



## Influence of apoptosis on the cutaneous and peripheral lymph node inflammatory response in dogs with visceral leishmaniasis

Pamela Rodrigues Reina Moreira<sup>a,1</sup>, Marcio de Barros Bandarra<sup>a,1</sup>,  
Geórgia Modé Magalhães<sup>a,1</sup>, Danísio Prado Munari<sup>b</sup>, Gisele Fabrino Machado<sup>c</sup>,  
Marcelo Martinasso Prandini<sup>a,1</sup>, Antonio Carlos Alessi<sup>a,1</sup>,  
Rosemeri de Oliveira Vasconcelos<sup>a,\*,1</sup>

<sup>a</sup> Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias (FCAV/UNESP), Jaboticabal, São Paulo, 14884-900, Brazil

<sup>b</sup> Departamento de Ciências Exatas, FCAV/UNESP, Jaboticabal, São Paulo, Brazil

<sup>c</sup> Departamento de Clínica, Cirurgia e Reprodução Animal, Faculdade de Medicina Veterinária de Araçatuba (FMVA/UNESP), Araçatuba, São Paulo, Brazil

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### ABSTRACT

In canine visceral leishmaniasis (CVL), the abnormalities most commonly observed in clinical examination on the animals are lymphadenomegaly and skin lesions. Dogs are the main domestic reservoir for the protozoon *Leishmania (L.) chagasi* and the skin is the main site of contamination by the vector insect. Some protozoa use apoptosis as an immunological escape mechanism. The aim of this study was to correlate the presence of apoptosis with the parasite load and with the inflammatory response in the skin and lymph nodes of dogs naturally infected with *Leishmania (L.) chagasi*. Thirty-three dogs from the municipality of Araçatuba (São Paulo, Brazil) were used, an endemic area for CVL. Muzzle, ear and abdominal skin and the popliteal, subscapular, iliac and mesenteric lymph nodes of symptomatic (S), oligosymptomatic (O) and asymptomatic (A) dogs were analyzed histologically. The parasite load and percentage apoptosis were evaluated using an immunohistochemical technique. Microscopically, the lymph nodes presented chronic lymphadenitis and the skin presented plasmacytic infiltrate and granulomatous foci in the superficial dermis, especially in the ear and muzzle regions. The inflammation was most severe in group S. The parasite load and apoptotic cell density were also greatest in this group. The cause of the lymphoid atrophy in these dogs was correlated with T lymphocyte apoptosis, thus leaving the dogs more susceptible to CVL. The peripheral lymph nodes presented the greatest inflammatory response. Independent of the clinical picture, the predominant inflammatory response was granulomatous and plasmacytic, both in the skin and in the peripheral lymph nodes. The ear skin presented the greatest intensity of inflammation and parasite load, followed by the muzzle skin, in group S. The ear skin area presented a non-significant difference in cell profile, with predominance of macrophages, and a significant difference from group A to groups O and S. It was seen that in these areas, there were high densities of parasites and cells undergoing apoptosis, in group S. The association between apoptosis and parasite load was not significant in the lymph nodes, but in the muzzle regions and at the ear tips, a positive correlation was seen between the parasite load and the density of cells undergoing apoptosis. The dogs in group S had the highest parasite load and the greatest number of apoptotic cells, thus suggesting that the parasite had an immune evasion mechanism, which could be proven statistically in the skin.

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\* Corresponding author. Tel.: +55 16 3209 2663.

E-mail address: [rosevasc@fcav.unesp.br](mailto:rosevasc@fcav.unesp.br) (R. de Oliveira Vasconcelos).

<sup>1</sup> Tel.: +55 16 3209 2663.

## 1. Introduction

Visceral leishmaniasis is a zoonosis with worldwide distribution, caused by protozoa of the genus *Leishmania*. In the Americas, the etiological agent involved is *Leishmania (Leishmania) chagasi*, an obligate intracellular parasite of cells of the mononuclear phagocytic system of vertebrate hosts. The main vector for *Leishmania* is the blood-sucking phlebotomine *Lutzomyia longipalpis* (Brasil, 2006). Canine visceral leishmaniasis (CVL) causes a chronic systemic condition that is more prevalent in the canine population than in humans, since human cases are preceded by canine cases (Santa Rosa and Oliveira, 1997; Ashford, 2000; Brasil, 2006).

Dogs with CVL present hypertrophy of the mononuclear phagocytic system, with proliferation of macrophages, which results in generalized lymphadenopathy in most cases. The lymph nodes of these dogs generally present chronic lymphadenitis. The inflammatory infiltrate is composed of macrophages, plasmacytes and lymphocytes, and is present in the capsule, subcapsular sinuses, cortical region and medullary region. In addition, follicular hyperplasia of the cortical region, hypertrophy and hyperplasia of macrophages in the medullary region, congestion, hemosiderosis and the presence of amastigote forms of the protozoon inside macrophages have been described (Lima et al., 2004; Giunchetti et al., 2008; Reis et al., 2009; Moreira et al., 2010).

In the lymph nodes of asymptomatic animals, it has been reported that lymphoid hyperplasia is prevalent in the cortical region, while in symptomatic dogs, atrophy of the cortical region has been found to be the most striking feature (Giunchetti et al., 2008; Moreira et al., 2010). Presence of plasmacytic infiltrate is expected, because of polyclonal activation and proliferation of B lymphocytes, which induces an increase in these cells in the cortical region, germinative center and medullary cords of the lymph nodes (Mylonakis et al., 2005).

Moreira et al. (2010) observed in the popliteal lymph node of symptomatic dogs that were naturally infected with *L. (L.) chagasi*, there was a negative association between macrophages and lymphocytes. When the density of macrophages (components of granulomas) was increased, the intensity of lymphoid reactivity in the cortical region diminished, with evolution in the more advanced stages of CVL to lymphoid atrophy.

The skin is the source of contamination of the vector insect of CVL, since infected dogs present greater quantities of parasites on the skin than skin humans (Brasil, 2006). On the skin of symptomatic dogs, inflammatory infiltrate composed mainly of parasitized lymphocytes, plasmacytes and macrophages has been observed. This infiltrate is more intense than what is seen in asymptomatic dogs. In asymptomatic dogs, parasites are absent from the skin and inflammatory infiltrate in the dermis is mild or absent (Solano-Gallego et al., 2004; Verçosa et al., 2008). Therefore, chronic inflammation and cutaneous parasitism are directly related to clinically severe disease (Giunchetti et al., 2006). Independent of the animal's clinical condition, the skin provides a good means of achieving parasitological confirmation of CVL. In studies conducted by Madeira et al.

(2009), no significant differences between skin of healthy clinical appearance (61.1%) and skin with characteristic lesions (60.5%) were observed.

Many pathogens use mechanisms to escape from the harmful action of the host's immune system. Prominent among these is apoptosis. This mechanism may be either useful or damaging to the host, since the pathogen uses it as a means of favoring its survival, either through inhibiting or through inducing apoptosis of cells of the immune system. The pathogen may induce resistance to apoptosis of infected host cells, or induce apoptosis of lymphoid cells, thereby affecting the profile of the cytokines produced and the regulation of their survival in the host. Infection by intracellular parasites such as *Trypanosoma cruzi* may direct the T lymphocyte response, by means of apoptosis of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes via cytokines (IL-2 and IL-4), thus worsening the infection (Dos Reis et al., 2007).

The importance of apoptosis in modulating the immune response in humans with cutaneous leishmaniasis remains uncertain. In a study on subtypes of human CD4<sup>+</sup> and CD8<sup>+</sup> T cells, Bertho et al. (2000) observed large numbers of cells at the initial stage of apoptosis, despite their normal morphological appearance. In patients who achieved spontaneous cure, there were a small number of apoptotic CD8<sup>+</sup> T cells. They suggested that the CD8<sup>+</sup> T lymphocytes could be related to cure mechanisms for localized cutaneous leishmaniasis.

In experimental studies on mice infected with *T. cruzi*, apoptosis of T lymphocytes was highlighted as an important factor for survival of the protozoon inside macrophages (Freire-De-Lima et al., 2000). In Chagas disease, destruction of these cells through this route would have the consequence of inhibiting the Th1 response, thereby favoring the parasite. In the same way, release of pro-apoptotic molecules by the protozoon into the extracellular environment would induce apoptosis of macrophages and release of viable infective forms of *T. cruzi* into tissue interstices (Lopes and Dos Reis, 2000).

In studies on experimental infection of mice by *Leishmania donovani*, apoptosis of uninfected cells of the immune system (CD4<sup>+</sup> T lymphocytes) was induced, possibly through the influence of the lipophosphoglycan (LPG) present in the cell wall of the parasite. From these results, it was suggested that this would be a mechanism for evasion of the host's immune system (Lüder et al., 2001).

Because CVL is controlled by culling seropositive dogs, it has not been possible to observe the evolution of the disease. It is known that the predominant response in some cases of advanced disease may be humoral and involve anti-inflammatory cytokines, thus characterizing susceptibility to infection by the protozoon (Corrêa et al., 2007; Alves et al., 2009). The cell response would be inhibited or reduced and, for this reason, evaluating the presence of apoptosis in peripheral lymph nodes and in the skin of dogs infected with *L. (L.) chagasi* could make it possible to understand their resistance or susceptibility, according to the influence of this cell death mechanism. Through this, it would be possible to identify a possible immune evasion mechanism for the etiological agent of CVL. Therefore, the aims of this study were to correlate the presence of apoptosis with the parasite load and with

the inflammatory response in the skin and lymph nodes of dogs naturally infected with *Leishmania (L.) chagasi*.

## 2. Material and methods

Thirty-three dogs at the Zoonosis Control Center of Araçatuba (SP), a region that is endemic for CVL, were evaluated. Animals sent there by their owners or caught in the city's streets are put down every day at this location. A sample of this population was evaluated, without preference for breed, sex or age.

Culling animals with CVL was done in accordance with Decree No. 51.838 of the Brazilian Ministry of Health, which establishes that domestic animals with leishmaniasis must be culled. This, this was done to the dogs under barbiturate anesthesia (Thiopental; Cristália Itapira, SP), as recommended by the code of ethics for use in scientific research (AVMA, 2001), by means of intravenous injection of potassium chloride.

Necropsies were performed on the dogs immediately after death. In the external examination of the cadavers, macroscopic abnormalities were evaluated, which enabled classification into groups, using the categories specified by the Ministry of Health (Brasil, 2006): symptomatic dogs (skin lesions, onychogryphosis and weight loss), oligosymptomatic dogs (lymphadenomegaly and slight weight loss) and asymptomatic dogs (apparently healthy). Thus, the groups of dogs evaluated were composed of 9 symptomatic, 8 asymptomatic and 16 oligosymptomatic animals. The organs removed were subjected to histopathological and immunohistochemical analyses (Moreira et al., 2010).

A group composed of 10 healthy dogs that came from an area that was not endemic for CVL, was used as a control for immunolabeling of apoptotic cells. Skin fragments from different areas (muzzle, abdomen and ear tips), peripheral lymph nodes (popliteal and subscapular) and cavity lymph nodes (iliac and mesenteric) were collected for analysis under an optical microscope. The lymph nodes and skin samples were gathered with the aim of assessing the inflammatory response pattern and the presence of apoptosis at the different clinical stages of CVL. In addition, it was sought to evaluate whether there were any differences in inflammatory response and parasite load in different anatomical regions of the skin. The results regarding inflammatory lesions of the popliteal lymph node are described in Moreira et al. (2010). The present paper presents the results relating to apoptosis in this lymph node.

The tissues were fixed in 10% formol solution, buffered with phosphate (pH 7.2). After 24 h of fixing, the tissues were processed, embedded in paraffin, cut into sections of 5  $\mu\text{m}$  in thickness and stained with hematoxylin and eosin.

The immunohistochemical analysis to determine the parasite load was performed by means of the streptavidin–biotin–peroxidase complex (LSAB kit; Dakocytomation, code K0690, California, USA). The primary antibody used was hyperimmune serum from a dog positive for CVL (dilution 1:1000), in accordance with the protocol described by Tafuri et al. (2004) and modified by Moreira et al. (2010). The seropositivity of this dog was

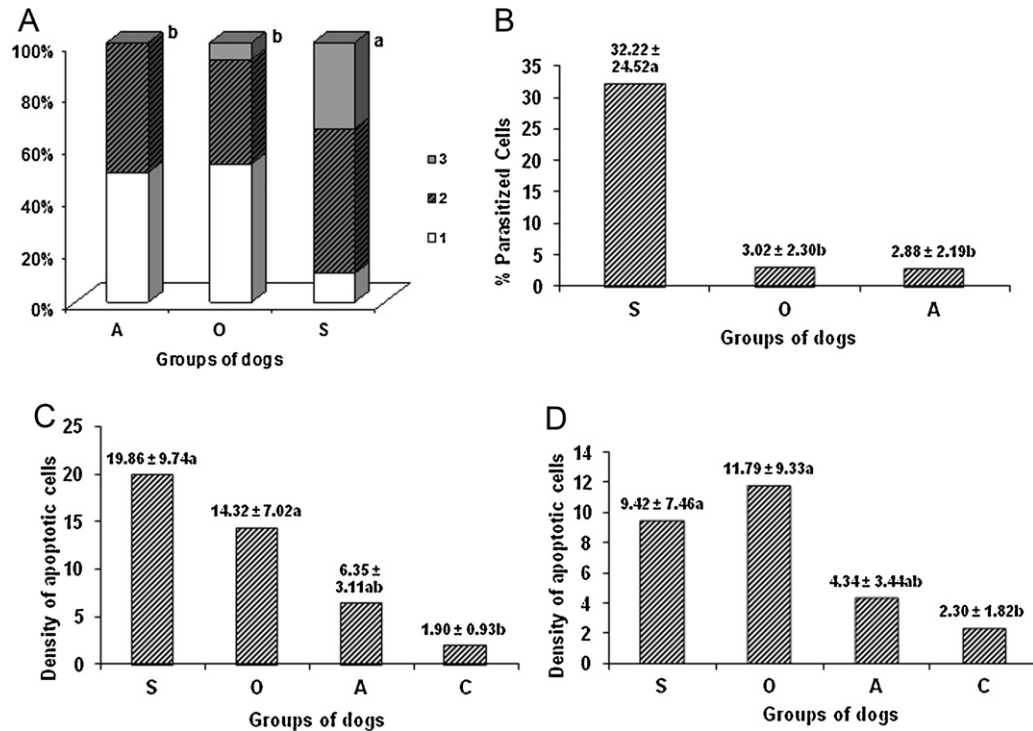
assessed using the ELISA technique (titer of 1:40,000). To determine the density of apoptotic cells, anti-caspase 3 cleaved antibodies were used (Cell Signaling, code 9661, Massachusetts, USA), at a dilution of 1:200. Both of these antibodies were incubated in a damp chamber for an 18-h period, at 4 °C. The antigen recovery system consisted of using steam (electric steaming pan, Philips Walita, Brazil), with a 10 mM sodium citrate solution (pH 6.0). Blocking of endogenous peroxidase was done using a solution of methanol and hydrogen peroxide (Synth, Brazil): 30 volumes at 3%, for 10 min. Blocking of the nonspecific immunolabeling was done using a commercial product (Protein Block; Dakocytomation, code X0909, California, USA), for 20 min of incubation. The chromogen used was diaminobenzidine (DAB; Dakocytomation, code K3468, California, USA) and this was counterstained using Harris hematoxylin (Synth, Brazil). The negative control chosen consisted of excluding the primary antibody from the reaction.

To determine the percentage of immunolabeled cells, five microscope fields were taken into consideration, using the 40 $\times$  objective lens. For the lymph nodes, all the anatomical areas were taken into account (capsule, subcapsular sinus, cortical region and medullary region). For the skin samples, areas on the dermis were chosen randomly. Using the values obtained from these fields, the mean number of immunolabeled cells per animal was determined. These means for the parasite load and the number of apoptotic cells were assessed for each group of infected dogs (asymptomatic, oligosymptomatic and symptomatic).

The mean numbers of immunolabeled cells for the parasite load and density of apoptotic cells in the skin and lymph nodes were subjected to analysis using the non-parametric Kruskal–Wallis and Dunn tests. The cell type scores found from the inflammatory infiltrate in the subscapular lymph node were evaluated by means of logistic regression, taking into consideration the group effect, and comparisons between groups were made using the non-parametric Kruskal–Wallis or Dunn tests. The association between parasite load and apoptotic cell density was evaluated by means of Pearson's simple correlation ( $P < 0.05$ ), in each lymph node or skin area, per group. The statistical analyses were performed using the computer program Graphpad Prism (version 4.0, 2003).

## 3. Results

Macroscopic examination of the peripheral lymph nodes showed that in the dogs in groups O and S, the volume was increased and the cut surface presented edema. The skin showed a variety of lesions according to the clinical evolution of CVL. There were no abnormalities among the dogs in group A. The dogs in group O showed slight sloughing of the skin (exfoliative dermatitis). The dogs in group S presented the most severe lesions, characterized by focal to generalized alopecia, sloughing of the skin, ulceration and formation of crusts. These features were more common in the head region (ear tips, muzzle and around the eyes) and limbs, or covered the entire body of the dog. Most of the animals presented ectoparasites (fleas and ticks).



**Fig. 1.** Density of inflammatory infiltrate, parasite load and cells undergoing apoptosis in peripheral lymph nodes in dogs with visceral leishmaniasis. (A) Proportion of macrophage infiltrate cell score (1: low; 2: moderate; 3: high) in the subscapular lymph node. Proportion of macrophage infiltrate cell score by group of dogs followed by different letters indicate significant differences according to the Kruskal–Wallis test ( $P < 0.05$ ). (B) Means and respective standard deviations for the characteristic of parasite load per group of infected dogs, in the subscapular lymph node. Means followed by different letters indicate significant differences according to the Kruskal–Wallis test ( $P < 0.05$ ). (C)/(D) Means and respective standard deviations for the density of apoptotic cells per group, regarding the popliteal (C) and subscapular (D) lymph nodes. Means followed by different letters indicate significant differences according to the Kruskal–Wallis test ( $P < 0.05$ ) (A: asymptomatic, O: oligosymptomatic, S: symptomatic and C: control).

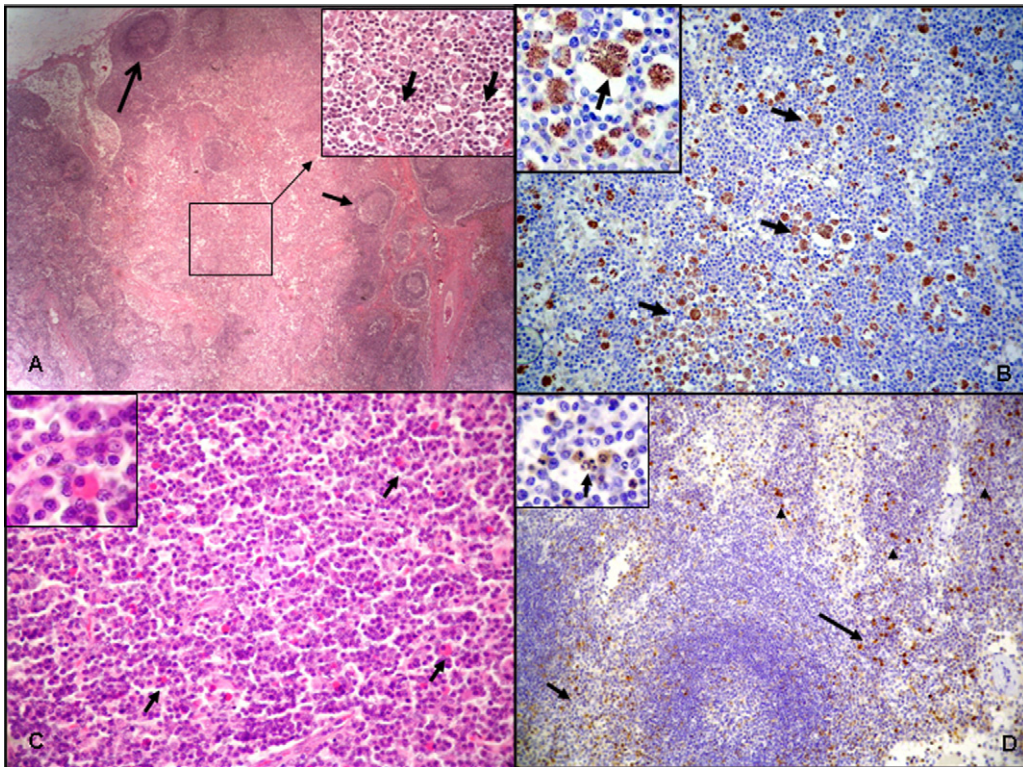
Microscopically, the cavity lymph nodes (iliac and mesenteric) did not present any significant abnormalities ( $P > 0.05$ ; Kruskal–Wallis test) with regard to inflammation, parasite load and apoptosis, in the three groups of infected dogs. In relation to the subscapular lymph node, it was observed that the symptomatic group presented the highest scores for the intensity of macrophage infiltrate (granulomas) and differed significantly from the other groups ( $P < 0.05$ ; Kruskal–Wallis test) (Fig. 1A). Regarding the density of lymphocytes and plasmacytes, no statistically significant differences were observed between the groups. In group S, marked lymphoid atrophy and predominance of diffuse granulomas starting from the cortical region were observed, with distortion of the medullary architecture (Fig. 2A). The granulomas observed in the subscapular lymph node, in the three clinical groups, were composed of macrophages and large numbers of lymphocytes (Fig. 2A). The parasite load in this lymph node (Fig. 2B) was greatest in group S ( $P < 0.05$ ; Kruskal–Wallis test), in comparison with the other groups (Fig. 1B).

The presence of cells with hyperchromatic retracted nuclei and acidophilic cytoplasm (apoptosis), and the formation of apoptotic bodies, was observed in both of the lymph nodes and in the skin, in association with the inflammatory infiltrate. In the popliteal and subscapular lymph nodes, cells with these morphological characteristics appeared in the paracortical region and in the cords

of the medullary region, although they have also been observed in the inflammatory infiltrate of the capsule and at the center of the lymphoid node (Fig. 2C). Immunolabeling of apoptotic cells occurred mainly in the cytoplasm of the cells, with predominance of lymphocytes in the paracortical region (Fig. 2D). Regarding the density of apoptotic cells, the popliteal lymph node of the infected dogs in this study did not differ statistically in the comparison between groups S and O ( $P > 0.05$ ), but only in relation to the control group (Fig. 1C; Kruskal–Wallis test;  $P = 0.0002$ ). In the subscapular lymph node, the greatest density of apoptosis occurred in group O (Fig. 1D). The groups O and S did differ statistically with de control group of dogs (Kruskal–Wallis test;  $P = 0.0030$ ). In the analysis on the degree of association between the variables of parasite load and density of apoptotic cells in the lymph nodes, there was no significant correlation between the groups of infected dogs ( $P > 0.05$ ).

In the present study, the cutaneous inflammatory reaction was limited to the superficial dermis and around the adnexa, and only rarely reached the deep dermis (Fig. 3A). Only in animals with very high parasite load was it possible to observe the presence of inflammation and highly parasitized macrophages around the hypodermis vessels. The granulomas were atypical, with poorly defined borders (Fig. 3A), as also observed in other organs of infected dogs. In many situations, especially in the ear region, the





**Fig. 2.** Photomicrographs of lymph nodes of dogs with visceral leishmaniasis. (A) Diffuse granulomatous reaction (□), with lymphoid atrophy in the cortical region (arrows), in the subcapsular lymph node. In the detail, a lymphocyte-rich granuloma is shown (arrow; 4× objective lens; hematoxylin and eosin; symptomatic dog). (B) Same lymph node with marked immunolabeling of parasitized macrophages (arrows) in the medullary region (40× objective lens; streptavidin–biotin–peroxidase complex). (C) Cortical region of the lymph node with several apoptotic cells (arrows). In the detail, note the pyknotic nucleus and acidophilic cytoplasm (subcapsular lymph node; asymptomatic dog; hematoxylin and eosin; 40× objective lens). (D) Paracortical region (arrows) and medullary region (arrowhead) with immunolabeled apoptotic cells (subcapsular lymph node, symptomatic dog; 20× objective lens). In detail, note apoptotic bodies (arrow, 40× objective lens, streptavidin–biotin–peroxidase complex).

inflammation was intense and diffuse with poor definition of the granulomas (Fig. 3B).

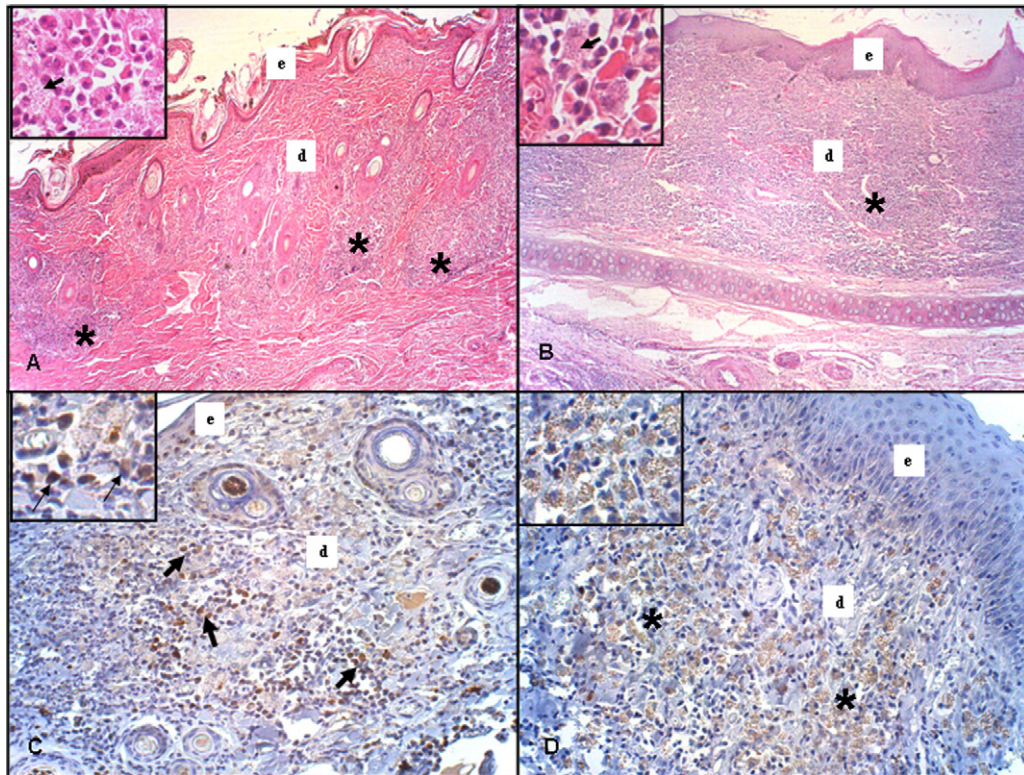
In the cutaneous tissue, in relation to the predominant cell profile, it was observed that in asymptomatic dogs, lymphocyte infiltrate in the dermis was absent or mild. The same type of cell and intensity of infiltrate were observed in group O. In group S, there were higher proportions of lymphocytes, plasmacytes and macrophages. The symptomatic group presented higher proportions of lymphocytes, plasmacytes and macrophages. Some animals did not have clinically and microscopically detectable skin inflammation (17/33), as follows: 3/9 in group S; 7/8 in group A; and 7/16 in group O. The other dogs had inflammation scores going from moderate to high (16/33): six in group S; one in group A; and nine in group O.

The skin area with significant differences regarding cell profile was the ear skin (regression logistic test;  $P=0.001$ ), with predominance of macrophage infiltrate and significant differences between groups A and O (regression logistic test;  $P=0.005$ ) and between A and S (regression logistic test;  $P=0.001$ ). In the same area, there was also a high density of lymphocytes, which not differed between these groups (regression logistic test;  $P>0.05$ ). In the muzzle, these differences between groups were not found and there was predominance the macrophages. It was found that in this area there were high densities of parasites

(Kruskal–Wallis test;  $P=0.0143$ ) and cells undergoing apoptosis (Kruskal–Wallis test;  $P=0.6974$ ), in group S.

In relation to the cutaneous density of apoptotic cells, non significant differences were observed between infected and control groups of dogs (Kruskal–Wallis test;  $P>0.05$ ), in the three skin areas. The cells that were immunolabeled for activated caspase 3 were lymphocytes of the cutaneous inflammatory infiltrate (Fig. 3C). In relation to parasite load (Fig. 3D), significant differences appeared in the three skin areas ( $P<0.05$ ) and were evident between groups S and A (muzzle/ $a=0.0143$  and abdomen/ $P=0.001$ ; Kruskal–Wallis test), and between S and O (abdomen; Kruskal–Wallis test;  $P=0.0001$ ). The variation between the means for this parameter was very high, because some animals did not present any inflammatory reaction or any presence of parasites in the skin. An association was found between the parameters of parasite load and density of apoptotic cells in the three anatomical areas of the skin, in group S (Spearman correlation coefficient analysis;  $P<0.05$ ). It was observed that there was a positive correlation between the muzzle, ear tip and abdominal regions regarding the presence of apoptosis (Kruskal–Wallis test;  $P<0.05$ ), i.e. increases in the numbers of cells undergoing apoptosis occurred in all three regions. There was also a correlation between parasite load and density of cells undergoing apoptosis, but only for the muzzle





**Fig. 3.** Photomicrograph of the skin of dogs with visceral leishmaniasis. (A) Muzzle skin showing granulomas with poorly defined borders (\*), around the adnexa of the dermis (d). In the epidermis (e), note discrete hyperkeratosis. (B) Ear skin showing diffuse granulomas (\*) in the dermis (d), with discrete acanthosis of the epidermis (e). In the detail of the dermal granulomas in muzzle and ear skin, note macrophages with parasitized cytoplasm (arrows A and B). (C) Immunostaining for inflammatory cells in apoptosis (arrows). Detail shows apoptotic lymphocytes (arrows) in the dermal infiltrate (d). (D) Immunolabeling of parasitized macrophages (\*) in the superficial dermis (d). Symptomatic group. (A) and (B): hematoxylin and eosin; 10× objective lens. (C) and (D): streptavidin–biotin–peroxidase; 40× objective lens.

(Kruskal–Wallis test;  $P=0.003$ ) and ear tip (Kruskal–Wallis test;  $P=0.006$ ). Thus, when there was an increase in the density of parasites, there was also an increase in the density if cells undergoing apoptosis in these areas.

#### 4. Discussion

In this study, it was observed that in the subscapular lymph node, increased numbers of parasites coincided with increased numbers of granulomas and with worsening of the disease (symptomatic group). [Moreira et al. \(2010\)](#) analyzed the lesions of the popliteal lymph node in the same animals as in the present study, and observed that the symptomatic dogs had the highest scores for macrophage infiltrate (granulomas) and simultaneously presented severe lymphoid atrophy. In the present study, the granulomatous reaction observed in the subscapular lymph node was rich in lymphocytes, thus differing from the reports of [Moreira et al. \(2010\)](#) in the popliteal lymph node, in group S.

[Reis et al. \(2009\)](#) showed different response profiles in different organs of dogs with CVL, and named these differences the compartmentalized response of each organ to the infection. This response pattern may be related to proliferation or control of the multiplication of the parasite in the infected organs. Even when different lymph nodes

were compared, these differentiated responses appeared. In the case of the subscapular lymph node in the present study, there was greater reactivity and lower intensity of lymphoid atrophy, compared with the reports of [Moreira et al. \(2010\)](#) from studying the popliteal lymph node. [Lima et al. \(2004\)](#) studied peripheral lymph nodes in dogs with CVL and observed that the cervical lymph node was the one most affected. This was because of the area that it drained, i.e. the head region, which is the greatest target of the vector of CVL. In the case of the present study, the subscapular lymph node also drains this region and therefore presented greater reactivity. The greater lymphoid atrophy observed in the popliteal lymph node ([Moreira et al., 2010](#)), in dogs with advanced disease, may have occurred because this lymph node would be affected later than seen in the lymph nodes of the cervical region ([Lima et al., 2004](#)) and in the subscapular lymph node of the present study. Therefore, this suggests that the inhibiting effects induced by the protozoon would have action times that differed between the lymph nodes affected.

[Lima et al. \(2004\)](#) and [Alves et al. \(2009\)](#) observed that in the pre-scapular lymph nodes, there was lymphoid reactivity and macrophage proliferation in the medullary region, thus resulting in lymphadenopathy. This occurred more frequently than in the axillary and popliteal lymph nodes. In our study, we also observed greater lymphoid

reactivity in the subscapular lymph node, which drains the same region as the pre-scapular lymph nodes described by these authors.

In the subscapular lymph node, there was a marked difference in parasite density between group S and groups O and A ( $P < 0.05$ ). Moreira et al. (2010) observed that in the popliteal lymph nodes of these same animals, groups S and O differed from group A. It can be suggested that these observed differences between the peripheral lymph nodes of the same animals may be related to the predominant cell composition in the granulomas. In the subscapular lymph node, there was a significant proportion of lymphocytes mixed with the macrophages. In our study, it is possible that in groups O and A, the response to parasite multiplication was more efficient in the subscapular, since the parasite load in this lymph node was much lower (group O =  $3.02 \pm 2.30$ ; group A =  $2.88 \pm 2.19$ ), in relation to the popliteal lymph node (group O =  $29.59 \pm 8.44$ ; group A =  $10.16 \pm 2.90$ ), as described by Moreira et al. (2010).

When apoptosis was evaluated in the lymph nodes, the significant differences were most evident between groups O and S in the popliteal and subscapular lymph nodes (Fig. 1D). Despite the biological evidence of an association between increased parasite load and apoptosis in group S, there was no significant correlation between these parameters. It is possible that *L. (L.) chagasi* may use immune evasion mechanisms, through participating in inducing the granulomatous reaction and lymphoid atrophy in animals with clinical conditions of greater severity. In this manner, these dogs would be more susceptible to infection and incapable of responding with cell-type immunity. In groups O and A, the difference in parasite load between the lymph nodes was very large. In the same way, the means for the density of cells undergoing apoptosis were higher in the popliteal lymph node (Fig. 1C and D). It is possible that the popliteal lymph node had a greater number of parasites because of the higher rate of apoptosis of T lymphocytes, in comparison with the subscapular lymph node. It is evident that the presence of lymphocytes mixed with the granulomas in the subscapular lymph node inhibited the multiplication of the parasite in groups O and A, and thus there was greater lymphoid reactivity.

In the cutaneous tissue, it was observed that in the asymptomatic and oligosymptomatic dogs, lymphocytic infiltrate was absent or mild. In asymptomatic dogs, Solano-Gallego et al. (2004) observed absence of this inflammatory infiltrate and parasites in the skin, similar to what was observed in uninfected dogs.

In the present study, the symptomatic group presented higher chronic skin inflammation and coincided with the findings of Giunchetti et al. (2006) and Reis et al. (2009), who observed these aspects, independent of the clinical group.

In the present study, three different anatomical regions of the skin were evaluated (muzzle, abdomen and ear tips). The abdominal region was the area in which most of the animals presented absence of inflammation or parasites. The group S and the ear tip were most involved in these processes. Giunchetti et al. (2006) found that the frequency of inflammatory infiltrate in the skin was similar in groups A and O, but the group O showed a higher density of

inflammatory cells. Solano-Gallego et al. (2004) observed a higher degree of skin inflammation in the muzzle of clinically infected dogs than in asymptomatic dogs. The group A did not show any significant skin abnormalities.

Moura et al. (2008) analyzed the differences in inflammatory reaction and parasite load between two regions of the ear (tip and middle). They observed that the tip of the ear was more parasitized than the middle area. The inflammatory reaction was also more intense at the extremity, with a positive correlation between parasitism and the inflammatory response, independent of the animal's clinical stage. In the muzzle, there was lower intensity of inflammation and parasite load. These findings are in agreement with the observations of the present study, since the most marked cutaneous lesions were in the ear and muzzle. We also observed greater parasitism coinciding with higher intensity of cutaneous inflammatory response.

In the present study, the cutaneous inflammation was always more intense in the symptomatic dogs, which had high levels of parasitized macrophages, with distribution going from multifocal to diffuse in the muzzle, and only diffuse in the ear. Giunchetti et al. (2006) also observed that in the symptomatic dogs there was intense diffuse dermal inflammatory infiltrate, with high parasite load. However, they did not observe any granuloma formation but, rather, an infiltrate rich in lymphocytes, plasmacytes and macrophages. Their report differs from the present study, since the granulomas had the lepromatous appearance common to several organs of dogs with CVL. Solano-Gallego et al. (2004) and Santos et al. (2004) also described the presence of granulomatous inflammation in infected dogs.

Santos et al. (2004) reported that most of the dogs with localized inflammatory infiltrate did not present cutaneous parasitism. In addition, in the symptomatic dogs, there was a positive correlation between the parasite load and the intensity of the inflammatory infiltrate. The more focal the inflammation was, the lower the parasite density was. This feature was not observed in our study, although diffuse inflammatory processes predominated in the ear and this area presented the greatest parasite load. In the other areas of the skin, in which the lesions were focal to multifocal, this association with greater or lesser numbers of parasites was not found.

In the asymptomatic dogs in the present study, cutaneous inflammatory reactions were absent or mild, and this coincided with absence of parasites in the cytoplasm of the macrophages in all the asymptomatic dogs. Verçosa et al. (2008) also observed an absence of cutaneous parasites in this group. Giunchetti et al. (2006) reported that dogs without symptoms seemed to be more resistant than clinically affected dogs were, probably because of cell activation of greater efficiency. These authors suggested that chronic skin inflammation and cutaneous parasitism were directly related to severe clinical disease.

In the present study, significant differences in parasite load appeared in the three skin areas, in groups S and O. Out of the 33 infected dogs, 12 presented parasites that were detectable under microscopic examination of the skin: 8/9 in group S and 4/16 in group O. Group A did not present immunolabeled parasites in the different areas of the skin. The intensity of the inflammatory infiltrate was related to

the parasite load in the skin, as also described by Giunchetti et al. (2006) and Moura et al. (2008). The findings of the present study were more frequently in the head region, which is location providing easier access for the vector insect of CVL, for its blood meals. The greater density of parasites in the muzzle and ear tips was possibly due to dogs' habits of sniffing out the environments to which they have access. The preferred habitats of sand flies (the vectors for CVL) are shaded localities that are rich in organic matter (Brasil, 2006). When dogs have access to these places, their habits facilitate their contact with the vector, and the head region forms the flies' main target. For this reason, it has been suggested that dogs' skin is an important site for multiplication of the parasite. This was proven in the present study, in which a positive correlation was seen between parasite load and the muzzle and ear tip regions of infected dogs in the three clinical groups.

Among the cutaneous tissues, there was only a significant difference in the density of apoptotic cells between groups S and A. The lymphocytes of the inflammatory infiltrate were the cells that presented immunolabeling for apoptosis. In the muzzle, it was observed that in the areas with an inflammatory reaction, there were also high densities of parasites and cells undergoing apoptosis, in group S. This is discordant with the findings of Verçosa et al. (2008), who observed occurrences of apoptosis in the skin of all the groups analyzed, but with greatest frequency in the asymptomatic animals. In our study, there was a positive correlation between increased parasite load and the number of cells undergoing apoptosis in group S. This proved that with worsening disease, the skin became an important target for the vector insect, since it provided shelter for larger numbers of protozoa and its immune response was impaired by the elimination of T lymphocytes that was mediated by the parasite.

Goto and Lindoso (2004) only observed apoptosis in the initial stage of experimental infection with *L. chagasi* in hamsters. For this reason, it was concluded that with evolution of CVL, the action of apoptosis on cells of the immune system might diminish. In the present study, the evolution of CVL was chronic and the level of apoptosis in the peripheral lymph nodes of dogs in the symptomatic group was significant. Likewise, there was marked apoptosis in lymphocytes of the inflammatory infiltrate in the muzzle and ear, which were the regions most affected and presenting the greatest density of parasites. For this reason, it is important to affirm that the response of dogs to infection by *L. (L.) chagasi* is quite different to that of the experimental models, possibly because the latter type of host does not experience the immunomodulatory effects of the saliva of the insect vector of the protozoon, as occurs with dogs.

In the present study, the predominance of apoptotic cells in the cortical region of the lymph node was observed in the paracortical region (T lymphocytes) and in the medullary cords. Freire-De-Lima et al. (2000) observed apoptosis of T lymphocytes in mice infected with *T. cruzi*, and suggested that this process was important for the survival of the protozoon inside macrophages, through inhibition of the Th1 response. Other authors have observed that release of pro-apoptotic molecules by

the protozoon, into the extracellular environment, would induce apoptosis of macrophages and release of viable infective forms of *T. cruzi* (Lopes and Dos Reis, 2000). The results from the present study are in agreement with these authors, since the parasite load and density of apoptotic cells were greater in the symptomatic dogs. These findings may be related to the cause of lymphoid atrophy of the lymph nodes, thereby leaving them more susceptible to CVL. The same feature was observed in the skin, since the dogs with advanced disease had greater density of apoptosis in areas of skin that were more parasitized and that had been more accessible to the vector insect. In this anatomical region, there may be a similar process of modulation of the inflammatory response, thus favoring multiplication and diffusion of the parasite to distant organs.

One final hypothesis for explaining the survival of the protozoon *Leishmania* sp. in susceptible hosts would be that this parasite is efficient at deceiving the host's immune system, since it has already been proven that the population of CD4+ T lymphocytes becomes reduced in animals that are infected by *L. donovani*, via apoptosis (Lüder et al., 2001). Likewise, it has been reported that the predominant cytokine profile in symptomatic dogs is type Th2 (anti-inflammatory cytokines). Through this, inhibition of the activation and microbicide activity of macrophages (which are stimulated mainly by IFN- $\gamma$ ) has been found (Corrêa et al., 2007).

For this reason, taking into consideration the results from the present study, it can be concluded that in the animals with advanced disease (symptomatic group), there was a greater parasite load and an extensive chronic inflammatory process in the peripheral lymph nodes and in the muzzle and ear tip skin. The predominant cell population in this infiltrate consisted of macrophages (granulomas) and plasmacytes. In the popliteal lymph node (Moreira et al., 2010) and in the subscapular lymph node of group S, there was a drastic reduction in the lymphoid population, thus resulting in severe atrophy. This atrophy coincided with the greater density of cells undergoing apoptosis in this group. In the skin, the lymphocytes of the inflammatory infiltrate were also the cells that underwent the apoptotic process. The subscapular lymph node seemed to respond more efficiently to the protozoon in groups A and O, because of the presence of lymphocytes in the granulomas, thus resulting in lower parasite loads in these groups. Therefore, we conclude that the dogs in group S were less efficient in responding to the infection by the protozoon and in setting up a type Th1 immune response. It is possible that *Leishmania (L.) chagasi* uses the apoptotic process as an immune evasion mechanism. The reliability of the results for this investigation can be improved by real-time PCR strategy for parasite load quantification and by increasing the sample size.

#### Ethical standards

The design for this study was approved by the Ethics and Animal Welfare Committee (CEBEA no. 000599-08), of FCAV/UNESP, Jaboticabal, State of São Paulo, Brazil.



## Conflict of interest

The authors declare that they have no conflict of interest.

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