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Apoptosis in T lymphocytes from spleen tissue and peripheral blood of *L.* (*L.*) *chagasi* naturally infected dogs

Valéria Marçal Felix de Lima[a,*], Karina Reinaldo Fattori[b], Fausto de Souza[a], Flavia Rezende Eugênio[a], Paulo Sérgio Patto dos Santos[a], Daniele Bernadete Rozza[a], Gisele Fabrino Machado[a]

[a] *Departamento de Clinica, Cirurgia e Reprodução Animal, Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Rua Clóvis Pestana, 793, ZIP 16050-400 Araçatuba, São Paulo, Brazil*[1]

[b] *Faculdade de Ciências Agrárias e Veterinárias Programa de Pós-graduação em Microbiologia Agropecuária, Universidade Estadual Paulista, Via de Acesso Professor Paulo Donato Castellane s/n, ZIP 14884-900 Jaboticabal, São Paulo, Brazil*

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ABSTRACT

Dogs are the main domestic reservoirs of *L.* (*L.*) *chagasi*. Once in the vertebrate host, the parasite may cause visceral leishmaniasis, which can also be transmitted to humans. Infected symptomatic dogs show disorganization in the white pulp in spleen tissue and a reduction in T lymphocytes in peripheral blood. To investigate whether apoptosis is involved in white pulp disorganization and diminished T cell counts in peripheral blood, apoptotic T cells from the spleen and peripheral blood of dogs naturally infected with *L.* (*L.*) *chagasi* and presenting clinical manifestations were quantified and compared with healthy dogs. Thirteen symptomatic adult dogs infected by *L.* (*L.*) *chagasi* and six healthy dogs from a nonendemic area (controls) were included in the study. Samples from spleen and peripheral blood were used to quantify apoptosis in CD3 lymphocytes by flow cytometry using Anexin V and Multicaspase kits; the results were compared using the Mann Whitney test. The percentage of total T cells was lower in *Leishmania* infected dogs compared to healthy controls ($P < 0.05$). Apoptosis levels in T cells from PBMC and spleen were higher in infected dogs than in controls ($P < 0.05$). The least squares method test was used to determine the effect between the degree of structural organization of spleen white pulp and the percentage of apoptosis in the spleen. A significant effect on the level of white pulp morphological disorganization and percentage of apoptosis in spleen T cells was observed ($F = 20.45$; $P = 0.0014$). These data suggest that apoptosis is an important for the immunopathogenesis of canine visceral leishmaniasis.

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1. Introduction

Leishmaniasis is caused by a protozoan of the trypanosomatidae family, genus *Leishmania*. It occurs in 88 countries of which 65 present the visceral form. Most cases (90%) of visceral leishmaniasis (VL) in humans occur in the rural or suburban areas of five countries, including Brazil (Desjeux, 2004). In Brazil, like other countries in South America, migration into urban areas contributed to the expansion of VL (Desjeux, 2004). In Brazil, this expansion occurred especially in the northeast (Dantas-Torres, 2006) and southeast areas (Santiago et al., 2007). Besides the high incidence and broader distribution that the expansion itself represents, the spread into new areas also carries the threat that severe and lethal forms of the disease may

* Corresponding author. Tel.: +55 18 36363285.
E-mail address: vmflima@fmva.unesp.br (V.M.F.d. Lima).
[1] Tel.: +55 18 3636 14 22.

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emerge when associated with malnutrition (Gontijo and Melo, 2004) and HIV/AIDS infection (Ashford, 2000).

Dogs are considered the main domestic reservoirs of *L.* (*L.*) *chagasi* (Moreno and Alvar, 2002). The parasite is transmitted from one infected dog to another through the bite of phlebotominae and possibly through other arthropod vectors such as fleas and ticks (Ferreira et al., 2009; Coutinho et al., 2005), and through blood transfusions, as reported by Owens et al. (2001). After the phlebotominae feed on the dogs' blood, the parasites rapidly spread into the lymph nodes and spleen through the lymph and blood and eventually reach the kidneys and liver. They may also affect the reproductive organs, skin, digestive and respiratory systems and the bladder (Molyneux and Ashford, 1983).

Once in the vertebrate host, the parasite can cause lesions and symptoms that are characteristic of canine visceral leishmaniasis, although some infected dogs may be oligosymptomatic or asymptomatic (Mancianti and Meciani, 1988), and some may evolve to spontaneous cure (Fisa et al., 1999). The most frequent signs of VL are lymphadenopathy, onychogryphosis, cutaneous lesions, weight lost, cachexia and locomotor abnormalities (Semião-Santos et al., 1995). The asymptomatic form represents 20–40% of the serum-positive population, of which 80% actually develops the disease (Noli, 1999). In the Brazil in urban area of the northeast region, the asymptomatic form represent 30% of the serum positive population (Queiroz et al., 2009).

The suppression of cellular immunity is the most important aspect in the pathogenesis and progression of canine disease. The absence of T cell response to antigens of *Leishmania* sp. is observed *in vivo*, with a negative leishmanin skin test (Dos Santos et al., 2008). In dogs infected with *Leishmania infantum*, a reduction in the number of T lymphocytes in PBMC occurs (Bourdoiseau et al., 1997) and disorganization of white pulp in spleen tissue has been previously described (Santana et al., 2008), but the mechanisms that are responsible for these changes have not yet been elucidated.

In human acute infection, the reduction in T lymphocytes and mononuclear cells of peripheral blood and failure in immunity has been associated with apoptosis (Potestio et al., 2004). In mice experimentally infected with *Leishmania donovani*, an increase in the level of spontaneous apoptosis in the spleen and liver compared to noninfected mice was also observed (Alexander et al., 2001). *In vitro* findings also suggest the involvement of apoptosis in the mechanism of suppression observed in visceral leishmaniasis, the infection of macrophages by *L. donovani* increased the level of FAS in the membrane and sFASL in the culture supernatant, a mechanism that may contribute to increased sensitivity to apoptosis for T cells specific for *Leishmania* sp. (Eidsmo et al., 2002).

To investigate whether apoptosis is involved in the reduction in lymphocytes in peripheral blood and alterations in white pulp, apoptosis was quantified in dogs naturally infected with *L.* (*L.*) *chagasi* presenting clinical manifestations and the structural disorganization in white pulp was correlated with the percentage of apoptosis in T cells. If proven, such findings could contribute to improving our present understanding of the immunopathogenesis in infected dogs.

2. Materials and methods

2.1. The study area

The county of Araçatuba (21°12′32″ S; 50°25′38″ W), with an area of 1,167,311 km^2, is located in the state of São Paulo, Brazil. It is a region known to be endemic for canine VL.

2.2. Ethical issue

This study was approved by the institutional Ethics and Animal Welfare Committee (*Comissão de Ética em Experimentação Animal*, CEEA, UNESP, process number 02232).

2.3. Animals and diagnosis of VL

A total of 13 adult dogs were used, males and females, aged between 2 and 4 years-old, of undefined breed and different weights, from the Zoonosis Control Center of Araçatuba (CCZA). The dogs were symptomatic and showing at least three clinical signs of canine VL. These could include fever, dermatitis, lymphoadenopathy, onychogryphosis, weight loss, cachexia, locomotor abnormalities, conjunctivitis, epistaxis, hepatosplenomegaly, edema, and apathy. Briefly, the diagnosis of VL was confirmed by detecting anti-*Leishmania* antibodies for *L.* (*L.*) *chagasi* by indirect ELISA, according to Lima et al. (2003), and simultaneously positivity in rapid test rK39 and in PCR amplification of *Leishmania* spp. DNA in spleen tissue.

A group of 6 healthy dogs, both males and females, from a nonendemic area (Londrina, State of Paraná, Brazil) were included in the study as negative controls. These dogs were serum negative for *L.* (*L.*) *chagasi* by indirect ELISA (Lima et al., 2003), negative in the rapid test rK39 and in PCR amplification of *Leishmania* spp. DNA in spleen tissue.

2.4. Sample collection

Samples of spleen from both groups were removed by surgical excision. The dogs were premedicated with the combination of morphine (0.4 mg kg^{-1} IM) and acepromazine (0.05 mg kg^{-1} IM). Fifteen minutes later, propofol (4.0 mg kg^{-1} IV) and midazolam (0.1 mg kg^{-1} IV) were used for induction. Immediately, the dogs were positioned in dorsal recumbency and anesthesia was maintained with isoflurane (1.5 V%). The heart and respiratory rates, the systolic arterial blood pressure and end-tidal CO_2 measurements were monitored during all anesthetic procedure. The samples were maintained in RPMI 1640 supplemented with 10% (v/v) fetal calf serum (Sigma) at 4 °C and processed immediately to evaluate apoptosis.

From each dog, 4 ml of blood was collected from the cephalic veins, clotted at room temperature for 4 h and subsequently centrifuged to extract the serum. The serum samples were stored at–20 °C prior to analysis and

additional blood samples were collected with sodium EDTA and processed immediately to evaluate apoptosis.

2.5. Kalazar detect rapid test rK-39 (InBios International, USA)

To perform this test, blood samples were collected by venipuncture and centrifuged and the serum was separated. The procedure of the test was performed in accordance with the manufacturer's recommendations.

2.6. PCR

DNA obtained from spleen samples was extracted by freezing and thawing the cells 3 times and washing them in 1× SSC buffer solution (NaCl 3 M, sodium citrate 0.3 M, pH 7.0). For cell lysis and protein digestion, 300 μl of lysing solution was added (10% SDS in 0.2 M sodium acetate) together with 20 μg/ml proteinase K. Samples were incubated at 56 °C for 2 h and the DNA was extracted using the phenol/chloroform/isoamyl alcohol method (25:24:1), according to Sambrook et al. (1989). After extraction, DNA was resuspended in 50 μl TE (10 mM Tris–HCl pH 8.0, 1 mM EDTA pH 8.0) and incubated for 3 min at 60 °C. The material was stored at −20 °C until used. The 13A (3′-GTG GGG GAG GGG CGT TCT-5′) and 13B (3′-ATT TTA CAC CAA CCC CCA GTT-5′) primers were used (Rodgers et al., 1990) to amplify a 120 bp fragment located in the constant region of the kinetoplast minicircles in all *Leishmania* species. The PCR was performed in a 60 μl volume containing 30 pmol of each primer (Invitrogen®), 0.2 mM DNTPs (Invitrogen®), 1.5 mM $MgCl_2$ (Invitrogen®), 5 U Taq DNA Polymerase (Invitrogen®), 50 nM buffer solution, milliQ water and DNA. Amplification was performed in an Eppendorf® Mastercycler Thermocycler gradient with initial heating to 95 °C for 5 min, followed by 33 cycles at 95–57–72 °C for 1.5 min, 1.5 min, and 2 min, respectively. Extension was performed at 72 °C for 10 min and the final product was stored at −20 °C until analysis. Reaction mixes containing either no DNA or DNA extracted from a *L. chagasi* promastigote culture (MHOM/BR00/MER02) were used as negative and positive controls, respectively. The amplified 120 bp product was analyzed by electrophoresis on acrylamide gels followed by silver staining.

2.7. Apoptosis evaluation in T cells

Peripheral blood cells were stained following the separation of mononuclear cells on a Ficoll-Paque gradient (Amersham Biosciences). Spleen cells were processed after lysis of red blood cells. For staining, the cells were suspended in PBS containing 1% bovine serum albumin, 0.1% azide and 20% fetal bovine serum to block the Fc receptor (FCR). Anti-canine CD3 monoclonal antibodies (Serotec, UK) were added and incubated for 30 min. Isotype control (Serotec, UK) antibody was added in a separate tube to control for nonspecific labeling. Following incubation, the tubes were centrifuged at 1000 × *g* for 3 min at 4 °C. The supernatant was discarded by quick inversion and the cell pellet briefly vortexed to resuspend the cells. The cells were washed four times with ice-cold PBS with 10% bovine calf sera. After the final wash, the cells were resuspended with PBS.

After immune staining for CD3 in PBMC and in leucocytes from spleen the apoptosis was detected using two different methods. The Nexin assay, which uses Annexin V, is a calcium-dependent phospholipid binding protein with high affinity for phosphatidylserine, a membrane component normally located in the internal face; however, during early activation of apoptotic pathways, these molecules are translocated to the outer surface of the cell membrane, where Annexin V can bind directly to them (Vermes et al., 1995). The second method, MultiCaspase SR kit, detects caspase pathways by a fluorescent labeled inhibitor of caspase reagent that specifically identifies active caspases. These methods have been used in similar studies (Colgate et al., 2007).

The percentage of apoptosis was determined using the Nexin assay Kit (Guava, Hayward, CA) and Mutilcaspase SR kit (Guava, Hayward, CA). The procedure of each test was performed in accordance with the manufacturer's recommendations. Proper instrument performance was verified by running the Guava Check application with Guava Check reagents. Data were acquired regarding the Guava EasyCyteMini system using CytoSoft software, as described in the Guava PCA User's Guide and respective package inserts. For Guava MultiCaspase and Guava Nexin, 10,000 events were usually acquired. Negative and positive control Camptothecin (Sigma, USA) (0.15 μg/mL) in DMSO (Sigma) (Tao et al., 2004) were used to verify reagent performance and set analysis markers, delineating the negative and positive populations.

2.8. Spleen samples and histological analysis

Three to 4 mm-thick tissue slices were cut transversally to the capsule and fixed in 10% formalin. After fixation, tissue slides were embedded in paraffin. Four to 5 μm-thick sections were cut and stained with hematoxylin and eosin (HE). These sections were examined by at least two of the authors who were blind to previous knowledge of the dogs' health and identities. The authors scored the level of white pulp organization: (1) slightly disorganized, with either hyperplastic or hypoplastic changes leading to a loss of definition of any of the regions of the white pulp and (2) for moderately or extensively disorganized, when the white pulp regions were poorly individualized or indistinct.

2.9. Immunohistochemistry (IHC)

IHC stains were performed using the standard streptavidin–biotin peroxidase (HRP) immunostaining procedure with polyclonal antibody. Anti-CD3 (A0452) (DAKO, CA, USA) was used to detect T lymphocytes. This antibody was previously used to stain CD3 in canine lymphomas (Sueiro et al., 2004). Slides were deparaffinized and hydrated. Antigen retrieval was achieved by steam heating in citrate buffer for 30 min. For inhibition of endogenous peroxidase, slides were incubated with 2% (v/v) hydrogen peroxide 30 vol diluted in 50% (v/v) methanol for 30 min and nonspecific binding was blocked with 3% (w/v) nonfat dry milk in PBS for 30 min. Primary antibody against CD3

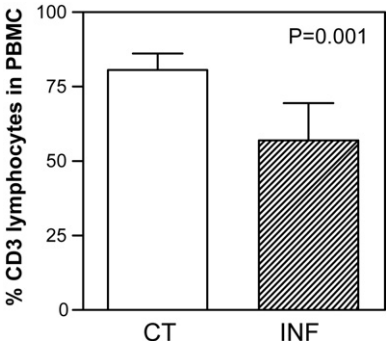

Fig. 1. Percentage of CD3 lymphocytes in PBMC from *Leishmania* infected dogs (INF) and healthy controls (CT). The results are expressed as the mean percentage ± SD. $P < 0.05$: significant differences between the mean values for *Leishmania* infected dogs and healthy controls.

(1:100) was applied for 18–22 h at 4 °C in a humidified chamber. Slides were washed in PBS, incubated with a biotinylated secondary antibody (LSAB$^+$ Kit, DAKO K0690, CA, USA) for 45 min at room temperature, washed once more with PBS, and incubated with streptavidin–HRP complex (LSAB$^+$ Kit, DAKO K0690, CA, USA) for 45 min at room temperature. The reaction was developed with 3,3′-diaminobenzidine (DAKO K3468, CA, USA). The slides were counterstained with Harris's hematoxylin, dehydrated, cleared and mounted with coverslips. Spleen tissue from healthy dogs was used as a positive control.

2.10. Statistical analysis

The data were analyzed by a nonparametric test. Group means were compared using Mann Whitney test. The least squares method was used to evaluate the effect of group (degree of correlation for the structural organization of white pulp) and quantitative variable (percentage of apoptosis). The results were considered significant when $P < 0.05$. The SAS software was used (SAS 9.1, SAS Institute, Cary, NC, USA) for all statistical analyses performed in this study.

3. Results

3.1. Decrease in T lymphocytes in PBMC

Flow cytometry analysis of CD3 lymphocytes in PMBC from infected dogs showed significantly lower numbers (58 ± 12, mean ± SD) compared to healthy controls (80.6 ± 5, mean ± SD) (Fig. 1) ($P = 0.001$, Mann Whitney test).

3.2. Apoptosis in T cells from peripheral blood and spleen

To examine apoptosis in T cells from PBMC and spleen of *L. chagasi*-infected dogs, PBMC and spleen samples were evaluated immediately following collection. Apoptosis of T cells from PBMC and spleen was detected using commercial kits for both, Annexin V (Guava, Hayward, CA) and Caspases (Guava, Hayward, CA) and simultaneously anti-CD3 mAbs (Serotec, UK).

The percentage of apoptotic T cells was evaluated after CD3 positive cells were gated in Plot 1 (Fig. 2a), followed by detection double stained for apoptosis detection in cells gated in Plot 1 (Fig. 2b). The PBMC showed higher numbers of apoptotic T cells in infected dogs (Fig. 2c) compared to healthy dogs (Fig. 2d) and in the spleen, similar results were obtained (Fig. 2e and f).

Data presented in Fig. 3 clearly indicate that T lymphocytes from PBMC and spleen tissue of infected dogs showed a significantly higher level of apoptosis compared to that observed in healthy dogs ($P < 0.05$, Mann Whitney test). In general both kits used showed similar values of apoptotic cells in controls and infected dogs (Fig. 3).

3.3. White pulp structural organization and CD3 IHQ

The percentage of dogs with slightly disorganized white pulp, with either hyperplastic or hypoplastic changes leading to a loss of definition of any of the regions of the white pulp areas was 63.6%, while 36.3% dogs showed white pulp areas that were moderately or extensively disorganized and were poorly individualized or indistinct. The CD3 IHC confirmed the structural disorganization of the white pulp in infected dogs compared to that observed in healthy dogs (Fig. 4). A significant effect on the level of white pulp morphological disorganization and percentage of apoptosis in T cells from the spleen was observed ($F = 20.45$; $P = 0.0014$, least squares method).

4. Discussion

The level of apoptosis in T cells from PBMC and spleen from VL dogs from an endemic area was analyzed and the values compared to uninfected control dogs to investigate the role this mechanism plays in the pathology and infectious process of the disease, as well as in the host immune response.

Apoptosis is essential for homeostatic control of lymphocyte numbers, particularly following the development of an immune response to an invasive microorganism (Scaffidi et al., 1999). However, some microbes are able to either directly manipulate this response or have developed strategies to survive in the host until homeostatic mechanisms are activated to reduce effector T cell numbers (Freire-de-Lima et al., 2000).

The apoptosis level in T cells from the spleen and peripheral blood were higher in infected dogs compared to that of healthy canines, suggesting that the infection by *L.* (*L.*) *chagasi* could induce apoptosis in T cells. Since the progression of infection is related to the impairment of cell-mediated immunity (Dos Santos et al., 2008), the detection of T cell apoptosis may contribute to inefficient cell immune response during *L. chagasi* infection.

Several mechanisms of T cell apoptosis have been proposed. Deprivation of growth factors, cytotoxicity by TNF-α and FAS and FAS-L interaction are among the major mediators (Scaffidi et al., 1999). *Leishmania* spp. infection studies have shown that macrophages infected by *L. donovani* increased the level of FAS in the membrane and sFASL in the culture supernatant (Eidsmo et al., 2002) and in *L. donovani* infected Balb/c mice, low IL-2 levels in the T cell culture

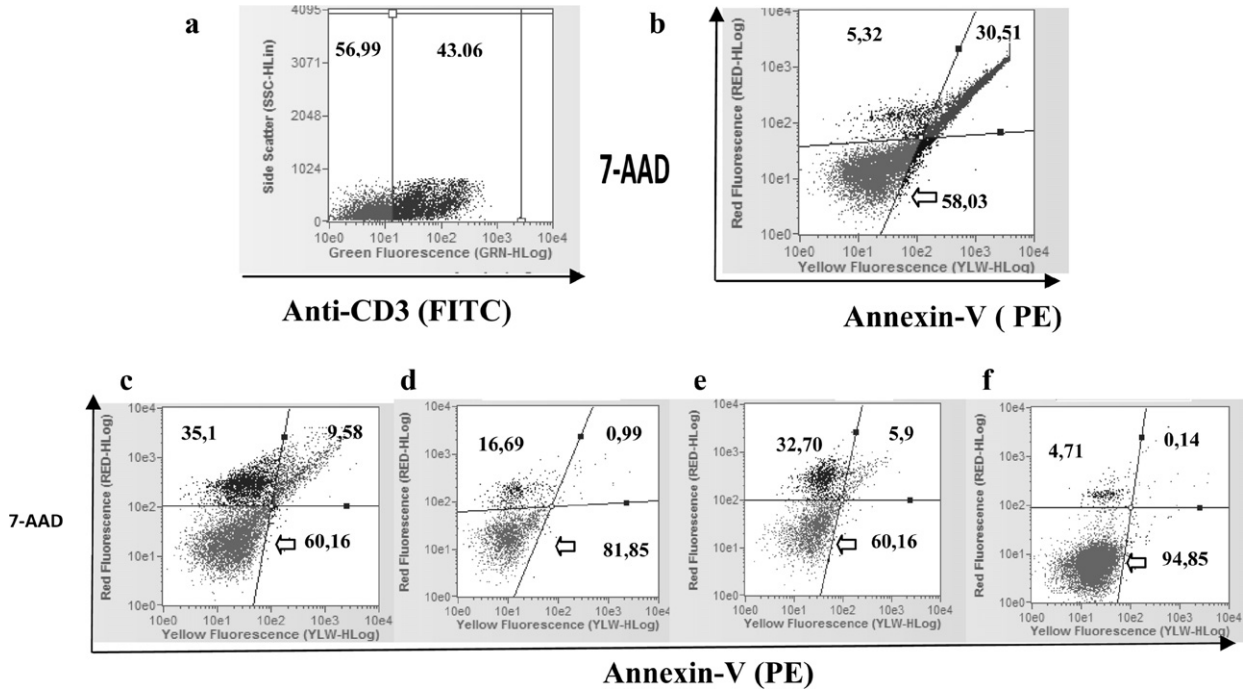

Fig. 2. Flow cytometry analysis: (a) fluorescence plot for gated lymphocytes from *Leishmania* infected dogs (Anti-CD3 monoclonal antibody FITC). Percentage inside upper right quadrants correspond to lymphocyte CD3 positive cells; (b) a representative fluorescence plot for gated lymphocytes and stained for apoptosis (positive control, the cells were incubated with apoptosis inductor Camptothecin); (c) a representative fluorescence plot for gated lymphocytes from PBMC from *Leishmania* infected dogs and stained for apoptosis; (d) a representative fluorescence plot for gated lymphocytes from PBMC from healthy dogs and stained for apoptosis; (e) a representative fluorescence plot for gated lymphocytes from the spleen of *Leishmania*-infected dogs and stained for apoptosis; and (f) a representative fluorescence plot for gated lymphocytes from the spleen of healthy dogs and stained for apoptosis. Percentage inside upper right quadrants correspond to lymphocytes stained with Annexin V-PE and 7-AAD fluorescence (double stained), late stage of apoptotic cells. Similarly dot plot figures were observed using the multicaspase kit.

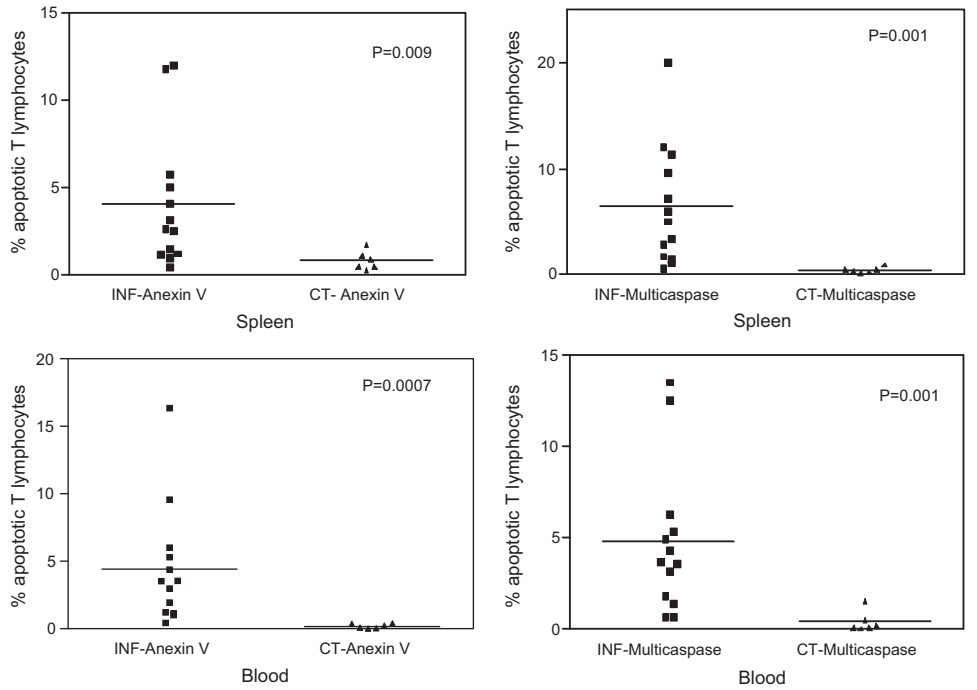

Fig. 3. Apoptosis of T lymphocytes in infected dogs (INF) and control dogs (CT). Each circle represents an individual dog. Bars represent the mean value in each group. $P < 0.05$: significant differences between the mean values for *Leishmania* infected dogs and healthy controls.

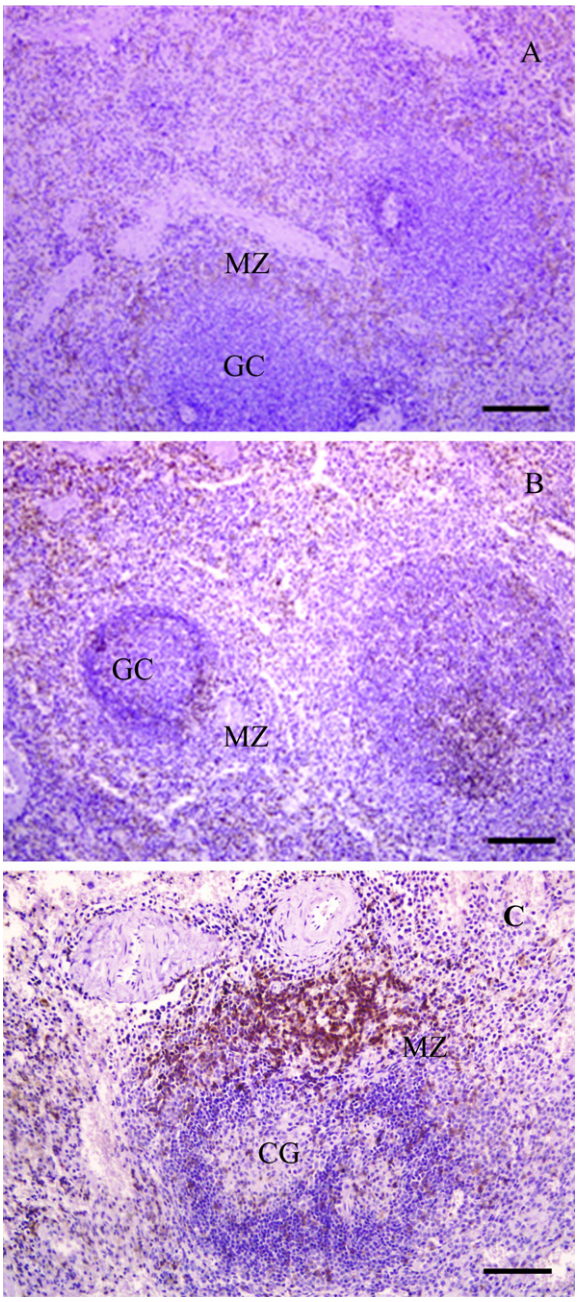

Fig. 4. Immunohistochemical analysis of CD3+ lymphocytes in the spleen of *Leishmania* infected dogs (a) and (b) and healthy control dogs (c): (a) the slight disorganization of the regions of white pulp; and (b) the extensive disorganization of the regions of white pulp. The lymphoid follicles showed a germinal center (GC) and a marginal zone (MZ). Avidin-biotin immunoperoxidase stained. In (a)–(c) the bars represent 200 μm.

supernatant cause apoptosis of Th1-like cells (Das et al., 1999), the mechanism that induces apoptosis in T cells from dogs naturally infected with *Leishmania* spp. has not yet been established and remains to be defined.

Few studies in the literature have investigated apoptosis in trypanosomatid infection, though in myocarditis of experimental canine chagas disease, abundant apoptosis of lymphocytes was observed (Zhang et al., 1999) similar to the present results. The possibility that apoptosis may contribute to the pathogenesis and clinical status of leishmaniasis has recently been suggested. *Leishmania* and its membrane constituents have been shown to result in activation-induced apoptosis of CD4 T cells *in vitro* (Wolday et al., 1999). Similarly, in mice infected intravenously with *L. donovani*, significant T cell apoptosis was detected in spleen tissue compared to controls (Alexander et al., 2001).

Lymphoid disorganization in the white pulp in spleen was present in the infected dogs examined in this study and cachexia was frequently observed, similar to that observed by Santana et al. (2008), who reported loss of lymphoid follicle definition in underweight dogs. Tissue lymphoid disorganization is not only related with cachexia, TNF-α is also related with wasting in visceral leishmaniasis (Pearson et al., 1990) and the TNF-α production mediates loss of the architectural structure of spleen tissue in murine models of visceral leishmaniasis (Engwerda et al., 2002). Alike mice, in dogs naturally infected by *Leishmania* spp. high levels of TNF-α are produced by spleen cells indicating that the presence of *L.* (*L.*) *chagasi* induces an immune response with relevant expression of this cytokine (Michelin et al., 2011). TNF-α is also involved in apoptosis mechanisms (Kanaly et al., 1999). The relation observed between a high percentage of apoptosis and the structural disorganization of white pulp suggests that apoptosis is involved in lymphoid tissue disorganization and the role of TNF-α in both processes should be clarified in the future.

The higher levels of apoptosis observed in T cells from the spleen and PBMC of infected dogs appear to be a physiological response to persistent immune activation; the mechanisms involved have yet to be studied. The depletion of T cells probably reflects the low T cell immunity response verified in symptomatic dogs that presented high parasitism in the spleen (Sanchez et al., 2004). The participation of T lymphocytes in the granuloma formation to control *Leishmania* sp. infection has been shown (Murray, 2001). In infected symptomatic dogs, the lack of mature and well organizated granulomas in the spleen (Sanchez et al., 2004) could be related to apoptosis, the infiltrated T cells in the spleen of symptomatic dogs represent a nonstructural nonfunctional granuloma, such as those observed in T cell deficient mice (Murray, 2001).

It is possible that apoptotic mechanisms may eventually develop into nonspecific T cell depletion, making dogs with VL more susceptible to other infections, especially ehrlichiosis and babesiosis. Such coinfections are frequently observed in VL symptomatic dogs (Oliveira et al., 2008) and, occasionally, neoplastic disease, especially hematopoietic tumors, are involved (Foglia Manzillo et al., 2008).

Observation of apoptosis in T lymphocytes from symptomatic naturally infected dogs, confirming studies in patients with acute visceral leishmaniais where CD4+ T cells from PBMC undergo significant levels of apoptosis (Potestio et al., 2004). Similarities observed in immunological response between naturally infected dogs and patients with acute VL suggest that the dog is an excellent model for studying new therapies.

Taken together, these data indicate that in infected dogs, the immunosuppression associated with chronic infection

is due to accelerated rates of T cell apoptosis and this mechanism could contribute to white pulp disorganization in the spleen and diminished T cell levels in peripheral blood. The present results could contribute to improving current understanding of the immune response in dogs infected with *L.* (*L.*) *chagasi*, while additional studies would further our understanding concerning apoptosis and other immune mediators in dogs naturally infected with this disease.

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