Cytokine 121 (2019) 154736

Contents lists available at ScienceDirect

Cytokine

journal homepage: www.elsevier.com/locate/cytokine

# Does Prolactin treatment trigger imunoendocrine alterations during experimental *T. cruzi* infection?

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ARTICLE INFO

*Keywords:*
Prolactin, *Trypanosoma cruzi*
Cytokines
Immune response

ABSTRACT

Prolactin (PRL) is a pleiotropic polypeptide hormone produced by the anterior pituitary gland and negatively controlled by dopamine. Some researchers have associated the immune regulatory functions of PRL with some infectious diseases like *Toxoplasma gondii* and *T. cruzi*. This work aimed to analyze the possible immuno-modulatory effects of this hormone through the subcutaneous administration of PRL during the experimental Chagas disease. On the 14th day post-infection (dpi), PRL triggered increased percentages of NK cells in treated infected animals as compared to the infected and untreated ones. For early and late apoptosis, our results showed that in chronically infected groups, PRL counteracted splenocyte apoptosis as revealed by the reduced percentages of both, early and late apoptosis. Reduced percentages of spleen $CD4^+$ and $CD8^+$ T cells were detected in infected PRL treated rats (60 days post-infection). Concerning to B cells, a significant increased percentage of these cells was found for all PRL treated infected animals (14th dpi), but no statistically significant alteration was observed on the 60th days post-infection. Furthermore, PRL treatment triggered a significant increase in the percentage of $CD4^+$ T lymphocytes IFN-γ producers, while on the 60th dpi, a reduced percentage of IFN-γ in these cells was observed in prolactin-treated rats compared to infected and untreated ones. Enhanced serum IL-12 levels were detected in infected and PRL treated subjects (60th dpi). Only on 7th day post-infection, the flow cytometric analysis of CFSE-stained $CD3^+$ T cells showed an enhanced proliferation of polyclonal stimulated T cells in PRL-treated and infected animals. In this study, we demonstrated that PRL can influence many aspects of the immune response during the experimental Chagas' disease, and this substance could be used as a supporting trial along with the conventional drug treatment.

## 1. Introduction

Prolactin (PRL) is a pleiotropic polypeptide hormone produced by the anterior pituitary and negatively controlled by dopamine [4]. Its functions exceed the well-known actions on the development of the mammary gland and initiation and maintenance of lactation, also exhibiting immunomodulatory activities, the latter function being orchestrated by the presence of PRL receptors, which are widely distributed throughout many different cells and tissues [8]. Apart from the anterior pituitary gland, PRL plays a role in many other functions according to the expression of its receptors in extra-pituitary tissues such as endothelium, skin, immune cells and peripheral blood mononuclear cells, mainly lymphocytes [5]. Prolactin and its neuroendocrine interactions occur predominantly at the level of the hypothalamic–pituitary axis and its actions play a major role in the immunoneuroendocrine network, similar to a crossroad that allows the endocrine, immune and nervous systems to communicate with each other [25].

Based on molecular and functional evidences, the extra-pituitary PRL shows different biological effects as compared to the pituitary hormone [25]. The autocrine/paracrine PRL is structurally similar to members of the cytokine hematopoietic family, like GM-CSF and IL-2 [3,17]. Thus, it acts like a cytokine and interacts with the immune system, stimulating immune cell proliferation and survival [8,23]. The immune regulatory properties exerted by PRL are achieved through specific binding to receptors that are found in lymphoid organs, including the thymus and spleen and several cells such as natural killer (NK), macrophages, T and B lymphocytes [8,51].

Some researchers have associated the immune regulatory functions

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https://doi.org/10.1016/j.cyto.2019.154736
Received 22 August 2018; Received in revised form 8 May 2019; Accepted 24 May 2019
Available online 01 June 2019

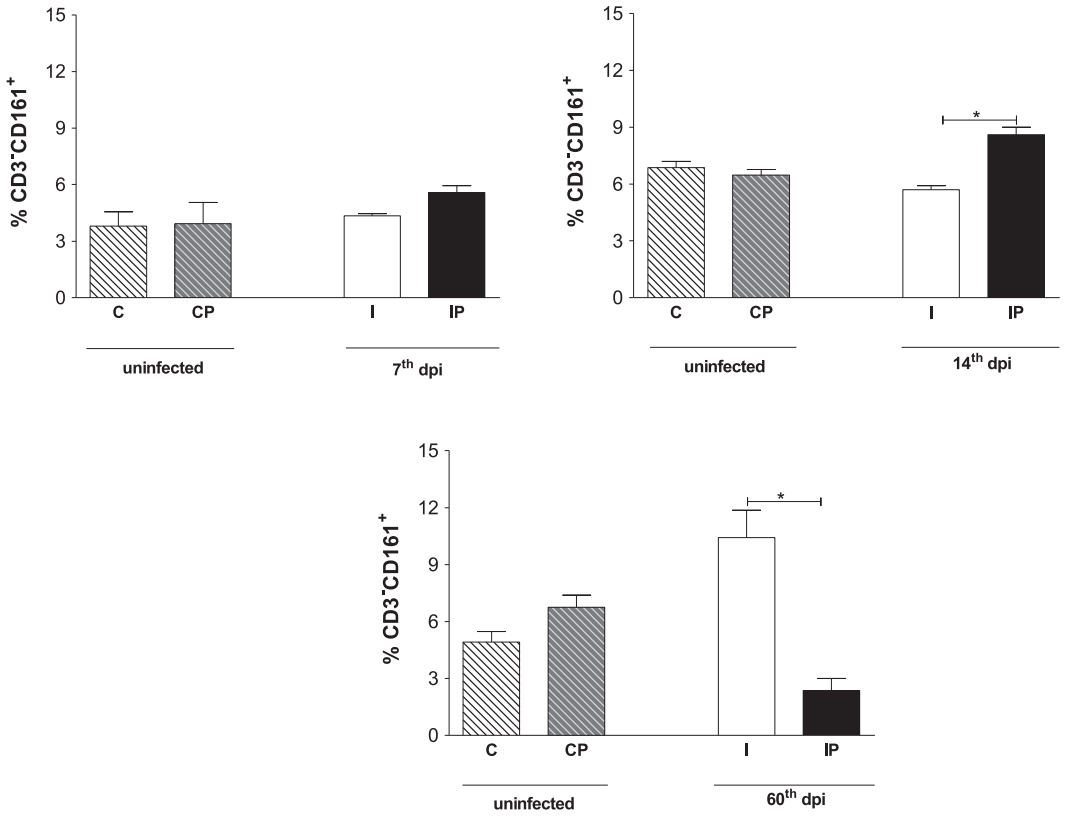


**Fig. 1.** The bars represent the mean percentage values of NK ($CD3^{-}CD161^{+}$) cells isolated from the spleen of uninfected and *T. cruzi*-infected Wistar rats. The animals were randomized into the following groups: control (C); control PRL treated (CP); infected (I); and infected PRL treated (IP). Data are representative of 3 days of experiments (7, 14 and 60 days) using five rats per group in each experiment. Statistical analysis was done by One-way ANOVA followed by Bonferroni's multiple comparison ($p < 0.05$).

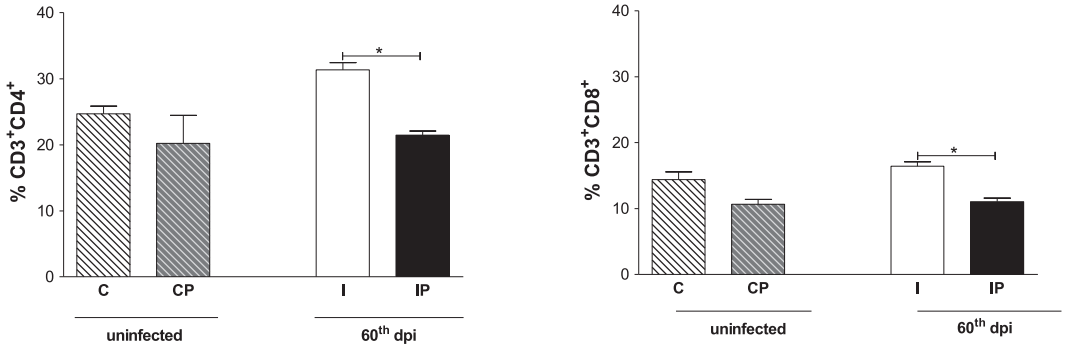


**Fig. 2.** CD4 and CD8 lymphocyte percentages from the spleen of uninfected and *T. cruzi*-infected Wistar rats, randomized among the following groups: control (C); control PRL treated (CP); infected (I); and infected PRL treated (IP). APC-conjugated anti-rat CD3, PE-Cy7-conjugated anti-rat CD4 and PerCP-conjugated anti-rat CD8 were used to identify T helper lymphocytes ($CD3^{+}CD4^{+}$) and cytotoxic T lymphocyte cells ($CD3^{+}CD8^{+}$). The experiments are performed after 60 days post infection using five rats per group. All graphical results shown are mean percentages and standard error of mean. Statistical analysis was done by One-way ANOVA followed by Bonferroni's multiple comparison ($p < 0.05$).

of PRL with some infectious and autoimmune diseases. Benedetto and coworkers described the protective role of PRL in a murine experimental toxoplasmosis model, as well as its anti-parasitic activity *in vitro*, being able to inhibit *Toxoplasma* proliferation in cell cultures [7]. Previous studies carried out by our group involving *Trypanosoma cruzi* (*T. cruzi*), demonstrated a reduced number of blood trypomastigotes during PRL treatment [23].

*T. cruzi* induces an imbalance in the hosts homeostasis characterized by intense parasitemia during the early infection and tissue damage at the chronic stage [28,52]. During the early infection, the host's organism reacts with a potent immune response involving the local and systemic release of several mediators, including cytokines, nitric oxide (NO) and chemokines [29,37].

Intensive research based on diverse immunomodulatory compounds with a broad range of potential therapeutic actions has been carried out for multiple types of infections, such as the important and neglected Chagas' disease, for which few effective compounds are currently available [55]. To evaluate the trypanocidal activity, compounds were chosen based on a variety of different actions, and although new substances have been identified, several *in vitro* and *in vivo* analysis and new drug candidates for the chronic Chagas' disease treatment have failed. Additionally, some of these substances display trypanocidal activity but are improper due to the unsuitable and undesirable side effects [16,30,2].

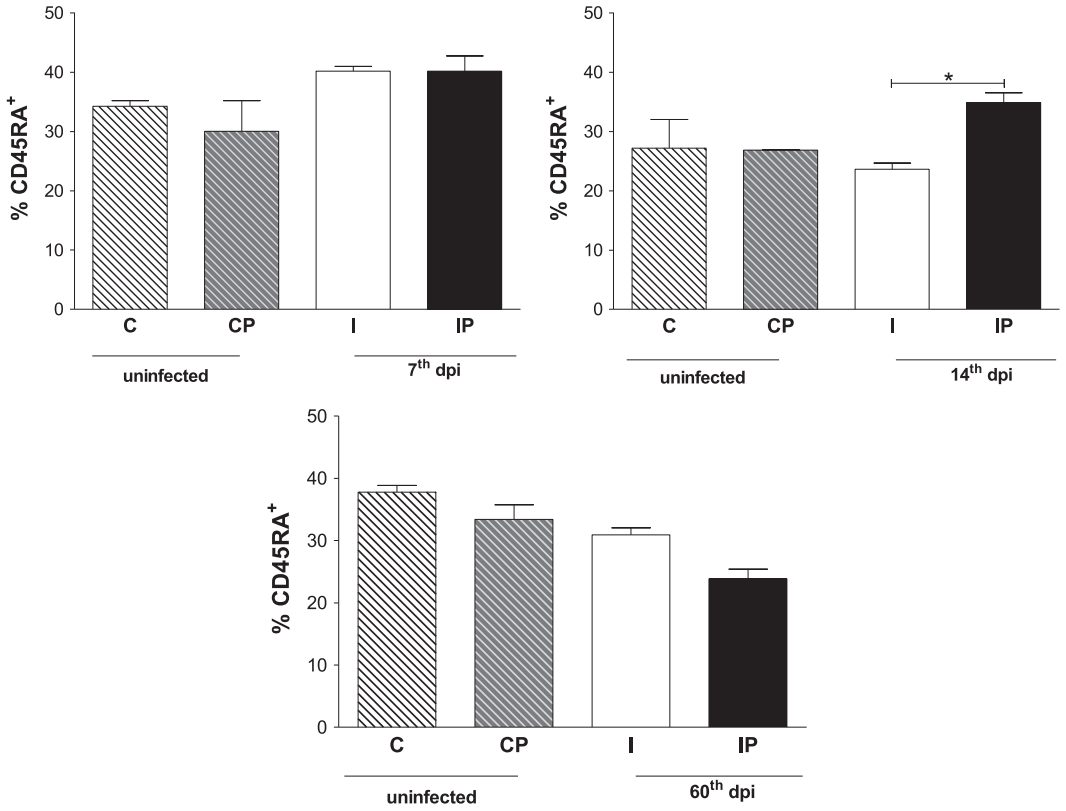


**Fig. 3.** Percentage of CD45RA$^+$ lymphocytes from the spleen of uninfected and *T. cruzi*-infected Wistar rats, for the following groups: control (C); control PRL treated (CP); infected (I); and infected PRL treated (IP). Data are representative of 3 days of experiments (7, 14 and 60 days) and results are shown as the means ± SEM of n = 5 rats. Statistical analysis was done by One-way ANOVA followed by Bonferroni's multiple comparison ($p < 0.05$).

Thus, aiming at examining the immunomodulatory effect, the present authors evaluated the influence of PRL on the immune response during acute and chronic experimental Chagas disease. The immune cells were characterized by stained surface markers using multicolor flow cytometry and the Th1 and Th2 cytokine profiles were also evaluated.

## 2. Materials and methods

### 2.1. Experimental groups

All assays were performed as per the guidelines of the National Council for the Control of Animal Experimentation (Conselho Nacional de Controle de Experimentação Animal – CONCEA-Brazil) after the necessary approval of the Ethics Committee (11.1.1211.53.9) on Animal Use of the University of São Paulo, *Campus* of Ribeirão Preto. Sixty male *Wistar* rats (5 weeks old), weighing between 100 and 120 g, were randomized into the following groups: control (C); control PRL treated (CP); infected (I); and infected PRL treated (IP). Five animals were used per group/per day of experiment (7th, 14th and 60th dpi). As the experimental schedule was performed twice, the total number of animals used was 120. Five *Wistar* rats were kept per cage, at environmental temperatures with water and rodent chew *ad libitum*. To avoid high percentages of ammonia from urine, cage straw was changed three times a week.

### 2.2. Prolactin treatment

Ovine PRL (40 µg/ Sigma–Aldrich Chemicals), were administered subcutaneously for 60 consecutive days, in 0.1 ml of saline solution (0.9%) [23,62,65]. Drug treatment started at the same day of the infectious inocule.

### 2.3. Parasite extracts

*T. cruzi* antigens were purified as described by Camargo et al. [11], with some modifications. At the peak of parasitemia, the hearts of Balb/c mice were bled and bloodstream trypomastigote forms were separated. Afterwards LLC-MK2 cells (monkey kidney epithelial cells) were infected and kept in culture to establish the intracellular cycle, kept at 37 °C in RPMI-1640 medium with fetal calf serum (5%) and incubated (5% $CO_2$ atmosphere). After 5 to 9 days, tissue culture-derived trypomastigotes (TCTs) were harvested from the supernatants of LLC-MK2 cultures and purified by centrifugation, for cellular debris separation. Afterwards they were centrifugated and the resulting pelleted was washed in cold PBS, and subjected to five times of freeze-thawing followed by 5 sonication rounds (30 s, 20KHz, 30 W). Cellular debris were removed by centrifugation at 20,000*g*. Protein levels were determined using a Bio-Rad protein assay. Samples were stored at −70°c until use.

### 2.4. Parasites and euthanasia

Wistar rats were intraperitoneally (i.p.) inoculated with $1 \times 10^5$ blood trypomastigotes of the Y strain of *T. cruzi*. Animals were submitted to tribromoethanol (250 mg/kg, i.p.) anesthesia and soon after animal lost of consciousness, decapitation was performed.

### 2.5. Preparation of single splenic and peritoneal cell suspension

To obtain spleen suspensions, the spleen tissue was mechanically cut into small fragments and dispersed by extrusion through a 70 µm Nylon Cell Strainer and macerated in RPMI 1640 medium to produce a single cell suspension from which the cells extruded spontaneously. The cells were washed in a hypotonic buffer (160 mM $NH_4Cl$, 10 mM Tris-HCl, pH = 7.4) for red blood cell lysis. The cells were centrifuged at

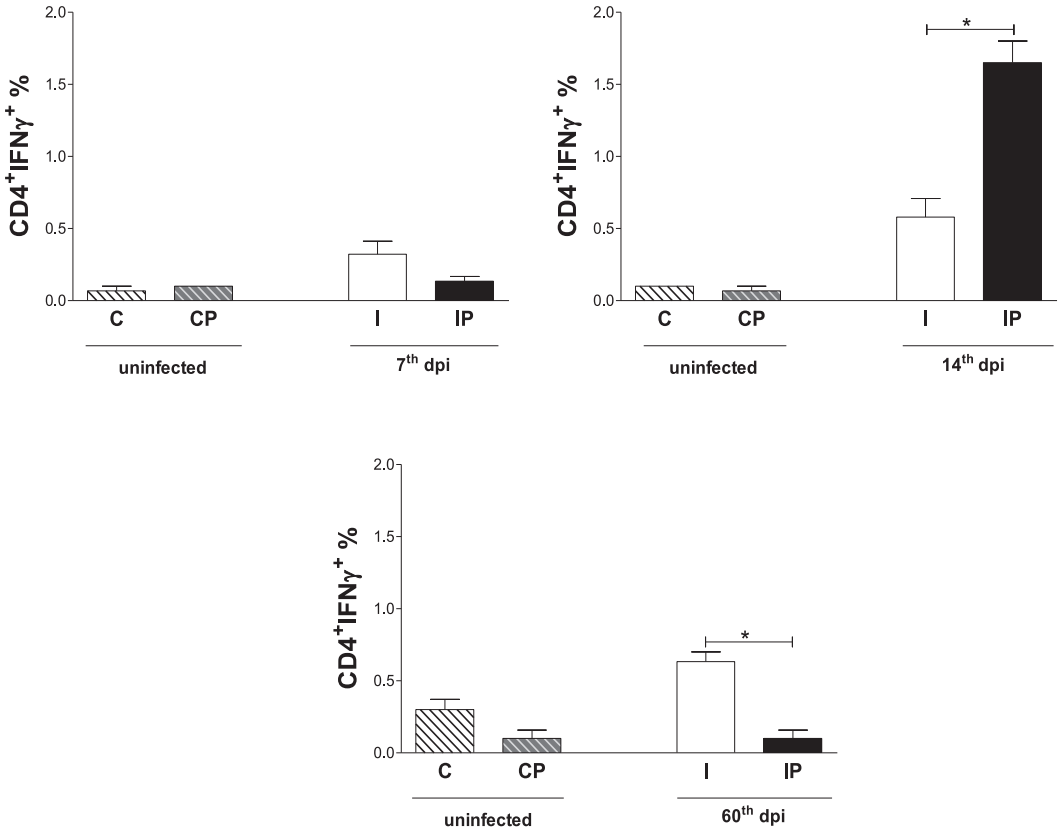


**Fig. 4.** Percentage of Intracellular Th-1 cytokine IFN-γ expressions in $CD4^+$ T cells. Splenocytes derived from uninfected and *T. cruzi*-infected Wistar rats were stimulated *in vitro* with phorbol 12-myristate 13-acetate (PMA), ionomycin and brefeldin A. The animals were randomized among the following groups: control (C); control PRL treated (CP); infected (I); and infected PRL treated (IP). All graphical results shown are mean percentages and standard error of mean of CD3+ CD4+ lymphocytes with intracellular IFN-γ production. Data are representative of 3 days of experiments (7, 14 and 60 days) using five rats per group in each experiment. Statistical analysis was done by One-way ANOVA followed by Bonferroni's multiple comparison ($p < 0.05$).

410*g* for 15 min, the pellet was re-suspended in RPMI 1640 medium and the remaining tissue fragments were eliminated by sedimentation, after diluted (1:10) with Trypan solution (0.4%), and the cells were counted in a Neubauer, to assess the viability of splenic and peritoneal cells.

### 2.6. FACS analysis

Cells from the spleen were plated at a density of $2 \times 10^6$ cells/well in polystyrene tubes for cytofluorometric analysis. Following Fc receptor blocking, cells were incubated with color combinations of the monoclonal antibodies anti-CD3- Allophycocyanin (APC), anti-CD4-phycoerythrin-Cy7 (PE-Cy7), anti-CD8-peridinin-chlorophyll-protein complex (PerCP), anti-CD45RA -PE (phycoerythrin) and anti-CD161-PE. Stained cells were stored for analysis in PBS containing 1% paraformaldehyde, in sealed tubes held in the dark. All steps were performed at 4 °C. Analysis of these cells was performed using a Becton Dickinson FACScan flow cytometer with DIVA-BD software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). All conjugated monoclonal antibodies were purchased from BD Biosciences (BD Biosciences, San Jose, CA, USA).

### 2.7. Stimulation with PMA/ionomycin in the presence of Brefeldin A

Splenocytes were placed in 96-well plates and stimulated, *in vitro*, using ionomycin 500 ng/ml (Sigma) and phorbol 12-myristate 13-acetate 50 ng/ml (PMA). The cells were incubated for 4 h at 37 °C in 5%$CO_2$ atmosphere. After, cells were against cultured in the presence of brefeldin A (10 μg/ml), that has an inhibitory effect on protein secretion, for the least 2 h.

### 2.8. Intracellular staining

Cells were washed in *Stain Buffer BSA* and blocked for nonspecific binding using *Fc block*. Surface staining was performed with fluorochrome-conjugated monoclonal antibodies directed against rat membrane markers: anti-CD3-FITC and anti-CD4-PECy7. Briefly, cells were fixed and permeabilized with Cytofix/Cytoperm solution followed by washing in Perm/Wash solution and then, stained intracellularly with mAbs against cytokine (anti-IFN-γ-PE). Finally, they were washed in Perm/Wash solution, fixed in 1% paraformaldehyde in PBS and analyzed by flow cytometry in a FACS Canto flow cytometer (Becton Dickinson Immunocytometry Systems) by using FACSDiva software. BSA, GolgiPlug reagent, containing Brefeldin A and reagents for cell fixation/permeabilization (Cytofix/Cytoperm and Perm/Wash) as well as all antibodies (anti-CD3-FITC, anti-CD4-PECy7 and anti-IFN-γ-PE) and appropriate isotype controls were obtained from BD Biosciences (BD Biosciences, San Jose, CA, USA).

### 2.9. In vitro CFSE proliferation assay

Spleens were aseptically and individually removed as previously detailed. Splenocytes cells were washed in warm PBS and were labeled with CFSE using Cell Trace CFSE Cell Proliferation kit (Molecular Probes, Life Technologies USA) and cultured at $2 \times 10^6$ $mL^{-1}$ cells in RPMI1640 culture medium with or without ConA (5 μg $ml^{-1}$, a T-cell mitogen) for 10 min at 37 °C. Dye was then quenched by washing in ice-cold medium. Splenocytes were cultured in a humidified atmosphere of 5% $CO_2$ at 37 °C for 5 days. Cells were collected and incubated with anti-Fc receptor monoclonal antibody, and then stained with anti-CD3-Allophycocyanin (APC) monoclonal antibody. The data were acquired

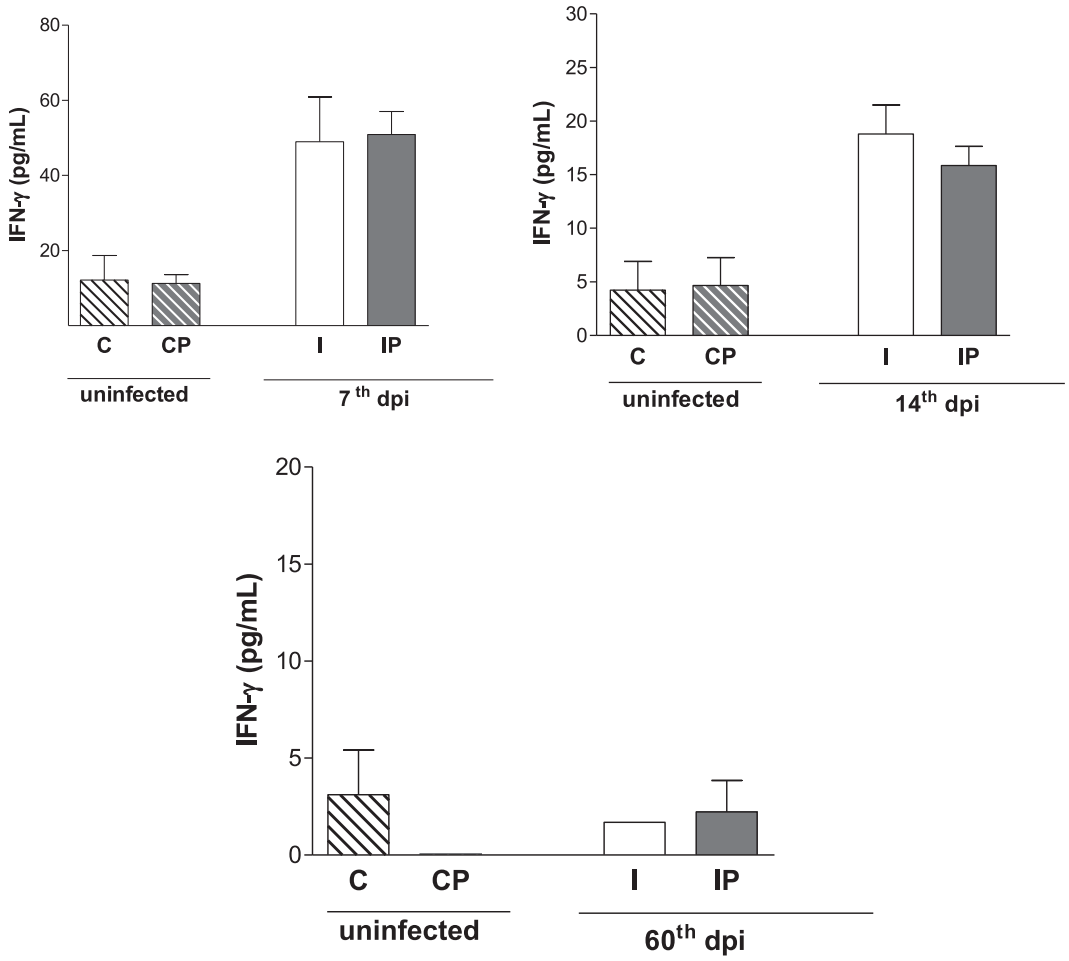


**Fig. 5.** IFN-γ serum concentrations (pg/mL) in male Wistar rats infected with $1.0 \times 10^5$ blood trypomastigotes of the Y strain of *T. cruzi* on the 7th, 14th and 60th day post infection of experimental disease, for the following groups: control (C); control PRL treated (CP); infected (I); and infected PRL treated (IP). All data are expressed as mean + S.E.M. n = 5/group/day of experiment. Statistical analysis was done by One-way ANOVA followed by Bonferroni's multiple comparison.

by gating on $CD3^+$T cell population, assessed by flow cytometry using FACSCan and FACSDiva software (BD Biosciences). The sequential loss of CFSE fluorescence ($CFSE^{low}$) was used to measure cell division, providing a more robust quantification of the proliferation by a cell population.

### 2.10. Cytokine assays

Serum were collected during experimental *T. cruzi* infection and used to evaluate cytokine production (IL-2, IL-12 and IFN-γ) using the high sensitivity enzyme-linked immunosorbent assay (ELISA) Kits from IBL (IBL International GmbH, Hamburg, Germany). The technique followed the manufacturer's guidelines with reference standard curves, using known amounts of the specific labeled antibodies. All samples were processed individually and assayed in duplicate, and were read at 450 nm. Cytokine levels were expressed in pg/mL.

### 2.11. Early and late apoptosis

Apoptotic splenocytes were detected *in vitro* by Phosphatidylserine (PS) exposure on the outside of the plasma membrane, which is a common feature during apoptosis and forms the basis for the annexin V binding assay to detect apoptotic cells. Annexin-V and propidium iodide (PI) assays were conducted with the BD Pharmingen kit (San Diego, CA, USA), according to the manufacturer's instructions. Splenocytes were incubated with annexin V, for 15 min, before analysis. Early apoptotic cells were those that were positive for FITC-annexin V and negative for propidium iodide. The percentage of $Annexin^+PI^+$ cells represents the late apoptotic cells.

### 2.12. Statistical analysis

The results were expressed as the mean ± standard error of mean (SEM). Statistical differences among groups were determined by one-way ANOVA followed by Bonferroni's post test. All statistical analyses were performed using Graph Pad Prism version 5.0 (GraphPad Software, Inc., San Diego, CA, USA).

## 3. Results

### 3.1. NK cells

On the 7th day of experimental infection, no significant results were observed in the percentages of NK cells for all PRL-treated rats. However, on the 14th dpi, we observed increased NK percentages in PRL-treated rats were as compared to infected and untreated ones. Oppositely during the chronic infection, a reduction in NK cells was noted in the infected and treated animals, as compared to untreated ones (Fig. 1).

### 3.2. CD4 and CD8 T cells

On the 60 days post-infection, $CD4^+$ and $CD8^+$T cell subsets were significantly reduced in PRL treated and infected rats as compared to

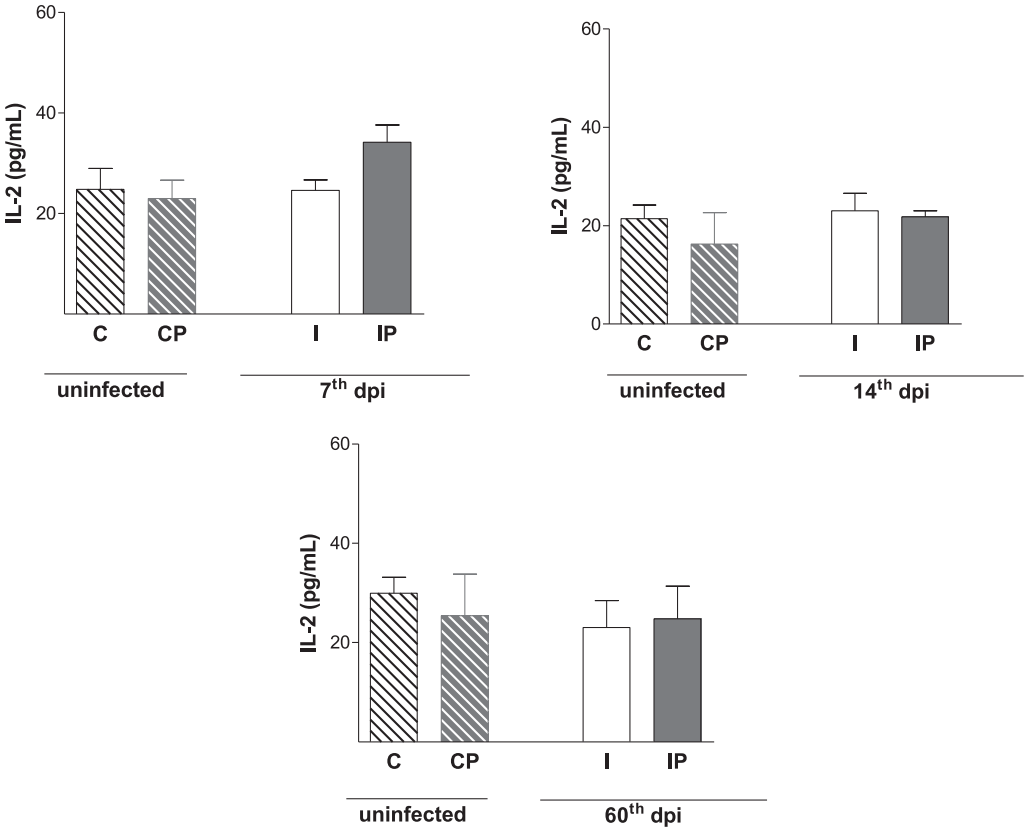


**Fig. 6.** IL-2 serum concentrations (pg/mL) in male Wistar rats infected with $1.0 \times 10^5$ blood trypomastigotes of the Y strain of *T. cruzi* during the course of experimental infection, for the following groups: control (C); control PRL treated (CP); infected (I); and infected PRL treated (IP). Statistical analysis was done by One-way ANOVA followed by Bonferroni's multiple comparison. All data are expressed as mean + S.E.M. n = 5/group/day of experiment.

untreated animals. No significant results were observed for $CD4^+$ and $CD8^+$ T cells percentages in PRL treated control groups (Fig. 2).

### 3.3. B lymphocytes

A significant increase in the percentage of B cells was observed 14 days post-infection for infected PRL-treated group when compared to untreated one. On the 60th days post-infection, no significant results for B cell percentages was found in PRL infected and treated animals (Fig. 3).

### 3.4. Intracellular staining of IFN-γ and Serum levels of IFN-γ, IL-2 and IL-12

On the 14th dpi, PRL promoted an upregulation in the production of IFN-γ, within the $CD4^+$ T compartment, although no changes in the levels of this cytokine were observed for the same groups on 7th dpi (Fig. 4)

Additionally, no difference in IFN- γ (intracellular or serum) production was detected in all PRL-treated animals (7 th, 14th and 60th dpi) (Figs. 4 and 5). In contrast, 60 days post-infection, lower percentage of IFN-γ in $CD4^+$T cells was observed in prolactin-treated rats comparing to infected and untreated animals (Fig. 4).

Additionally, no changes were detected on IL-2 production during all phases of the experimental infection (Fig. 6). Furthermore, Fig. 7 shows that the *T. cruzi* infection per se was accompanied by a significant increase in IL-12 production (14th dpi) as compared to control animals. In the same experimental day, PRL treatment did not alter IL-12 concentrations. However, on the 60th dpi, enhanced serum levels of IL-12 were observed in infected and treated subjects when compared to untreated animals (Fig. 7). (See Fig. 8.).

### 3.5. T cells proliferation

On 7th day post-infection, the flow cytometric analysis of CFSE-stained $CD3^+$ T cells showed an enhanced proliferation of polyclonal stimulated T cells in PRL-treated and infected animals, with enhanced $CD3^+$ T cells percentages expressing lower levels of CFSE as compared to untreated counterpart.

### 3.6. Apoptosis after T. cruzi stimulation

*T. cruzi* antigens and splenocytes were cultured. Four hours later, cells were collected and incubated for 15 min with FITC-Annexin V conjugate and propidium iodide. On 60th dpi, our results showed that, significantly reduced levels of early and late apoptotic splenocytes, from infected prolactin treated animals as compared to untreated rats ($P < 0.05$). PRL treated animals showed enhanced percentage of viable cells as compared to untreated counterparts. PRL control uninfected animals displayed no significant results for the apoptosis detected by Annexin-V-FITC.

On 7 days-post infection, a significant reduction in the percentages of the early apoptosis was observed for PRL treated and infected animals as compared to untreated rats (see Fig. 9).

## 4. Discussion

The influence of PRL in maintaining homeostasis of the immune system has been the subject of several clinical and experimental studies, evidencing that this hormone has stimulatory properties on immune cells [43,64]. Some authors have described the protective effects of PRL in a variety of infections, such as those caused by *Salmonella typhimurium* [21] and *Toxoplasma gondii* [6,7]. However, there is little data concerning the effects of PRL during T. cruzi infection. Previously, our research group has showed that PRL supplementation in infected rats is associated with an improvement of the immune response, as

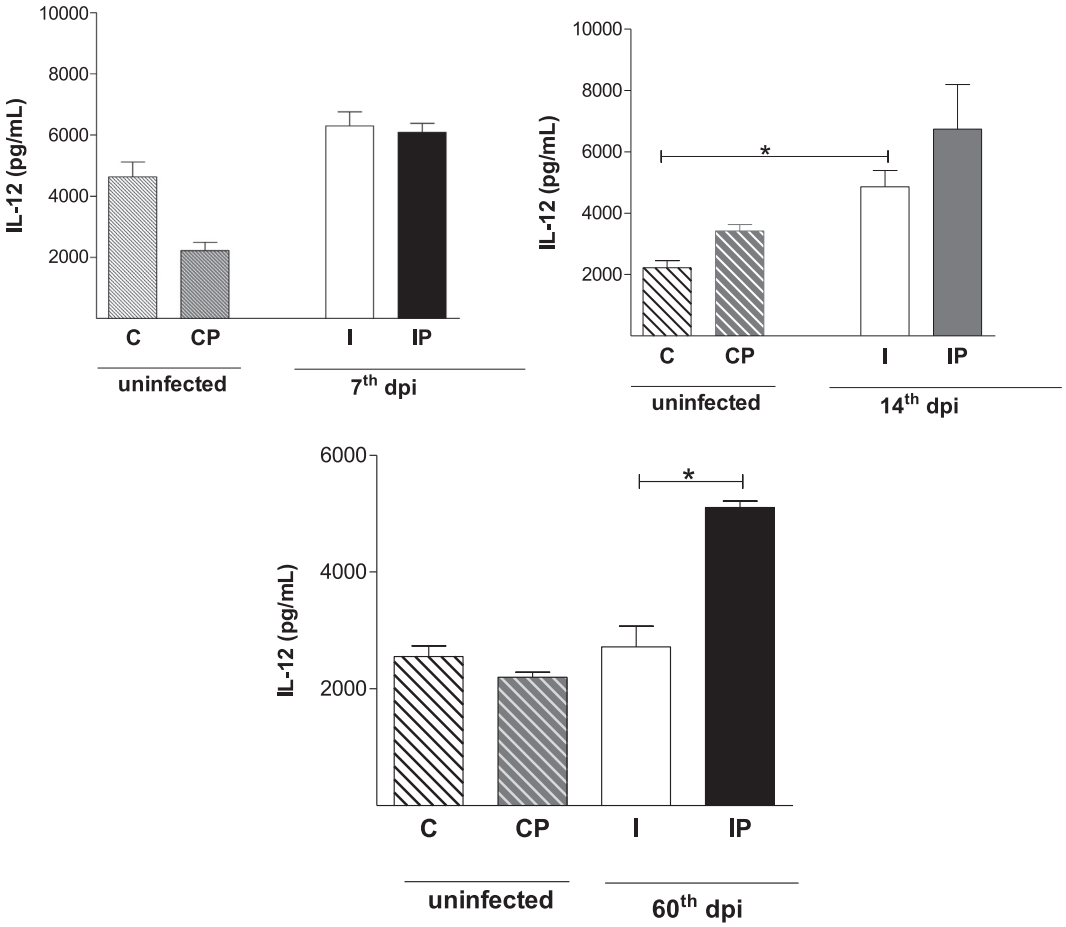


**Fig. 7.** IL-12 serum concentrations (pg/mL) in male Wistar rats infected with $1.0 \times 10^5$ blood trypomastigotes of the Y strain of *T. cruzi* during the course of experimental infection, for the following groups: control (C); control PRL treated (CP); infected (I); and infected PRL treated (IP). Statistical analysis was done by One-way ANOVA followed by Bonferroni's multiple comparison ($p < 0.05$). All data are expressed as mean + S.E.M. n = 5/group/day of experiment.

demonstrated by increased percentages of both $CD4^+$ and $CD8^+$ T cells [23] plus a significant reduction in the levels of parasitemia during the 7th and 14th day after infection [23]. The importance of the study during the acute phase of infection is based that many late lesions are influenced by the course of the disease at this stage [45].

The recruitment of NK cells and T lymphocytes during the initial phase of *T. cruzi* infection and their involvement in the activation of the innate immune response are important in host defense against multiplication of the parasite [9,58,23]. The role of PRL in the immune system was previously described and increased percentages of $CD4^+$ and $CD8^+$T cells in *T. cruzi* infected *Wistar* rats were found [23]. Others studies in humans and rodents have reported the influence of PRL on NK cells, PRL triggered an enhanced proliferative response and cytotoxic action against tumors of these cells [14,49,40–42]. Our data show a stimulatory action of PRL, with a significant enhanced percentage of NK cells in the infected and PRL-treated group, as compared with their infected and untreated counterparts (14th dpi).

During early infection, NK cells are primarily responsible for the synthesis of IFN-γ, a cytokine that acts on macrophages, stimulating the production of IL-12, TNF-α and NO [26]. Furthermore, on being challenged by *T. cruzi*, other immune cells also produce various cytokines, including IL-12, that induces the Th1 cytokine profile. IL-12 is an important cytokine that shares many biological functions with IL-2 [57], promoting a functional activation of NK cells with the release of IFN-γ. Interestingly, Matera and Mori [42] demonstrated the synergistic effect of PRL on the IL-2-induced activation of NK cells. Additionally, Michailowsky et al. [44] demonstrated a relevant role of the IL-12 in the resistance during *T. cruzi* infection, since its absence or reduced production is crucial for the reactivation of *T. cruzi* infection in cardiac and central nervous tissues. Furthermore, the gradual decline in IL-12 levels induced by HIV-1 infection may explain, in part, the reactivation of *T. cruzi* infection in these patients. According to Matalka [39] PRL enhances the production of IL-12 p70 from whole blood in a stimulus-specific manner, although no significant difference in the IL-2 production was found. For PRL treated infected subjects, on the 60th dpi, increased levels of IL-12 were observed as compared to their untreated groups.

Although *in vitro* analysis demonstrate that NK cells are the major source of IFN-γ during the early *T. cruzi* infection, and during this phase we cannot exclude the possibility of $CD4^+$ and $CD8^+$ T cells also contribute to IFN-γ synthesis [63]. Rodrigues et al. [54] showed that IFN-γ plays a critical role in the control of acute infection with the G strain of *T. cruzi*. On the 14th dpi, a significant increase in IFN-γ $CD4^+$T producing cells was found as compared to untreated ones. These data corroborate our previous studies which reported the stimulatory action of PRL on the production of IFN-γ. Studies by Cesario et al. [13] using cultures of peripheral blood mononuclear cells demonstrated that at certain concentrations, PRL was able to increase the production of this cytokine.

The persistence of high levels of IFN-γ during the late phase of Chagas disease may be related to the emergence of cardiomyopathy [19,54]. Interestingly, during the chronic stage of the disease, $CD8^+$ T cells seem to participate in the pathogenesis of *T. cruzi* infection. They develop specific cytotoxic activity triggering the death of *T. cruzi* infected host cells [38], resulting an intense inflammatory reaction involving a massive presence of immune cells such as macrophages and

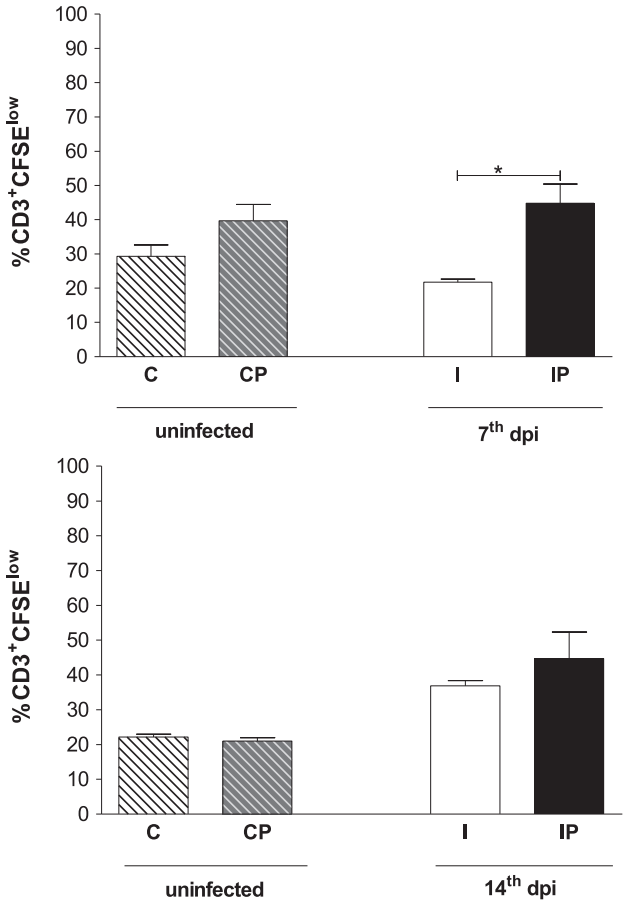


**Fig. 8.** Effect of PRL on the CD3+ cells proliferation exposed for 5 days to soluble trypomastigotes antigens (25 μg/mL). CD3+ cells labeled with CFSE, derived from the spleen of Wistar rats, were randomized among the following groups: control (C); control PRL treated (CP); infected (I); and infected PRL treated (IP). The lymphocytes were cultured for 5 days in the presence of *T. cruzi* antigens and analyzed by FACS. Graphs show the level of T-cell proliferation, as determined by CFSE fluorescence loss in the culture conditions as indicated in methods section. The experiments are performed after 7 and 14 days post infection using five rats per group. All data are expressed as mean + S.E.M. n = 5/group/day of experiment. Statistical analysis was done by One-way ANOVA followed by Bonferroni's multiple comparison ($p < 0.05$).

TCD8$^+$ lymphocytes. In our experiments, during the acute phase of *T. cruzi* infection, a characteristic TH-1 immune pattern was observed in cultured splenocytes. Chavez-Rueda and co-workers [15] evaluated the effects of PRL on the secretion of IFN-γ by CD4$^+$ T lymphocytes and found that the addition of anti-hPRL antibodies to the culture reduced the production of this cytokine. PRL kept on acting in a dual fashion enhancing the Th1 immune response during the acute phase and decreasing the percentages of CD4$^+$ and CD8$^+$ T cells and IFN-γ concentrations during the late phase of infection (60dpi). Then, PRL induced an inverse modulation indirectly protecting the affected organs from an exacerbated immune response during the late phase.

PRL seems to be involved in the maintenance of cell integrity and in the proliferation of several cell types such as T cells, adipocytes, pancreatic and mammary epithelial cells [61,20,48,22]. According to some authors, PRL regulates lymphocyte activation and proliferation, preventing glucocorticoids (GCs) induced apoptosis [33,10,60]. Some authors have demonstrated [18,36] a progressive decrease in systemic PRL during murine *T. cruzi* infection was observed in parallel with enhanced concentrations of GCs, which increase as long as the disease progresses. PRL and GCs display antagonistic actions. During adverse *stimuli* such as infections GCs normally show increased systemic levels [47,46]. The same authors also stated that increased PRL

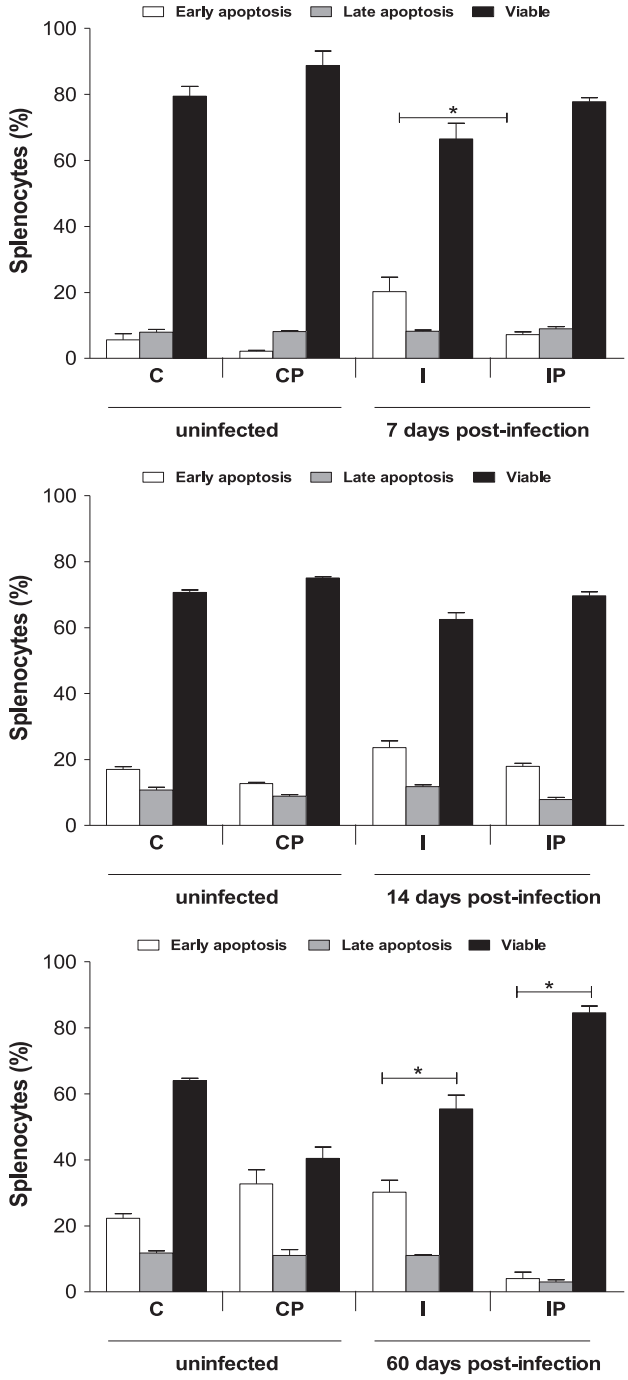


**Fig. 9.** Apoptosis assessment in splenocytes of uninfected and *T. cruzi*-infected Wistar rats, randomized among the following groups: control (C); control PRL treated (CP); infected (I); and infected PRL treated (IP). Cells were treated with soluble trypomastigotes antigens (25 μg/mL) for 4 h and the apoptotic cell fractions were measured using flow cytometry post staining with Annexin V-FITC/propidium iodide (PI). Early apoptotic cells were positive for FITC-annexinV and negative for PI. The percentage of Annexin + PI + and double negative (Annexin-PI-) cells represent late apoptotic cells and viable ones, respectively. Data are representative of 3 days of experiments (7, 14 and 60 days) using five rats per group in each experiment. All graphical results shown are mean percentages ± SEM. ($p < 0.05$).

concentrations could protect the host from an exacerbated immunosuppressive response mediated by glucocorticoids. Our results fully agree with all the papers prior cited, with enhanced splenocyte proliferation observed amongst the PRL-treated and infected animals.

*In vitro* studies reported the anti-apoptotic effect of PRL being able

to reverse glucocorticoids induced cell death in Nb2 cell line derived from lymphoma [59,24,33]. According to Krishnan et al. [31] animals with elevated plasma PRL levels showed a decrease in the exposure of phosphatidylserine on the surface of splenocytes and thymocytes, inhibiting caspase-3 activation and reducing DNA fragmentation. The stimulation of certain T lymphocyte subsets by PRL is probably the result of the increased expression of anti-apoptotic genes of the Bcl family such as bcl-2 and bcl-x [10,31]. These genes inhibit apoptosis by preventing the release of cytochrome *c* reducing the caspase-9 activation [1]. Leff et al. [35] demonstrated increased the expression of bcl-2 cultured cells when PRL was added. Thus, our experiments corroborate the studies cited above, since splenocytes from infected animals had higher percentages of early and late apoptosis, and reduced viability, when compared to control uninfected animals. PRL treated and infected rats displayed increased splenocyte viability and significantly reduced percentage of early and late apoptosis as compared to the untreated animals.

Some authors described B cell importance in the resistance to *T. cruzi* infection in which infected and genetically deficient B cell animals showed increased susceptibility to infection [12] parasitemia [32] and reduced number of memory T lymphocytes. Pascutti et al. [50] also demonstrated that the resistance of acute *T. cruzi* infected was linked with their ability to antibody production. One of the hallmarks of the acute phase of Chagas' disease is the hypergammaglobulinemia, triggered by the excessive activation of polyclonal B cells, with unspecific response, impairing the activation of antigen-specific responses [27,53].

The stimulatory effect of PRL on immune cells has been linked to several autoimmune diseases characterized by enhanced activation of B cells, such as systemic lupus erythematosus [56]. Ledesma-Soto et al. [34] demonstrated PRL receptor on the surface of early stage B cells, thus enhancing its actions on proliferation and maturation. For that we used CD45RA molecule which characterizes rat B cell subsets. During the early phase of infection (7th dpi) no significant results were observed. As long as the infection progressed, PRL treated and infected animals displayed a significant enhancement of the B cell population. During chronic infection (60 days) an opposite effect was seen, with a significant drop in the percentage of $CD45RA^+$ cells confirming the immunomodulatory actions of PRL.

The mechanism of PRL action proposed in this paper could be based on the plurality of PRL actions. During the acute phase, PRL triggers higher antibody production by B cells, as well as an intense TH-1 immune response in order to contain parasite replication. Contrarily, in the late phase of infection, less antigenic stimulus is observed along with a depressed TH-1 immune which is extremely important to maintain host cell homeostasis in this phase of the infection.

Based on our results we propose that PRL could play a supporting role in conventional drug treatment, although it is important to consider the risk of its continuous use, toxicity and dose schedules. The mechanisms of PRL actions on the immune system are not yet fully understood and further additional studies still need to be undertaken in this field.

## Declaration of Competing Interest

The authors declared that there is no conflict of interest.

## Acknowledgments

We thank Cristiana Gonçalez of Faculty of Pharmaceutical Sciences of Ribeirão Preto (FCFRP), University of São Paulo for technical. This study was supported by fellowships from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, 2010/19895-0).

## Statement of authorship

We hereby certify that it is an original publication and the manuscript has not been previously submitted or published elsewhere. MF, VB, FS, CC, MT and FM conceived the study design and/or conducted the studies. MF, VB and JP performed the statistical analyses, participated in the study design, and helped in drafting of the manuscript. All authors have made substantial contributions and final approval of the conceptions, drafting, and final version.

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