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Research Article

Cardiac Protection by Immunization with *Phytomonas serpens* in Trypanosoma *cruzi*-Infected Mice is Related to Nitric Oxide Production

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ABSTRACT

The genus Phytomonas includes parasites that are etiological agents of important plant diseases, especially in Central and South America. These parasites are transmitted to plants by an infected phytophagous hemipteran bite. The existence of shared antigens between pathogenic and non-pathogenic trypanosomatids opened the possibility that species non-infectious to humans, who are economically feasible and easy to culture at large scale, could be potentially useful as antigen sources for immunization to Chagas disease. Our goal was to analyze the effect of immunization of mice with P. *serpens*, isolated from tomato fruit on Nitric Oxide (NO) production in the heart and plasma in the early of Trypanosoma *cruzi* infection. C57BL/6 mice were immunized by intra peritoneal (i.p) route with living forms of P. *serpens*15T. Each inoculum consisted of $1x10^7$ living parasites per 0.1mL in 15 mM PBS, pH 7.2 and was given four times at one-week intervals. Seven days after the last immunization, were infected i.p with a non-lethal dose of trypomastigotes ($5x10^3$ cells/animal).Control mice received PBS alone. Parasitamia was determined beginning at the 3th day after challenge. On day 12 post-infection, plasma and heart were obtained from uninfected and *T. cruzi*-infected mice and analyzed. We found that infected mice present higher blood and heart parasitism than immunized-infected mice ($P < 0.05$). This resistance correlated with increased NO production in the heart and in the plasma when compared with unimmunized mice ($P < 0.05$). It was observed that the levels of IL-1β, TNF-α, IL-6, and IL-12 were higher in the plasma of immunized and *T. cruzi*-infected mice compared to non-immunized and infected mice ($P < 0.05$). These findings open a new avenue for comparative investigations into Trypanosomatidae biology and provides an alternative safer of immunogenic agents for therapeutic in Chagas heart disease.

INTRODUCTION

Currently, only Benznidazole (BZ) and nifurtimox are recognized by the World Health Organization as drugs for treatment of Chagas disease, both are very toxic and have limited efficacy [1]. Thus, an immune therapy that will control the Trypanosoma *cruzi* transmission and Chagas cardiomyopathy is urgently needed. In last few decades,

significant research efforts have led to the development of several experimental vaccines that have shown promising results in small animal models of *T. cruzi* infection and Chagas disease [2].The acute phase is usually mildly symptomatic and often misdiagnosed as a febrile illness of childhood. Severe acute infection occurs in approximately 1% of the patients and is manifested by fever, chills, rash, liver function abnormalities, acute myocarditis, pericardial effusion, acute heart failure and/or meningoencephalitis [3].

The parasites of the Trypanosomatidae family include several genera comprising monoxenous insect trypanosomatids of *Crithidia, Leptomonas, Herpetomonas, Blastocrithidia, Angomonas, and Strigomonas*, and heteroxenous parasites such as Phytomonas, the etiological agent of plant diseases [4-7]. Included among the hetero xenious trypanosomatids are species responsible for a broad spectrum of human and animal diseases, such as *Trypanosoma cruzi*, the etiological agent of Chagas disease [7,8]. The combined number of people infected by kinetoplastids pathogens is estimated to be over 20 million, resulting in various health problems and more than 100,000 deaths each year [8]. Studies aimed at clarifying the affinities between the members of the Trypanosomatidae family have been performed by several researchers [9-14]. Data also showed that crude extract of *Crithidia* and *Leptomonas* have epitopes similar to Leishmania (L.) chagasi [7]. In addition, antigens from *Phytomonas serpens and Leptomonas sp.* were tested for vaccine purposes and induced relevant immune protection against *T. cruzi* infection in mice [12,15-17]. Recently, it was revealed a new range of proteins possibly responsible for immunological cross-reactivity between P. *serpens* and *T. cruzi* [18].T. *cruzi* infection have shown that a robust inflammatory response is triggered in the acute phase, with production of inflammatory cytokines, such as IFN-γ, TNF-α, IL-1β, IL-6 and IL-12related to the control of parasitism [19] and cardiac disease [20].

During acute phase of T. *Cruzi* infection, TNF-α alone or in association with other cytokines control parasite replication via the release of nitric oxide (NO) by macrophages [21] and cardiomyocytes [22]. There is no information about how cardiac protective immunity induced by *P. serpens* could be modulating the NO and cytokines production during acute phase of *T. cruzi* experimental infection. Therefore, the aim of this study was to investigate the effects of immunization of mice with *P. serpens*, isolated from tomato fruit, on NO production in the heart and plasma associated with resistance.

MATERIALS AND METHODS

Ethics Statement

This study was carried out in strict accordance with the principles and guidelines adopted by the Brazilian National Council for the Control of Animal Experimentation (CONCEA) and the technical procedures were approved by the Ethical Committee on Animal Use (CEUA), State University of Londrina (CEUA/UEL: protocols 20779.2018.59 and 35/2011). All the animals were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) and euthanized by cervical dislocation. All surgical procedures were performed under anesthesia, and care was taken to minimize animal suffering.

Animals

Six to 12- week- old C57BL/6 female and male mice were supplied by the Multi Institutional Center for Biological Investigation (CEMIB), State University of Campinas, Brazil. Mice (n=40) were maintained under standard conditions in the animal house of the Department of Pathological Sciences, Londrina State University, Londrina, Brazil. Commercial rodent diet (Nuvital CR1, Quimtia, Colombo, and Parana, Brazil) and sterilized water were available ad libitum.

Parasites

T. cruzi (Y strain) [23] as maintained by weekly intra peritoneal (i.p) inoculation of Swiss mice with $2x10^5$ trypomastigotes. To conduct our experiments, blood from previously inoculated Swiss mice was obtained by cardiac puncture with heparinized syringes. *P. serpens* 15T, isolated from tomato fruit (Lycopersicum esculentum) [24] was cultured in GYPMI medium (glucose, yeast extract, peptone, and meat infusion) [15] at 28°C.

Immunization of mice and challenge with *T. cruzi*

For immunization of C57BL/6, living forms of *P. serpens* 15T collected during log phase growth were washed 3 times by centrifugation at 3000 *g* for 5 min in 15 mM PBS (phosphate-buffered saline, pH 7.2) and administered by (i.p) inoculation. Each inoculums consisted of 1 $x10^7$ living parasites/ 0.1 mL 15 mM PBS, pH 7.2 given 4 times at 1-week intervals [15,25]. Seven days after the last immunization with P. *Serpens*, C57BL/6 mice were infected i.p. with a non-lethal ($5x10^3$

cells/animal) dose of trypomastigotes. Control mice received PBS alone (Figure 1).We used 5 mice per group and the total number of mice used was 40.

Nitrite determination

Nitric oxide (NO) concentration in plasma and heart obtained from uninfected and T. *cruzi*-infected mice on day 12 dpi was estimated by measuring nitrite, as described previously [26,27]. All reagents for the nitrite assay were obtained from Sigma Chemical Co.

Multiplex bead-based cytokines

Multiplex analysis of cytokines in mouse plasma samples was performed using a bead-based multiplexing kit (Invitrogen Mouse Cytokine Magnetic 10- Plex Panel). For magnetic bead assays our instrument (Luminex MAGPIX® with exponent software) was calibrated with the MAGPIX® Calibration Kit (MPX-CAL- K25) and performance verified with the MAGPIX® Performance Verification Kit (MPX- PVER- K25), according to the manufacturer's standards.

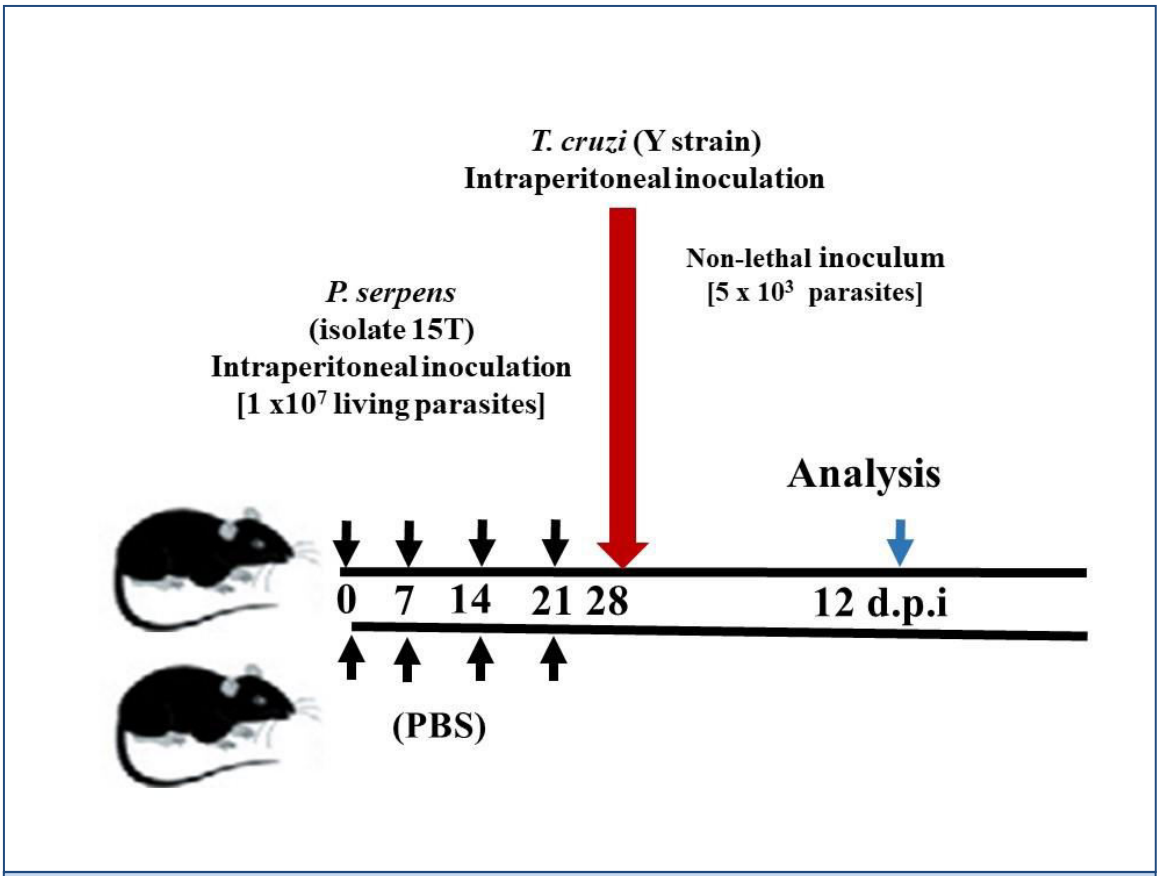

Figure 1: Experimental design.

C57BL/6 mice were immunized with 1 x 10^7 living forms of *P. serpens* 15T given 4 times at 1-week intervals. Seven days after the last immunization, mice were infected by via intra peritoneal (i.p.) with a non-lethal (5 x 10^3 cells/animal) dose of blood trypomastigotes of the *T. cruzi* (Y strain) and analysis at 12 days post-infection (dpi), according experimental protocol. Control mice received PBS alone.

RT-qPCR

Real time qPCR was performed to determine the tissue parasite burden in controls (only infected-mice) and *T. cruzi*-infected/immunized animals. Heart tissues were collected from mice at 12 dpi, weight and washed in PBS. The genomic DNAs were purified using the lyses buffer [50mM Tris-HCl pH7.6, 10 mM EDTA, 0.5% SDS, 0.2mg/mL of proteinase K (Invitrogen, Carlsbad, CA)] followed by phenol/chloroform extraction. Samples were mechanically homogenized (Ultra stirrer, Scientific SDN BHD, Malaysia) heated for 12h at 55°C, and extract twice with phenol: chloroform: isoamyl alcohol (25:24:1). Cold ethanol (Merck), twice the volume of the extracted sample, was then added to the aqueous phase and samples were stored at -20°C for 12h. Then, samples were centrifuged for 30 min at 10,000 *g*, washed with 70% ethanol, dried at room temperature and re suspended in 10 mM Tris HCl pH 8.5.Real-time PCR was performed using the Platinum SYBR Green qPCR Super Mix UDG with ROX reagent (Invitrogen Corporation, New York, USA) with 100 ηg of total gDNA. The primer sequences used were TCZ-F 5'-GCTCTTGCCCACAMGGGT GC-3' and TCZ-R 5'CCAAGCAGCGGATAGTTCAGG-3' [28]. The samples were amplified in a thermal cycle Corbett Rotor-Gene ™ with the following PCR conditions: first step (2 min at 50°C), second step (10 min at 95°C) and 40 cycles (30 s at 95°C, 30 s at 57°C, 30 s at 72°C, 15 s at 82°C), followed by a dissociation stage. The results were based on a standard curve constructed with DNA from culture samples of T. *cruzi* epimastigotes (Y strain).

Statistics

The results were expressed as mean ± standard error of the mean (SEM). Significance was evaluated by analysis of variance (ANOVA) followed by Bonferroni or Tukey's multiple comparison tests; all differences mentioned were significant compared to controls ($P < 0.05$). All statistical analyses were conducted with Graph Pad Prism version 5.0 (Graph Pad Software, San Diego, CA).

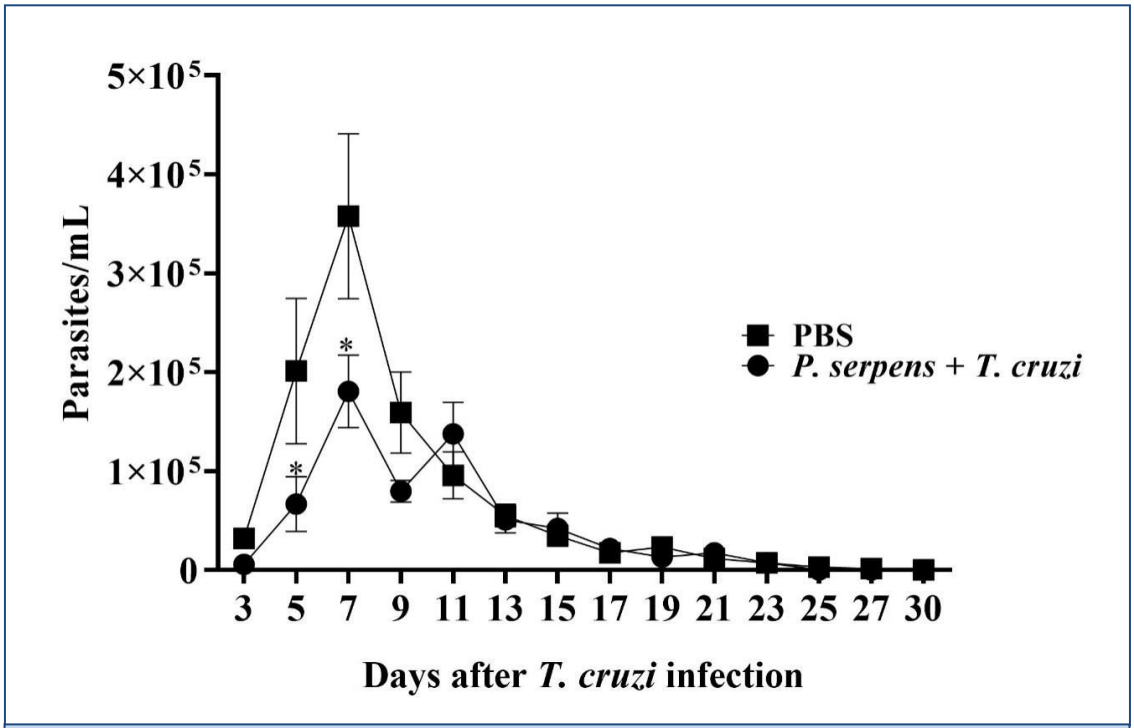

Figure 2: Course of infection with the Y strain of T. cruzi in C57BL/6 immunized or not with P. serpens.

C57BL/6mice received 1X 10^7 living forms of *P. serpens* 15T i.p, four times at weekly intervals and an i.p. challenge 1 week later with 5X10^3 blood trypomastigotes, respectively. Parasitemia was quantified as trypomastigotes per milliliter of blood. Results are expressed as mean ± SEM from 5 mice per group, in an experiment representative of three similar experiments. Results were analyzed by Analysis Of Variance (ANOVA) followed by Bonferronior Tukey's multiple comparison tests.*$P < 0.05$, significant difference in parasitaemia, C57BL/6 immunized versus non-immunized

RESULTS

Effects of immunization on the course of *T cruzi* infection

On 3 dpi, parasitemia was similar between the infected and immunized-infected mice (Figure2). The parasitic load in the blood increased considerably from day 5 and peaked on 7 dpi and was significantly higher in infected mice than in immunized-infected group ($P < 0.05$). Parasitemia declined sharply over the next 4 days (ie, 9 and 11 dpi), and by 17 dpi, parasite burden in the blood appeared to be well controlled regardless of infection (Figure2). On the 30 dpi, all mice were alive and remained so until the end of the experiment (data not shown). *T. cruzi*-infected C57BL/6 mice present higher heart parasitism than immunized-infected mice following i.p. infection with strain Y blood forms (day 12 after infection), C57BL/6 mice developed higher cardiac parasitism than immunized and infected mice (Figure 3).

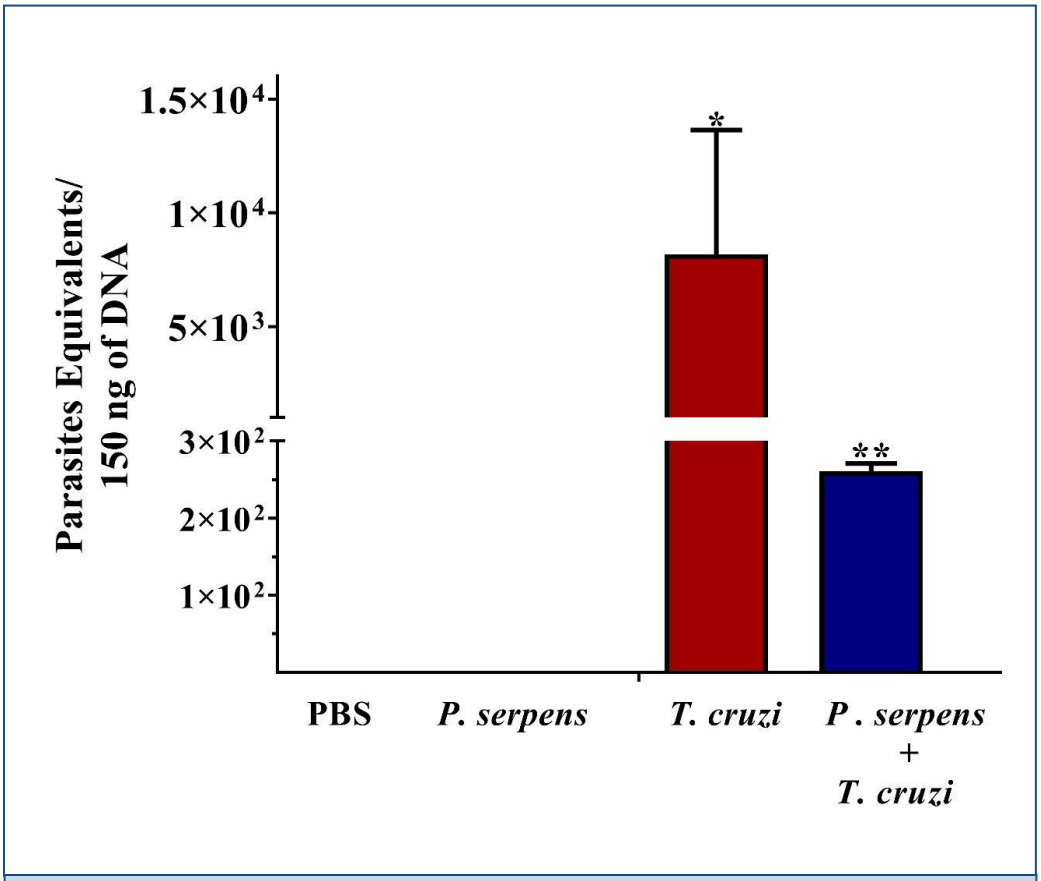

Figure 3: Parasite DNA content in cardiac tissue.

Real-time PCR analysis of hearts from C57BL/6 mice immunized with 1 x 10^7 living froms of *P. serpens* 15T, infected with 5 x 10^3 blood trypomastigotes of the *T. cruzi* (Y strain) or both (immunized and infected). Hearts were extracted 12 days after infection. Controls received PBS. * $P < 0.0001$ compared to non-infected mice. ** $P < 0.01$ compared to infected mice.

Immunization induce differential nitric oxide response in heart

As NO is important effector molecule in the destruction of *T. cruzi*, we sought to compare its production in both groups of mice. NO production in heart obtained after 12 days of infection was twofold higher in C57BL/6 infected mice than uninfected mice. Immunization with P. *Serpens* increased NO production in the heart compared with unimmunized mice (Figure4).

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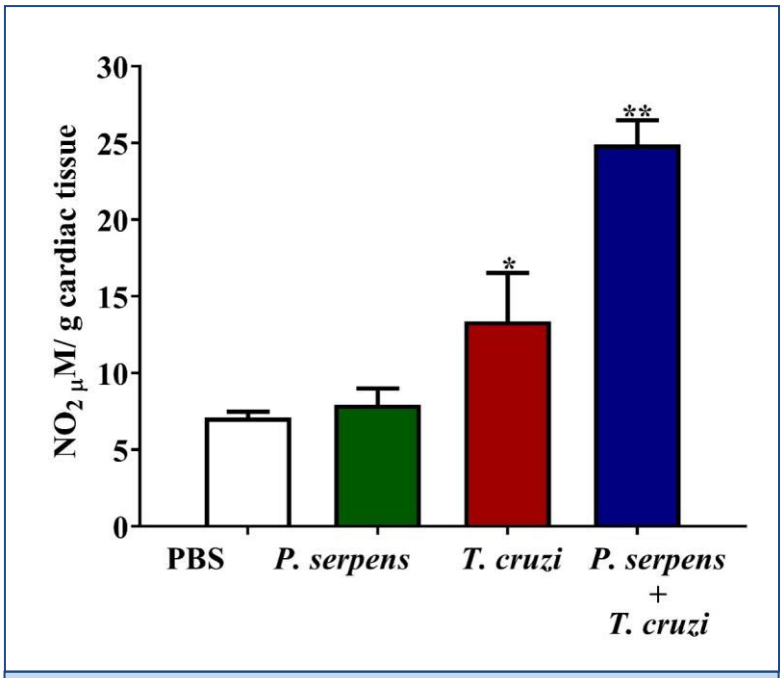

Figure 4: Nitric oxide (NO) levels in the cardiac tissue.

Using modified Griess methodology we determined the concentrations of nitrite/nitrate in the hearts from C57BL/6 mice immunized with 1 x 10^7 living forms of *P. serpens*15T, infected with 5 x 10^3 blood trypomastigotes of the *T. cruzi* (Y strain) or both (Immunized and infected). Hearts were extracted 12 days after infection. Controls received PBS. *$P < 0.005$ compared to non-infected and immunized mice. ** $P < 0.05$ compared to infected.

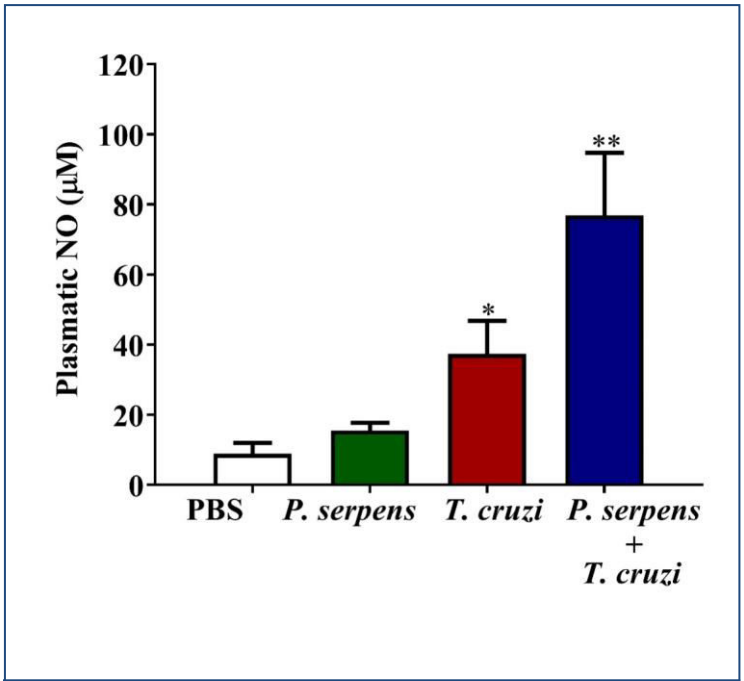

Figure 5: Nitric oxide (NO) levels in the plasma.

Using modified Griess methodology we determined the concentrations of nitrite/nitrate in the plasma from C57BL/6 mice immunized with 1 x 10^7 living forms of *P. serpens*15T, infected with 5 x 10^3 blood trypomastigotes of the *T. cruzi* (Y strain) or both (Immunized and infected). Hearts were extracted 12 days after infection. Controls received PBS. * $P < 0.005$ compared to non-infected and immunized mice. ** $P < 0.05$ compared to non-infected, immunized and infected mice.

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Resistance to *T. cruzi* infection in immunized mice correlates with higher production of NO and inflammatory cytokines

The resistance of heart parasitism was associated with increase of NO production in the plasma of immunized and infected mice (day 12 d.p.i) when compared with unimmunized mice (Figure5). In addition, we investigated comparatively the levels of IL-1β, TNF-α, IL-6, and IL-12 production in the plasma (Figure 6) of C57BL/6 mice, the immune mediators that are necessary to control the parasite. It was observed that the levels of cytokines analyzed were higher in the plasma of immunized and *T. cruzi*-infected mice compared to non-immunized and infected mice (Figure 6). Thus, immunization with *P. serpens* appears to modulate NO and inflammatory cytokines in favor of controlling the progression of the disease.

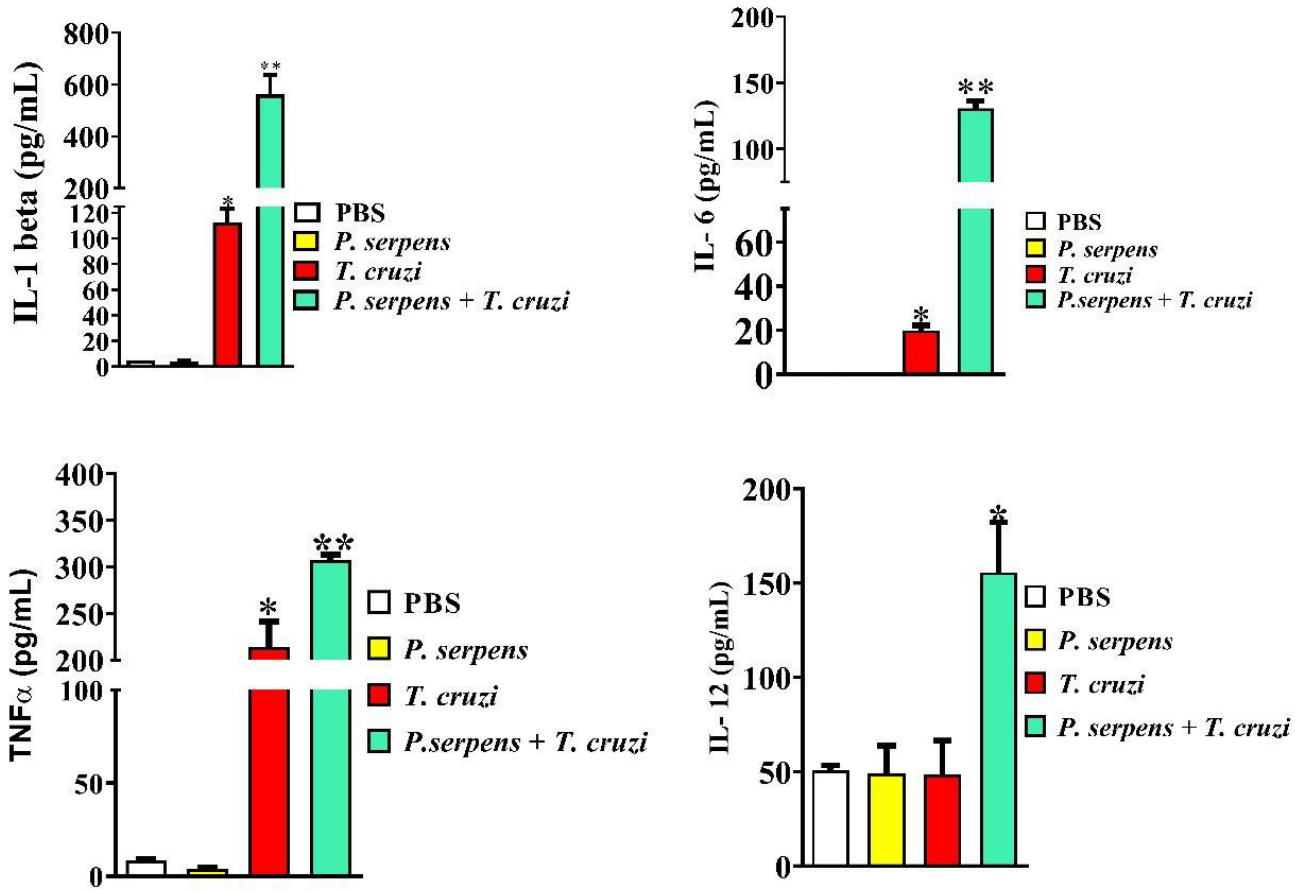

Figure 6: Cytokines levels in the plasma.

Concentrations of IL-1β, IL-6, and TNF-α and IL-12 were measured using a bead-based multiplexing kit (Invitrogen Mouse Cytokine Magnetic 10 - Plex Panel). C57BL/6 mice were immunized with 1 x 10^7 living forms of *P. serpens* 15T, infected with 5 x 10^3 blood trypomastigotes of the *T. cruzi* (Y strain) or both (immunized and infected). Plasma was obtained 12 days after infection. Controls received PBS. *P <0.001 **P < 0.05 compared to immunized and infected mice.

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DISCUSSION

The affinities between the members of the Trypanosomatidae family have led several researchers to use non-pathogenic trypanosomatids to investigate biochemical, cellular and immunological similarities between these species and the human pathogenic species [7,10,11,13,15,16,18]. Although lower trypanosomatids considered as non-pathogenic to humans (*Crithidia, Herpetomonas and Leptomonas* species), reports have shown that they can infect people, particularly those infected with Human Immunodeficiency Virus (HIV) [29,30].

The pathophysiology of Chagas disease is associated with enhanced TNF-α and NO production [31,32]. High NO levels in the serum are associated with the severity of heart injury and electrical and echocardiography alterations in rhesus monkeys chronically infected with *T. cruzi* [33]. In acute infection, NO plays a beneficial role as trypanocidal agent [34,35]. Moreover, *T. cruzi* infection, especially occurring in a cytokine-enriched milieu, induces NO expression by macrophages and cardiomyocytes [21,22,36,37]. Despite all the evidence, suggesting antigenic similarities between *T. cruzi* and plant trypanosomatids *P. serpens* only one previous study investigated the role of NO in the protective immune response against *T. cruzi* induced by *P. serpens* [16]. INOS Knockout (KO) mice and controls treated with amino guanidine (NO inhibitor), infected with *T. cruzi*, were used to demonstrate that NO is of primary relevance to the resistance of acute *T. cruzi* infection in the mice orally immunized with *P. serpens* (15T).

The results obtained in the present study showed that mice infected with *T. cruzi* exhibited higher plasma levels of NO in comparison with uninfected animals. Although iNOS is not considered essential for the control of *T. Cruzi*-infection [38], the elimination of trypomastigotes forms in the acute phase is dependent on a number of factors [39] and [40], one of which is the production of NO catalyzed by iNOS [41-44]. In this study, we evaluated whether intra peritoneal inoculation of *P. serpens* in mice induces heart protection facilitated by NO and cytokines production in *T. Cruzi*-infected mice. Our results revealed that *T. Cruzi*-infected C57BL/6 mice present higher heart parasitism than immunized-infected mice. These data corroborate with those found in previous studies [16]. This protective response is associated with higher production in the plasma of NO and inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-12), necessary to control the parasite. The increase of levels of NO observed in plasma of the mice-immunized-infected group could be related to the high expression of iNOS in the heart that have direct contact with blood. In this manner, such compartments could contribute to the systemic increase in NO in the infected group. On the other hand, the differences in profile of plasma NO observed between the infected and immunized-infected group could arise from the Phytomonas-host interaction initially established. The mechanism by which NO production is induced by *P. serpens* in heart remains to be determined. One possibility is that *P. serpens* antigens, especially the *cruzi* pain-like cysteine peptidases [45] and calmodulin (CaM) [12] may induce the enzyme directly. Alternatively, iNOS expression may result from autocrine stimulation by cytokines and chemokines released by cardiomyocytes after exposure to *P. serpens*. In immunized mice, the production of TNF-α, IL-1β, IL-6 and IL-12 increase compared with controls mice. TNF-α increased on day 12 after *T. cruzi* infection, suggests that they might have been the stimulus for NO production. In summary; we showed that the previous immunization with *P. serpens* decreased the cardiac parasitism and it was associated with NO overproduction and pro-inflammatory cytokines in the early of *T. Cruzi*-infection. These findings support the idea of the development of whole-organism-based vaccines targeting phytoflagellate trypanosomatids species as a safer source of immunogenic agents for therapeutic options in Chagas heart disease.

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COMPETING FINANCIAL INTERESTS

The authors have declared that no conflict of interest exists.

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