Rev. Inst. Med. Trop. Sao Paulo
2016;58:48
http://dx.doi.org/10.1590/S1678-9946201658048

ORIGINAL ARTICLE

In situ CUTANEOUS CELLULAR IMMUNE RESPONSE IN DOGS NATURALLY AFFECTED BY VISCERAL LEISHMANIASIS

Claudio Nazaretian ROSSI(1), Thaise Yumie TOMOKANE(2), Luis Fábio da Silva BATISTA(3), Mary MARCONDES(4), Carlos Eduardo LARSSON(1) & Márcia Dalastra LAURENTI(2)

SUMMARY

Thirty-eight dogs naturally affected by visceral leishmaniasis were recruited in *Araçatuba, São Paulo* State, Brazil – an endemic area for visceral leishmaniasis. The animals were distributed into one of two groups, according to their clinical and laboratory features, as either symptomatic or asymptomatic dogs. Correlations between clinical features and inflammatory patterns, cellular immune responses, and parasitism in the macroscopically uninjured skin of the ear were investigated. Histological skin patterns were similar in both groups, and were generally characterized by a mild to intense inflammatory infiltrate in the dermis, mainly consisting of mononuclear cells. There was no difference in the number of parasites in the skin (amastigotes/mm²) between the two groups. Concerning the characterization of the cellular immune response, the number of positive inducible nitric oxide synthase (iNOS$^+$) cells was higher in the dermis of symptomatic than in asymptomatic dogs ($p = 0.0368$). A positive correlation between parasite density and macrophages density ($p = 0.031$), CD4$^+$ T-cells ($p = 0.015$), and CD8$^+$ T-cells ($p = 0.023$) was observed. Furthermore, a positive correlation between density of iNOS$^+$ cells and CD3$^+$ T-cells ($p = 0.005$), CD4$^+$ T-cells ($p = 0.001$), and CD8$^+$ T-cells ($p = 0.0001$) was also found. The results showed the existence of a non-specific chronic inflammatory infiltrate in the dermis of dogs affected by visceral leishmaniasis, characterized by the presence of activated macrophages and T-lymphocytes, associated to cutaneous parasitism, independent of clinical status.

KEYWORDS: Canine visceral leishmaniasis; Cutaneous lesion; Histopathology; Cellular immune response.

INTRODUCTION

Visceral leishmaniasis (VL) is a zoonotic disease caused by a protozoon of the genus *Leishmania.* In Brazil, *Leishmania (Leishmania) infantum* [syn. *Leishmania* (*Leishmania) chagasi*] is considered the etiologic agent of the disease; its transmission amongst vertebrate hosts primarily occurs through the bite of the insect vector *Lutzomyia* longipalpis[1].

Epidemiologically, domestic dogs (*Canis familiaris*) are considered the main domestic reservoir of the parasite, since both symptomatic and asymptomatic infected dogs are highly able to transmit the protozoa to its natural vector[2]. The skin is the tissue through which the etiological agent is delivered at the moment of a sand fly bite[3]; it is also one of the main sources of parasites to the vectors[4]. However, little is known about the mechanisms underlying the parasite resistance in the skin of animals affected by VL, which is an important parasite reservoir tissue, both in clinically healthy and sick infected dogs[5]. Apparently, the ears represent the most frequently infected tegumentar areas, probably due to its lower hair density and cerumen attractiveness, leading to an increased exposure to the insect vector's bite[6].

Regarding the skin histopathological pattern observed in dogs with leishmaniasis, the occurrence of superficial or deep perivascular mononuclear dermatitis is frequently observed[7,8]. However, periglandular dermatitis; lichenoid, nodular, or pustular dermatitis; lobular panniculitis; suppurative folliculitis; and necrotizing vasculitis may also occur, though these are much rare[9]. Regarding the occurrence of cutaneous parasitism and its possible correlation with cutaneous histologic patterns in infected dogs, some studies have found that the type of inflammatory infiltrate present in the skin of parasitized animals may reflect the profile of the host's systemic immune response against the parasite; therefore, this infiltrate can be considered a morphological marker of the host's susceptibility or resistance to infection[10]. Some reports have also suggested that there may be a relationship between clinical symptoms and skin histological patterns; as such, the understanding of immunopathological aspects of the skin in infected animals may also help to understand the relationship between the presence of infection and its transmission[9].

(1) University of Sao Paulo, Department of Clinics, School of Veterinary Medicine and Animal Science. *São Paulo, SP,* Brazil.
(2) University of Sao Paulo, Department of Pathology (LIM-50), Medical School. *São Paulo, SP*, Brazil.
(3) University of Sao Paulo, Department of Pathology, School of Veterinary Medicine and Animal Science. *São Paulo, SP*, Brazil.
(4) Sao Paulo State University, Department of Animal Health and Production, School of Veterinary Medicine. *Araçatuba, SP*, Brazil.
Correspondence to: Márcia Dalastra Laurenti, Departamento de Patologia, Faculdade de Medicina, Universidade de São Paulo. Av. Dr. Arnaldo 455, 1° andar, sala 1209, Cerqueira César 01246-903 São Paulo, SP, Brasil.
Tel/Fax: +55 11 30618339. E-mail: mdlauren@usp.br

Canine VL (CanL) can be regarded as an immune-mediated disease due to the parasite's ability to modify the immune system of the parasitized host[11]. Since *Leishmania* is an obligate intracellular parasite, the host's defenses are highly dependent on cell-mediated immunity, with a central role played by $CD4^+$ T-cell subsets, which are able to produce Th1 and Th2 cytokines[12]. The effective response against the parasite is based on Th1- type response, and the resistance to infection is dependent on the ability of $CD4^+$ T-cells to secrete interferon gamma (IFN-γ)[12,13]. This Th1 cytokine profile induces macrophage activation[13], resulting in the production of nitric oxide (NO) by the enzyme inducible nitric oxide synthase (iNOS), which appears to play an important role in the control of *Leishmania* infection in dogs[14,15]. Concurrently, $CD8^+$ T-lymphocytes are also important in the development of the immune response and are considered essential for the immunity to infection in dogs[16]. Therefore, the aim of the present study was to correlate the clinical features in dogs that are naturally affected by VL with the skin histological pattern, as well as to determine the association between the *in situ* inflammatory response and the intensity of cutaneous parasitism.

MATERIALS AND METHODS

This project was approved by the Ethics Committee on Animal Use of the School of Veterinary Medicine and Animal Science, University of *São Paulo,* under the protocol number 2649/2012. The procedures were performed in accordance with the guidelines of the Brazilian College of Animal Experimentation.

Studied population and samples

Dogs destined for euthanasia after CVL diagnosis, in compliance to a Brazilian federal law, at the Center for Zoonosis Control, *Araçatuba, São Paulo* State, Brazil, a high LV-endemic area constituted the canine population used in this study. Thirty-eight stray mongrel dogs of different ages and sexes naturally affected by CVL, confirmed by parasitological diagnosis on lymph node smears, were enrolled in this study. Following clinical evaluation, animals were anesthetized with intravenous sodium pentobarbital (25 mg/kg; Fontoveter, *Campinas*, Brazil); blood was then obtained by cardiac puncture to perform serology and biochemical analysis, and a biopsy from macroscopically uninjured skin of the central region of the right ear was collected using a 6 mm punch for histological and immunohistochemistry studies. According to the Brazilian program for VL control implemented by the Ministry of Health, infected dogs were euthanized via intravenous injection of 19% potassium chloride (Darrow, *Rio de Janeiro*, Brazil). Skin biopsies of dogs (n = 6) from a non-endemic VL region were used as negative controls.

Visceral leishmaniasis diagnosis

After the clinical evaluation and the laboratory exams were performed, dogs were divided into two groups: symptomatic (n = 24) dogs presenting clinical signs of leishmaniasis such as weight loss, lymphadenomegaly, hepatosplenomegaly, pale mucous membranes, skin lesions, onychogryphosis, epistaxis, ocular lesions, as well as laboratory abnormalities suggestive of VL such as hyperproteinemia and high serum creatinine levels, and asymptomatic (n = 14) dogs without visible clinical signs, with serum protein < 8.5 mg/dL, and serum creatinine within normal limits; according to the International Renal Interest Society (IRIS, 2006) guidelines.

Parasitological diagnosis was based on cytological examination of popliteal lymph node samples stained with fast Romanovsky-type dye (Panoptico Rápido®; Laborclin, *Paraná*, Brazil) and observed under an optical microscope at 100× magnification for the presence *Leishmania* amastigotes. Dogs were also evaluated by serology for the detection of anti-*Leishmania* antibodies through an enzyme-linked immunosorbent assay (ELISA) using the total *Leishmania (L.) infantum* antigen (MHOM/72/BH46) and the alkaline phosphatase anti-canine immunoglobulin (Ig)G conjugate, as previously described[17].

Inflammatory pattern of skin according to histological examination

Skin fragments obtained by excisional biopsy of the right ear were fixed in a 10% formaldehyde buffered solution with 0.01 M phosphate, and processed by standard histological techniques. Paraffin sections were hematoxylin-eosin (HE) stained for histological examination by light microscopy in order to determine the presence and the degree of parasitism, as well as the characteristics of the inflammatory process in the skin tissue of the ear. Regarding the histopathological study, a semi-quantitative analysis of the HE-stained sections was performed according to Solano-Gallego *et al*., 2004, and results were assigned according to the intensity of the different characterized processes: (–) negative for absence; (+) mild -1 to 10; (++) moderate - 11 to 30; and (+++) intense > 30 parasites or cell types[9].

Skin parasitism and cellular response assessed by immunohistochemistry

Immunohistochemistry was performed to quantify the tissue parasitism and cellularity of the skin immune response using antibodies against *Leishmania* (produced in the Laboratory of Pathology of Infectious Diseases FM-USP, *São Paulo*, Brazil), anti-CD3 (DakoCytomation® A0452; Dako Denmark A/S, Glostrup, Denmark), anti-CD4 (Novocastra® NCL-L-CD4-368; Leica Microsystems, Wetzlar, Germany), anti-CD8 (Novocastra® NCL-L-CD8-295; Leica Microsystems), anti-NOS2 (NOS2® SC-651; Santa Cruz Biotechnology Inc., Dallas, TX, USA), and anti-macrophage (Serotec® MCA874G; AbD Serotec, Kidlington, UK).

The immunohistochemical reaction for the detection of parasites was performed on paraffin-embedded tissue sections using polyclonal anti-*Leishmania* antibodies produced in mice and diluted to 1:500 in phosphate buffered saline (PBS) (0.01 M) containing 1% bovine serum albumin (BSA), and LSAB kit (Dako Denmark A/S) according to Moreira *et al.*[18] (2007). To control the immunohistochemical reaction, sparse to moderate *Leishmania*-parasitized dog tissues were used as positive controls, and the omission of the primary antibody was used as the negative control[18].

To evaluate the cellular immune response of the skin, tissues were deparaffinized and hydrated; followed by antigen recovery using a citrate buffer (10 mM, pH 6.0) at 95 °C for 30 minutes in a water bath. After blocking endogenous peroxidase with a 3% hydrogen peroxide solution, as well as a non specific link with 6% PBS and a 0.15 M skim milk solution (Molico®; Nestlé, *São Paulo*, Brazil), primary antibodies were applied (anti-CD3 and anti-macrophage at 1:50 and 1:100 dilutions, respectively). For the development of the reaction, LSAB kit (DakoCytomation® K0690; Dako Denmark A/S) was used followed by chromogenic substrate DAB+H_2O_2 (diaminobenzidine with hydrogen

peroxide; DakoCytomation® K3468; Dako Denmark A/S). Sections were counterstained with Harris hematoxylin; after dehydration, slides were mounted with resin and glass slides. For the development of anti-NOS2, anti-CD4, and anti-CD8 antibody reaction, Novolink kit (Novocastra® RE7280-K; Leica Microsystems) was used. Antigen recovery for anti-CD4 antibody was performed using EDTA buffer (1 mM, pH 8.0); wash buffer consisted of TRIS (0.05 M, pH 7.2). For anti-CD8 antibody, Tris-EDTA buffer (10 mM Tris Base, 1 mM EDTA; pH 9.0) was employed.

Tissue parasitism and immunostained cell density were assessed by quantitative morphometric analysis using a light microscope (Axioskop 2 plus®; Carl Zeiss Meditec AG, Jena, Germany) connected to a microcomputer. To this end, 10 fields of each histological slice, which represented one animal, were photographed with a 40X objective. The amount of parasites and immunostained cells were counted using the AxionVision 5.0® software (Carl Zeiss Meditec AG). Subsequently, the average number of amastigotes and immunostained cells per square millimeter in each section were calculated.

Statistical analysis

Statistical analysis was performed using the PRISM 5 for Windows®; (GraphPad Software, Inc., La Jolla, CA, USA). To assess the correlation between clinical status (asymptomatic and symptomatic), cellular immunity, and skin parasite load, the Spearman's correlation test was performed. The nonparametric Mann-Whitney *U* test was used to evaluate differences related to the clinical characterization of cellular immune response and the parasitism of the dogs' skin. Differences were considered significant when $p < 0.05$.

RESULTS

Clinical, parasitological, and serological diagnosis

Based on the clinical presentation, the distribution of the thirty-eight evaluated dogs according to age and sex is shown in Table 1.

Among the symptomatic dogs, the most frequently observed clinical manifestations were lymphadenomegaly (95.8%), cutaneous lesions (91.7%), splenomegaly (66.7%), weight loss (62.5%), hepatomegaly and onychogryphosis (54.2%), alopecia and exfoliative dermatitis (68.2%). Considering the biochemical features, 63% (15/24) of symptomatic animals showed increased serum creatinine (> 1.4 mg/dL) and 54% (13/24) had hyperproteinemia (> 9.0 g/dL), while asymptomatic dogs showed serum protein and creatinine levels within the normal limits. According to the serological diagnosis, 36/38 (94.7%) of the infected dogs were reagent by ELISA; one symptomatic (4.2%) dog and one asymptomatic (7.1%) dog was seronegative. The mean of serological titers were higher in symptomatic when compared to the asymptomatic dogs ($p = 0.0411$). As expected, all the control dogs, from a non-endemic VL area were seronegative and *Leishmania* amastigotes were not identified in their lymph node smears.

Histological evaluation of the skin inflammatory pattern

Histological changes in the skin tissue of the ear were characterized by the presence of inflammatory infiltrates in the superficial and perianexal dermis, composed mainly of mononuclear cells, such as macrophages, lymphocytes, and plasma cells. The inflammatory infiltrate varied from mild to intense, and from focal to diffuse (Fig. 1). The histological pattern of healthy skin did not show any tissue damage or parasitism in 25% (6/24) of symptomatic and 36% (5/14) of asymptomatic animals. In spite of the fact that an inflammatory reaction had been observed in 18/24 (75%) of the symptomatic dogs, the presence of *Leishmania* sp. amastigotes was detected in only 11/24 (46%) of these animals; in

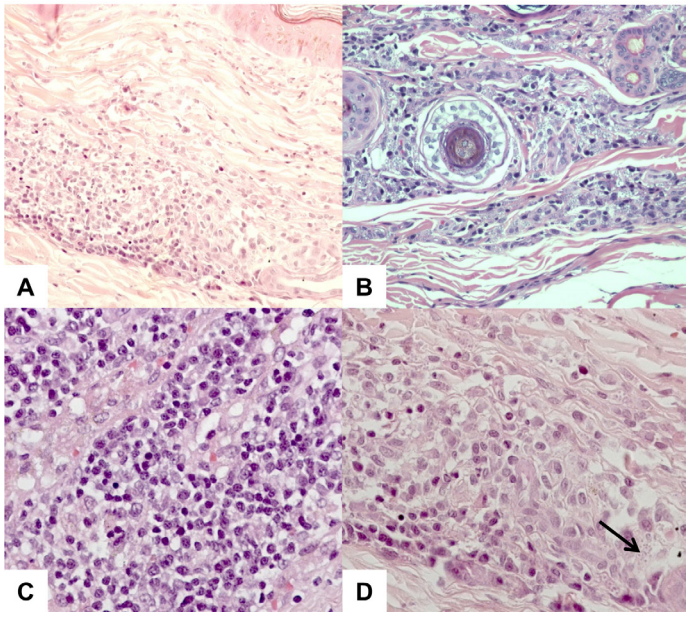

Fig. 1 - Histological section of the ear skin tissue stained by hematoxylin and eosin showing an intact epidermis and an inflammatory reaction in the dermis (A), a perianexal inflammatory infiltrate (B), a dermal infiltrate formed by mononuclear cells (C), and amastigotes inside the macrophages in the dermis (D) (arrow) in symptomatic dogs affected by visceral leishmaniasis (objective 20× A and B, 40× C and D).

Table 1
Number (%) of asymptomatic or symptomatic dogs affected by visceral leishmaniasis, according to age and gender

Clinical status	Gender N (%)		Age (months) N (%)		
	Male	Female	6-24	25-72	>72
Asymptomatic	6 (33)	8 (40)	5 (33)	6 (37.5)	3 (43)
Symptomatic	12 (67)	12 (60)	10 (67)	10 (62.5)	4 (57)
Total	18 (47)	20 (53)	15 (40)	16 (42)	7 (18)

the same way, skin inflammation was observed in 9/14 (64%) of the asymptomatic dogs, but skin parasitism was confirmed only in 5/14 (36%) animals by the histopathological examination of HE-stained sections (Table 2).

Skin parasitism and cellular immunity evaluation

The presence of parasites in the skin of the ear assessed by immunohistochemistry was observed in 14/24 (58%) of the symptomatic dogs and 5/14 (36%) of the asymptomatic ones. Although more parasites were observed in symptomatic dogs, the number of amastigotes/mm² (mean ± standard deviation) in the skin of asymptomatic (537.28 ± 378.75) and symptomatic (1336.37 ± 866.77) animals did not show a significant difference ($p = 0.1584$) (Figs. 2 and 3).

Regarding the *in situ* cellular immune response in the skin of the ear, and in spite of the average number of macrophages, it was found that the number of $CD3^+$, $CD4^+$, and $CD8^+$ T-cells was also higher in symptomatic animals than in asymptomatic animals; although no significant difference was observed between the clinical groups. The mean and standard deviation of the number of macrophages/mm² was 344.56 ± 51.81 and 249.18 ± 28.47 ($p = 0.2147$); of $CD3^+$ cells/mm² was 380.60 ± 114.55 and 243.12 ± 105.57 ($p = 0.1285$); of $CD4^+$ cells/mm² was 382.35 ± 84.65 and 146.08 ± 42.20 ($p = 0.0510$); and of $CD8^+$ cells/mm² was 130.33 ± 31.15 and 58.13 ± 25.40 ($p = 0.0500$) in symptomatic and asymptomatic, respectively (Figs. 2 and 3).

Finally, the density (cells/mm²) of the inducible nitric oxide (iNOS) cells was significantly higher in the dermis of symptomatic dogs when compared to asymptomatic ones ($p = 0.0368$). The mean and standard deviation of the $iNOS^+$ cells/mm² in symptomatic animals was 669.90 ± 114.07, while in asymptomatic dogs it was 346.43 ± 76.41 (Figs. 2 and 3).

Table 2
Semi-quantitative analysis of the inflammatory infiltrate and tissue parasitism in the ear skin biopsies of asymptomatic (n = 9) and symptomatic (n = 18) dogs that presented microscopic skin lesions due to visceral leishmaniasis

Animals	Skin inflammatory reaction								Parasite	
	Intensity		Localization		Celular type				HE	IHC
	Intense	Discrete	Focal	Diffuse	PMN	MAC	LY	PC		
A-108	-	+	+	-	-	+	+	-	+	+
A-112	+	-	+	-	-	++	+	-	-	-
A-113	-	+	+	-	-	+	-	-	-	-
A-114	-	+	+	-	-	+	-	-	-	-
A-115	-	+	+	-	-	+	+	+	+	+++
A-118	-	+	-	+	-	+	+	-	+	++
A-123	-	+	+	-	+	+	-	-	+	+++
A-124	-	+	+	-	-	+	+	-	+	-
A-144	-	+	-	+	-	+	+	+	-	+
S-102	-	+	+	-	-	+	+	-	+	+
S-103	+	-	-	+	-	++	+	+	-	-
S-104	+	-	-	+	+	++	+	+	+	++
S-105	-	+	+	-	-	+	+	+	+	+
S-106	+	-	-	+	-	+++	+	++	+	++
S-109	-	+	+	-	-	+	+	+	+	+
S-110	-	+	-	+	-	+	+	+	+	+++
S-111	-	+	+	-	-	+	+	-	-	-
S-116	-	+	+	-	-	+	-	-	-	-
S-119	+	-	-	+	-	+++	+	+	+	++
S-130	-	+	+	-	-	+	+	-	+	++
S-131	-	+	+	-	-	+	+	+	-	+++
S-134	+	-	-	+	-	++	+	+	+	++
S-138	-	+	+	-	-	+	+	-	-	++
S-139	+	-	-	+	-	+++	+	+	+++	+++
S-140	-	+	+	-	-	+	-	-	-	-
S-142	-	+	-	+	-	+	+	+	-	++
S-143	-	+	-	+	-	+	+	-	+	+++

A = Asymptomatic; S = Symptomatic; PMN = polymorphonuclear leukocyte; MAC = Macrophage; LY = Lymphocyte; PC = Plasma cells; HE = Hematoxylin Eosin staining; IHC = Immunohistochemical reaction; (-) negative; (+) discrete; (++) moderate; (+++) Intense.

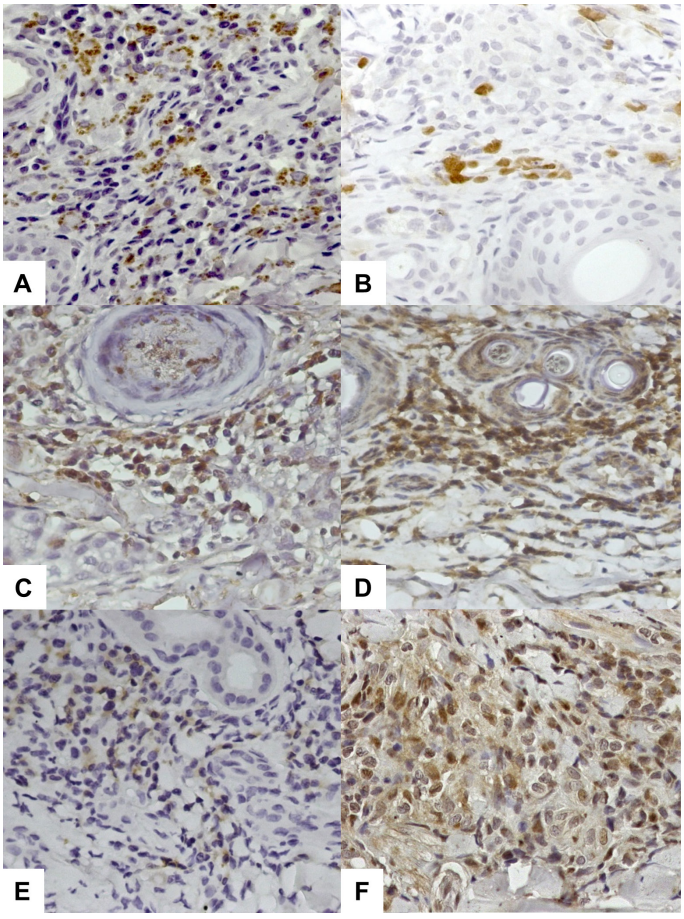

Fig. 2 - Histological section showing amastigote forms of parasites (A), macrophages (B), $CD3^+$ (C), $CD4^+$ (D), $CD8^+$ (E), and $iNOS^+$ (F) cells detected by immunohistochemistry in the ear skin tissue of symptomatic dogs affected by visceral leishmaniasis (objective 40×).

A moderate positive correlation was observed between the density of parasites and macrophages ($r = 0.3501$, $p = 0.031$), parasites and $CD4^+$ cells ($r = 0.4007$, $p = 0.015$), parasites and $CD8^+$ cells ($r = 0.3773$, $p = 0.023$). A moderate positive correlation was also observed between the densities of $iNOS^+$ and $CD3^+$ cells ($r = 0.4522$, $p = 0.005$), $iNOS^+$ and $CD4^+$ cells ($r = 0.5475$, $p = 0.001$), $iNOS^+$ and $CD8^+$ cells ($r = 0.5946$; $p = 0.0001$). There was a strong positive correlation between the densities of $CD3^+$ and $CD4^+$ cells ($r = 0.7738$, $p = 0.0001$), as well as $CD3^+$ and $CD8^+$ cells ($r = 0.7309$, $p = 0.0001$). The density of skin parasites showed a moderate positive correlation with anti-*Leishmania* antibody titers ($r = 0.3316$, $p = 0.042$).

DISCUSSION

The present study was designed to evaluate and compare the histological pattern, the parasite load, and the type of cellular infiltrate present in the skin of VL naturally infected dogs, correlating these elements with the dogs' clinical status. In spite of the fact that 22 animals (91.7% of the symptomatic dogs) had skin lesions, only a fragment of macroscopically uninjured skin collected from the right ear of all the 38 dogs was evaluated.

The most frequent clinical signs observed in the 24 symptomatic dogs were those related to the lymph nodes (lymphadenomegaly in 95.8%), skin (mainly characterized by alopecia and exfoliative dermatitis in 91.7%), and viscera, such as spleen (splenomegaly in 66.7%) and liver (hepatomegaly in 54.2%). These findings corroborated those from the literature, especially regarding the skin[7,8,19]. This tissue is considered, from a clinical point of view in CanL, as the most exposed and most visibly affected organ by the protozoan. When considering the sex, males and females had a homogeneous distribution, and neither a difference between clinical groups nor a correlation with parasitism or cutaneous cellular infiltrate was observed. The evaluated population had a predominance of young adult dogs, as is expected in an endemic area where euthanasia is the main measure for LV control. As euthanized dogs are usually replaced by their owners by younger dogs, the dog's population turnover rate increases, leading to a younger population that might be more susceptible to a variety of other infectious diseases in addition to CanL[20].

Although the parasite was observed in the lymph nodes of all the 38 symptomatic and asymptomatic animals by direct parasitological examination, the parasite detection in the skin was only confirmed in 58.3% of symptomatic (14/24) and 35.7% of asymptomatic (5/14) dogs by immunohistochemistry. Our results differed from those described by Lima *et al*.[21] (2010), who found higher densities of parasites in the skin rather than in the lymphoid organs, regardless of the clinical status of the dogs. In our study, the number of amastigotes/mm² detected in the macroscopically uninjured ear skin did not show a significant difference between symptomatic and asymptomatic dogs ($p = 0.1584$), confirming the observations of Madeira *et al*.[22] (2004). However, these data diverge from those of other studies, which showed higher parasite load in symptomatic than in asymptomatic animals[23,24]. It is worth highlighting that Solano-Gallego *et al*.[9] (2004) documented the presence of cutaneous parasitism only in symptomatic animals. These controversial results could be attributed to the time of disease progression, since cutaneous signs are usually seen in a late stage as has been shown in a prospective study that evaluated clinical signs in dogs naturally infected by *L. infantum*[25]. Our data are related to a cross sectional study without reference to the time of infection evolution. Moreover, the period of the year in which the samples were taken may have influenced the results, especially in the studies conducted in the Mediterranean region where the transmission is more seasonal[26]. However, the host's immunological and genetic background, has probably the major role in the development of clinical disease.

In spite of the fact that there is no significant difference in the parasite load found in skin of the ear between the two clinical groups, serological titers were higher in symptomatic when compared to asymptomatic dogs ($p = 0.0411$). In addition, a positive correlation between cutaneous parasitism and anti-*Leishmania* IgG antibody titers was observed ($r = 0.3316$, $p = 0.042$), which has been previously reported by other authors[22,27]. A correlation between serological titers and clinical status has already been described[5,12,28], and has been associated to the chronic course of disease[22], through the stimulation of an exacerbated humoral immune response, as is commonly found in symptomatic dogs[29].

Regarding the histopathological pattern observed in macroscopically uninjured skin of the ear in infected dogs, our data corroborate those described in the literature. Therefore, the predominance of inflammatory infiltrates in the superficial dermis, primarily characterized by

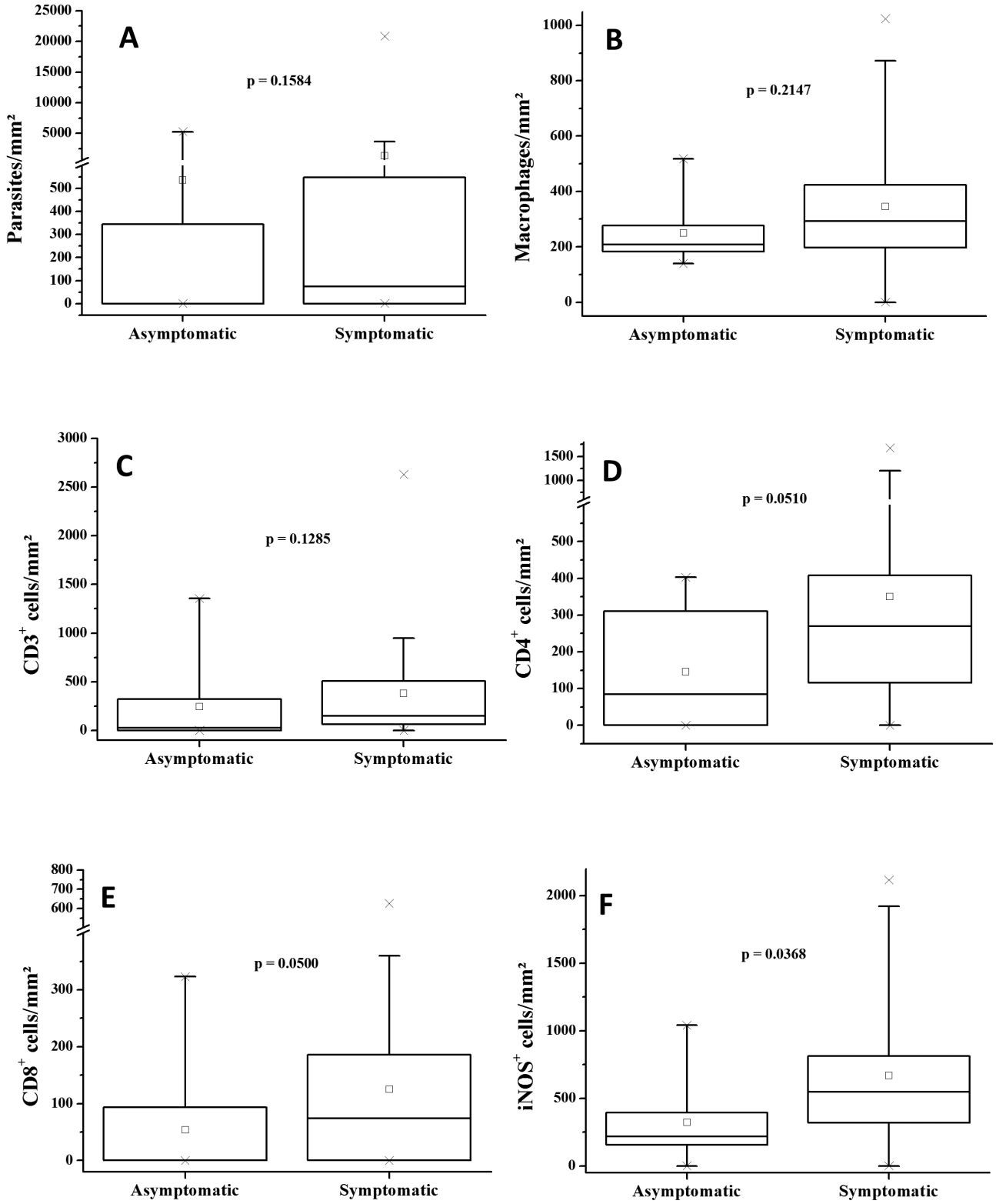

Fig. 3 – Box plot graph showing the mean, median, maximum, and minimum values, as well as the 95% confidence intervals, of the number of amastigotes (A), macrophages (B), and $CD3^+$ (C), $CD4^+$ (D), $CD8^+$ (E), and $iNOS^+$ (F) cells per area (mm^2) detected by immunohistochemistry in the ear skin tissue of asymptomatic and symptomatic dogs affected by visceral leishmaniasis.

mononuclear cells (macrophages, lymphocytes, and plasma cells), ranging from mild to severe, as well as from focal to diffuse, has already been described by other authors[8,9,19]. It is important to mention that skin inflammation was associated to cutaneous parasitism, and among those animals in which parasites were not found in the skin, the inflammatory process has always been mild and focal. This latter finding could be attributed to additional conditions, such as bites from other insects and the presence of ectoparasites, which are relatively common throughout the year in tropical countries[30], and also to trauma[31]. It is worth noting that in skin biopsies of the ear that were evaluated in this study, neither epidermal injury nor cartilage tissue injury were observed. Skin with a normal histological pattern, and with no tissue damage, was observed in 25% (6/24) of the symptomatic and in 36% (5/14) of the asymptomatic dogs, which is in accordance with what has already been demonstrated by Solano-Gallego *et al*.[5] (2004).

With respect to the cellular infiltrate, the number of macrophages, $CD3^+$, $CD4^+$ and $CD8^+$ cells/mm^2 was higher in the skin of symptomatic dogs when compared to asymptomatic ones ($p = 0.2147$, $p = 0.1285$, $p = 0.0510$, and $p = 0.0500$, respectively) although no significant difference was observed regarding the density of these cells between the clinical groups. In spite of the similarity in the number of inflammatory cells between symptomatic and asymptomatic dogs, their function may differ. Thus, inflammatory cells present in the skin of symptomatic dogs could represent predominantly a Th2 profile which is ineffective to eliminate the parasite, while, lymphocytes in the dermis of asymptomatic dogs could be mainly Th1, able to activate macrophages and reduce the tissue parasitism[12]. A mixed cytokine profile was observed in asymptomatic dogs as well as during active CVL, however, high levels of expression of IFN-γ, TNF-α, IL-13 and levels of transcription factors GATA-3 and FOXP3 were correlated with asymptomatic disease; while high levels of IL-10 and TGF-β, concomitant with low expression of IL-12, were associated to the persistence and replication of parasites in the skin[32].

This finding might be also related to the cutaneous parasite load identified in these animals, which could in turn stimulate a high influx of inflammatory cells[13]. However, it is pertinent to consider the compartmentalization of the immune response[33]; in this way, a systemic response can be different from a cutaneous response and the local immune response of the skin may not reflect what occurs in viscera. However, it is important to mention that a positive correlation was observed between the parasite density and the macrophages density ($p = 0.031$), $CD4^+$ T-cells ($p = 0.015$), and $CD8^+$ T-cells ($p = 0.023$) in the skin, showing that the greater the cutaneous parasitism, the more intense the inflammatory response of these cell types.

Concerning the underlying leishmanicidal mechanisms, the density of $iNOS^+$ cells was higher in symptomatic dogs ($p = 0.0368$) that exhibited more intense tissue parasitism and inflammatory processes; iNOS cell density has also shown a positive correlation with the density of $CD3^+$ ($p = 0.005$), $CD4^+$ ($p = 0.001$), and $CD8^+$ ($p = 0.0001$) cells. These findings indicate that macrophage activation, in addition to subsequent production of NO, could be directly related to the intensity of the cellular immune response[34]. Some studies suggest that the activation of an effective cellular immune response with the production of NO by the enzyme iNOS may play an important role in the control of *Leishmania* infection in dogs[14]. Interestingly, it has been shown that iNOS expression in the spleen of dogs naturally infected by *L. (L.) infantum chagasi* is associated to clinical manifestations and higher parasite loads[35]. Moreover, the arginase activity has been detected in *Leishmania*. Since arginase and iNOS compete for the use of L-arginine as a substrate, the availability of this amino acid for both pathways is critical for the parasite replication. Therefore, the parasite must escape from several microbicidal mechanisms, such as NO production mediated by iNOS, to survive within the macrophages of vertebrate hosts[36]. Moreover, Panaro *et al*., 1998 evaluating canine peripheral blood cells suggested that monocytes lack NO-mediated killing capacity. They demonstrated that phagocytosis, killing capacity and superoxide anion (O^-_2) production had significantly increased in the monocytes stimulated by IFN-γ. So, *in vivo* host-parasite interaction is a complex phenomenon which undergo the influence of several elements that contribute to the effectiveness of the immune system[37].

CONCLUSION

The results obtained in this study have shown that the main histological changes observed in macroscopically uninjured skin of the ear in dogs affected by CanL included the presence of a nonspecific chronic inflammatory infiltrate in the superficial dermis, characterized by the presence of macrophages, T-lymphocytes ($CD4^+$ and $CD8^+$ cells) and cells expressing inducible nitric oxide synthase. The inflammatory response of the skin was directly correlated to the tissue parasitism, but not to the clinical status.

ACKNOWLEDGMENTS

This project was supported by grants #2004/07965-2 and #2012/50285-9 from the *São Paulo* Research Foundation; #476479/2012-6 from CNPq; and LIM50 HC-FMUSP. Márcia D. Laurenti and Mary Marcondes are Research Fellows from CNPq, Brazil. English-language editing of this manuscript was provided by Journal Prep.

REFERENCES

1. Dantas-Torres F, Brandão-Filho SP. Visceral leishmaniasis in Brazil: revisiting paradigms of epidemiology and control. Rev Inst Med Trop Sao Paulo. 2006;48:151-6.

2. Laurenti MD, Rossi CN, da Matta VLR, Tomokane TY, Corbett CEP, Secundino NFC, et al. Asymptomatic dogs are highly competent to transmit *Leishmania* (*Leishmania*) *infantum chagasi* to the natural vector. Vet Parasitol. 2013;196:296-300.

3. Kemp M, Theander TG, Kharazmi A. The contrasting roles of CD4+ T cells in intracellular infections in humans: leishmaniasis as an example. Immunol Today. 1996;17:13-6.

4. Camargo BJ, Troncarelli MZ, Ribeiro MG, Langoni H. Leishmaniose visceral canina: aspectos de saúde pública e controle. Clín Vet. 2007;71:86-92.

5. Solano-Gallego L, Morell P, Arboix M, Alberola J, Ferrer L. Prevalence of *Leishmania infantum* infection in dogs living in an area of canine leishmaniasis endemicity using PCR on several tissues and serology. J Clin Microbiol. 2001;39:560-3.

6. Travi BL, Tabares CJ, Cadena H, Ferro C, Osorio Y. Canine visceral leishmaniasis in Colombia: relationship between clinical and parasitologic status and infectivity for sand flies. Am J Trop Med Hyg. 2001;64:119-24.

7. Papadogiannakis EI, Koutinas AF, Saridomichelakis MN, Vlemmas J, Lekkas S, Karameris A, et al. Cellular immunophenotyping of exfoliative dermatitis in canine leishmaniosis (*Leishmania infantum*). Vet Immunol Immunopathol. 2005;104:227-37.

8. Saridomichelakis MN, Koutinas AF, Olivry T, Dunston SM, Farmaki R, Koutinas CK, et al. Regional parasite density in the skin of dogs with symptomatic canine leishmaniosis. Vet Dermatol. 2007;18:227-33.

9. Solano-Gallego L, Fernández-Bellon H, Morell P, Fondevila D, Alberola J, Ramis A, et al. Histological and immunohistochemical study of clinically normal skin of *Leishmania infantum*-infected dogs. J Comp Pathol. 2004;130:7-12.

10. Fondevila D, Vilafranca M, Ferrer L. Epidermal immunocompetence in canine leishmaniasis. Vet Immunol Immunopathol. 1997;56:319-27.

11. Miranda S, Martorell S, Costa M, Ferrer L, Ramis A. Characterization of circulating lymphocyte subpopulations in canine leishmaniasis throughout treatment with antimonials and allopurinol. Vet Parasitol. 2007;144:251-60.

12. Quinnell RJ, Courtenay O, Shaw MA, Day MJ, Garcez LM, Dye C, et al. Tissue cytokine responses in canine visceral leishmaniasis. J Infect Dis. 2001;183:1421-4.

13. Coura-Vital W, Marques MJ, Giunchetti RC, Teixeira-Carvalho A, Moreira ND, Vitoriano-Souza J, et al. Humoral and cellular immune responses in dogs with inapparent natural *Leishmania infantum* infection. Vet J. 2011;190:e43-7.

14. Zafra R, Jaber JR, Pérez-Écija RA, Barragán A, Martínez-Moreno A, Pérez J. High iNOS expression in macrophages in canine leishmaniasis is associated with low intracellular parasite burden. Vet Immunol Immunopathol. 2008;123:353-9.

15. Sisto M, Brandonisio O, Panaro MA, Acquafredda A, Leogrande D, Fasanella A, et al. Inducible nitric oxide synthase expression in *Leishmania*-infected dog macrophages. Comp Immunol Microbiol Infect Dis. 2001;24:247-54.

16. Pinelli E, Gonzalo RM, Boog CJP, Rutten VPMG, Gebhard D, Del Real G, et al. *Leishmania infantum*-specific T cell lines derived from asymptomatic dogs that lyse infected macrophages in a major histocompatibility complex-restricted manner. Eur J Immunol. 1995;25:1594-600.

17. Laurenti MD, de Santana Leandro MV, Tomokane TY, De Lucca HRL, Aschar M, Souza CSF, et al. Comparative evaluation of the DPP® CVL rapid test for canine serodiagnosis in area of visceral leishmaniasis. Vet Parasitol. 2014 ;205:444-50.

18. Moreira MA, Luvizotto MCR, Garcia JF, Corbett CEP, Laurenti MD. Comparison of parasitological, immunological and molecular methods for the diagnosis of leishmaniasis in dogs with different clinical signs. Vet Parasitol. 2007;145:245-52.

19. Figueiredo MM, Moura EP, Costa MM, Ribeiro VM, Michalick MS, Tafuri WL, et al. Histopathological and parasitological investigations of ear healthy skin of dogs naturally and experimentally infected with *Leishmania* (*Leishmania*) *chagasi*. Histol Histopathol. 2010;25:877-87.

20. Nunes CM, Lima VMF, Paula HB, Perri SH, Andrade AM, Dias FEF, et al. Dog culling and replacement in an area endemic for visceral leishmaniasis in Brazil. Vet Parasitol. 2008;153:19-23.

21. Lima LVR, Carneiro LA, Campos MB, Chagas EJ, Laurenti MD, Corbett CEP, et al. Canine visceral leishmaniasis due to *Leishmania* (*L.*) *infantum* chagasi in Amazonian Brazil: comparison of the parasite density from the skin, lymph node and visceral tissues between symptomatic and asymptomatic, seropositive dogs. Rev Inst Med Trop Sao Paulo. 2010;52:259-65.

22. Madeira M de F, Schubach A de O, Schubach TMP, Leal CA, Marzochi MC. Identification of *Leishmania* (*Leishmania*) *chagasi* isolated from healthy skin of symptomatic and asymptomatic dogs seropositive for leishmaniasis in the Municipality of Rio de Janeiro, Brazil. Braz J Infect Dis. 2004;8:440-4.

23. Giunchetti RC, Mayrink W, Genaro O, Carneiro CM, Corrêa-Oliveira R, Martins-Filho OA, et al. Relationship between canine visceral leishmaniosis and the *Leishmania* (*Leishmania*) *chagasi* burden in dermal inflammatory foci. J Comp Pathol. 2006;135:100-7.

24. Reis AB, Teixeira-Carvalho A, Vale AM, Marques MJ, Giunchetti RC, Mayrink W, et al. Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite density in Brazilian dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi*. Vet Immunol Immunopathol. 2006;112:102-16.

25. Foglia Manzillo V, Di Muccio T, Cappiello S, Scalone A, Paparcone R, Fiorentino E, et al. Prospective study on the incidence and progression of clinical signs in naive dogs naturally infected by *Leishmania infantum*. PLoS Negl Trop Dis. 2013;7:1-8.

26. Vlkova M, Rohousova I, Drahota J, Stanneck D, Kruedewagen EM, Mencke N, et al. Canine antibody response to *Phlebotomus perniciosus* bites negatively correlates with the risk of *Leishmania infantum* transmission. PLoS Negl Trop Dis. 2011;5:e1344.

27. Dos-Santos WLC, Jesus EE, Paranhos-Silva M, Pereira AM, Santos JC, Baleeiro CO, et al. Associations among immunological, parasitological and clinical parameters in canine visceral leishmaniasis: emaciation, spleen parasitism, specific antibodies and leishmanin skin test reaction. Vet Immunol Immunopathol. 2008;123:251-9.

28. Da Costa-Val AP, Cavalcanti RR, de Figueiredo Gontijo N, Michalick MS, Alexander B, Williams P, et al. Canine visceral leishmaniasis: relationships between clinical status, humoral immune response, haematology and *Lutzomyia* (*Lutzomyia*) *longipalpis* infectivity. Vet J. 2007;174:636-43.

29. Alexandre-Pires G, de Brito MTV, Algueró C, Martins C, Rodrigues OR, da Fonseca IP, et al. Canine leishmaniosis. Immunophenotypic profile of leukocytes in different compartments of symptomatic, asymptomatic and treated dogs. Vet Immunol Immunopathol. 2010;137:275-83.

30. Prats N, Ferrer L. A possible mechanism in the pathogenesis of cutaneous lesions in canine leishmaniasis. Vet Rec. 1995;137:103-4.

31. Mozos E, Pérez J, Day MJ, Lucena R, Ginel PJ. Leishmaniosis and generalized demodicosis in three dogs: a clinicopathological and immunohistochemical study. J Comp Pathol. 1999;120:257-68.

32. Menezes-Souza D, Corrêa-Oliveira R, Guerra-Sá R, Giunchetti RC, Teixeira-Carvalho A, Martins-Filho OA, et al. Cytokine and transcription factor profiles in the skin of dogs naturally infected by *Leishmania* (*Leishmania*) *chagasi* presenting distinct cutaneous parasite density and clinical status. Vet Parasitol. 2011;177:39-49.

33. Reis AB, Martins-Filho OA, Teixeira-Carvalho A, Giunchetti RC, Carneiro CM, Mayrink W, et al. Systemic and compartmentalized immune response in canine visceral leishmaniasis. Vet Immunol Immunopathol. 2009;128:87-95.

34. Gantt KR, Goldman TL, McCormick ML, Miller MA, Jeronimo SM, Nascimento ET, et al. Oxidative responses of human and murine macrophages during phagocytosis of *Leishmania chagasi.* J Immunol. 2001;167:893-901.

35. Dos Santos FR, Vieira PMA, Correa-Oliveira R, Giunchetti RC, Carneiro CM, Reis AB, et al. Qualitative and quantitative immunohistochemical evaluation of iNOS expression in the spleen of dogs naturally infected with *Leishmania chagasi*. Parasitol Res. 2011;108:1397-403.

36. Da Silva MFL, Floeter-Winter LM. Arginase in *Leishmania*. In: Santos A, Branquinha M, d'Avila-Levy C, Kneipp L, Sodré C, editors. Proteins and proteomics of *Leishmania* and *Trypanosoma*. Netherlands: Springer; 2014. p. 103–17. Available from: http://link.springer.com/10.1007/978-94-007-7305-9_4

37. Panaro MA, Lisi S, Mitolo V, Acquafredda A, Fasanella A, Carelli MG, et al. Evaluation of killing, superoxide anion and nitric oxide production by *Leishmania infantum*-infected dog monocytes. Cytobios. 1998;95:151-60.

Received: 21 September 2015
Accepted: 17 December 2015