

**EXPERIMENTAL INFECTION WITH *Leishmania chagasi* IN
IMMUNOSUPPRESSED BALB/c MICE: CYTOKINES AND PARASITE BURDENS**

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ABSTRACT: The immune response in leishmaniasis may result in a polarization of the T lymphocyte subpopulation, altering cell phenotype and resulting in immune protection or disease exacerbation. *Leishmania* may persist in the body either during asymptomatic infections or after treatment, which represents high risk under immunosuppression. The objective of this study was to evaluate the effect of infection with immunosuppression by dexamethasone associated with pentoxifylline on animal weight, spleen weight, spleen and hepatic parasitic load and immunopathology, as well as the IFN- γ and IL-10 production in spleen cell culture of Balb/c mice infected with *Leishmania chagasi*. The infection did not cause body weight gain in animals, but both the weight and size of the spleen were increased. The immunosuppression using dexamethasone associated with pentoxifylline affected body weight gain and spleen weight and size in both infected and non-infected animals. The immunosuppression did not significantly alter the course of the splenic or hepatic parasite burden. Dexamethasone and pentoxifylline significantly affected cytokine production, but did not influence the Th1/Th2 ratio in infected animals.

KEY WORDS: *Leishmania chagasi*, immunosuppression, cell-mediated response, parasite burden, experimental infection.

CONFLITS OF INTERESTS: There is no conflict.

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INTRODUCTION

Leishmaniasis is caused by intracellular protozoa of the genus *Leishmania*, order Kinetoplastida, family Trypanosomatidae. They are heteroxenous parasites that require two hosts to complete their life cycle, namely a vertebrate and an invertebrate, particularly hematophagous flies of the genus *Phlebotomus*, order Diptera (1). *Leishmania* presents a spherical form and are non-mobile and without flagellum when in vertebrate hosts, called amastigote, and a flagellated form in mosquitoes, denominated promastigote (2).

In experimental infections by the genus *Leishmania*, host resistance or susceptibility to the parasite is determined by differentiation of T lymphocytes into the subpopulations Th1 or Th2 (3). Recent studies – especially concerning leishmaniasis agents in humans, i.e. *L. major* – define the Th1/Th2 paradigm as indicator of the resistance/susceptibility to the infection as well as indicate the role of IL-12 and IL-4, which is to provoke the development of Th1 and Th2 cells (4). However, similar patterns have not been observed in infections provoked by *L. donovani* and *L. chagasi*, both agents of visceral leishmaniasis in humans (5).

The most studied visceral leishmaniasis model is the Balb/c mouse lineage infected with *L. donovani* or *L. chagasi* (6). The course of visceral infection by *L. chagasi* in a Balb/c mouse strictly mimics the infection by *L. donovani*, since the mouse initially expresses a susceptible phenotype, but subsequently develops its self-cure (7). Most studies employing strains of visceral leishmaniasis have been performed with 10^7 parasites per mouse, although variability among injection doses has limited the comparison of results (8). Infection by *L. donovani* in mice results in early increase of parasite burden and its subsequent control during the infection course (7).

The hepatic infection is usually self-limited – an example of granulomatous inflammatory response predominantly composed of mononuclear cells – with multiple cytokines including mainly IFN- γ , IL-12, IL-4 and moderate levels of TNF- α , all produced in the hepatic granuloma (9). Simultaneous to the hepatic infection resolution, a growth of amastigote forms occurs in the spleen (10). The persistence of parasites in this organ is followed by failure to form granulomas and splenomegaly provoked by alteration of lymphatic tissue microstructure and augmentation of hematopoietic activity (11).

While immunological response of experimental cutaneous leishmaniasis has been extensively studied, the characteristics of immune response to visceral leishmaniasis are still not fully understood (12).

The protective role of IL-12 in visceral leishmaniasis was attributed to its ability to activate macrophages, thus increasing killing activity. IL-12 produced by infected macrophages induces natural-killer-cell (NK) activation, Th1 cell differentiation and IFN- γ production (4). INF- γ , in turn, stimulates production of nitric oxide by macrophages, which is responsible for parasite destruction and infection resistance (13). Failure to produce IL-12 or alternative production of IL-4/IL-13, concurrently with IL-10, may increase parasite replication within infected cells (4).

IL-10 was originally described as a cytokine specifically expressed by TCD4⁺Th2 cells; however, recent studies have demonstrated that this interleukin may be secreted by either Th1 or Th2 cells. New reports have proven that the IL-10 production by effector Th1 cells aid in restricting collateral damage caused by an exacerbated inflammation. Nevertheless, this control may also limit immune response efficiency, resulting in a breakdown of the pathogen elimination process (14). Although TGF- β expression is induced by a latent form of parasitism and plays a significant role in the maintenance of the visceral disease, IL-10 is the main immunosuppressive cytokine found in visceral leishmaniasis that may be produced by host cells, Th2 cells and/or regulatory T cells (4).

Leishmania persistence in hosts after treatment, in asymptomatic infections, might be considered positive factors for the maintenance of immunological memory in T cells, which promotes resistance against reinfections. On the other hand, this situation may represent a risk under immunosuppressive conditions, in the context of several reports from recent decades have defined leishmaniasis as an opportunistic infection (15).

Glucocorticoids, including hydrocortisone acetate and dexamethasone, are immunosuppressive antiinflammatory drugs that, when used alone or in combination, may alter the course of infection by *Toxoplasma gondii* and leishmania (16). Dexamethasone is well known for its numerous immunosuppressive properties, especially regarding production of pro-inflammatory cytokines such as IL-2 e IFN- γ (17). However, their effects on the Th1/Th2 profile are still unclear (15).

Immunomodulating properties of pentoxifylline and its synergetic inhibitory effects with glucocorticoids make this drug a potential supporting agent for treatment of

human autoimmune diseases (17). Several pentoxifylline effects at the cellular level comprise modulation of cytokine production in a dose-dependent pattern namely TNF- α synthesis by macrophages, synthesis of IL-2, IL-12 and INF- γ , and influence on the production of other cytokines including IL-4, IL-6 and IL-10. These properties indicate that pentoxifylline may act by stimulating alterations on Th1 cellular response to Th2 (3).

In the face of the growing incidence and severity of visceral leishmaniasis cases in immunosuppressed hosts, knowledge on immunological behavior is extremely important. Experimental studies are fundamental to better understand how host immune response works, which may enable the development of vaccines to prevent the disease in humans and other animals and permit the use of exogenous cytokines that may aid the recovery of infected immunosuppressed patients (18).

In the present study, the effects of leishmania infection and immunosuppression on body and spleen weight, splenic and hepatic parasite burden as well as immunopathological aspects related to IFN- γ and IL-10 production in spleen cell culture were applied to comparatively evaluate the infection in immunosuppressed and non-immunosuppressed animals.

MATERIALS AND METHODS

Animals

Isogenic male Balb/c mice aged seven weeks were utilized. Animals were divided into four experimental groups, Group 1 (G1): 16 non-infected and non-immunosuppressed mice; Group 2 (G2): 21 non-infected mice immunosuppressed by dexamethasone associated with pentoxifylline; Group 3 (G3): 17 *Leishmania chagasi*-infected mice; and Group 4 (G4): 20 *Leishmania chagasi*-infected mice immunosuppressed by dexamethasone associated with pentoxifylline.

Infection

In each of the two experiments, amastigote forms of *Leishmania chagasi* strain M6445 – kindly provided by the Protozoa Laboratory of São Paulo Institute of Tropical Medicine, School of Medicine, University of São Paulo – were employed for infection. The strain was carried by Golden Syrian hamsters (*Mesocricetus auratus*), according to the infection protocol by Stauber *et al.* (19).

On the infection date, denominated day zero, groups G3 and G4 were anesthetized following the protocol of Curl and Peters (20). Then, each animal was infected with 10^7 amastigotes intravenously in the infraorbital foramen. Groups G1 and G2 were also anesthetized and inoculated with water, at the same volume and by the same route.

Immunosuppression

Animals of groups G2 and G4 were immunosuppressed for 30 days, from the 60th to 90th post-inoculation (PI) day, with 200 μ L/day containing 15 mg/kg of dexamethasone disodium phosphate, intraperitoneally, and 300 μ L/day containing 150 mg/kg of pentoxifylline, subcutaneously injected in the scapular dorsal region (16). G1 and G3 received only water according to the same immunosuppressive protocol.

Sampling

Four mice from each group were euthanized in a saturated isoflurane vapor chamber, on the 30th, 60th, 75th and 90th PI days, totaling 16 animals per day. On the 75th PI day, G2 and G4 animals were on their 15th day of immunosuppression, and on the 90th PI day, they had been immunosuppressed for 30 days. All animals were weighed on day 60, 75 and 90 PI, after which their spleens were removed, photographed and weighed.

Parasite burden

Parasite burden was assessed by the culture microtitration technique as described by Buffet *et al.* (21).

Spleen cell culture supernatant

In order to obtain the supernatant of spleen cell culture, approximately half of the spleen from each mouse was removed, placed on Petri dishes containing 5 mL of RPMI 1640 cell culture medium and crushed. Material was collected and centrifuged in 15-mL Falcon tubes for 10 minutes at 1,500 rpm, at 4°C. Subsequently, supernatant was discharged and the cell sediment was harvested by homogenization in 2 mL of cell culture complete medium [CCCM: RPMI 1640 added to 10% inactive/sterile bovine fetal serum, 1% penicillin/streptomycin (respectively, 10,000

UI/mL and 10 mg/mL) and enriched with 1% L-glutamine]. After counting, the final concentration was adjusted to 5×10^6 cells/mL. Cells were tested in triplicate on 48-well microculture plates, immediately complemented with 100 μ L of CCCM, and stimulated with 10 μ g/mL of mitogen concanavalin-A and 10 μ g/mL of *Leishmania chagasi* antigen (8). The plates were incubated at 37°C, with 5% CO₂ for 48 hours, after which supernatant was harvested and stored at -80°C for subsequent cytokine processing and analysis.

The antigen was obtained from a culture of *Leishmania chagasi* promastigotes in 30 to 40 mL of Schneider's Drosophila medium, supplemented with 20% inactive/sterile bovine fetal serum and penicillin/streptomycin (10,000 UI/mL and 10 mg/mL, respectively), for 10 to 15 days at 28°C. After this period, culture was centrifuged at 3,000 rpm for 10 minutes in 50 mL Falcon tubes. Media were discharged and sediment was re-suspended in 10 mL of phosphate buffered saline solution (PBS), 0.01 M, pH 7.2, sterile. The material was re-centrifuged at 3,000 rpm for 10 minutes, thus to discharge the supernatant. This process was repeated twice and final supernatant was homogenized, aliquoted and stored at -20°C until sonicated.

Sonicator was adjusted for six more cycles of 50% potency, at 4°C, for 30 seconds. Next, this solution was examined in an optical microscope (200x) to verify the fragmentation of promastigote forms. The suspension was centrifuged at 5,000 rpm for 15 minutes at 4°C, filtered in a micropore membrane of 0.22 μ m; then, 1-mL aliquots were stored in microtubes at -20°C.

To stimulate the cellular culture with antigens, the soluble protein concentration in the filtrate was determined to adjust the stimulus concentration to 10 μ g/mL, according to the Lowry *et al.* method (22).

Levels of INF- γ and IL-10 were detected on spleen cell culture supernatant, with and without stimulus, by ELISA following the manufacturer's protocol (R&D Systems, USA).

Statistical analysis

To compare groups of all variables in the present study (body weight, spleen weight, cytokines and parasite burden) at each observation moment, Kruskal-Wallis test was applied, followed by Dunn test for multiple comparisons and Mann-Whitney test. To compare observation moments for same variables in each group, a Friedman test for

dependent samples was employed, followed by Dunn's test for multiple comparisons and Wilcoxon test for dependent samples (23, 24).

RESULTS AND DISCUSSION

Results presented herein refer to the more representative of the two independently performed experiments.

Figure 1 reveals that on the 60th PI day groups did not differ regarding median body weight. Up to that day no group had been immunosuppressed and G3 and G4 had been infected for 60 days. From the 75th to 90th days, median body weights in G2 rose from 25.1 to 26.4 g, while in G4 they jumped slightly from 26.3 to 27.2 g, which remained lower than their respective control group medians. The median mouse weight in G1 was 30.1 g (day 75) and 30.75 g (day 90) and in G3, 31.85 g (day 75) and 32.95 (day 90). These results suggest that dexamethasone and pentoxifylline may experimentally reduce the body weight gain of mice.

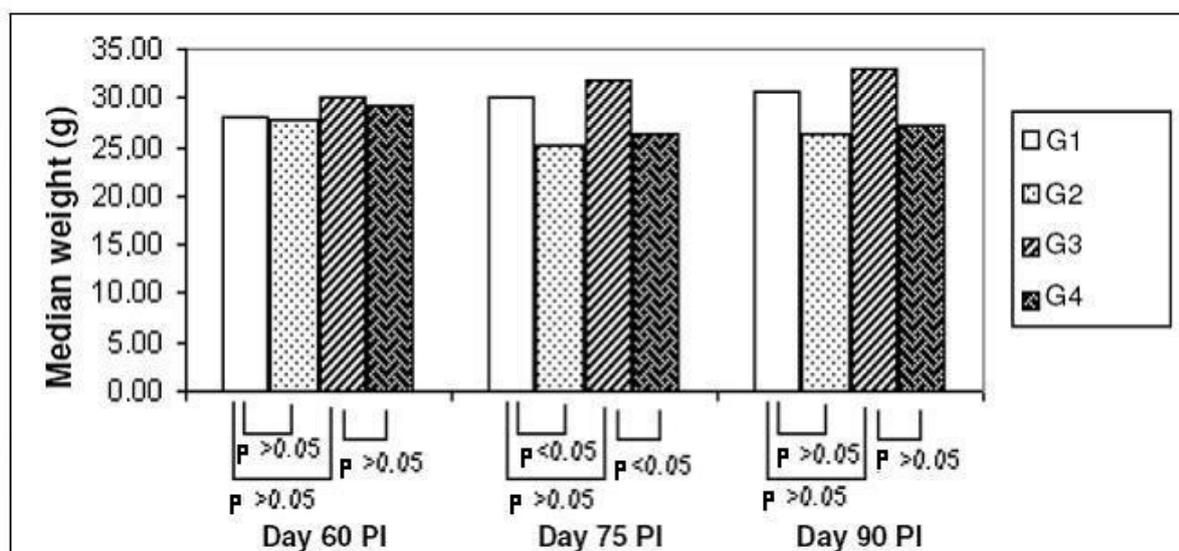


Figure 1. Median body weight, in grams, of each group on days 60, 75 and 90 post-inoculation.

G1: non-infected and non-immunosuppressed animals; G2: non-infected animals immunosuppressed for 30 days; G3: animals infected for 90 days; G4: animals infected for 90 days and immunosuppressed for 30 days.

Spleen weight and size greatly influenced both infection and immunosuppression, as shown in Figures 2 and 3. The median spleen weights of G3 and G4 on the 60th day of infection were 0.92 and 0.83 g, respectively. In G1 and G2, the median spleen weight was similar on day 60, 0.13 g. Splenomegaly observed in infected groups may

be a consequence of viscerotropic leishmania infection, in this case, *Leishmania chagasi* (9, 25). Rousseau *et al.* (26) observed spleen enlargement in mice infected with *Leishmania infantum*, from 140 to 198 mg 18 days after the infection, and 442 mg on the 70th day. According to Kaye *et al.* (11), parasite persistence in the spleen is accompanied by failure to form granulomas and by a variety of pathological alterations including splenomegaly, modification of lymphatic tissue microstructure and augmentation of hematopoietic activity.

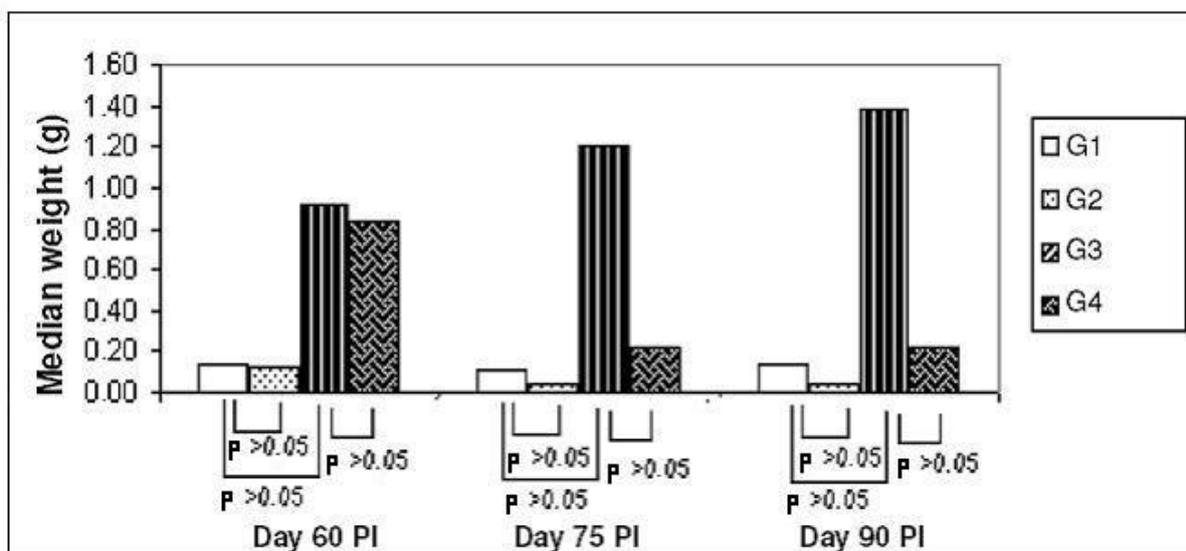


Figure 2. Median spleen weight, in grams, of each group on days 60, 75 and 90 post-inoculation.

G1: non-infected and non-immunosuppressed animals; G2: non-infected animals immunosuppressed for 30 days; G3: animals infected for 90 days; G4: animals infected for 90 days and immunosuppressed for 30 days.

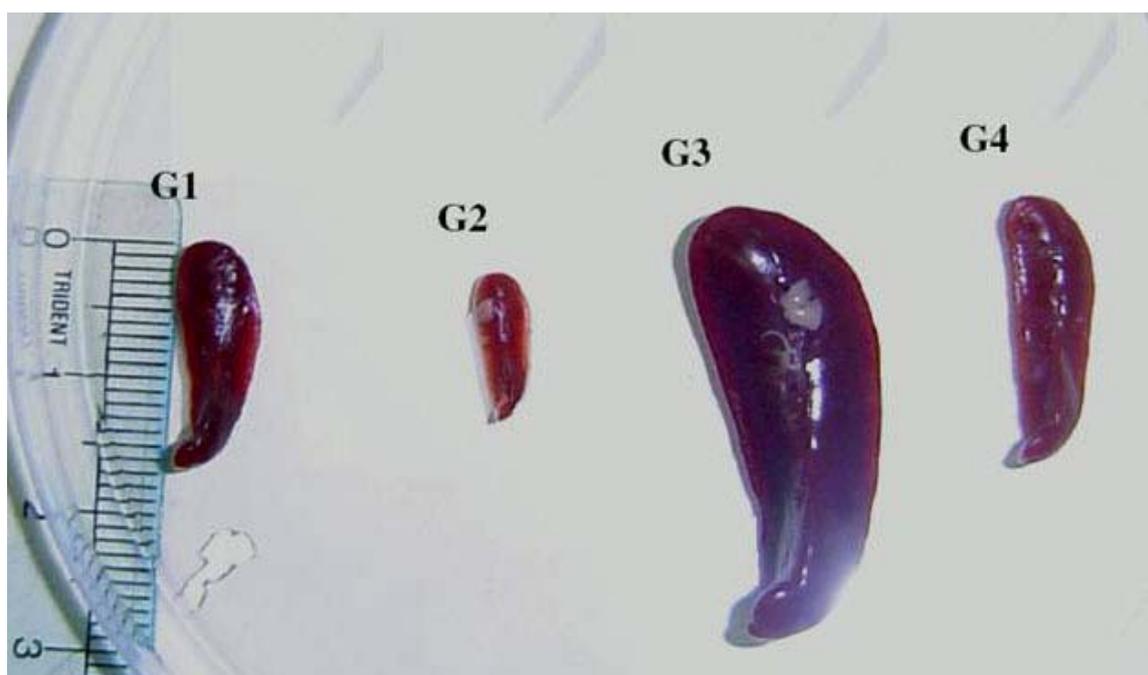


Figure 3. Spleen sizes of each animal group on the 90th day post-inoculation.

G1: non-infected and non-immunosuppressed animals; G2: non-infected animals immunosuppressed for 30 days; G3: animals infected for 90 days; G4: animals infected for 90 days and immunosuppressed for 30 days.

A drop of weight and size in both non-infected (G2) and infected (G4) groups (Figures 2 e 3) was observed, reflecting the effect of immunosuppression on the spleen. The median weights in G2 on the 75th and 90th days were 0.04 g and 0.05 g, less than 0.13 g on the 60th day, before the immunosuppression protocol. In G4, the median weight on day 60 was 0.83 g, decreasing to 0.22 g on day 75 and 0.21 g on day 90. Similarly, Lebec *et al.* (27) also observed that several parameters of cell-mediated immunity were affected by dexamethasone treatment and that the drug modified immunopathological patterns, including significant dose-dependent reduction of spleen weight after drug administration.

As shown in Figure 4, the parasite burden in the G3 liver decreased during the study period, probably due to particular characteristics of this organ, specifically its ability to generate granulomas, which strengthens hepatic resistance against leishmaniasis (10, 11). On the 30th day, the hepatic burden was higher than the splenic burden (Figures 4 and 5). On the 60th PI day, an inversion – i.e. splenic parasite burden increase and hepatic burden decrease – was observed and persisted until the 90th day, which may have occurred because during *L. chagasi* infection in mice, amastigotes rapidly multiply in the liver for the first four weeks. In the spleen, the increase of parasite number is slower and the liver apparently serves as initial

location for parasite expansion, while the spleen may be responsible for infection persistence (13, 28).

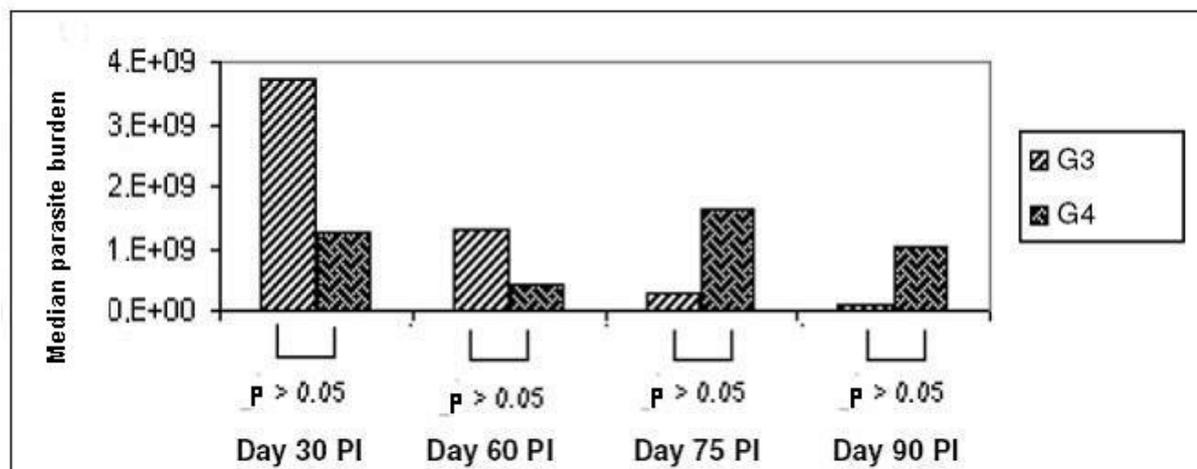


Figure 4. Median parasite burden in the liver, number of parasites per gram of tissue, in G3 and G4, on days 30, 60, 75 and 90 post-inoculation. G3: animals infected for 90 days; G4: animals infected for 90 days and immunosuppressed for 30 days.

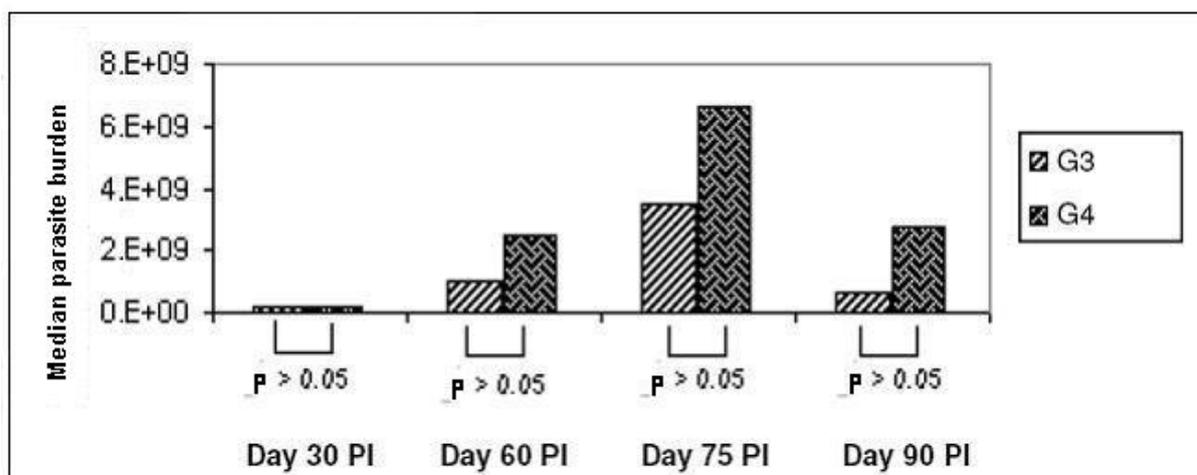


Figure 5. Median parasite burden in the spleen; number of parasites per gram of tissue, in G3 and G4, on days 30, 60, 75 and 90 post-inoculation. G3: animals infected for 90 days; G4: animals infected for 90 days and immunosuppressed for 30 days.

A higher parasite burden in G4 (immunosuppressed group) was observed on days 75 and 90 (Figures 4 and 5), which suggests that the drugs had affected the infection control in these animals. Despite immunosuppression, no statistical difference was observed between parasite burdens in G3 and G4, which differs from results obtained by Gangneux *et al.* (16). In the current study, mice treated with dexamethasone and pentoxifylline from the 10th to the 40th PI day presented a

significantly increased parasite burden in both the spleen and liver. Analysis revealed a smaller cytokine production of IFN- γ compared to the control group, whereas IL-4 concentration did not differ among the three groups. These results suggest that augmented parasite burden may not be associated with either specific cell immunophenotype of the spleen or with Th1 and Th2 cytokine secretion profile.

Similar levels of IFN- γ and IL-10 were observed in both non-stimulated (Figures 6 and 7) and antigen-specific-stimulated cultures (Figures 8 and 9), showing that cytokine production can be activated by different pathways.

IFN- γ levels were not statistically associated with a diminished parasite burden ($p > 0.05$) (data not shown). However, detectable IFN- γ levels were observed (Figure 6) on days 75 and 90 only in G3 and not in G4 (immunosuppressed group), which may be due to the role of IFN- γ in the parasite burden of these groups, which was lower in G3 and higher in G4 (Figures 4 and 5).

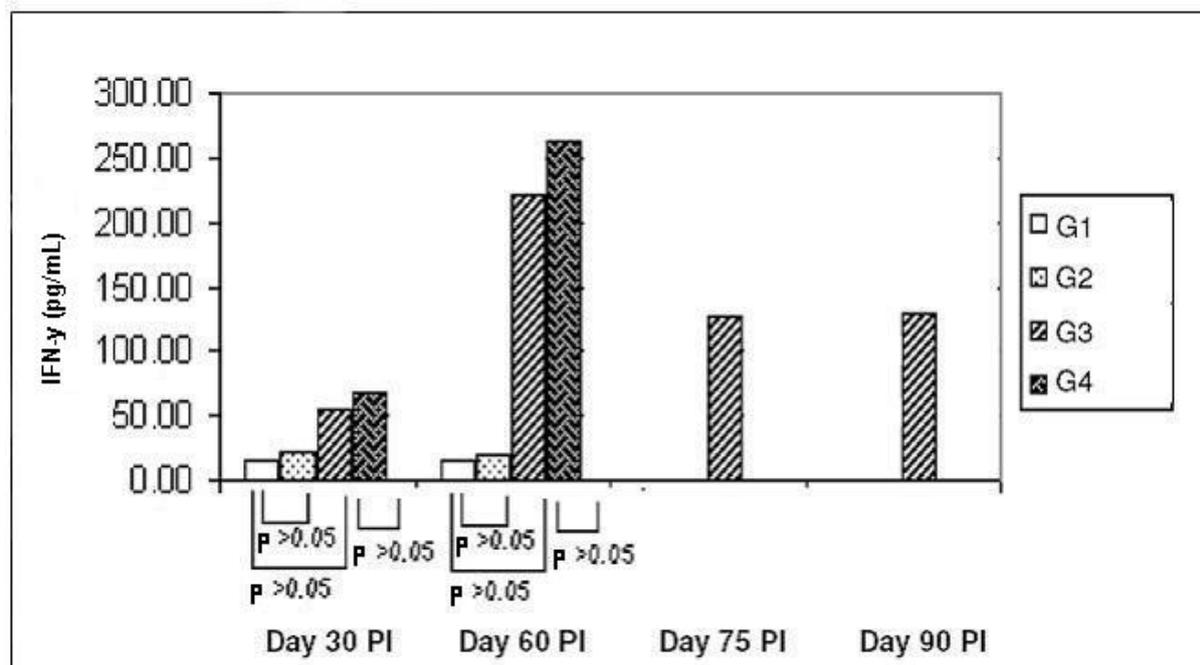


Figure 6. Levels of IFN- γ (pg/mL) in spleen cell culture of Balb/c mice, non-stimulated, in groups 1, 2, 3 and 4 on the 30th, 60th, 75th and 90th days post-inoculation.

G1: non-infected and non-immunosuppressed animals; G2: non-infected animals immunosuppressed for 30 days; G3: animals infected for 90 days; G4: animals infected for 90 days and immunosuppressed for 30 days.

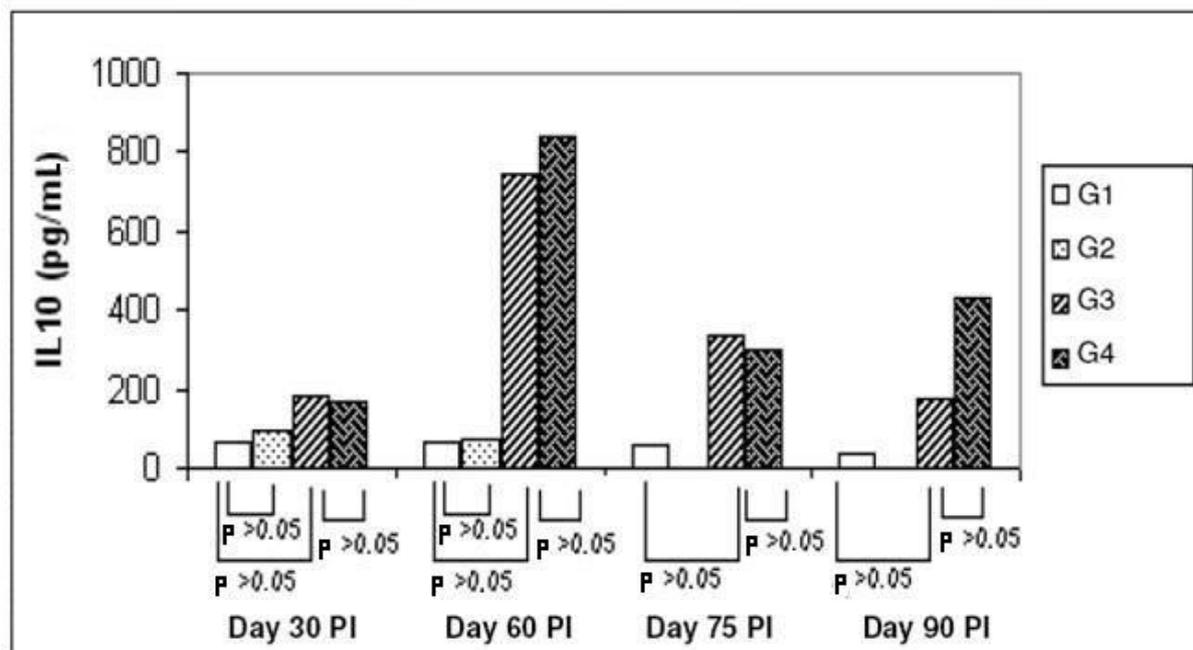


Figure 7. IL-10 levels (pg/mL) in spleen cell culture of Balb/c mice, non-stimulated, in groups 1, 2, 3 and 4 at the 30th, 60th, 75th and 90th days post-inoculation.

G1: non-infected and non-immunosuppressed animals; G2: non-infected animals immunosuppressed for 30 days; G3: animals infected for 90 days; G4: animals infected for 90 days and immunosuppressed for 30 days.

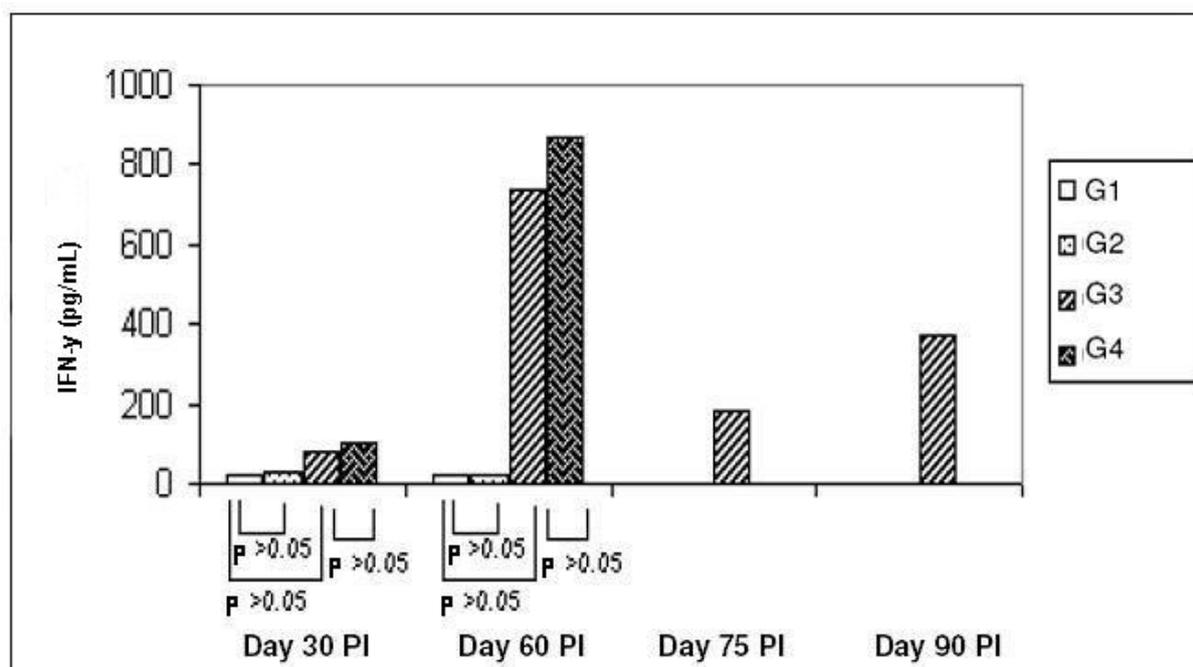


Figure 8. IFN-γ levels (pg/mL) in spleen cell culture of Balb/c mice stimulated with sonicated antigen of *Leishmania chagasi*, in groups 1, 2, 3 and 4 on the 30th, 60th, 75th and 90th days post-inoculation.

G1: non-infected and non-immunosuppressed animals; G2: non-infected animals immunosuppressed for 30 days; G3: animals infected for 90 days; G4: animals infected for 90 days and immunosuppressed for 30 days.

Melby *et al.* (25) observed a significantly augmented number of cells that express INF- γ when compared to the control group on the 28th day of infection, while Rolão *et al.* (29) reported that high INF- γ levels were accompanied by parasite burden reduction. However, Ansari *et al.* (30) suggested that despite the presence of elevated INF- γ levels during infection, the host may fail to control the disease due to an incomplete response to INF- γ . The peak levels on the 60th day in groups G3 and G4 may be related to infection control, since some reports have shown that this control initiates approximately four weeks after the infection (6, 9, 31).

IL-10 was detectable during the whole experiment in G3 and G4 (Figures 7 and 9). Melby *et al.* (31) also observed that the production of IL-10 and TGF- β was more intense at the beginning of the infection by *L. infantum*. After the 28th day, they noticed that the visceral parasite burden (spleen and liver) began to decline and the number of spleen cells that produced IL-10 returned to basal levels, even with high production of IFN- γ and drastically increase of IL-12-producing cells.

In the present study, IL-10 and IFN- γ were detected in G3 at all moments (Figures 6, 7, 8 and 9). Gomes-Pereira *et al.* (32) concluded that IL-10 levels were not sufficient to inhibit IFN- γ production by hepatic leukocytes or subsequent parasite destruction, suggesting that IL-10 may not directly inhibit IFN- γ function in experimental visceral leishmaniasis. These findings agree with the present study results.

IFN- γ and IL-10 were present during the entire experiment in all groups stimulated with concanavalin A (conA), a non-specific stimulus (Figures 10 and 11). Studies on immunosuppression in hamsters infected by with *L. chagasi* showed that conA induces lymphoproliferative response throughout the experimental period (12). However, IL-10 levels are lower in the infected groups (G3 and G4) than in G1 and G2, on the 30th and 60th days.

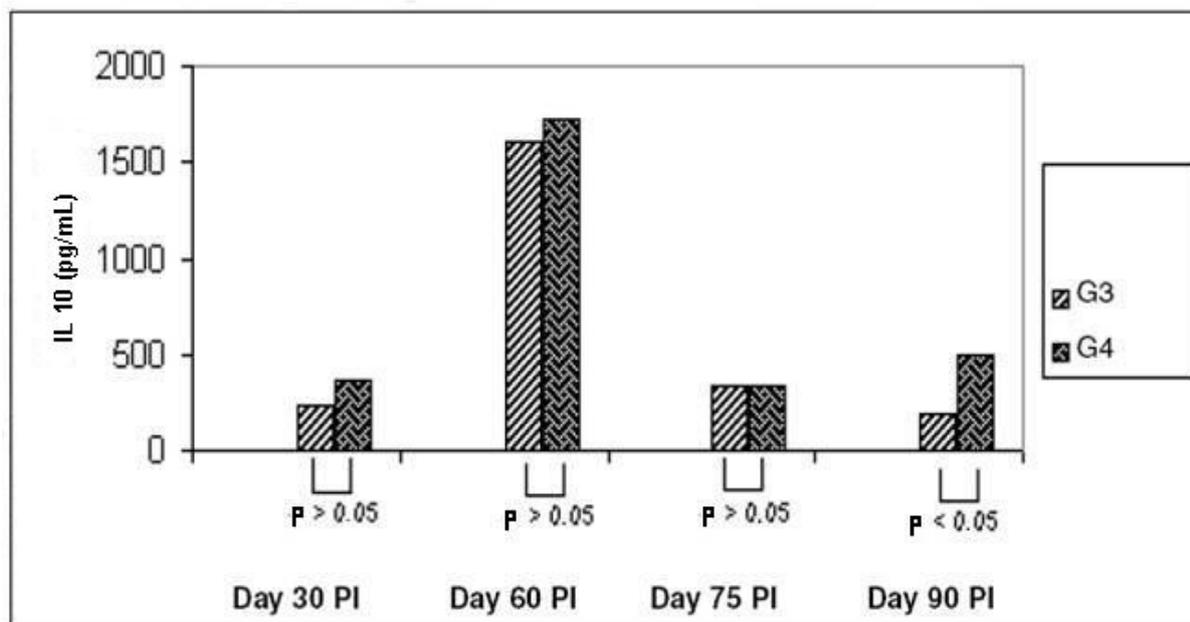


Figure 9. IL-10 levels (pg/mL) in spleen cell culture of Balb/c mice stimulated with sonicated antigens of *Leishmania chagasi*, in groups 1, 2, 3 and 4 on the 30th, 60th, 75th and 90th days post-inoculation.

G3: animals infected for 90 days; G4: animals infected for 90 days and immunosuppressed for 30 days.

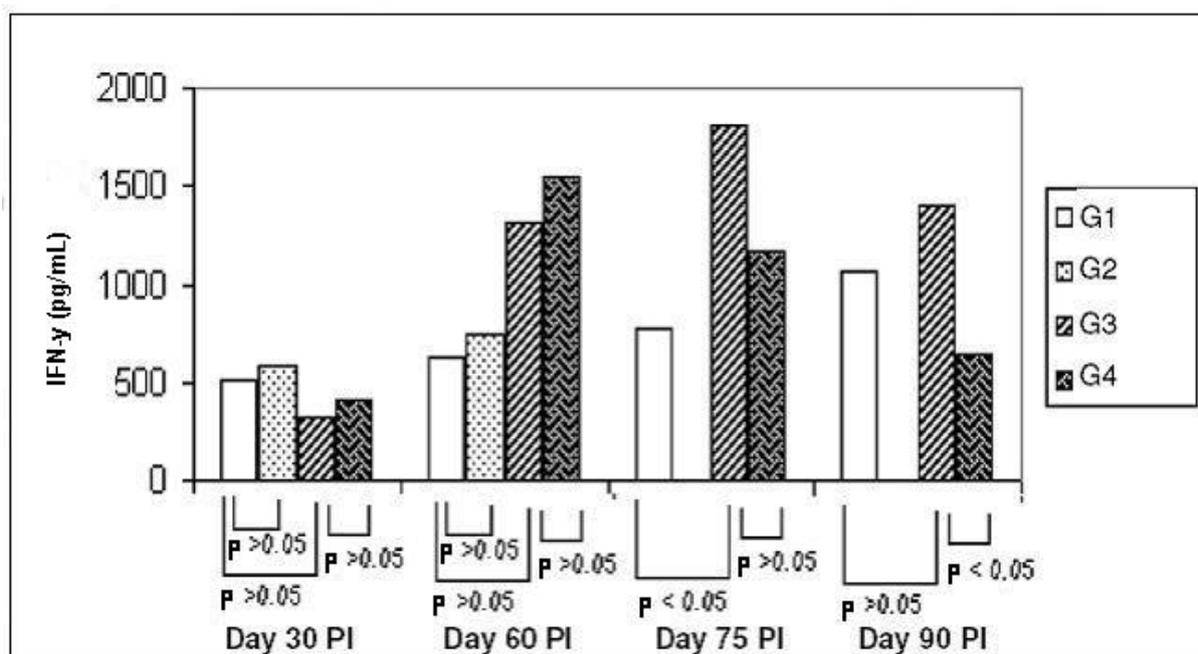


Figure 10. IFN- γ levels (pg/mL) in spleen cell culture of Balb/c mice stimulated with concanavalin A, in groups 1, 2, 3 and 4 on the 30th, 60th, 75th and 90th days post-inoculation.

G1: non-infected and non-immunosuppressed animals; G2: non-infected animals immunosuppressed for 30 days; G3: animals infected for 90 days; G4: animals infected for 90 days and immunosuppressed for 30 days.

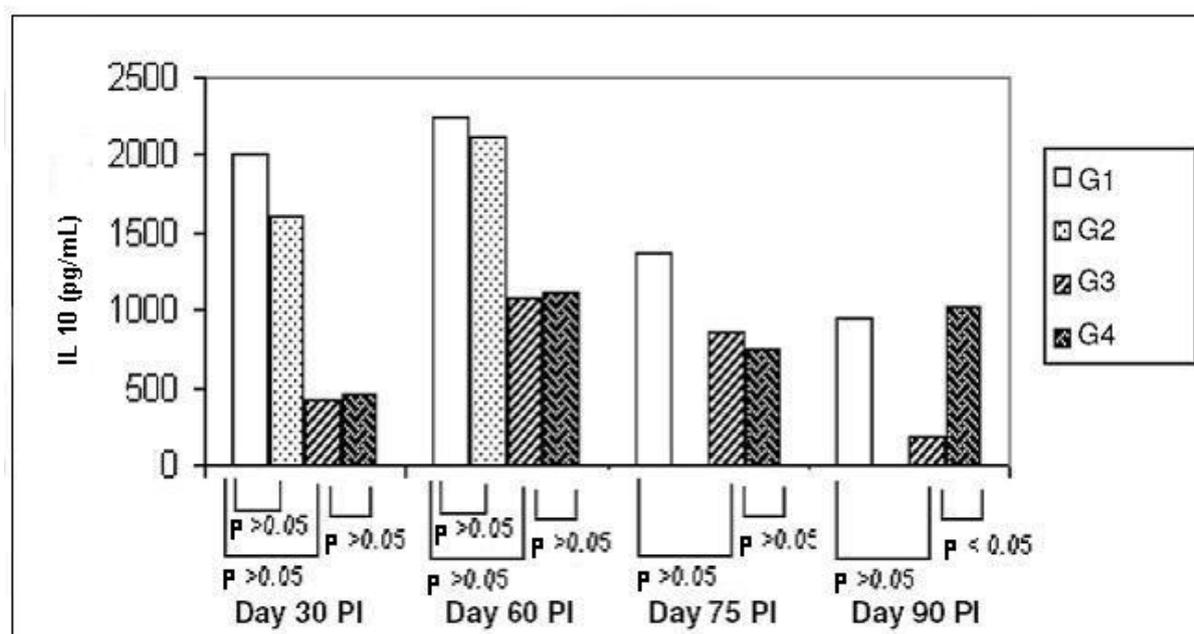


Figure 11. IL-10 levels (pg/mL) in spleen cell culture of Balb/c mice stimulated with concanavalin A, in groups 1, 2, 3 and 4 on the 30th, 60th, 75th and 90th days post-inoculation.

G1: non-infected and non-immunosuppressed animals; G2: non-infected animals immunosuppressed for 30 days; G3: animals infected for 90 days; G4: animals infected for 90 days and immunosuppressed for 30 days.

According to Mookerjee *et al.* (33), hamsters may present lymph proliferative damage in mitogens when infected by visceral leishmaniasis.

Due to the spleen size in G2 animals on the 75th and 90th days, the minimum number of 5×10^6 cells/mL could not be reached, which prevented cell culture. In the infected and immunosuppressed group (G4), IFN- γ was not detected in either non-stimulated or antigen-stimulated cultures (Figures 6 and 8). G4 presented not only detectable levels of IL-10 on the 75th and 90th days, but also a median level of this cytokine that surpassed G3 (Figures 7 and 9). As shown in Figures 8 and 9, immunosuppressed animals (G4) initially presented a Th0 profile, on days 30 and 60, that had become a Th2 profile by days 75 and 90, since no detectable levels of IFN- γ were found thereafter.

According to the present results, a mixed Th1/Th2 pattern can be observed in the cytokine production until the 60th day PI.

In the *L. chagasi* model, the initial suppression of Th1 activity (IFN- γ secretion) was not apparently related to IL-4 or IL-10 (24). For Alexander *et al.* (4), the paradigm Th1/Th2 of resistance/susceptibility to intracellular parasites appeared to be a oversimplification of one of the most complex immune chains of regulatory/counter-

regulatory interactions, which differ mainly according to the leishmania species and experimental model employed.

IL-10 was detected on the 15th and 30th days after immunosuppression, respectively, days 75 and 90 (Figures 9 and 11). These results do not corroborate those obtained by Franchimont *et al.* (34) and Kunicka *et al.* (35), who observed inhibitory effects of dexametasone on IL-10 production of stimulated cell culture.

Another important aspect is physical stress resulting from daily manipulation for immunosuppressive drug injections, which may influence cytokine production.

Moynihan *et al.* (36) demonstrated that physical or psychological stress may influence IL-2, IL-4 and IFN- γ production by cells of the spleen or lymph nodes *in vitro*. These authors also observed that dexametasone suppresses the production of the three aforementioned cytokines. Data suggest that glucocorticoids may regulate the cytokine production of Th1 or Th2 patterns. Moreover, they indicate that, under stressful conditions, in which the production of cytokines and effector function is affected, other neuroendocrine factors, besides glucocorticoids, may be relevant.

CONCLUSIONS

The infection of mice with *Leishmania chagasi* strain M6445 did not alter body weight gain, but influenced on size and weight of the spleen. Immunosuppression with dexamethasone associated with pentoxifylline modified both body weight and the size and weight of the spleen of infected and non-infected animals. Immunosuppression with dexamethasone and pentoxifylline did not significantly alter the parasite burden course in the spleen or liver. However, it affected tested cytokines, without altering the Th1/Th2 profile response in infected animals.

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ETHICS COMMITTEE

The present work was approved by the Ethics Committee on Animal Experimentation (CEEA), Registration N° 143/2005, of the Veterinary Medicine and Animal Husbandry School, São Paulo State University, UNESP, Botucatu, São Paulo State, Brazil.

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