 is crucial to reduce the intensity of myocarditis.

It has been suggested that the effector lymphocytes recruited in response to the presence of the parasite in the myocardium may display altered tolerance mechanisms, leading to self-damage in an autoreactive fashion (10, 11, 19, 37) because activated T cells are not properly cleared out from circulation or because an altered peripheral tolerance is induced by the pathogen. Thus, it is possible that the inflammatory damage of cardiomyocytes cause an imbalanced expression of PD-L1, known to maintain the tolerance to cardiac troponin I (26), which is a protein exclusively expressed by cardiomyocytes. These hypotheses are currently under further investigation.

The role for PD-1 in maintaining immunological tolerance during acute myocarditis induced by T. cruzi was showed in this study by the treatment of mice with blocking antibodies against PD-1, PD-L1, or PD-L2. These treatments induced increased inflammation that was more remarkable in the cardiac tissues but not in other tissues where the presence of the parasites has been described during this infection. We also demonstrated that the blockade of PD-1 and PD-L1 was more effective in worsening myocarditis than the blockade of PD-L2. These data are in agreement with those from previous studies on the role of PD-1 in the immune response against intracellular pathogens and suggest that PD-L1 has a predominant regulatory role over PD-L2 during this parasitic infection. Although spontaneous cardiomyopathy has been described in BALB/c mice as a consequence of PD-1 deficiency, this autoimmune disorder has not been described in C57BL/c mice. We did not observe any myocarditis in uninfected C57BL/c PD-1−/− mice. In addition, we did not detect any cell migration to the heart tissue of normal C57BL/6 mice after treatment with anti-PD-1 MAb, indicating that PD-1 is one of the factors responsible for the deregulation of the immune response in the myocardium after T. cruzi infection.

The mechanism by which PD-1 regulates the immune response to T. cruzi appears to involve regulation of T cell proliferation and apoptosis. The induction of PD-1 expression in lymphocytes is known to be induced through the signaling by common γ-chain cytokines IL-2, IL-7, IL-15, and IL-21. We showed that deletion of PD-1 leads to increased Stat-5 phosphorylation, which is crucial to T cell proliferation. In addition, the blockade of PD-1 and PD-L1 restored the proliferative capacity of lymphocytes obtained from infected mice. This result is very important since it explains one of the mechanisms that causes the inhibition of T cell proliferation that is well known to be induced after T. cruzi infection, which induces a large production of NO, which is responsible for induction of apoptosis (22).

It has been shown that the blockade/deletion of PD-1 leads to an improved immune response to intracellular pathogens (2), which correlates with our findings of reduced parasite load, an increased number of inflammatory infiltrates in the myocardium, and increased levels of the proinflammatory cytokines TNF-α and IFN-γ and chemokines CCL3, CCL5, and CCL2. These data support the regulatory role for PD-1 signaling, mainly through the PD-L1 ligand in the infected myocardium during the acute phase of T. cruzi infection.

In spite of favoring parasite persistence, PD-1 signaling is important for the survival of the infected host, as mice receiving the anti-PD-1 treatment die earlier than the control group or the mice receiving other treatments. We hypothesized that the increased mortality rate of these mice could be associated with the uncontrolled, intense myocardial inflammation observed as a consequence of the PD-1 blockade. This reduced tolerance to myocardial inflammation results from increased lymphocyte proliferation, proinflammatory cytokine production, and reduced apoptosis of T cells. The participation of regulatory T cells in the mechanisms involving PD-1 regulation during this infection was not studied and is an interesting issue for future reports.

In conclusion, our data demonstrated that PD-1 and PD-L1 participate in T. cruzi-induced myocarditis and that they regulate the inflammatory immune response.

Finally, recent studies have started to suggest that immune therapy involving soluble PD-1 could be beneficial under inflammatory conditions, as is the case with Chagas’ heart disease. Soluble PD-1 does not appear to be produced under normal conditions. However, an alternative splicing variant of the PD-1 gene (PD-1Deltaex3), which leads to production of soluble PD-1, has recently been described (39). We did not perform any assays to test the presence of PD-1 or its ligands in mouse plasma. We hypothesize that a delicate manipulation of this signaling pathway on T cells of T. cruzi-infected hosts could become a potential strategy to design future therapeutic approaches for Chagas’ heart disease.

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