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Research Article

Immunodetection of CD4 and CD8 T Cell in Dogs with Visceral Leishmaniasis

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Abstract

The aim of this study was to analyze different organs of dogs with VL for the presence of parasite and the proportion of two lymphocyte subtypes (CD4/CD8). Symptomatic (S=17) and asymptomatic (A=12) dogs from endemic area for VL were used. Control group (C=6) had dogs from non-endemic area. The detection of parasite DNA (RT-qPCR) and T cells (immunohistochemistry) was done on skin, lymph nodes, spleen and liver. The results were compared by group. The spleen of symptomatic dogs showed the highest levels of parasite DNA, followed by lymph nodes, skin and liver. CD4/CD8 T cells predominated in asymptomatic dogs and differed significantly from symptomatic and control groups ($P<0.05$). The CD4 T cells immunodetection was higher than CD8 in all organs of infected dogs. The skin had low parasite load and high immunodetection of T-cells subpopulations in the asymptomatic group. The same group showed positive correlation between CD4 T cells and parasite load in skin. The symptomatic group had negative correlation between CD4 and parasite load on lymph nodes, and CD8 and parasite load in spleen and lymph nodes. In the symptomatic group, the lymphoid organs had the highest parasite load and it suggesting most susceptibility to infection by the Leishmania spp. This organ had low number of CD8 T-cells in the symptomatic group, suggesting an inefficient cellular response. Possibly, part of the sub-population of CD4 could be regulatory T-cells and this will be contributing to the maintenance of the infection in susceptible organs, such as the spleen.

INTRODUCTION

In visceral leishmaniasis (VL), the main organs of dogs infected by parasite *Leishmania infantum* are lymph nodes, spleen, liver, skin and bone marrow. Some these organs are more resistant and others are more susceptible to multiplication of the parasite [1-7]. The differences between resistance and susceptibility to *Leishmania* sp. infection are influenced by proportion of Th1 and Th2 immune response, including CD4 T lymphocyte subpopulations. The resistance to infection is associated with activation of Th1 cells that promote protective cellular immune response, through production of cytokines, mainly IL-2, INF-γ and TNF-α, which activate the macrophages and stimulate them to synthesize toxic substances to promote lysis of the parasite [8]. The susceptibility is related to the activation of Th2 cells, that produce humoral immune response, characterized by cytokines, mainly IL-4 and IL-10, that suppress the action of the macrophages and stimulate the B lymphocytes to produce antibodies, being inefficient against the parasite, allowing the progression and consequently the persistence of the disease [8,9].

The CD8 cytotoxic T cells are responsible for lysis of the macrophages infected with the *Leishmania* sp. and these active cells increase the synthesis of INF-γ and TNF-α [8], because it recognize the foreign antigens present on the surface of the infected cells of the host and secrete perforin that destroys the target cell and directly induces the effector phase of apoptosis [10]. Moreira et al. [11], observed that liver and spleen of infected dogs with VL presented significant lymphoid apoptosis and suggest that it contributing towards the survival of parasite in these organs, due to deficient activation of cellular immunity.

The analysis of the immune response in different compartments during a chronic infection allows the understanding of mechanisms of resistance and susceptibility of each microenvironment in response to the presence of a pathogen. There are few studies that have described the responses to injury in the different organs of dogs infected with *Leishmania infantum* [3,7] as well as, no studies were found that evaluate the presence of CD4 and CD8 in different organs of the same animal by immunohistochemistry. Most articles perform ELISA tests, flow cytometry and PCR. Therefore, the objective of the present

study was to analyze peripheral lymph nodes, liver, spleen and skin of dogs naturally infected by Leishmania infantum, in order to investigate the relationship between the presence of levels of parasite DNA and the subpopulation of T cells (CD4 and CD8) by immunohistochemistry.

MATERIAL AND METHODS

Material collection

The dogs investigated in this study were originated from the Zoonosis Control Center in Araçatuba, (São Paulo State, Brazil), a region that is endemic for VL [1]. The design for this study was approved by the Ethics and Animal Welfare Committee (CEUA nº. 028220/12), of FCAV / UNESP, Jaboticabal, State of São Paulo, Brazil.

We evaluated 29 dogs with VL, without preference for age, breed or gender. The infected dogs were divided into two groups according to physical examination and classified as asymptomatic (n = 12), without clinical signs of the disease and in symptomatic (n = 17), with clinical signs of the disease as described by Figueiredo et al., [12]. The animals were euthanized using an intravenous (IV) overdose of barbiturate, followed by IV administration of potassium chloride (decree number 51.838 of the Brazilian Ministry of Health and Resolution number 714, of June 20, 2002, of the Federal Veterinary Medicine Council). A veterinarian was being presence (Pamela Rodrigues Reina Moreira and number of the veterinarian's Regional Council of Veterinary Medicine is 18769). The necropsy of the dogs was performed immediately after their death. The control group consisted of six dogs from the routine of the Department of Veterinary Pathology Jaboticabal-SP, Brazil, and a non-endemic area for VL [13]. Infected dogs and control dogs were selected, following confirmation or not of disease respectively, by RIFI and ELISA.

Fragments of peripheral lymph node, liver, spleen and skin were collected. For Immunohistochemical analyses the fragments were fixed in 10% formalin solution and for the RT-qPCR technique [7], a fragment of each organ was collected and stored in liquid nitrogen at -196ºC until the analysis by this technique.

Immunohistochemical analysis

To perform the immunohistochemical technique, the fragments were fixed in 10% formalin solution, buffered with 0.15 molar phosphate at pH 7.2 and, after 24 hours of fixing, they were dehydrated, processed, embedded in paraffin and cut into sections of 5 µm of thickness. Antigenic recovery was performed by heat using a Pascal pressure chamber (Dako Cytomation, Carpinteria, USA) and a 10 mM sodium citrate buffer solution (pH 6.0). The blocking of endogenous peroxidase was performed using a commercial product (Hydrogen Peroxide Block Spring, reference DHP-125), for 20 minutes after the primary antibody incubation.

To double block nonspecific reactions was utilized a commercial product (Protein Block, Dako Cytomation, Catalog number X0909, Carpinteria, USA), for 30 minutes of incubation, subsequently was added to 8% of skim powdered milk solution and used over the tissue sections. Thereafter, the primary antibodies were CD4 T cells (Mouse monoclonal, Serotec, catalog number MCA1998S) and CD8 T cells (Mouse monoclonal, Serotec, catalog number MCA1999S) and both were added at 1:150 dilution and incubated for 18 hour at 4ºC. The secondary antibodies utilized was Polymer complex linked to peroxidase (Kit Advance HRP®, Dako Cytomation, reference K406889-2), in the last step, DAB substrate-chromogen solution (3,3-diaminobenzidine; Dako Cytomation, reference K3468-1) was used for the color development.

Negative controls consisted of immunohistochemical reactions performed as described above, except that the primary antibody was omitted and only the antibody diluent (Dako Cytomation, reference S302283-2) was used. Positive controls were produced using tissues suggested by the manufacturer of the antibody.

To determine the number of immunostained cells, ten microscope fields were analyzed (Nikon Eclipse E200) with a 40x objective lens [4], which presented an area of approximately 0.19625 µm2. From the values obtained in these fields, an average number of immunostained cells was determined for each group, infected (symptomatic and asymptomatic) and controls dogs.

Reagents used: Sodium citrate buffer solution (pH 6.0)

Blocking of endogenous peroxidase (Hydrogen Peroxide Block Spring, reference DHP-125)

Protein Block (Dako Cytomation, catalog number X0909, Carpinteria, USA)

Primary antibodies were CD4 T cells (Mouse monoclonal, Serotec, catalog number MCA1998S) and CD8 T cells (Mouse monoclonal, Serotec, catalog number MCA1999S)

Secondary antibodies: Polymer complex linked to peroxidase (Kit Advance HRP®, Dako Cytomation, and reference K406889-2)

DAB substrate-chromogen solution (3,3-diaminobenzidine; Dako Cytomation, reference K3468-1)

Antibody diluent (Dako Cytomation, reference S302283-2)

Statistical analysis

The average of immunostaining cells for each antibody was evaluated by the Kruskal-Wallis nonparametric test, and comparisons between groups within each organ were evaluated using Dunn's test. To analyze the number of DNA copies of the parasite in each organ, the Mann-Whitney nonparametric test was used to compare the groups of dogs with VL (Asymptomatic and Symptomatic).

The verification of the association between the CD4 and CD8 variables with the parasite load was compared among the infected groups, within each organ, that were submitted to the Spearman Correlation Coefficient. The coefficients obtained between: 0.65 and 1.00 indicate strong positive or negative correlation; between 0.30 and 0.65 present a mean positive or negative correlation and between 0.00 to 0.30 a weak positive or negative correlation. Differences were significant when $P < 0.05$. The analyses were performed using the Graph Pad Prism software (version 5.00, 2007).

RESULTS

The immunolabeling of CD4 (Figure 1) and CD8 T lymphocytes

(Figure 2) were observed on the lymphocyte membrane. In the lymph nodes, CD4 T cells were present mainly in the paracortical and medullary region; CD8 T cells were observed mainly in the lymph node medullary region, in both groups and in smaller proportion in the paracortical region. In the liver, CD4 and CD8 T cells were observed in sinusoids and in hepatic granulomas. In the spleen the two subpopulations of lymphocytes were verified in the transition of white pulp with red pulp (mantle layer of the splenic corpuscle), in red pulp and rarely in the white pulp. In the skin, these lymphocytes were observed in the middle of the granulomatous inflammation and around skin adnexa. When comparing the groups by organ, significant differences were observed for both lymphocytes in asymptomatic group that presented the highest medians. This group showed significant differences of the symptomatic and control groups (Figure 3).

The correlation between the parasite load and the number of CD4 T cells were significant in the lymph nodes ($P = 0.0048$ / $r = - 0.51$) of symptomatic dogs and skin ($P = 0.0299$ / $r = 0.64$) of asymptomatic dogs (Figure 4).

The correlation between the number of DNA copies of *Leishmania* and the number of CD8 T cells were significant in the spleen ($P = 0.036$ / $r = - 0.39$) and lymph nodes ($P = 0.015$ / $r = -0.45$) in the symptomatic group (Figure 5).

The correlation between CD4 and CD8 T cells, were significant in all organs of infected dogs. Where in the lymph nodes ($P < 0.0001$ / $r = 0.85$), spleen ($P < 0.0001$ / $r = 0.73$), liver ($P < 0.0001$ / $r = 0.67$) and skin ($P < 0.0001$ / $r = 0.72$) were observed (Figure 6).

DISCUSSION

In the present study, it was observed that the subpopulations of T cells (CD4 and CD8) were in highest proportion in the dogs of the asymptomatic group in all the organs, with significant differences between the symptomatic and control dogs. These results agreed with the findings of Pinelli et al., which describe that cytokines (mainly IL12) produced by the Th1 response in resistant (asymptomatic) dogs, influence the increase in production and proliferation of lymphocytes and in the cytotoxic activity of CD8 T cells, maintaining the healthy clinical appearance of the animal. Figueiredo et al. [12], observed in the jejunum of dogs naturally infected with *Leishmania infantum*, a low parasite load, with increased frequency and expression of CD4, CD8 and Foxp3. Reis et al. [13], verified that asymptomatic dogs had high numbers of CD8 T cells in peripheral blood and low parasite load in bone marrow. These results suggest resistance to VL in these dogs. As observed in the present study, dogs asymptomatic had a low parasitic load and a higher density of CD4 and CD8 T cells in all organs.

In the present study, there were strong negative correlations between parasite load and CD4 and CD8 T cells in lymphoid organs (spleen and lymph nodes). When the parasitism increased was observed that the lymphocyte density decreased. This suggests a greater susceptibility of these organs to VL. Moreira et al. [5,7], observed that the high density of parasite load in the lymphoid organs was associated with a greater number of apoptotic lymphocytes, which were not able to destroy *Leishmania infantum*, which allowed the persistence of the infection in the canine tissues. Giunchetti et al. [14], observed positive correlation between parasite load and highest levels of CD8 T cells in popliteal lymph node. These authors suggests a possible selective migration of type2 CD8 T cells to the lymph node, possibly associated to high levels of IL-10 that suppressing the cell mediated immune response in dogs with VL. Corrêa et al. [15], observed high levels of IL-10 in spleen of dogs with VL.

Stanley e Engwerda [16] reported that the spleen is the organ that suffers the greatest lesions. These lesions are mediated by the direct loss of specific cell populations and alterations in the tissue microenvironment, leading to an inability to generate effective immune responses. In addition, there is an inability of the splenic macrophages to exert their leishmanicidal mechanisms or to recruit cytotoxic T lymphocytes (CD8) and NK cells. Therefore, the highest parasite load in this organ is justifiable. These results corroborate with those observed in our study, because the spleen was also the organ that presented the highest parasitism in both groups of infected dogs, even the asymptomatic had high parasite

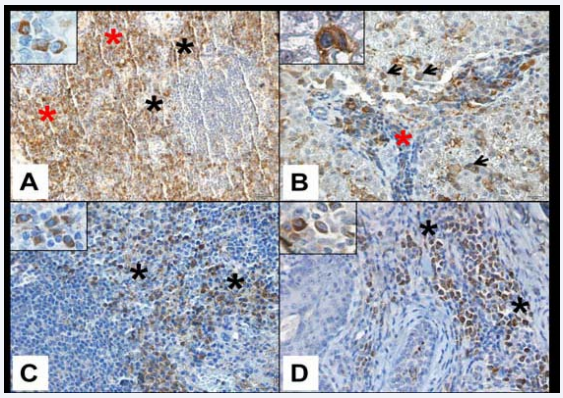

Figure 1 Photomicrograph of immunostaining CD4 T cells in dogs with Visceral Leishmaniasis. (A) In lymph node note positive lymphocytes in the regions of cortico-medullary transition (* black) and medullary (* red / 20x objective lens). In detail note CD4 T cell positive (100x objective lens). (B) In the hepatic sinusoids (arrows), inflammatory infiltrate (*) and granulomas were observed the presence of CD4 cells (40x objective lens; detail: 100x objective lens). (C) Note positive splenic lymphocytes in the transition from white pulp to red pulp (* / detail / 40x objective lens). (D) Note the CD4 immunostaining in the inflammatory infiltrate of the skin (* / detail / 40x objective lens). Polymer complex linked to peroxidase.

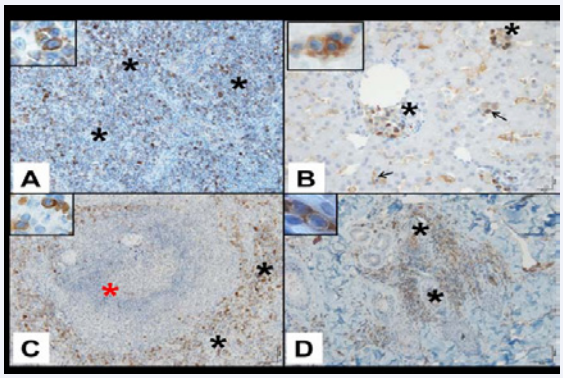

Figure 2 Photomicrograph of immunostaining of CD8 T cells in dogs with visceral leishmaniasis. (A) Observe in the lymph node the presence of CD8 T cells in the medulla region (* / 20x objective lens; detail: 40x objective lens). (B) In the liver observed these cells in sinusoids (arrows) and hepatic granulomas (*; 40x objective lens). (C) Note in the spleen, the presence of greater proportion these cells in the transition region of the white pulp with red pulp (* black) and discreetly in the germinal center of the white pulp (* red / 20x objective lens). In detail is observed immunostaining in the membrane of lymphocytes (100x objective lens). (D) Note the immunostaining of CD8 T cells in the inflammatory infiltration around of the skin adnexa (* / 20x objective lens). Observed the immunostaining in membrane of lymphocytes (detail; 100x objective lens). Polymer complex linked to peroxidase.

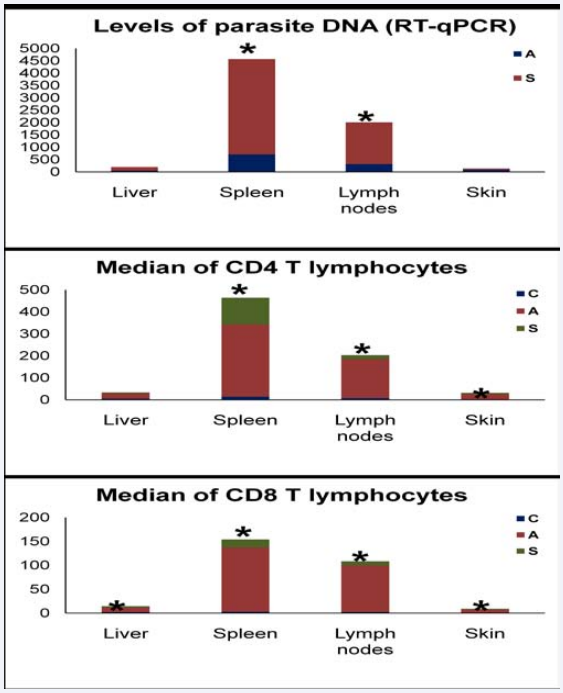

Figure 3 (a) Median of levels of parasite DNA (RT-qPCR) in different organs analyzed in the symptomatic (S), asymptomatic (A) groups of dogs with VL. Note the significant differences between the groups in organs of infected and control dogs. (b) Median of CD4 T cells immunostaining in different organs analyzed in the symptomatic (S), asymptomatic (A) and control (C) groups of dogs. Note the significant differences between the groups in organs of infected and control dogs. (c) Median of CD8 T cells immunostaining in different organs analyzed in the symptomatic (S), asymptomatic (A) and control (C) groups of dogs. Note the significant differences between the groups in organs of infected and control dogs. Nonparametric Kruskal-Wallis and Dunn tests ($P<0.0001$).

load. Possibly, the disorganization of the splenic architecture caused by the presence of diffuse granulomas with parasitized macrophages and the presence of a microenvironment rich in Th2 cytokines may contribute to failures in the activation of cellular immunity, resulting in persistent infection.

The liver presented low proportion of CD4 and CD8 T cells and these differences were observed between the groups only for CD8 T cells. Asymptomatic dogs presented the highest number of this cell, differing only from symptomatic dogs. This organ also presented low parasite load, probably in these organs, the CD8 T cells has cytotoxic action, efficiently destroying the parasite, making this organ resistant to infection. Agreeing with the reports of Moreira et al. [11], who observed a predominance of lymphocytes infiltrated in hepatic granuloma and low parasitism

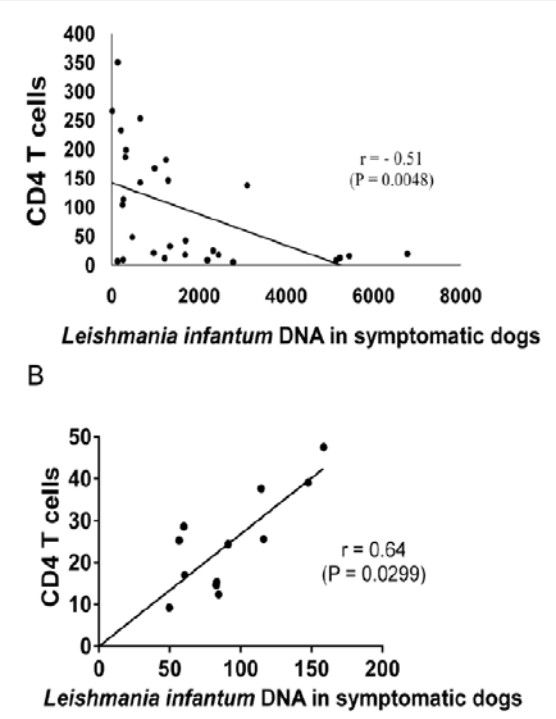

Figure 4 Correlation between the medians of CD4 T cells and the number of DNA copies of Leishmania infantum in the lymph nodes (A) and skin (B) determined by linear regression analysis and the Spearman correlation coefficients (r).

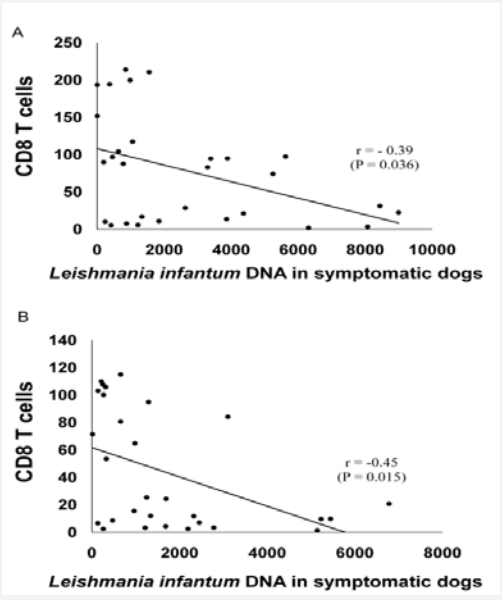

Figure 5 Correlation between the medians of CD8 T cells and the number of DNA copies of Leishmania infantum in the spleen (A) and lymph nodes (B) determined by linear regression analysis and the Spearman correlation coefficients (r).

when compared to the spleen that showed high density of parasitized macrophages and apoptotic lymphocytes. However, Giunchetti et al. [14], described direct relationship between liver changes and parasite load in symptomatic dogs. The symptomatic dogs of present study had minor hepatic parasite load when compared with the others organs. Possibly, the hepatic resistance to the multiplication of the parasite *Leishmania* sp. could be associated with the activation of the hepatic stellate cells present in the space of Disse, which when activated are able to produce extracellular matrix (fibrosis) and express an inflammatory phenotype and produce cytokines, chemokines, nitric oxide, express adhesion molecules and present antigens that stimulate lymphocytes T [17]. In humans with VL, hepatic fibrosis has been described [18]. The hepatic infection may also

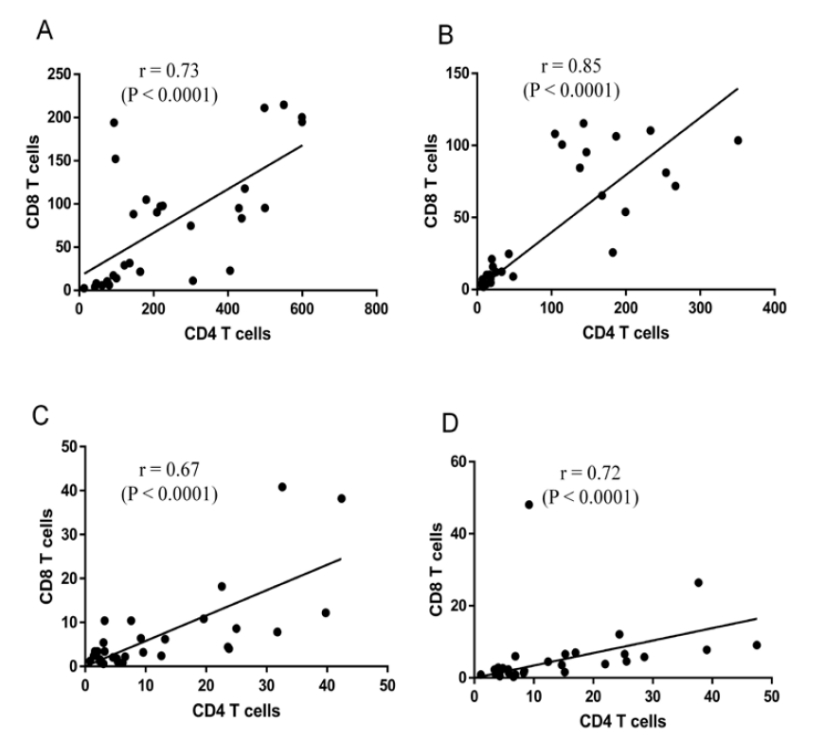

Figure 6 Correlation between the medians of CD4 and CD8 T cells in the spleen (A), lymph nodes (B), liver (C) and skin (D) determined by linear regression analysis and the Spearman correlation coefficients (r).

be self-resolving by the action of Kupffer cells, Pit cells (liver killer cells) and T lymphocytes (CD4 and CD8), resulting in elimination of the protozoan [19]. However, Moreira et al. [20], observed low detection of natural killer (NK) cells in the liver and spleens of dogs with VL, suggesting the suppression of the cytotoxic action of these cells. Murphy et al. [21], describe that NK cells probably express different receptors that are able to secrete cytokines with cytotoxic action or are able to inhibit this activity by protecting healthy cells from cytolysis. In the present study, the liver was the organ with the lowest parasitic load, possibly because of a cell population capable of eliminating circulating pathogens. However, the cytokine profile present in the hepatic microenvironment could also influence the activation of the cells responsible for hepatic clearance. Corrêa et al. [15], verified high levels of IFN-γ (ex-vivo) in spleen of symptomatic dogs and in liver of asymptomatic dogs.

In the present study, the asymptomatic group presented high density of CD4 and CD8 with significant differences between symptomatic and control, on skin. In this organ, the symptomatic dogs too presented significant differences with the control group, for the CD4 T cells variable. This organ presented low parasite load. Few studies have demonstrated the involvement of CD8 cells in resistance to canine VL in naturally infected dogs. Barbiéri related that these lymphocytes were detected in asymptomatic dogs experimentally infected with *L. infantum* but not in symptomatic animals, suggesting that direct lysis of *L. infantum* infected macrophages by cytotoxic T lymphocytes represents an additional effector mechanism in resistance to VL [8]. In the present study, in the skin of asymptomatic group, there were strong positive correlations between parasite load and CD4 T cells. Probably in this organ, the CD4 T cells are stimulating a Th2 immune response, similar to that described by Ackermann that reporting the macrophage activation pathway through CD4 T cells depends on predominant cytokines profile. Esch et al. [22], related that control of *Leishmania infantum* infection is dependent upon Th1 CD4 T cells to promote macrophage intracellular clearance of parasites. Moreira et al. [7], observed in skin of dogs with VL, that there was detection of macrophages with M2 phenotype, suggesting that the cutaneous immune responses are inefficient in controlling the *Leishmania* infection, favoring the progress of infection.

In all organs of this study, a positive correlation was observed between CD4 and CD8 T cells; however it suggested that in lymphoid organs, CD4 T cells are favoring a Th2 immune response, because these organs had highest parasitism. Kemp et al. [23-25], describe that CD8 T cells can present two subpopulations of cells (types 1 and 2). CD8 T cells (type 1) secrete Th1 cytokines (INF-y) and type 2 secretes Th2 cytokines (IL-4, IL-5 and IL-10). Probably, in the present study, organs with low parasite load (liver and skin) had CD8 T cells (type 1), which cause the lysis of macrophages parasitized with *Leishmania*, resulting in protective immunity. In lymphoid organs an inefficient cellular response was confirmed by the negative correlation between parasitism and CD8 T cells density in the spleen. In addition, CD8 T lymphocytes could be type 2, capable of secreting Th2 cytokines that favor persistence of infection.

CONCLUSION

The spleen of infected dogs had higher detection of the parasite, suggesting that the splenic microenvironment is

favorable to survival of the protozoa. CD8 T cells were reduced in the symptomatic dogs showing a possible inefficient cellular immune response. Probably, part of the sub-population of CD4 could be regulatory T-cells and this will be contributing to the maintenance of the infection in susceptible organs, such as the spleen, due to immunosuppressed microenvironment.

ETHICAL STANDARDS

The design for this study was approved by the Ethics and Animal Welfare Committee (CEUA nº. 028220/12), of FCAV / UNESP, Jaboticabal, State of São Paulo, Brazil.

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