



C57BL/6 Mice Pretreated With Alpha-Tocopherol Show a Better Outcome of *Trypanosoma cruzi* Infection With Less Tissue Inflammation and Fibrosis

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Chagas disease is accompanied by a multisystem inflammatory disorder that follows *Trypanosoma cruzi* infection. Alpha-tocopherol has been described as an antioxidant and a potential adjuvant to enhance immune responses to vaccines. Therefore, we have evaluated the immune response to *T. cruzi* infection upon alpha-tocopherol pre-administration. The results show that administration of alpha-tocopherol before the infection results in lower parasitemia and lower mortality of C57BL/6 mice infected with the *Tulahuen T. cruzi* strain. Alpha-tocopherol administration in normal C57BL/6 mice resulted in higher levels of IFN- γ production by T and NK cells before and after the infection with *T. cruzi*. More importantly, previous administration of alpha-tocopherol increased the production of IL-10 by T and myeloid suppressor cells and the formation of effector memory T cells while decreasing the expression of PD-1 on T cells. These results suggest that alpha-tocopherol may limit the appearance of dysfunctional T cells during the acute and early chronic phases of *T. cruzi* infection, contributing to control infection. In addition, alpha-tocopherol could diminish tissue inflammation and fibrosis in late acute disease. These results strongly suggest that alpha-tocopherol may be a helpful agent to be considered in Chagas disease.

Keywords: Chagas' disease, *Trypanosoma cruzi*, fibrosis, immunomodulation, adjuvants, interleukin-10, interferon- γ , inflammation

INTRODUCTION

Trypanosoma cruzi is the etiological agent of Chagas disease or American Trypanosomiasis (1). It is estimated that approximately 6 to 7 million people are infected worldwide, mainly in Latin America. Later, the disease does appear on other continents (2). Furthermore, 100 million people are at risk of contamination, and 10000 deaths annually can be attributed to the infection (3). Chagas disease is a significant public health problem worldwide (2, 4).

The disease is characterized by two distinct phases: acute and chronic. In the acute phase, trypomastigotes are detected in the peripheral blood. The acute infection may be asymptomatic in humans (5). However, general symptoms like fever, tachycardia, mild splenomegaly and adenomegaly may be found (6). Trypomastigote forms can invade many organs and tissues, but the usual targets are different muscle tissue types, macrophages, and neurons (7).

After infection, a strong immune response is built to control the parasite numbers (4). The control of *T. cruzi* acute infection requires activating and establishing several innate and adaptive effector mechanisms. The roles of the immune system in the dynamics of the disease include containment of parasitic replication, control of the propagation of the parasite in target tissues, tissue inflammation, and its regulation afterward (8). The inflammatory process is crucial in establishing severe disease (8, 9).

NK cells, neutrophils, dendritic cells, and macrophages are important components of the innate response during acute infection (10). Antibodies and cytokines produced by B lymphocytes and the activation of CD4⁺, CD8⁺, $\gamma\delta$ ⁺, and NK⁺ T lymphocytes are critical for infection control and progression to the chronic phase (8, 10).

In addition to cells, pro-inflammatory cytokines may be essential during the acute *T. cruzi* infection. The cytokines associated with the phenotype of resistance are interferon- γ (IFN- γ) (11), interleukin-12 (IL-12), and tumor necrosis factor- α (TNF) (12). IFN- γ participates in activating phagocytic cells, stimulating these cells to produce reactive oxygen species (ROS) and nitric oxide and polarizing the CD4 T lymphocyte response to a Th1 profile (13).

Other studies reinforce the role of interleukin-10 (IL-10) as a cytokine with activity related to the profile of resistance and protection against uncontrolled immune response during immune responses against protozoan infections (14, 15). In this case, the immune response is down-modulated by IL-10, preventing the pathology from occurring. IL-10 may also help control acute infection, as previously demonstrated (16).

Presently, a couple of drugs are used to treat the acute Chagas disease, and they appear to have no great effects on established conditions (17). Also, vaccines are unavailable, and there are no alternative treatment options currently available. Therefore, strategies or potential drugs are highly desirable to diminish inflammation and fibrosis during the infection (18).

Alpha-tocopherol is an isomer of vitamin E, described as the most potent biological form among the isomers (19). Vitamin E has been shown to have an adjuvant effect when conjugated with antigens, as in the study by Karlsson et al. (20). This study evaluated the vaccination in mice with plasmids encoding proteins for the influenza virus using alpha-tocopherol as an adjuvant. The results indicated a more potent and balanced response to IgG1 and IgG2c using plasmids together with alpha-tocopherol (20). Furthermore, immunization with tetanus toxoid and alpha-tocopherol increased specific anti-tetanus toxin antibodies and splenocyte levels of IFN- γ and IL-4 compared to a conventional adjuvant (21). In addition, alpha-tocopherol is an immunomodulatory compound, helping

to control tumor size in many different models (22–24). Vitamin E has also shown *in vitro* biological activity. For instance, mouse splenic T lymphocytes cultured in the presence of vitamin E had an increased cell proliferation and IL-2 production (25).

Vitamin E deficiency is associated with sepsis in children and might contribute to the development of septic shock during different bacterial infections (26). One study showed myocarditis aggravation and augmented sympathetic heart denervation in vitamin E-deficient rats during *T. cruzi* infection (27). Therefore, suggesting that vitamin E could be beneficial to control the inflammatory condition found in the *T. cruzi* infection.

As *T. cruzi* infection causes an exacerbated immune response with tissue damage in the host, the present study sought to investigate whether pre-treatment with alpha-tocopherol can promote the control of pro-inflammatory conditions during acute infection. We have demonstrated that previous alpha-tocopherol administration can induce a better control of *T. cruzi* infection and efficiently modulate the immune response in tissues, such as the heart and skeletal muscles. This study indicates that alpha-tocopherol might help promote a better disease evolution.

MATERIALS AND METHODS

Animals

C57BL/6 mice 4 to 6 weeks old, bred and maintained in the Animal Facility of the Gonçalo Moniz Institute (IGM), Fiocruz, Bahia. All experiments were conducted according to protocols approved by the Animal Use Ethics Committee (CEUA L-IGM-007/2017) of the IGM.

Experimental Groups

Experimental groups were divided according to the intervention performed. The groups were: Control, with no intervention; Veh/Infected: animals that received only the vehicle and were infected or Aphotoc/Infected (treated only with alpha-tocopherol) before the infection. The administration scheme of these components in the different groups was one dose per week, subcutaneously, for one month. Groups were infected after 30 days from the last injection.

Treatment With Alpha-Tocopherol

Alpha-tocopherol (Sigma-Aldrich, cat# T3634) was administered subcutaneously before infection with *T. cruzi*. The alpha-tocopherol was diluted in DMSO, and the solution was kept in an ultrasound bath to help homogenization. New solutions were prepared before use. The volume per injection was adjusted for a dose of 100 mg of alpha-tocopherol/Kg/injection/animal (28). Different organs were analyzed for acute or chronic alphotoc toxicity, using the dose/scheme described above, and no signs of toxicity were found (data not shown).

Parasites and Infection

T. cruzi Tulahuen strain was used (29). The parasites were maintained by serial passage in mice before inoculation.

The infection of the experimental groups was carried out as previously described (30). Mice were injected intraperitoneally with 50 or 1×10^3 trypomastigotes. Mice were euthanized and evaluated 60 days post-infection (dpi).

Determination of Parasitemia

The number of circulating parasites was determined on different days post-infection. Counting was performed under an optical microscope, evaluating the number of parasites in 100 microscopic fields (40x magnification). 5 μ L of blood was collected from the animals' tails (30).

Cell Cultures

Spleens from animals in the different experimental groups were aseptically removed, washed, and resuspended to determine the number of cells per milliliter. Splenocytes were plated in 24-well culture plates. Each well contained 5×10^6 cells/mL, which were incubated in RPMI containing 10% FBS in a 5% CO₂ at 37°C. Brefeldin-A was added 4 hours before the cells were harvested to stain them for flow cytometric analysis. After 12 hours of culture, the cells were plated in 96-well plates containing 2×10^6 cells in each 100 μ L. Subsequently, the plates were centrifuged at 2000 rpm for 3 seconds and cells stained.

Flow Cytometric Analysis

Cells from the spleen and skeletal muscle were resuspended in FACS buffer (PBS, 2% FBS, and 0.1% sodium azide). Aliquots of the cell suspensions were incubated for 20 minutes and protected from light, with the following mAbs: anti-CD11b (Clone M1/70) FITC, anti-TCR $\alpha\beta$ (Clone B20.1) FITC, anti-CD8 (Clone 53-6.7) PerCP-Cy 5.5 or FITC, anti-CD4 (Clone GK1.5) PerCP-Cy 5.5 or FITC, anti-Gr1 (Clone RB6-8C5) Pe Cy 5.5, anti-NK1.1 (Clone PK-136) Pe-Cy 5.5, anti-CD62L (Clone DREG-56) PE, anti-PD1 (Clone J43.1). PE-labeled mAb were from Invitrogen or eBioscience. A rat anti-mouse isotypes were also used as controls. For intracellular cytokine-staining, the previously stained cells were fixed with 1% paraformaldehyde for one hour and washed three times in BD Perm/Wash buffer. The intracellular cytokine staining was performed with anti-IFN- γ (Clone XMG1.2, Invitrogen), anti-IL10 (Clone JES5-16E3, eBioscience). Antibodies were diluted in Perm/Wash buffer, and the cells were incubated for 1 hour. They were washed twice with Perm/Wash and once more with FACS buffer. A BD LSRFortessa or FACScan instruments were used for readings.

Gating Strategies for the Flow Cytometry Analysis

To obtain cytokine-producing CD4⁺ and CD8⁺ T cells, lymphocytes were gated in FSC x SSC plots. CD4⁺ or CD8⁺ T cells were identified in FL3 or FL1, respectively. Then, cytokine-producing cells were studied in FL2. Cytokine-producing MDSCs were gated and analyzed as follows: Gr1^{high} cells were identified in FL1 and CD11b^{high} in FL3. Gated Gr1^{high}CD11b^{high} MDSCs were examined in FL2 for the presence of IL-10. A total of 200.000 events were collected per sample. Results were analyzed using Flowjo software.

Histopathological and Quantitative Morphological Studies

The skeletal muscle and heart fragments were collected and fixed in Millonig formalin, embedded in paraffin to obtain 5 μ m sections, stained in Hematoxylin and Eosin (H&E) or by Picrosirius red method. Later, the equipment photographed the slides at a maximum magnification of 200X (Virtual Slide Microscopy VS120, Olympus, Japan). Five images of each slide were taken, corresponding to an animal sample. The ImageJ Pro Plus software (computer) was used for the analyses. The morphometric determination of the inflammatory infiltrate considered the cell count throughout the image and subsequently calculated the total area of the tissue. The total number of cells was divided by the total tissue area in mm², and the value was represented by cells/mm². The images were also analyzed for fibrosis analysis using the ImageJ software with the Threshold Color plug-in. The areas based on color were selected and measured to quantify the percentage of collagen marked in red. Afterward, the total tissue area was measured, and then the percentage of collagen area in the entire area was calculated, as evidenced by "Pale pink" (Picrosirius red).

Statistical Analysis

The Kolmogorov-Smirnov test was used to verify the normality of the sample distribution, using IBM SPSS Statistics (IBM) software. Other studies were performed using GraphPad Prism software, version 6.0. The samples then had a non-normal distribution, and the Mann-Whitney test was used. For analysis of mortality progression, the Log-rank test was used. P values less than 0.05 were considered significant.

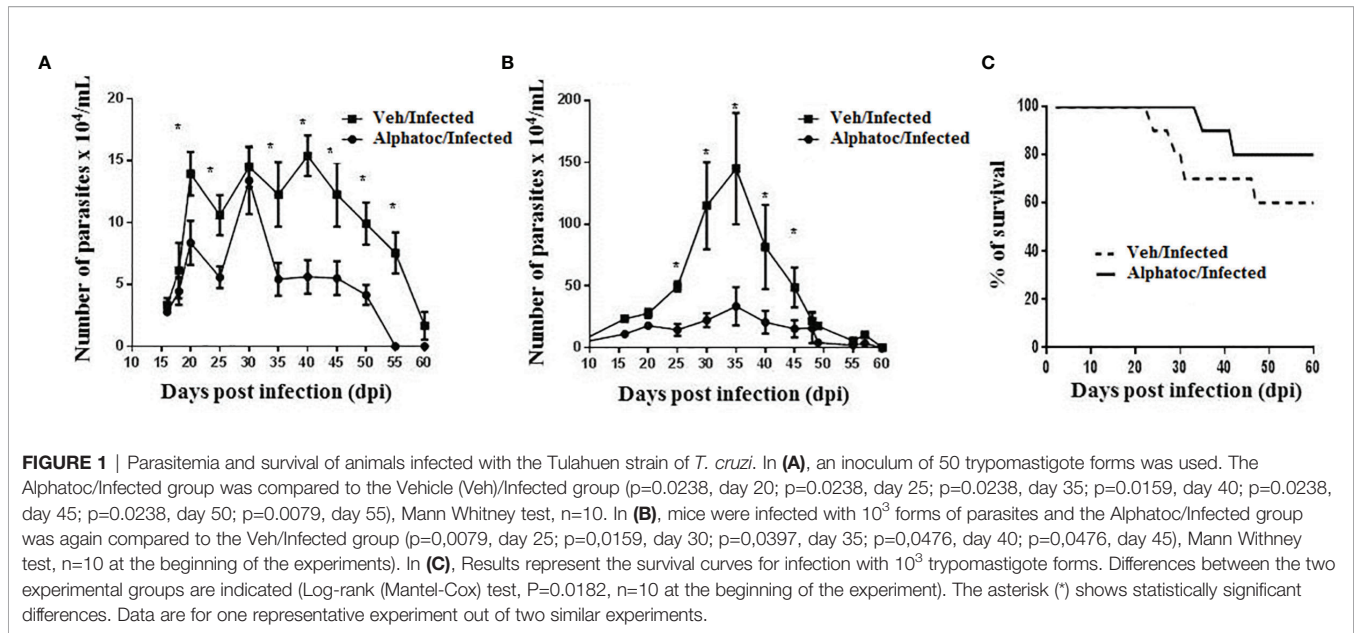
RESULTS

Alphatoc Administration Induces a Better Control of Parasitemia and Increases the Resistance to Infection

C57BL/6 mice were previously treated with alphatoc and then infected with 50 or 1×10^3 trypomastigotes (**Figure 1**). **Figures 1A, B** show lower parasitemia in mice treated with alphatoc than in the untreated infected group of mice at many time points after initial infection. **Figure 1C** shows higher survival in the alphatoc-pretreated infected mice than in the untreated control. The results show a 60% mortality rate in the vehicle-pretreated infected animals, and only 20% of alphatoc-pretreated and infected mice died within 60 days post-infection. No mortality was recorded when 50 forms were used to infect the mice in either group. Thus, alphatoc treatment before the infection induces higher resistance to these animals.

Alphatoc Pre-Treatment Increases the Numbers of IFN- γ -Producing Cells in Both Infected and Uninfected Animals

Figures 2A, C show the total numbers of splenic NK⁺ and NKT⁺ cells producing interferon- γ in uninfected mice (**Figure 2A**) or infected animals (**Figure 2C**). Pre-treatment with vehicle or



alphatoc is indicated. Splenic numbers of $NK^+IFN-\gamma^+$ and $NKT^+IFN-\gamma^+$ cell numbers were augmented when uninfected mice were pretreated with alphatoc compared to vehicle-treated mice. However, no difference in the splenic numbers of $NK^+IFN-\gamma^+$ cells was detected after infection (**Figure 2C**). Also, the total numbers of splenic $NKT^+IFN-\gamma^+$ cells were increased in the group previously treated with alphatoc (**Figure 2C**). **Figure 2B** shows the whole numbers of $CD4^+IFN-\gamma^+$ and $CD8^+IFN-\gamma^+$ splenic T cells in uninfected animals pretreated or not with alphatoc. The results show that alphatoc pre-administration augmented the total splenic $CD4^+$ but not $CD8^+$ T cells expressing $IFN-\gamma$ in mice pretreated with vehicle. Infected groups pretreated with alphatoc had higher numbers of $CD4^+$ and $CD8^+$ T cells producing $IFN-\gamma$ than vehicle pretreated and infected mice (**Figure 2D**).

Infected Mice Pretreated With Alphatoc Present More Effector Memory Splenic $CD8^+$ T Cells

The expression of L-selectin ($CD62L$) was studied to analyze the effector profile of $CD4$ and $CD8$ splenic T lymphocytes. Sixty days after *T. cruzi* infection with 1000 forms of trypomastigotes, the alphatoc/infected and vehicle/infected groups presented a higher number of splenic $CD4^+CD62L^{neg}$ cells when compared to the control groups (**Figure 3**). However, effector memory $CD8^+CD62L^{neg}$ T cells were higher in infected mice previously treated with alphatoc than in the other experimental groups (**Figure 3**).

PD-1 Expression in Splenic T Cells of Alphatoc or Vehicle Pretreated and Infected Mice

As we detected an increased production of interferon- γ by T cells and an augmented formation of effector T cells upon alphatoc pre-administration, we evaluated the expression of the PD-1

molecule, a marker for dysfunctional or anergic T cells, in mice pretreated with vehicle or alphatoc and infected afterward. After 60 days of infection, both the total numbers of $CD3^+PD-1^+$, $CD4^+PD-1^+$, and $CD8^+PD-1^+$ T cells were decreased in the alphatoc/infected compared to the veh/infected mice (**Figures 4A, B**), thus denoting that alphatoc may prevent T cell dysfunction/anergy during the acute *T. cruzi* infection.

Alphatoc Pre-Treatment Diminished Tissue Inflammation and Fibrosis in the Late Acute *T. cruzi* Infection

Sixty days after the infection, skeletal and cardiac muscles were evaluated by histology (**Figure 5**). Representative photomicrographs (HE staining) from mice infected with 50 (**Figures 5A, C, E, G, I, K**) or 10^3 (**Figures 5B, D, F, H, J, L**) trypomastigotes are shown. After infection, vehicle-treated animals presented a diffuse inflammatory infiltrate in the atrium (**Figures 5A, B**). In alphatoc pretreated mice, a reduced inflammatory cell infiltrate was found in the atrium (**Figures 5C, D**).

In infected vehicle pretreated animals, inflammation spread among skeletal, muscular fibers (**Figures 5E, F**). In alphatoc pretreated animals (**Figures 5G, H**), inflammatory infiltrate was restricted to the perivascular interstitium. Interstitial fibrosis (Picrosirius red staining) was evident in the vehicle pretreated animals (**Figures 5I, J**) and was minimal or absent in alphatoc pretreated animals (**Figures 5K, L**). Thus demonstrating that mice treated with alphatoc before the infection are more protected against tissue lesions than untreated mice.

Quantitative studies show the number of inflammatory cells in the heart or skeletal muscle sections from alphatoc-treated or vehicle-treated infected mice, as indicated in **Figures 5M-P**, respectively. The results show that pre-treatment with alphatoc led to diminished heart or skeletal muscle inflammatory cells.

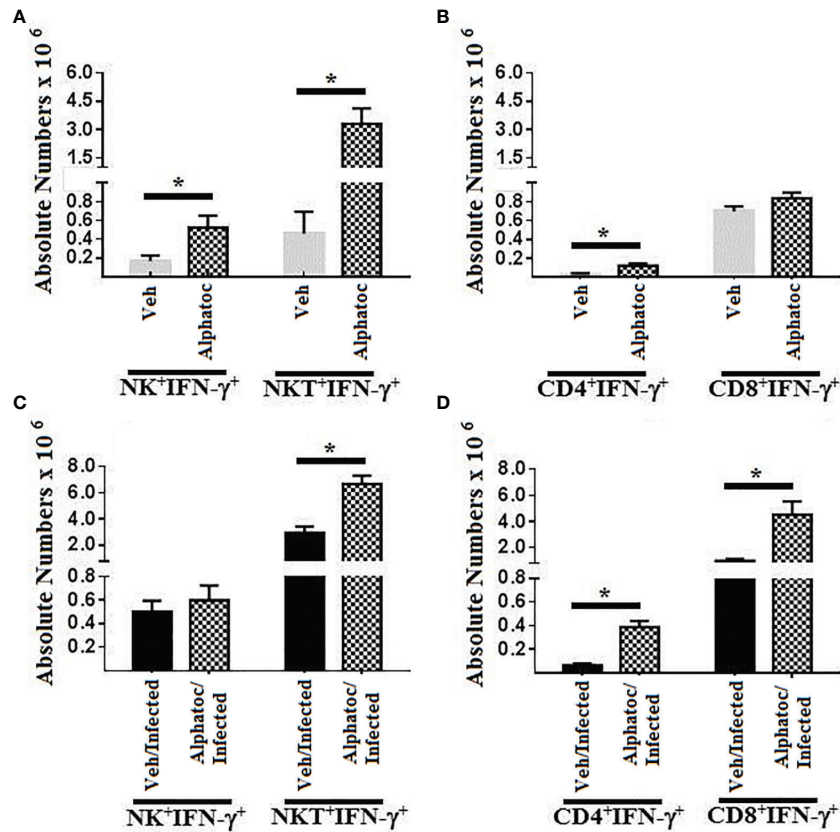


FIGURE 2 | IFN- γ production by NK and T cell splenocytes. In **(A)**, IFN- γ -producing NK and NKT cells in uninfected animals pretreated with vehicle or alphatoc were compared. **(B)**, CD4 or CD8 T cell splenocytes producing IFN- γ are shown in the vehicle or alphatoc pretreated animals. In **(C)**, NK and NKT cells producing IFN- γ are shown. The alphatoc/infected group was individually compared to the other groups. In **(D)**, the alphatoc/infected group was individually compared to the other groups (* $P < 0,05$, Mann Whitney test, $n=4$). Data are for one representative experiment out of three.

Despite the inoculum, fibrosis areas showed a considerable reduction of collagen deposition in skeletal muscle from mice pretreated with alphatoc (**Figures 5Q, R**).

Alphatoc Pre-Administration Increased the Numbers of Splenic CD8⁺ T Cells and Myeloid Suppressor Cells Expressing IL-10

Because alphatoc pre-treatment resulted in a more benign acute infection and less tissue inflammation, IL-10, a cytokine with anti-inflammatory functions, was studied. **Figure 6A** shows the number of splenic CD8⁺IL-10⁺ increased significantly in animals pretreated with alphatoc. In addition, the number of splenic myeloid suppressor cells able to produce IL-10 (CD11b⁺GR-1⁺IL-10⁺) also rose on day 60th post-infection (**Figure 6B**). The percentages (not shown) and numbers of CD4⁺IL-10⁺ T cells were not different from infected vehicle pretreated mice (**Figure 6A**).

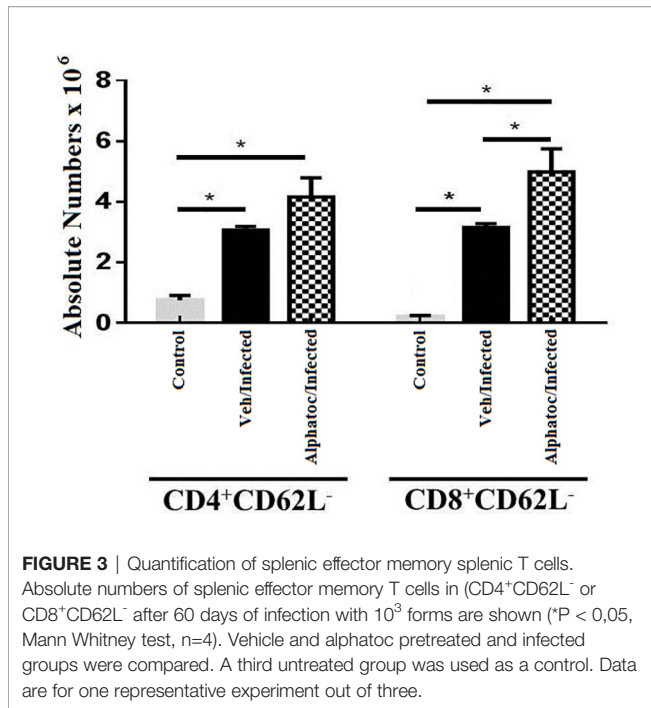
DISCUSSION

T. cruzi infection induces pro-inflammatory responses followed by robust and persistent immune responses that may culminate

in immunopathology and progress to more severe disease. In this context, we sought to assess whether alpha-tocopherol could alter the profile of regulatory, pro-inflammatory, or specific responses that help modulate the illness in the initial chronic phase of *T. cruzi* infection.

Over time, several experimental models were used to understand the dynamics of cell populations during experimental *T. cruzi* infection. C57BL/6 are susceptible to infection by the Tulahuen strain, with high mortality rates, especially when injected with a high inoculum of *T. cruzi* (29, 31).

The present study investigated the number of circulating parasites and mortality of animals of the C57BL/6 lineage infected with the Tulahuen strain of *T. cruzi*, previously treated with alphatoc. The animals were infected with 50 or 10³ trypomastigote forms (**Figures 1A, B**, respectively). The results show that parasitemia levels were lower in the groups treated with alphatoc than untreated. There was no mortality in the groups infected with 50 forms regardless of the treatment (data not shown). In infected mice with a higher inoculum parasite (10³), the longevity of animals treated with alpha-tocopherol and infected was 80% compared to only 40% of survival in untreated

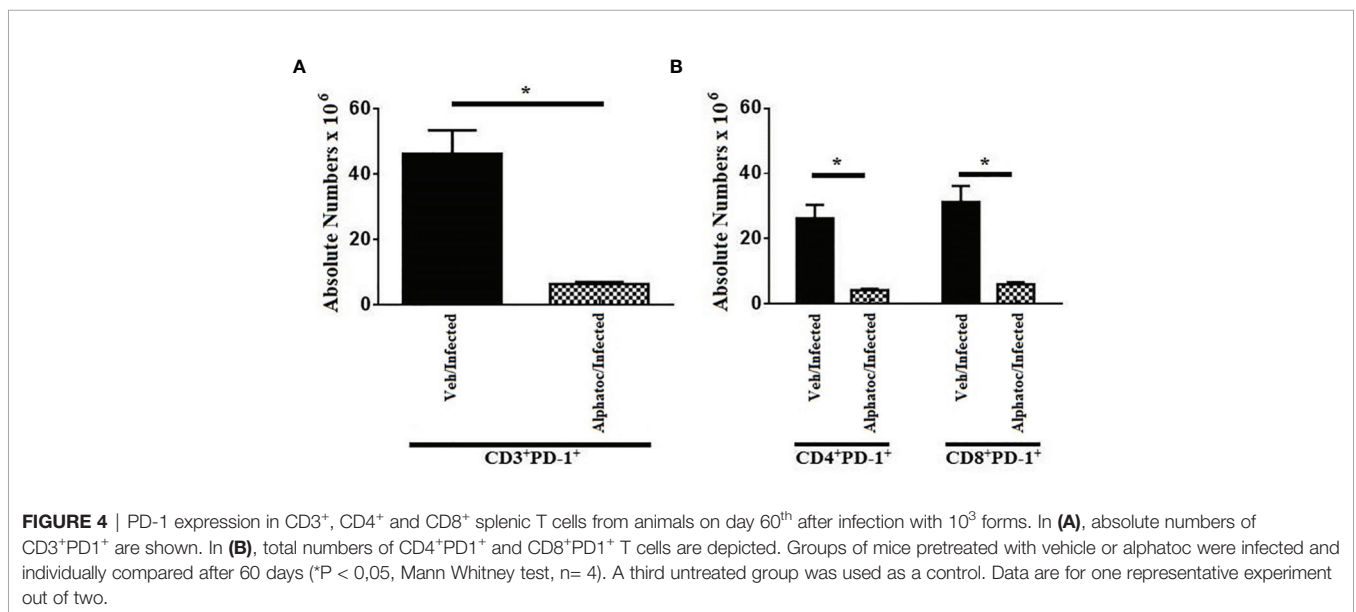


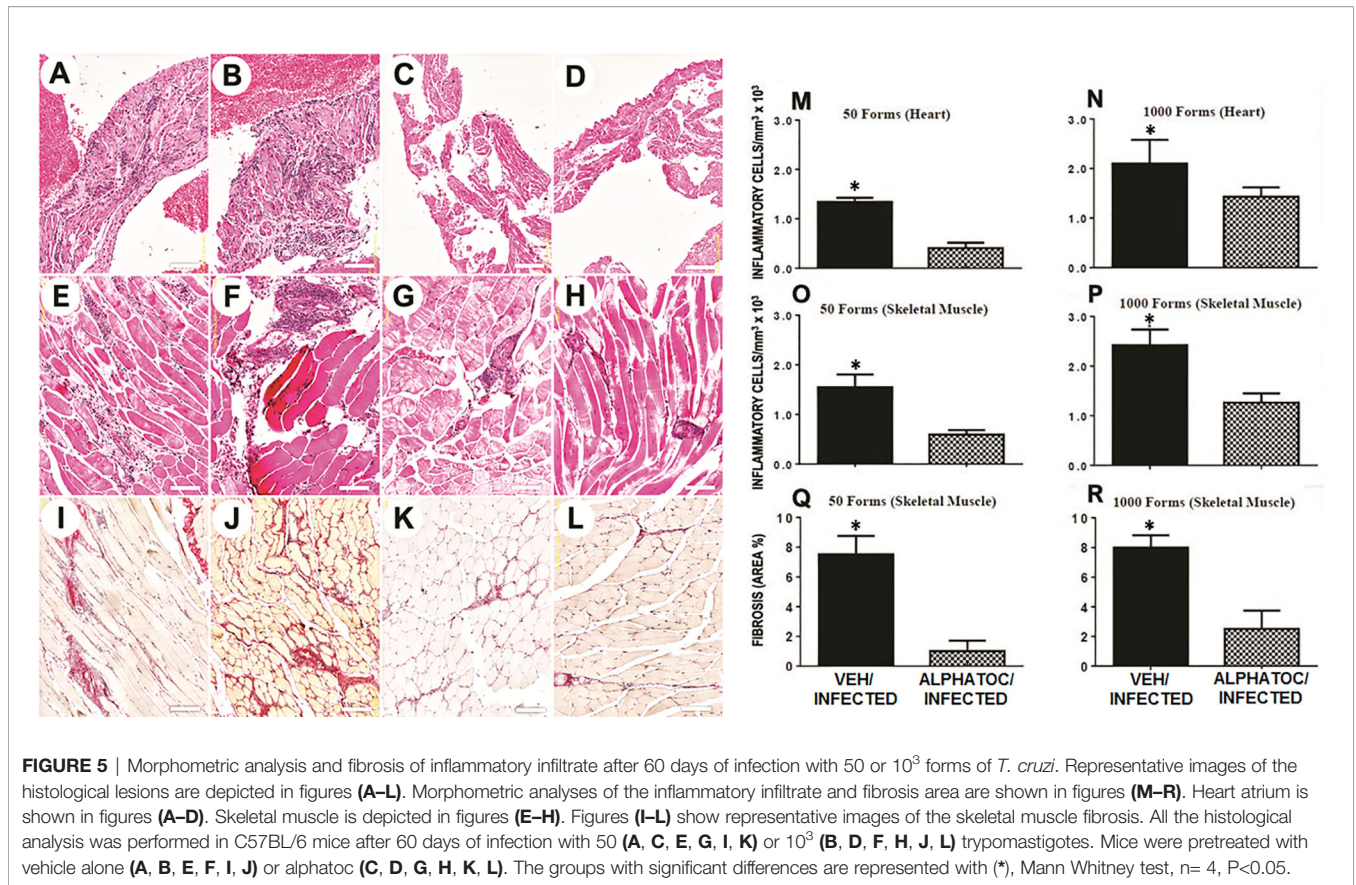
mice (**Figure 1C**). In addition, to describe alphatoc’s effect on parasitemia and survival, we have evaluated what could be associated with increased resistance in these alphatoc pretreated animals.

The parasitemia control and increased survival may be associated with the augmented functional activity of NK and T cells in the acute phase of infection, as previously shown in other studies (32–34). Alphatoc pre-treatment induced the expression of IFN-γ and increased the numbers of NK and T cells after its administration before the infection (**Figures 2A, B**).

The increased numbers of NKT, CD4⁺, and CD8⁺ cells were kept up to day 60th after inoculum, indicating a long-term effect (**Figures 2C, D**). Some studies suggest that the amount of memory T cells before the infection and the quantity of memory T cells may also correlate with resistance (29, 35). The relevance of effector T cells for protection during acute illness may be associated with a better capacity to produce cytokines such as IFN-γ and, therefore, activation of microbicidal mechanisms of phagocytic cells (36–38). Consequently, we have evaluated the numbers of splenic effector memory T cells on day 60 after infection. **Figure 3** shows increased splenic effector memory CD8⁺ T cells in mice pretreated with alphatoc compared to the vehicle pretreated group. Memory CD8⁺ T cells are crucial in infection control, especially in the infected tissues (38, 39). However, it was also described that memory T cells might acquire a non-functional phenotype in peripheral tissues either in tumor environments or during *T. cruzi* infection, the so-called exhausted or dysfunctional phenotype (40, 41). Therefore, the simple presence of increased numbers of effector memory T cells does not guarantee a better immune response. A marker usually expressed in exhausted or dysfunctional T cells is the PD-1 molecule (42). Therefore, PD-1 expression was evaluated in splenic T cells from mice treated with alphatoc previously to infection (**Figures 4A, B**). The results showed that the expression of PD-1 was greatly reduced in T cells from mice pretreated with alphatoc, thus indicating that alphatoc is inhibiting the generation of exhausted or dysfunctional T cells during the acute *T. cruzi* infection.

In the acute *T. cruzi* infection, treatment with monoclonal antibodies anti-PD1 in C57BL/6 mice can improve the cellular immune response and decrease the parasite load in the heart (43, 44). It has been shown that the high expression of PD-1 contributes to the evasion and establishment of parasites (43–45). Our results indicate that *in vivo* administration of alphatoc may induce a better infection outcome, increasing the

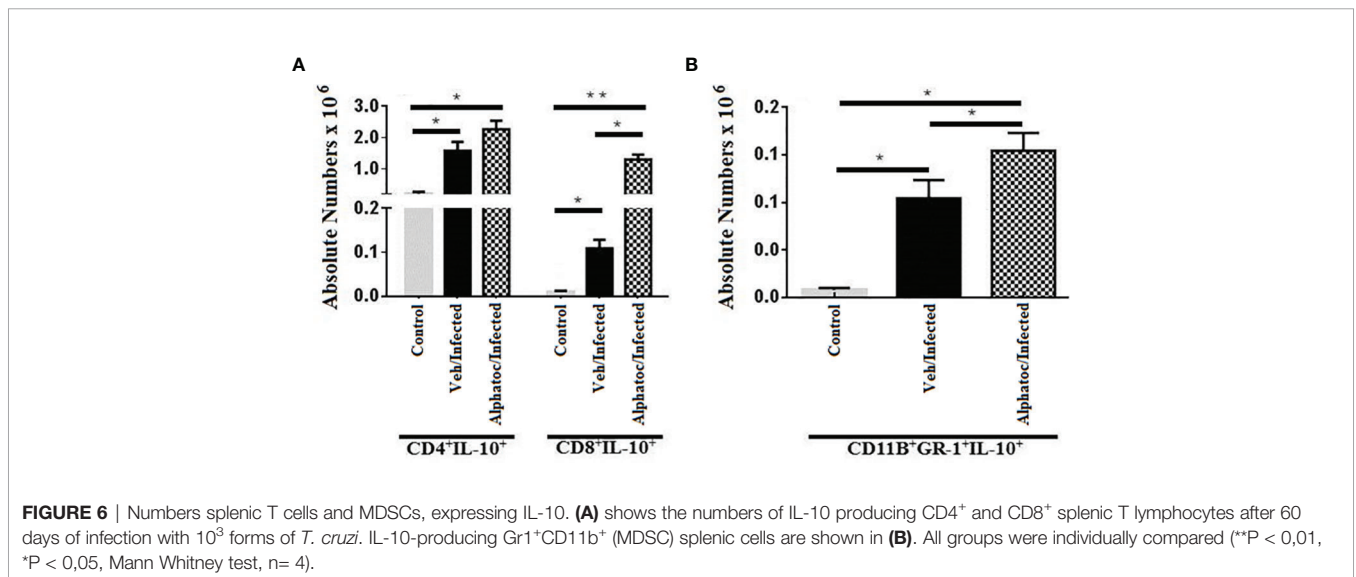




production of IFN- γ , effector memory T cells, and inhibiting the production of exhausted or dysfunctional T cells. Also, the mechanism responsible for this biological effect is unknown.

It was also described that PD-1 blocking during the acute phase of *T. cruzi* infection might increase tissue lesion despite a better parasite clearance (44). Therefore we conducted

histopathological evaluations in animals infected with 50 or 10³ trypomastigotes. These studies revealed that the group that received alphatoc had fewer inflammatory cells in the heart and skeletal muscle (Figure 5). Fibrosis was reduced in the alphatoc/infected group regardless of inoculum (Figure 5). In conclusion, the main benefits and treatments with alphatoc before *T. cruzi*



infection were reducing parasitemia, increasing survival, and reducing complications generated in the target tissues, such as intense inflammatory infiltrate and fibrosis.

Corroborating that regulatory mechanisms are active in alphatoc pretreated animals, an increased number of CD8⁺ T cells and MDSCs, expressing IL-10 were found (**Figures 6A, B**). The increase in IL-10 can promote a more balanced immune response in these animals, keeping the levels of pro-inflammatory cytokines within ranges compatible with infection control and regulating the inflammation caused by IL-12, IFN- γ , and TNF (12, 15, 46). IL-10 knockout mice infected with *T. cruzi* show increased susceptibility to lethal infection and more significant pathogenic responses associated with pro-inflammatory cytokines (47). In addition, these animals showed early mortality and more significant inflammation than wild-type mice. As a counterproof, this study also showed that IL-10^{-/-} animals treated with recombinant IL-10 increased survival, had a delay in mortality, and a reduction in IFN- γ produced by CD4⁺ T cells.

Similarly, Holscher et al. (47) showed that C57BL/6 IL-10^{-/-}, had a more vigorous production of pro-inflammatory cytokines. Therefore, there is a need for IL-10 to control a pathological immune response in the acute phase of infection. Yet, in the experiments described here, there was a rise in the production of IFN- γ and IL-10. This observation might reflect the activity of polyfunctional T cells that may secrete both cytokines simultaneously (48). The low levels of PD-1 expression may contribute to the appearance of polyfunctional T cells. In this case, it would be advantageous since IL-10 could prevent or diminish some of the harmful effects of high levels of inflammatory cytokines such as IFN- γ yet help resolve the infection.

We have shown herein that alpha-tocopherol pre-administration is associated with an improvement in the outcome of *T. cruzi* infection. A reduction in the inflammatory infiltrates and fibrosis in the heart and skeletal muscle was particularly impressive. However, the observed biological effects deal with the preventive aggravation of the disease, an experimental condition that may not be feasible in the real world. Still, it documents an important role for vitamin E as an

immunomodulator for *T. cruzi* infection, suggesting a potential use during the acute and chronic phases of the disease. Yet, unpublished studies from our group show that alpha-tocopherol administration during the chronic phase of *T. cruzi* infection has similar effects, particularly when associated with *T. cruzi* antigens (manuscript in preparation).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the involvement of animals was solely for the harvesting of cells for culturing and *in vitro* experiments. This study was conducted in accordance with the ethical conduct in the care and use of animals and in compliance with ARRIVE guidelines.

AUTHOR CONTRIBUTIONS

AS, MB, FC, and JM contributed to conception, and design of the study. AS, MB, JF, WD-S, FC, and JM organized the database. AS and MB performed the statistical analysis. FC and JM wrote the first draft of the manuscript. FC, WD-S, and JM wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version

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