Research paper

Cytokine and iNOS profiles in lymph nodes of dogs naturally infected with *Leishmania infantum* and their association with the parasitic DNA load and clinical and histopathological features

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ARTICLE INFO

ABSTRACT

In South America, visceral leishmaniasis is a zoonotic disease with severe evolution characteristics in humans, and dogs are its main reservoir. In this context, this study aimed to evaluate the clinical status of dogs from a Brazilian endemic area naturally, at Barra Mansa municipality, infected with *Leishmania infantum*, in conjunction with their histopathological profile and, in order to determine possible markers of susceptibility or resistance to the disease, parasitic DNA load, cytokine and iNOS mRNA expression profiles were investigated in lymph nodes. High levels of IFN-γ and IL-6 mRNA were detected. Both IFN-γ and IL-6 mRNA were associated with disorganization of the corticomedullary region. IFN-γ and TNF-α mRNA were associated with the absence of follicular hyperplasia. The regulatory pathway was remarkable with IL-10 mRNA detection and its significant association with the severity of the disease. Plasmacytosis and sinus histiocytosis were associated with high loads of parasitic DNA, but there was no significant association between the parasite DNA load and animal clinical alterations. Since high parasitic loads were found in animals with or without symptoms, clinical examination cannot be considered as a criterion for disease susceptibility assessment.

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1. Introduction

Leishmaniases are parasitic diseases caused by protozoa of the genus *Leishmania* and are transmitted by the bite of infected phlebotomine sand flies (Werneck, 2014). Their most serious form, the visceral leishmaniasis, presents clinical characteristics of severe evolution in humans (Brasil, 2006, 2009). In South America, it has expanded to medium and large urban areas where the zoonotic disease cycle occurs (Brasil, 2006; Werneck, 2010; Brasil, 2009; Werneck, 2014).

In this region, *Leishmania infantum* (syn = *Leishmania chagasi*) is the main etiological agent involved (Dantas-Torres, 2009) and the dog (*Canis familiaris*) is the main infection source (Brasil, 2006, 2009). This host can show a wide clinical spectrum, from asymptomatic to fully symptomatic (Mancianti et al., 1988), and the disease evolution and its clinical appearance are the consequences of complex interactions between parasite and host immune response (Santos-Gomes et al., 2002; Brachelente et al., 2005; Maia and Campino, 2012).

The skin is the site of parasite inoculation by the infected phlebotomine sand fly and thus the first immune barrier (Maia and Campino, 2012). After the multiplication within the macrophages, the parasites can leave the skin, and spread to the mononuclear phagocytes in different organs (Pinelli et al., 1994). After dissemination, the lymph nodes are regarded as the first relevant lymphoid

tissue to be affected, so that the study of their immune response against *Leishmania* spp. can help in the understanding the infection process (Maia and Campino, 2012).

The development of the infection depends on the ability of the host macrophage to destroy the parasite effectively. This activity is determined by the balance between a heterogeneous set of cytokines and free radicals (Santos-Gomes et al., 2002); therefore, comprehensive knowledge of the immune profile developed in infected dogs is crucial for the development of vaccines and immunomodulatory therapies (Maia and Campino, 2012), as well as for the understanding of the mechanisms associated with the immune evasion by *Leishmania* spp. and disease progression (Cecilio et al., 2014).

In this context, this research aimed to assess the clinical status of animals from a Brazilian endemic area naturally infected with *L. infantum*, in conjunction with their parasite load, histopathological profile, cytokine and inducible nitric oxide synthase (iNOS) expressions in lymph nodes, in order to determine possible markers of susceptibility or resistance to the disease.

2. Materials and methods

A cross-sectional study was conducted with a convenience sample of 26 dogs from a Brazilian endemic area, at Barra Mansa municipality, for canine visceral leishmaniasis (CVL). The study included animals with indication for euthanasia according to the guidelines of the Brazilian Ministry of Health (Brasil, 2006, 2009), after positive serological results for *L. infantum* by Immunochromatographic Rapid Test – Dual Path Platform (DPP®) – BioManguinhos®/FIOCRUZ and Enzyme-Linked Immunosorbent Assay (ELISA) performed by Public Offices. The animals were submitted to clinical evaluation and collection of biological specimens to perform the following procedures: parasitological culture, histopathological and immunohistochemical profile evaluation, and real-time polymerase chain reaction (RT-PCR) to determine the parasite DNA load and the expression profiles of cytokines and iNOS, with the latter conducted at the Pasteur Institute in Paris, France, and the former at FIOCRUZ, Brazil.

The project was registered at the Ethics Committees on the Use of Animals in Research under the numbers LW-47/12 (FIOCRUZ) and 688 (UFF).

The animals were submitted to clinical evaluation and classified according to the presence of clinical signs and symptoms suggestive of CVL in two different groups: animals with severe clinical disease (SC) and animals with mild or no clinical disease (MNC). To this end, the following determination criteria were used: animals presenting onychogryphosis, ophthalmologic abnormalities, generalized adenitis, cachexia, and hepatosplenomegaly, even if isolated and independent of association with other signs or symptoms, were included in the SC group; animals with no apparent clinical disease or only presenting isolated hepatomegaly or splenomegaly, and/or desquamation, crusted ulcer, or regional adenitis were included in the MNC group.

The animals were sedated and euthanized according to Silva et al. (2011). After confirmation of death, necropsy was performed and four popliteal lymph node fragments were collected and stored respectively at −196 °C, −20 °C, in 10% formalin and in buffered saline solution according to the techniques to be subsequently applied and which are described below.

The fragments stored in buffered saline were seeded in biphasic Novy-McNeal-Nicolle (NNN) medium with Schneider medium as liquid phase plus 10% fetal bovine serum. They were kept in biological oven at 26–28 °C and examined weekly for 30 days by fresh examination to highlight flagellated forms of *Leishmania* spp.

Leishmania isolates were identified by multilocus enzyme electrophoresis according to Almeida et al. (2011).

Tissue samples fixed in 10% formalin were submitted to histological analysis and stained with hematoxylin-eosin (HE) for histopathological evaluation (Carson and Hladick, 2009). The technique of immunohistochemistry anti-*Leishmania* was based on previous studies (Quintella et al., 2009; Dos Santos et al., 2015) and the amastigote count was obtained by the mean from three different magnification 40× fields count per sample, yielding an average count of amastigotes per tissue.

DNA extraction was performed on tissue samples stored at −20 °C and obtained using a tissue punch with 3 mm of diameter. The DNeasy Blood & Tissue Kit (Qiagen) was employed in a semi-automated system Qiacube (Qiagen). The DNA obtained was measured using the 2.0 Qubit® Fluorometer equipment together with the Qubit® kit dsDNA HS AssayKit (Invitrogen®). All procedures were performed according to the manufacturers' guidelines.

Real-time PCR was conducted in triplicate on StepOne™ platform (Applied Biosystems®) and the DNA quantity used was standardized and the same for all samples. The TaqMan® system was employed and a standard curve with the DNA obtained from culture of *Leishmania infantum* promastigotes (MHOM/BR/1974/PP75) was prepared as measurement methodology by comparison between the samples and the standard curve. We used primers and TaqMan MGB-probes designed for amplification of conserved kDNA regions of *L. infantum*, as previously described by Francino et al. (2006). To avoid the occurrence of false negative results by PCR inhibition factors, negative DNA samples were submitted to amplification of the canine constitutive gene encoding the β-subunit actin protein, using a validated Taqman® Gene Expression Assay System (Applied Biosystems, Foster City, CA) under the following temperature cycles: 95 °C for 15 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 31 s, and a final phase of 72 °C for 10 min.

Utilizing the samples of lymph nodes frozen at −196 °C, total RNA extraction was performed using Trizol reagent with the aid of the disperser ULTRA-TURRAX® T10 basic (IKA®, Germany), according to the manufacturer's instructions. Quantification of RNA and its qualitative assessment were performed by spectrophotometry at the optical density of 260 nm and the purity by the ratio 260/280 on NanoDrop 2000 (Scientific Terms) and by visualization of the 28 and 18 s RNA bands in electrophoresis in 1.2% agarose gel. The total RNA obtained was subjected to reverse transcription using 200 U of Moloney murine leukemia virus reverse transcriptase (Superscript II, Invitrogen), according to manufacturer's instructions.

For the Real-Time PCR, the canine house keeping gene of the enzyme hypoxanthine phosphoribosyl transferase (HPRT) was used for reaction normalization and the values were calculated as the n-fold difference with the level of expression in naïve organ. The primers and fluorogenic probes for cytokines and for the enzymes iNOS and HPRT were chosen at exon–exon junctions in the following sequences (with their access numbers at the National Center for Biotechnology Information − NCBI): IL-2 (NM_001003305.1), IL-4 (NM_001003159.1), IL-6 (NM_001003301.1), IL-10 (NM_001003077.1), IL-12p35 (U49085.1); INF-gamma (NM_001003174.1); TNF-alpha (EU249361.1); iNOS (AF077821.1); and HPRT (AY283372.1). The fluorogenic probes were synthesized using a FAM reporter molecule at its 5′ end and a TAMRA quencher attached to the 3′ end (Eurogentec, France). All primers and probes were designed using the Primer Express 3.0 software (Applied Biosystems) according to the required technical parameters. Table 1 shows the primers and probes and their respective gene sequences. The reaction was performed in Step One Plus TM Real-Time PCR System (Applied Biosystems), in a 20 μl reaction, under the following conditions of cycle and temperature: 2 min of initial incubation at 50 °C, followed

Table 1
Sequences of genetic primers and fluorogenic probes FAM-TAMRA used in the Real-Time Polymerase Chain Reaction for the evaluation of the cytokine and nitric oxide synthase expressions in dogs.

	Forward and Reverse Primers	Probes
HPRT	ATCCAAAGATGGTCAAGGTTGC TCCAACAAAGTCAGGTTTATAGCCA	CTTGCTGGTGAAAAGGACCCCTCGA
IFN-γ	CCAGTAAGAGGGAGGACTTCCTT CATTTATCGCCTTGCGCTG	CTGATTCAAATTCCTGTGAACGATCTGCA
iNOS	CACAGCCTATTCTCGCCTGC TGCGGAGCACCTGGCT	CCAGCCCAAGGTCTATGTTCAAGACATCC
IL-2	CAACTCTCCAGGATGCTCACATT TTCTGCTAGACATTGAAGGTGTGTAA	AAGTTTTACACGCCCAAGAAGGCCACAG
IL-4	AGCACTCACCAGCACCTTTGT CTTGACAGTCAGCTCCATGCA	TTGAACATCCTCACAGCGAGAAACGACTC
IL-6	GATGCTAAAGAGCAAGGTAAAGAATCA CCTGCGACTGCAAGATAGCC	ATGAAGTGACCACTCCTGACCCAACCA
IL-10	TCCCTGGGAGAGAAGCTCAA CCACCGCCTTGCTCTTATTC	CTGAGGCTGCGACGCTGTCACC
IL-12p35	ATCCCAAGAGGCAGATCTTTCTG CTTCAAGGGAGGATTTCTGTGG	TCGATGAGCTGTTACAGGCCCTGAATTT
TNF-α	TCGAACCCCAAGTGACAAGC CACGTCGGCTCAGCCACT	TAGCTCATGTTGTAGCAAACCCCGAAGC

by 10 minutes min of denaturation at 95 °C, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min each. Each reaction was conducted in the presence of an internal control for potential genomic DNA contamination and for the purity of the reagents used.

2.1. Statistical analysis

Statistical analysis was performed using the Kolmogorov-Smirnov test with Lilliefors significance correlation and the Shapiro-Wilk normality test for the continuous variables. Due to the small sample size and because the distributions were mostly not normal ($p < 0.05$), the Mann-Whitney test (p_{MW}) was employed for the evaluation between clinical groups and cytokine mRNA profile, as well as between clinical groups and parasite DNA load. The same test was used to assess the association between the profile of cytokines mRNA and the histopathological findings. By means of the Spearman correlation, the expressions of cytokines mRNA were evaluated among themselves and between them and the parasite DNA load. For the qualitative variables, the Pearson (p_P) or Fisher (p_F) chi-square tests were performed, associating the histopathology and the parasitic DNA load groups.

3. Results

Twenty-six animals were assessed, of which 14 composed the group of animals with severe clinical disease (SC), whereas 12 composed the group of animals with mild or no clinical disease (MNC).

Among the 26 tissue samples of lymph nodes evaluated, 18 (69.23%) tested positive in the parasitological culture. There were negative results in both clinical groups, equally divided between them.

With respect to the histological and immunohistochemical assessment, at least one of the following pathological findings was evidenced: follicular hyperplasia, disorganization of the corticomedullary region, plasmacytosis, sinus histiocytosis, presence of granulomas and amastigotes. The most frequent finding was plasmacytosis, which was observed in 84% of the analyzed samples, followed by amastigotes (80%), and granulomas (68%). Fig. 1 shows the percentage distribution of histopathology. Some histopathological findings are presented in Fig. 2.

Regarding the identification of amastigotes, their count in the immunohistochemistry presented wide variation between individuals, with the mean found by microscopic field ranging from 1.33 to 212.67. Amastigote detection is shown in Fig. 3.

Among the lymph node samples tested, all positive results for amastigote presence were confirmed by the parasitological culture, and three samples, which had previously presented negative results on the culture, tested positive on the parasitological examination by microscopy.

The DNA of *Leishmania infantum* was detected in 24 of the 26 samples of lymph nodes assessed. For the two remaining samples, one tested negative, whereas the other presented inconclusive result because the possibility of concomitant inhibitors could not be discarded in the qPCR for the canine β-actin target. These same two samples tested negative in parasitological culture and histopathological evaluation. Among the positive samples, the genomic equivalent numbers (gEq) found varied considerably, ranging from 0.00001 to 348.8. These results were organized into
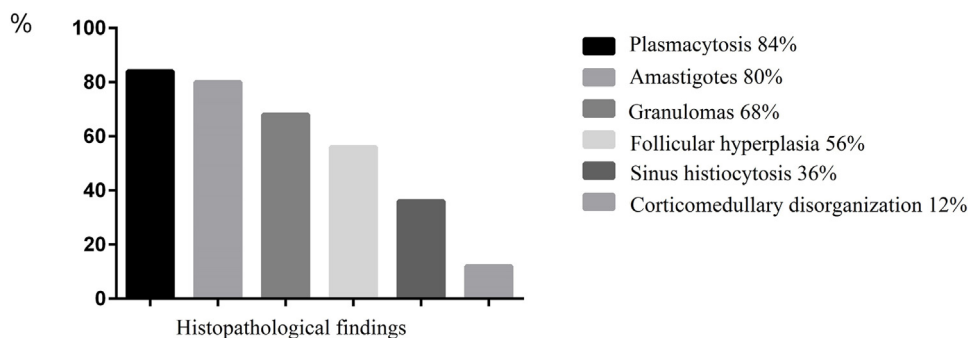

Fig. 1. Frequency distribution of histopathological findings in popliteal lymph nodes of dogs naturally infected with *Leishmania infantum*: plasmacytosis, presence of amastigotes and granulomas, follicular hyperplasia, sinus histiocytosis, and disorganization of the corticomedullary region.
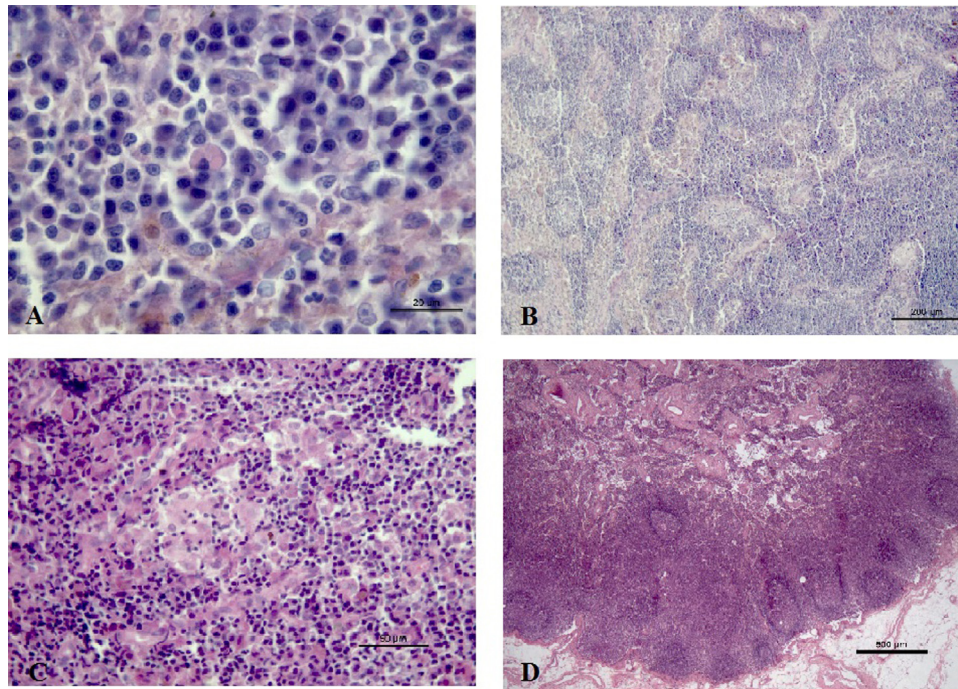
Fig. 2. Fragment sections of popliteal lymph node of dogs naturally infected with *Leishmania infantum*. Photomicroscopy of haematoxylin–eosin stained specimens: (A) Plasmacytosis, magnification 40x; (B) Sinus histiocytosis, magnification 10x, (C) Granuloma, magnification 40x; (D) Follicular hyperplasia, magnification 5x.
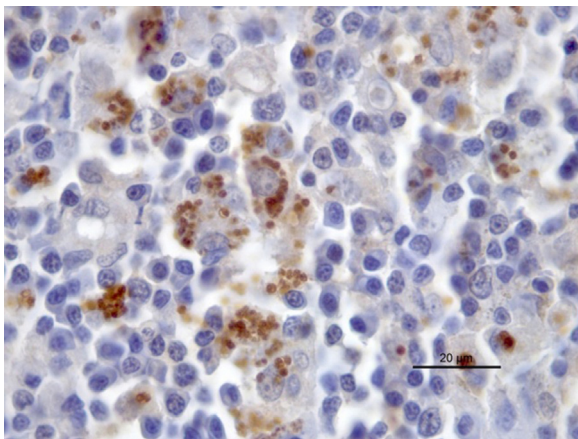

Fig. 3. Amastigotes in a fragment section of popliteal lymph node of a dog naturally infected with *Leishmania infantum*. Immunohistochemistry photomicroscopy, magnification 40x.

two groups according their parasitic DNA load: group 1, comprising 13 samples showing no or low parasitic DNA loads (gEq ≤ 10); and group 2, comprising 12 samples showing significant parasitic DNA loads (gEq > 10).

Regarding the expression profiles of cytokines and iNOS mRNAs, IFN-γ and IL-6 achieved the highest levels, presenting medians of 17.69 and 5.41, respectively, in relation to naïve counterpart. In particular, the dog with maximal expression of IL-6 also showed the highest parasitic DNA load, the highest mean score of amastigotes per field, relative low expression of IFN- γ, and the presence of granulomas in the histopathology, but no symptoms.

IL-10, IL-4, and IL-2 mRNAs were detected at lower levels. Medians were 2.87, 1.10, and 1.75, respectively, in relation to naïve counterpart.

The expression levels of TNF-α and IL-12p35 ranged from non-detection to 5.23 and 4.48, respectively. For iNOS, there was no or little detection in the evaluated samples (maximum of 2.04). Their median levels were 1.85, 0.98, and 0.50, respectively.

IFN-γ and IL-10 mRNA showed significant differences in their distribution among the clinical groups (p_{MW} = 0.031 and p_{MW} = 0.015). Both medians were higher in the SC group, with the IFN-γ median twice as high in the SC group compared with the MNC group. There were no significant differences in relation to other cytokine mRNA expressions and their distribution by clinical groups (p > 0.05). However, it is worth noting that, although not statistically significant, IL-6 mRNA presented a higher median in the SC group, approximately three times higher, compared with that of the MNC group.

Fig. 4A shows the distribution of the expression profile of cytokines and iNOS in the clinical groups.

There were no significant differences regarding the parasitic DNA load and its distribution by clinical groups (p_{MW} > 0.05). However, it is worth noting that the median in the SC group was almost twice as high than in the MNC group, but with individuals from the MNC group showing high parasitic DNA loads as well (Fig. 4B).

There was no significant difference in the association between the expression profiles of cytokines and iNOS and the parasitic DNA load groups (p_{MW} > 0.05).

Application of Spearman correlation between the cytokine and iNOS expression profiles and the individual values of parasitic DNA load, without division into groups, showed no significant correlation (p > 0.05); nevertheless, it is worth highlighting that the most positive correlation was found between the parasite DNA load and IL-10 (r Spearman 0.262).

Regarding the association between histopathology and parasitic DNA load, plasmacytosis was significantly associated (p_F = 0.032) with the highest parasitic DNA load group. Likewise, it was also observed significant association between this group and sinus histiocytosis (p_F = 0.04) and the presence of amastigotes (p_F = 0.032). There were no other significant associations between the histopathological findings and the parasitic DNA load groups. The significant associations are showed in the Table 2.
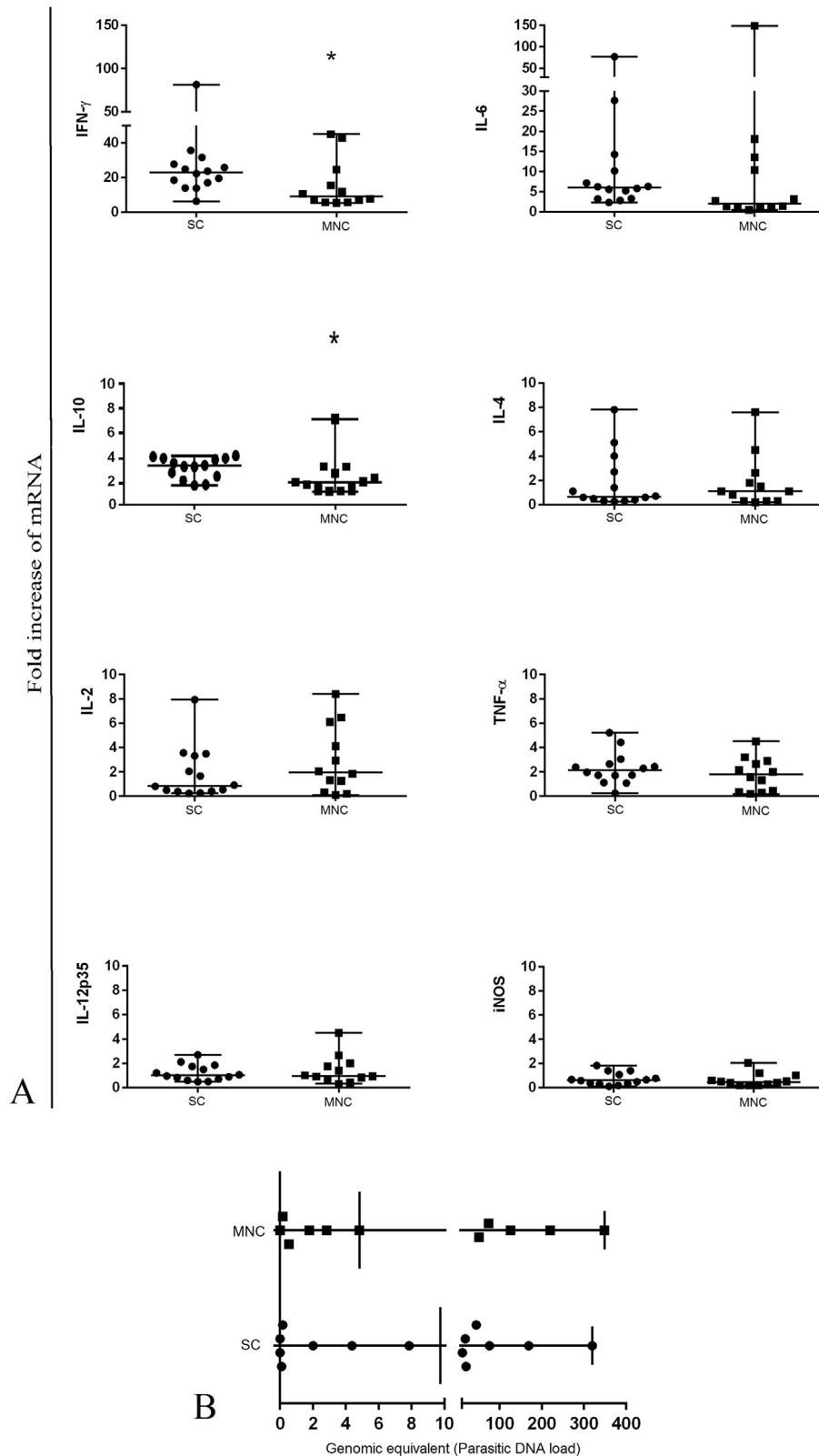
Fig. 4. Associations between the clinical groups of animals naturally infected with *Leishmania infantum* presenting severe clinical disease (SC) and mild or no clinical disease (MNC) and: A- mRNA expression profile of cytokines IFN-γ, IL-6, IL-10, IL-4, IL-2, TNF-α, and IL-12p35 and iNOS enzyme. Median values and 0–100 quartiles. *Significant difference between MNC and SC ($p_{MW} < 0.05$) by the Mann-Whitney test; B- the parasitic DNA load. There was no significant difference between SC and MNC ($p_{MW} > 0.05$) by the Mann-Whitney test.

As for the association between histopathology and the expression profiles of cytokines and iNOS mRNA, the medians related to IFN-γ and TNF-α expressions were significantly higher in the groups with no follicular hyperplasia ($p_{MW} = 0.013$ and $p_{MW} = 0.002$, respectively), whereas the medians related to IFN-γ and IL-6 were associated with the groups with presence of disorganization of

Table 2
Association between the percentages of histopathological findings plasmacytosis, sinus histiocytosis and presence of amastigotes with the parasitic DNA load groups, no or low parasitic DNA loads (gEq ≤ 10) and significant parasitic DNA loads (gEq > 10), in lymph nodes from dogs naturally infected with *Leishmania infantum*.

Histopathological finding		Parasitic DNA loads	
		gEq ≤ 10	gEq > 10
Plasmacytosis	Yes	40%	60%
$p_F = 0.032$	No	100%	0%
Sinus histiocytosis	Yes	11,1%	88,9%
$p_F = 0.04$	No	73,3%	26,7%
Presence of amastigotes	Yes	40%	60%
$p_F = 0.032$	No	100%	0%

Genomic equivalent number (gEq).

the corticomedullary region ($p_{MW} = 0.027$ and $p_{MW} = 0.014$, respectively). Particularly, IL-6 mRNA expression was almost nine times higher in dogs with such tissue disruption compared with others where this finding was not observed. There were no other significant associations in this evaluation.

4. Discussion

The clinical findings presented by the animals investigated in this study are characteristic of CVL and demonstrate the diversity of the symptomatic spectrum described for the disease. (Mancianti et al., 1988; Brasil, 2006; Dantas-Torres, 2009).

As for histopathology, the presence of plasmacytosis and histiocytosis is in agreement with the findings reported by Moreira et al. (2010), who observed such characteristics in a cytological study on lymph nodes of dogs infected with *L. infantum*. However, in the study aforementioned, the findings were independent of tissue parasitic load, whereas in this study, both plasmacytosis and histiocytosis were significantly associated with parasitic DNA loads. In the present study, the presence of granulomas points to the chronicity of the pathological process; in fact, chronic adenitis was also characterized by Alves et al. (2009) in lymph nodes during CVL. Granulomas were the most frequent pathological alteration in histopathology in the study by Moreira et al. (2010); the authors also identified significant distortions on the lymph node architecture, with loss of morphology between cortical and medullary regions, which are in agreement with our findings regarding the disorganization of the corticomedullary region. In this context, Cavalcanti et al. (2015) also demonstrated architecture modification in the spleens of infected dogs, but in this case, this modification was more evident in animals with higher parasitic loads, with correlation between high parasite loads and the injury of expression of both pro- and anti-inflammatory cytokines. Follicular hyperplasia, the fourth most frequent histopathological finding in our study, has also been reported in the scientific literature for lymph nodes of dogs infected with *L. infantum* (Lima et al., 2004; Moreira et al., 2010). Interestingly, some studies point to the identification of atrophy of lymph nodes in symptomatic animals (Giunchetti et al., 2008; Moreira et al., 2010), but this finding was not identified in any of our samples.

Detection of the cytokines IFN-γ and TNF-α can be related, besides others associations, to Th1 pathway response, which, when predominant, is associated to protection against canine leishmaniasis (Gradoni, 2015). However, these cytokines were significantly related to the absence of follicular hyperplasia, suggesting a lack of visible histological response to infection. Regarding the significant association between IFN-γ and disorganization of the corticomedullary region, maybe, at least at the time of evaluation, the Th1 pathway was not able to control the disease process, as proposed by Santos-Gomes et al. (2002). Also in this sense, the low- or

non-detection of iNOS indicates non-macrophage activation by the classic pathway, which would favor the subsequent parasite elimination by means of nitric oxide production (Wanasen and Soong 2008; Cecilio et al., 2014). Likewise, low detection of IL-12 reduces the maturation of T cells toward the Th1 response that would produce IFN-γ for such classic macrophage activation (Cecilio et al., 2014). In fact, the high detections of both IFN-γ and IL-6 can be, among others aspects, show a balance between the attempt to Th1 pathway parasitic elimination and the active disease (De Lima et al., 2007; Cecilio et al., 2014). Turchetti et al., 2015 also reported no measurable amounts of nitric oxide in macrophages derived from canine monocytes infected with *L. infantum*.

Still in this context, IL-4 and IL-10 detections can indicate, among others factors, macrophage activation by alternative pathway toward the parasite survival (Wanasen and Soong, 2008). IL-10 shows the regulatory activity detection in the immune response of the animals evaluated. In fact, this cytokine modulates both innate and adaptive immunity, primarily by anti-inflammatory effects (Moore et al., 2001; Trinchieri, 2007), helping to avoid collateral damage caused by excessive inflammation. However, such control can limit the effectiveness of the immune response, resulting in the failure of pathogen elimination (Trinchieri, 2007). Also, it has been demonstrated that visceral leishmaniasis may progress even in the presence of cytokines related to the Th1 type response, because their action can be neutralized by immunosuppressive factors such as IL-10 (Rodrigues et al., 2016). In this sense, the increased expression of IL-10 in the SC group and the association between this cytokine and the parasitic DNA load, although not significant, corroborate this assertion.

As for the IL-6 levels, their association with disorganization of the corticomedullary region, as well as the higher median found in the SC group, are in agreement with the findings reported by De Lima et al. (2007), who suggest that this cytokine is a good marker of active disease in peripheral blood of dogs infected with *L. infantum*. Also in this context, IL-6 levels in human serum have already been strongly associated with death by visceral leishmaniasis (Dos Santos et al., 2016). It is also important to highlight the presence of a dog in our study with maximum levels of IL-6, parasitic DNA load, and average amastigote count, but unlike this animal presented no signs or symptoms. Therefore, further studies on the expression of this cytokine in different dog tissues with larger samples are suggested in order to reveal more clearly its possible role as a marker of susceptibility to CVL.

In contrast, the same dog, despite the absence of signs or symptoms, presented granulomas in the histopathological evaluation. This finding suggests chronicity and, therefore, a possible longer infection course time. In fact, dogs infected with *L. infantum* can remain without clinical signs for a long period of time (Brasil, 2006). In addition, its high parasitic DNA load and intense viewing of amastigotes in the tissue confirm the existence of asymptomatic animals associated with high parasite loads (Lima et al., 2004; Marzochi et al., 2009). Still in this context, the absence of significant association between parasitic loads and canine clinical examination in the group evaluated, as well as the presence of individuals in the MNC group with significant parasitic DNA loads, demonstrate the impossibility of using animal clinical status as an evaluation criterion for susceptibility to CVL.

It is worth noting that the clinical examination, as well as the parasite load and the cytokine mRNA profile may vary throughout the infectious process, as shown in the experimental canine model (Costa et al., 2013). Therefore, in addition to the cross-sectional study, the clinical, parasitological and immunological field monitoring of naturally infected dogs would be important; nevertheless, this measure is not viable nowadays due to the need for euthanasia of the animal reservoir (Brasil, 2006, 2009).

5. Conclusions

No significant associations were found between the expressions of cytokines or iNOS and low parasitic loads or mild or absent animal disease through clinical evaluation. In addition, IFN-γ and TNF-α levels were associated with the absence of follicular hyperplasia.

The high levels of IFN-γ and IL-6 detected can, among others factors, indicate a balance between the effort to parasite elimination by Th1 pathway and the active disease. IL-6 is suggested as a marker of the latter condition; however, both cytokines were significantly associated with disorganization of the corticomedullary region.

The action of the regulatory pathway is evident in dogs naturally infected with L. infantum, which may contribute to the non-elimination of the parasite, being significantly associated with animals severely affected by the disease.

Plasmacytosis and sinus histiocytosis findings were significantly associated with high loads of parasitic DNA.

Animal clinical examination cannot be considered as an evaluation criterion in the process of disease susceptibility.

Acknowledgments

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