Evaluation of TNF-α, IL-4, and IL-10 and parasite density in spleen and liver of *L. (L.) chagasi* naturally infected dogs

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Dogs are the main domestic reservoirs of *L. (L.) chagasi*. Once in the vertebrate host, the parasite can cause visceral leishmaniasis, which can also be transmitted to humans. Cytokines are key elements of the host immune response against *Leishmania* spp. To investigate whether tumor necrosis factor (TNF)-α, interleukin (IL)-4 and IL-10 are associated with pattern infection in dogs, these cytokines were quantified in the spleen and liver of dogs naturally infected with *L. (L.) chagasi*, with or without clinical manifestations, and their levels were correlated with the parasite load verified in these organs. A total of 40 adult dogs naturally infected with *L. (L.) chagasi* were assessed, together with 12 uninfected control dogs. Samples from spleen and liver were used to determine the cytokine levels by capture ELISA and for quantifying parasite load by real-time PCR. Statistical analysis was performed using the minimum Chi square method and group means were compared using the Tukey test. TNF-α, IL-4 and IL-10 levels in infected dogs were higher than in control groups; the liver was the main cytokine-producing organ during infection. The level of splenic TNF-α showed correlation with parasite load and may represent an important marker for infection process evolution, with the participation of IL-10. These results may contribute to a clearer understanding of the immune response in dogs infected with *L. (L.) chagasi*, which may lead to the development of prophylactic or preventive measures for these animals.

INTRODUCTION

Leishmaniasis is caused by a protozoan of the Trypanosomatidae family, genus *Leishmania*. It occurs in 88 countries, 65 of which also 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, as in other countries in South America, migration into urban areas contributed to the expansion of VL (Desjeux, 2004) and this expansion occurred especially in the northeastern (Dantas-Torres, 2006) and southeastern regions (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 emerge when associated with malnutrition (Gontijo and Melo, 2004) and HIV/AIDS infection (Ashford, 2000).

Dogs are considered to be the main domestic reservoirs of *L. (L.) chagasi* (Moreno and

Alvar, 2002). The parasite is transmitted from one infected dog to another through the phlebotomine bites and possibly through other arthropod vectors, such as fleas and ticks (Coutinho *et al.*, 2005; Ferreira *et al.*, 2009; Dantas-Torres *et al.*, 2010) and through blood transfusions (Owens *et al.*, 2001). After the phlebotomine feed on the dog's 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 reach the reproductive organs, skin, digestive and respiratory systems and the bladder (Molyneux and Ashford, 1983; Ashford, 2000).

Once in the vertebrate host, the parasite can cause lesions and symptoms that are characteristic of canine visceral leishmaniasis (CVL), although some infected dogs may be oligosymptomatic or asymptomatic (Mancianti and Meciani, 1988), and some can evolve to spontaneous cure (Fisa *et al.*, 1999).

The most frequent signs of VL are lymphadenopathy, onychogryphosis, cutaneous lesions, weight loss, cachexia and locomotor abnormalities (Semião-Santos *et al.*, 1995). The asymptomatic form represents 20 to 40% of the serum-positive population, of which 80% actually develops the disease (Noli, 1999).

Cytokines have been recognized as key elements of the host immune response against *Leishmania* spp. Parasitized macrophages, lymphocytes, dendritic cells and natural killers produce cytokines that are involved with both the inflammatory and adapted responses to the parasite (Sacks and Sher, 2002; Brodskyn *et al.*, 2003).

It is known that cell-mediated immunity is able to control infection, leaving some dogs asymptomatic (Santos-Gomes *et al.*, 2002). The occurrence of dogs asymptomatic for VL has been associated with the activation of Th1 cells that produce interferon (IFN)-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-α (Pinelli *et al.*, 1994; Pinelli *et al.*, 1995). *Leishmania* parasites are killed by macrophages activated by IFN-γ producing lymphocytes

via a nitric oxide dependent mechanism (Vouldoukis *et al.*, 1996; Holzmuller *et al.*, 2005). However, other more recent studies have verified that both asymptomatic and symptomatic dogs produce cytokines belonging to Th1 and Th2 profiles, and that these coexist in CVL (Corrêa *et al.*, 2007), but cell mediated immunity observed in *L. infantum* infected asymptomatic dogs depended on the preferential expression of Th1 cytokines (Chamizo *et al.*, 2005).

The few studies that evaluated the effecter role of TNF-α have used infected dogs as models. In symptomatic dogs infected with *L. (L.) chagasi*, a correlation between the level of TNF-α in the serum and the active disease has not been identified (Lima *et al.*, 2007).

Expression of IL-4 has been detected in the bone marrow of dogs naturally infected with *L. (L.) chagasi*, specially in those more severely affected, which suggests an association between IL-4 and the disease (Quinnell *et al.*, 2001). Another study conducted with dogs infected with *L. (L.) infantum*, showed an increase in IL-4 expression in mononuclear cells of the peripheral blood after six months infection. This expression was quantitatively similar in both asymptomatic and symptomatic dogs (Manna *et al.*, 2006).

IL-10 has been indicated as one of the mainsuppressing cytokines of the protective immune response, both in murine models and in humans with VL (Barral *et al.*, 1993; Bacellar *et al.*, 2000). However, more recently, studies have indicated that the anti-inflammatory effects of IL-10 may limit the side effect caused by an excessive inflammatory response, while also interfering with the process of parasite elimination (Trinchieri, 2007).

To investigate whether TNF-α, IL-4 and IL-10 is associated with pattern infection in dog, these cytokines were quantified in the spleen and liver of dogs naturally infected with *L. (L.) chagasi*, with or without clinical manifestations, and their levels were correlated with the parasite density load verified in these organs.

ANIMALS AND METHODS

The Study Area

The county of Araçatuba ($21°12'32''S$; $50°25'38''W$), with an area of 1 167 311 km^2 (450 701 mi^2), is located in the state of São Paulo. It is a region known to be endemic for CVL (Nunes *et al.*, 2008).

Animals

A total of 40 adult dogs, aged between 2 and 4 years old, males and females, of undefined breed and of different weights, from the Zoonosis Control Center of Araçatuba, were serum positive for *L. (L.) chagasi* by indirect ELISA (Lima *et al.*, 2003).

The canines were divided in two groups: one group with 20 asymptomatic dogs; the other group with 20 symptomatic dogs showing at least three clinical signs of CVL. These could include fever, dermatitis, lymphadenopathy, onychogryphosis, weight loss, cachexia, locomotor abnormalities, conjunctivitis, epistaxis, hepatosplenomegaly, edema and apathy.

A group of 12 healthy dogs, also aged between 2 and 4 years old, both males and females, of undefined breed and of different weights, from a non-endemic area (Londrina, Paraná State, Brazil) were included in the study as negative controls. These dogs were serum negative for *L. (L.) chagasi* by indirect ELISA, according to the protocol described by Lima *et al.* (2003), and were PCR negative for this protozoan species.

The present research received approval from Research Ethics Committee for the use of Experimental Animals of the Faculdade de Odontologia de Araçatuba, Universidade Estadual Paulista (Brazil) protocol no. 57/06.

Sample Collection

Dogs were euthanized as follows: initially they were administered a tranquilizer with 0.05 mg/kg acepromazine maleate *i.v.*, followed by a 15-minute rest period and fast injection (20 seconds) of 15 mg/kg of sodium thiopental *i.v.*, followed by 10 ml of 19.1% potassium chloride through the same route. Total blood samples were collected for serological analysis, together with samples of spleen and liver, which were used to determine cytokine levels and for quantifying the parasite load.

Spleen and Liver Extracts

Extracts from spleen and liver were collected for quantitative analysis of TNF-α, IL-4 and IL-10 cytokines by capture-ELISA. To achieve this, 1 g of either spleen or liver and 2 ml of RPMI-1640 media (Sigma, St. Louis, MO, USA), pH 7.2, were used. The samples were refrigerated and grounded in a tissue homogenizer (Ultra Turrax T 8, Staufen, Germany) for about 5 minutes. The resulting homogenate was centrifuged at $10\,000 \times \textbf{\textit{g}}$ for 15 minutes at $4°C$ (Eppendorf 5810 R, Hamburg, Germany) and the supernatant was immediately stored at $-80°C$ (Revco, Golden Valley, MN, USA).

TNF-α, IL-4 and IL-10 Quantitative Analysis

The TNF-α, IL-4 and IL-10 cytokines from the spleen and liver extracts were quantified using anti-canine monoclonal antibody (mAb) and polyclonal antibody biotinylated (R&D System, Minneapolis, MN, USA). Canine recombinant TNF-α, IL-4 and IL-10 (R&D System, Minneapolis, MN, USA) were used to produce a standard curve. The ELISA assay was performed in accordance with the manufacturer's instructions. The test was developed with 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma, St. Louis, MO, USA). Plates were read with a Spectra Count apparatus (Pachard Bio Science Company, Piscataway, NJ, USA) using a 450 nm filter. The minimal sensitivity for detecting TNF-α was 3.12 pg/50 μl, for IL-4 was 78 pg/50 μl, and for IL-10 was 90.65 pg/50 μl.

Parasite Load

Parasite density was determined using real-time PCR following the protocol described by

Rolão *et al.* (2004). To achieve this, 35 mg of spleen or liver from each dog were used for DNA extraction with a commercial kit (PCR template Preparation Kit, Roche Diagnostics GmbH, Mannheim, Germany), in accordance with the manufacturer's instructions.

The promastigote form of *L. (L.) chagasi* was used to create the standard curve. Quantitative analysis of parasites was performed by counts with a Neubauer hemocytometer chamber (average of four counts). Parasite suspension was diluted in series of 10, varying from 1×10^6 to 10^{-1} parasites. These dilutions were then used for DNA extraction, as described above. Primers and internal probes were designed according to regions of the kinetoplast minicircle of *L. (L.) chagasi* (GenBank accession number AF169140), in accordance with Rolão *et al.* (2004). DNA samples were analyzed with the following (Applied Byosystems, Foster City, CA, USA): direct primer LshNRf (forward, 5′-GGTTAGCCGATGGTGG-TCTT-3′), reverse LshNRr (5′-GCTAT-ATCATATGTCCAAGCACTTACCT-3′), and internal probe LshNRp TaqMan® (5′-ACCACCTAAGGTCAACCC-3′). PCR was performed inan Icycler iQ thermocycler (Bio-Rad Laboratories, CA, USA). An aliquot of 2 µl of each DNA sample was added to a mix containing 10 µl of TaqMan Universal Master Mix, No AmpErase® UNG (Applied Biosystems, Foster City, CA, USA) and 2 µl of primers (900 nM) and TaqMan MGB probe (FAM^{TM} fluorophore) (200 nM) in a 20 µl final volume. Samples were assayed in duplicate and a negative control was included in each assay. PCR conditions were: 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds and 60°C for 60 seconds. The detection threshold was determined as the mean of the base line plus 10 x the standard deviation, representing the background level calculated from cycles 2 to 10.

Statistical Analysis

The variables studied (TNF-α, IL-4, and IL-10 levels and parasite density in the

FIG. 1. Optical density of serum samples tested by the enzyme immunoassays using crude protein derived from promastigotes of *Leishmania (L.) chagasi*, in the diagnosis of canine leishmaniasis in Araçatuba, SP, Brazil. The straight line represents mean of each group.

spleen and liver) were analyzed with the minimum Chi square method which considers the group effect. Group means were compared using the Tukey test, in which the data are tested for normality and homogeneity of variance, since these are prerequisites for this type of analysis. Correlation analysis was performed between the values for TNF-α, IL-4, IL-10 and the values corresponding to the parasite load in each group. Regression analysis was performed for parasite load in the spleen and liver, in each group. The results were considered significant when $P<0.05$. SAS (SAS 9.1 SAS Institute, Cary, NC, USA) was used for all statistical analysis performed in this study.

RESULTS

Production of Anti-*L. (L.) chagasi* Antibodies

The production of anti-Leishmania IgG antibodies was evaluated in 20 asymptomatic and 20 symptomatic dogs naturally infected by *L. (L.) chagasi*. The mean titers of anti-Leishmania IgG antibodies in asymptomatic (OD=1.191±SD 0.431) and symptomatic (OD=1.414±SD 0.484) were similar (Fig. 1), and no statistical difference was observed in anti-Leishmania antibody

production. In contrast, no serum reactivity occurred against this total antigen in 12 dogs from the non-endemic area (OD= $0.138 \pm$ SD 0.033) Student t test, $P < 0.0001$ (Fig. 1).

Quantitative Analysis of Cytokines

The mean TNF-α production was higher in the liver extracts, in both the asymptomatic and symptomatic groups compared to that from the spleen ($P < 0.05$). Similar results were observed for IL-4 and IL-10 (Table 1).

Observation also verified that the mean value of TNF-α in liver extracts from symptomatic dogs was significantly higher compared to that of asymptomatic dogs ($P < 0.05$); however, this difference was not observed in the spleen extract from dogs in the two groups. The mean value of IL-4 in the spleen and liver of asymptomatic dogs revealed no significant difference compared to the group of symptomatic dogs. Similar observations were obtained for IL-10 levels. The mean value determined in infected dogs, asymptomatic or not, both in the spleen and in the liver, was higher for the three cytokines tested, than those from the liver and spleen of control dogs ($P < 0.05$) (Table 1).

Quantitative Analysis of Parasite Load

The mean value of the parasite load from the spleen of asymptomatic and symptomatic dogs was higher than the parasite load from the liver of dogs in these two groups ($P < 0.05$). The mean value of the parasite load in asymptomatic dogs showed no difference compared to that from the spleen of symptomatic dogs, although a significant increase in the liver of symptomatic dogs was observed compared to that from the liver of asymptomatic dogs ($P < 0.05$) (Table 1).

Correlation Between Cytokine Levels (TNF-α, IL-4 and IL-10) and the Parasite Load by Pearson's Linear Correlation Coefficient

The results showed that a weak positive linear correlation existed between the levels of TNF-α in the spleen and the parasite load observed

TABLE 1. *Means* ($\bar{x}$) and standard deviation (s) of TNF-α, IL-4 and IL-10 in spleen and liver extracts compared to the parasite load verified in these organs, in both asymptomatic (n=20) and symptomatic (n=20) dogs naturally infected with *L. (L.) chagasi* compared to healthy dogs (n=12)

Variable	Group	Organ ($\bar{x} \pm s$)	
		Spleen	Liver
TNF-α (pg/ml)	Asymptomatic	0.174 ± 0.073 aB	0.599 ± 0.237 bA
	Symptomatic	0.183 ± 0.060 aB	0.763 ± 0.214 aA
	Control	0.041 ± 0.013 bA	0.146 ± 0.040 cA
IL-4 (pg/ml)	Asymptomatic	21.014 ± 3.679 aB	69.769 ± 16.894 aA
	Symptomatic	22.319 ± 6.009 aB	75.474 ± 23.283 aA
	Control	8.811 ± 2.637 bB	23.851 ± 7.472 bA
IL-10 (pg/ml)	Asymptomatic	7.178 ± 5.897 aB	99.774 ± 39.336 aA
	Symptomatic	9.584 ± 8.789 aB	107.532 ± 35.742 aA
	Control	0.723 ± 0.488 bB	26.059 ± 9.152 bA
Parasite load (log)	Asymptomatic	2.633 ± 1.138 aA	1.950 ± 1.150 bB
	Symptomatic	2.644 ± 1.242 aA	2.383 ± 1.288 aB

Means followed by distinct letters, with small caps representing comparisons between groups in the same organs and large caps between organs in the same group, are those that differed according to the Tukey ($p < 0,05$).
a; **b** - difference between asymptomatic and symptomatic group.
c - difference between asymptomatic, symptomatic and control group.
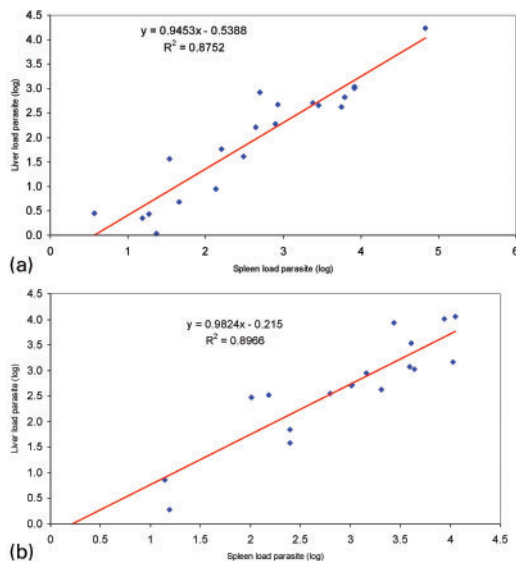
(a)

(b)

FIG. 2. Linear positive correlation between parasite load from DNA samples from spleen and liver from asymptomatic (a) and symptomatic (b) dogs naturally infected with *L. (L.) chagasi* ($P<0.0001$). (a) $r=0.93553$; (b) $r=0.94686$.

in this organ, in the group of symptomatic dogs. A weak positive linear correlation was also determined between the IL-10 level in the liver and the parasite load in the same organ, in asymptomatic dogs (Table 2).

Correlation Between Parasite Load in the Spleen and Liver by Pearson's Linear Correlation Coefficient

A strong positive linear correlation was observed between parasite load in the spleen and

liver in both groups studied; in asymptomatic dogs, r was 0.93553 and in symptomatic dogs r was 0.94686 (Fig. 2).

DISCUSSION

In this study, the cytokines of spleen and liver extracts from VL dogs from an endemic area were analyzed to investigate the role of these mediators in the infectious process of the disease. Analysis of the results verified that the TNF-α levels in the spleen and liver were higher in the two groups of infected dogs compared to those of healthy dogs. The higher levels verified in infected dogs indicate that the presence of *L. (L.) chagasi* induces an immune response with relevant expression of TNF-α. Confirming that TNF-α is produced when the protozoan is present, Carrillo *et al.* (2007) showed that in mononuclear leucocytes of peripheral blood of asymptomatic dogs infected with *L. (L.) chagasi* and stimulated with a soluble *Leishmania* antigen, an increase in the gene expression of TNF-α occurs.

In infected dogs, asymptomatic or not, the level of liver TNF-α was higher compared to the spleen from the same animals. These results are similar to those obtained with BALB/c mice infected with *L. (L.) donovani*, in which the level of liver TNF-α was higher than that observed in the spleen (Mukherjee *et al.*, 2003). In this study, observation

TABLE 2. *Correlation (r) between each cytokine and parasite load from spleen and liver of asymptomatic (N=20) and symptomatic (N=20) dogs naturally infected with* L. (L.) chagasi. *Presence of correlation is represented by* ★

Cytokine	Group	Organ (r)	
		Spleen	Liver
TNF-α	Asymptomatic	−0.361	−0.075
	Symptomatic	0,353 ★★	−0.231
IL-4	Asymptomatic	−0.188	0.098
	Symptomatic	−0.090	−0.100
IL-10	Asymptomatic	0.204	0.448★

★ $p<0.05$ significant correlation between parasite load in organ and cytokine.
★★ $p<0.1$ significant correlation between parasite load in organ and cytokine.

verified that the level of TNF-α was higher in the liver of the symptomatic dogs than in the asymptomatic. Similarly, Panaro *et al.* (2010) showed that TNF-α mRNA levels in peripheral blood mononuclear cell from dogs with natural *Leishmania infantum infection* were significantly increased in symptomatic *versus* asymptomatic. This increase in hepatic TNF-α was associated with an increase in the parasite load in this organ; in fact, histological and biochemical changes that take place in the liver during the course of the disease increase the parasite load in this organ, in dogs naturally infected by *L. (L.) chagasi* (Giunchetti *et al.*, 2008).

No difference in the level of TNF-α was observed when comparing the spleen of asymptomatic and symptomatic dogs. This can be explained by the ability of the spleen to express high levels of this cytokine, as TNF-α mediation is required for spleen tissue remolding when the parasite persists in this organ, as seen in mice infected with *L. (L.) donovani* (Engwerda *et al.*, 1998). These results are similar to those reported by Lage *et al.* (2007) in which no difference in the level of TNF-α in spleen cells was observed between symptomatic and asymptomatic dogs naturally infected with *L. (L.) chagasi.*

Similar to the results obtained for TNF-α, a significant statistical difference was also verified when values of IL-4 from spleen and liver from infected dogs were compared to those from non-infected canines. Similar results were observed in a study involving oligosymptomatic dogs experimentally infected with *L. (L.) infantum*, in which the level of IL-4 in the spleen was higher than that from non-infected dogs (Strauss-Ayali *et al.*, 2007). Additionally, levels of IL-4 in dogs naturally infected with *L. (L.) chagasi* were shown to be higher than those of non-infected dogs (Lage *et al.*, 2007). Compared to the levels verified in healthy canines, the increased level of IL-4 observed in the spleen of infected dogs indicates that this cytokine plays a key role in this infectious process. The persistence of the parasite in the spleen of dogs infected with *L. (L.) infantum* may be associated with the high levels of IL-4

(Strauss-Ayali *et al.*, 2007). A recent study conducted on mononuclear cells from the peripheral blood of dogs experimentally infected with *L. (L.) infantum* suggests that the lack of IL-4 expression, combined with a lack of IL-13 at the onset of the infectious process, may delay parasite multiplication (Sanchez-Robert *et al.*, 2008). The level of IL-4 was higher in the liver compared to the level verified in the spleen in the two groups of infected dogs. This result is different than that from other reports. In studies conducted on BALB/c mice infected with *L. (L.) donovani* (Mukherjee *et al.*, 2003; Rolão *et al.*, 2007), the level of IL-4 in the spleen was higher than that in the liver, during the course of infection. The difference between the two studies can be explained by the animal model used in each study and by the fact that the dogs in this study were naturally infected and therefore no information was available regarding when infection started or what moment the examination represented during the course of the disease.

No difference was detected between the levels of IL-4 in the spleen and liver of infected dogs in both groups. Similar results, regarding the levels observed in the spleen, were also reported by Lage *et al.* (2007). Lack of difference for this cytokine in dogs naturally infected with *(L.) infantum*, whether asymptomatic or not, was also observed in the peripheral blood by other authors (Manna *et al.*, 2006).

The lack of association between the level of IL-4 in the spleen and the clinical status of the dogs suggests that the cytokine may not be relevant to the evolution of the infectious process, although it may still play an important role during establishment of the infection (Strauss-Ayali *et al.*, 2007).

No linear correlation was detected between parasite load in the spleen and liver and the level of IL-4 in these organs. The high level of IL-4 observed may not enough to explain the progression from asymptomatic dogs to the symptomatic condition. However, it could contribute to polyclonal B cell activation and hypergammaglobulinemia, typical of the

disease. Lack of correlation could be attributed to the fact that the level of resistance or susceptibility for infection is preferentially related to the capacity to produce IFN-γ, rather than the presence of IL-4, as observed by Lehmann *et al.* (2000) in mice infected with *L. (L.) donovani*. This result differs from that obtained with BALB/c mice experimentally infected with *L. (L.) infantum*, in which a weak correlation between IL-4 and the parasite load in the spleen and liver was detected (Rolão *et al.*, 2007).

Similar to that verified for TNF-α and IL-4, in this study, a statistically significant difference was observed when comparing IL-10 levels in the spleen and liver from infected dogs with levels from non-infected dogs. These results are similar to those observed by Alves *et al.* (2008) in a study involving dogs naturally infected with *L. (L.) chagasi*. Studies on humans and mice have shown association between infection susceptibility and elevated levels of IL-10 and TGF-β (Kane and Moser, 2001; Trinchieri, 2007).

The level of IL-10 was higher in the liver compared to that in the spleen in both groups of infected dogs. Studies conducted on BALB/c mice infected with *L. (L.) donovani* (Mukherjee *et al.*, 2003; Rolão *et al.*, 2007) showed that the levels of this cytokine in the liver were higher than those verified in the spleen during the course of infection. The production of IL-10 and TGF-β was also observed in the liver and spleen of asymptomatic and symptomatic dogs infected with *L. (L.) chagasi* (Corrêa *et al.*, 2007; Lage *et al.*, 2007),

In addition, the level of IL-10 in the liver of asymptomatic dogs showed a positive linear correlation with the level of parasite load in this organ. This suggests that IL-10 in the liver may be participating in the regulatory process that increases the parasite load and thus interferes with the evolution of the infection.

Using real-time PCR, a powerful tool for evaluating *Leishmania (L.) chagasi* loads (Da Silva *et al.*, 2010), a strong positive correlation between parasite load in the spleen and in the liver was observed in the two groups of infected dogs in this study. These results suggest that the immune response to control the spread of the parasite in both organs is regulated by similar mechanisms, possibly without IL-4 participation. Similar results were observed in mice experimentally infected with *L. (L.) donovani* (Mukherjee *et al.*, 2003) or with *L. (L.) infantum* (Rolão *et al.*, 2007).

The liver of symptomatic dogs showed a higher parasite load compared to that in asymptomatic dogs. This difference could be related to physiopathological characteristics of this organ. An occurrence of intralobular liver fibrosis in symptomatic animals may contribute to an increase in parasite load (Melo *et al.*, 2008). In addition, the spleen is more susceptible to *L. (L.) chagasi*: a higher parasite load was detected compared to the liver. This difference may be associated with the reduction in CD5 + lymphocytes. Guerra *et al.* (2009) demonstrated that dogs with high splenic parasitism presented a significant decrease in absolute counts in this population of T lymphocytes in comparison with dogs presenting medium splenic parasitism.

Analysis of the results suggests that TNF-α plays an important role in the pathological process of VL, especially in the liver of symptomatic dogs. The association between the level of TNF-α and an increase in the parasite load observed in this group is an important indicator of the process of the evolution of VL infection. Moreover, the increased levels of TNF-α, IL-4 and IL-10 in the liver from infected dogs, compared to healthy canines, attribute to these cytokines an important role in the pathogenesis of VL.

These results may contribute to a clearer understanding of the immune response in dogs infected with *L. (L.) chagasi*, which could help in the development of vaccines or drugs.

Considering that in domestic areas dogs are the main hosts of the parasite and that they can transmit VL to humans, any new

measure or treatment that benefits the canine population will most likely benefit humans.

Additional studies may add to current understanding regarding the role of cytokines and other immune mediators in dogs naturally infected with *L. (L.) chagasi*.

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