Research Article

Immunodetection of MIF in Lymph Nodes of Dogs with Visceral Leishmaniasis

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Abstract

In canine Visceral Leishmaniasis (VL), the presence of generalized lymphadenopathy is commonly observed in animals with chronic disease. It is known that parasite *Leishmania (Leishmania) infantum* modulates host immune system, thereby favoring its multiplication. The aim of this study was to evaluate the immunodetection of macrophage migration inhibition factor (MIF) in lymph nodes (popliteal, subscapular, iliac and mesenteric) of naturally infected dogs (asymptomatic and symptomatic) by this protozoan. A set of 33 naturally infected dogs from an endemic area for VL were sampled and another set of five dogs negative for VL and from a non-endemic area were analyzed as the control group. Detection of MIF and parasite load of internal and peripheral lymph nodes of dogs with VL and controls was made by immunohistochemistry. Parasite load was observed only in lymph nodes of infected dogs and differed significantly (P<0.05) from control group. MIF cytokine was detected predominantly on macrophages and lymphocytes in all lymph nodes of all groups. Popliteal lymph node of symptomatic dogs had the highest number of immunomarked cells for MIF, especially in granulomas. Significant differences (P<0.05) among infected and control groups occurred only in popliteal and mesenteric lymph nodes. Additionally, amastigotes forms within macrophages were positive for MIF in symptomatic group. Therefore, it is concluded that MIF plays a favorable role to parasite, mainly in the symptomatic group and this protozoan possibly exerts immune mimicry, which favors its evasion of host immune system.

INTRODUCTION

Visceral Leishmaniasis (VL) is a zoonosis disease caused by protozoan *Leishmania spp*. that causes VL in the American, Europe and Asia [1]. The parasite has affinity for organs rich in mononuclear phagocytic system cells to multiply, such as spleen, lymph nodes, liver and bone marrow [2-7]. Infected dogs tend to develop generalized lymphadenopathy, being peripheral lymph nodes (popliteal, cervical and subscapular) the most affected [2-4,6,8-10].

VL can be considered as an immune-mediated disease, since the parasite has the ability to modulate host immune system [11,12]. Macrophages, dendritic cells, lymphocytes, cytokines (INF-γ, IL-12, TNF-α) and transcription factors (such as interferon regulatory factor 1/IRF-1) are crucial for resolution of infection in all *Leishmania* spp, for the development of lengthy protective immunity [13]. Macrophages are important in the transition from innate to acquired immunity, besides its microbicidal capacity, also feature antigens to lymphocytes and stimulate an effective immune response. These cells are activated by cytokines such as IFN- and TNF-α [14].

The macrophage migration inhibitory factor (MIF) is a cytokine that has as main function of immobilize mononuclear phagocytes and retain then in the site of inflammation [14]. This proinflammatory cytokine is an important activator of innate immune response, by bind to CD74 receptors expressed on the surface of antigen-presenting cells, resulting in activation of macrophages (expression of TNF-α, IL-1 and E2 prostaglandin). Macrophages activated by MIF are more efficient in destruction of intracellular pathogens. Likewise, the interaction between MIF and CD74 and CD44 receptors results in survival of B lymphocytes, by the suppression of apoptosis. These facts make MIF a bridge between innate and adaptive immune response [15]. Supernatants containing MIF, derived from stimulated lymphocytes, were capable of altering macrophage function and increase the mortality rate of microorganisms and tumor cells [16].

MIF is a unique structurally cytokine (12.5kDa) and a critical mediator of acute and chronic inflammatory diseases such as septic shock, rheumatoid arthritis, arteriosclerosis and cancer [17-19]. This cytokine is produced by activated T cells [14], macrophages and pituitary gland cells [20] and may act as a counter-regulator of glucocorticoid inflammatory and immunosuppressive response [21] and induce the release of nitric oxide in activated macrophages. Besides being an activator of innate immunity, also protects monocyte/macrophage from apoptosis [19].

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Elucidation of *Leishmania major* genome revealed two major genes which exhibit a significant similarity with mammalian cytokine MIF [22]. Primitive eukaryotic encode MIF genes, which show remarkable similarity to mammalians ones, such as humans nematodes *Brugia malayi* and *Ancylostoma ceylonicum*, as well as *Amblyomma americanum* ticks, anaplasmosis vector, which produces a ortholog MIF [23-25]. MIF has also been reported in *Eimeria, Trichinella* and *Plasmodium.* This ortholog cytokine possibly plays an important role in host-parasite interaction [26-29].

Endogenous role of MIF has also been evaluated in infections caused by protozoa such as *Trypanosoma cruzi* [30], in mice with experimental cutaneous leishmaniasis by *Leishmania major* [20,31] and in human patients with visceral leishmaniasis [32].

MIF orthologs cloning of *L. major* and functional characterization of ortholog Lm1740MIF of this parasite showed identity of 22% of this sequence with human MIF. Lm1740MIF interacts with CD74 receptor of MIF and present an anti-apoptotic activity, which may facilitate *Leishmania* persistence in macrophages [15]. MIF cytokine may act in increased survival and maintenance of macrophage function by suppression of p53-dependent apoptosis. MIF knockout mice, challenged with LPS, presented reduced of macrophage viability, increased of apoptosis and decreased of proinflammatory function [33]. Contradictorily, in mice experimentally infected with *Leishmania major* deficient in MIF -/-, the development of skin lesions was more severe, nitric oxide production was inhibited and there was a higher parasite load when compared to the same lineage MIF +/+ [31] .

Studies with recombinant MIF of *L. major*, identified two types of this cytokine with architecture similar to mammalian, but with some distinct structural features. One of them was found in all stages of *L. major* cycle (MIF2), already the other is exclusive of amastigotes forms (MIF1). Therefore, it was suggested that parasitic MIF modulates macrophage response of host, promoting parasite survival, possibly with participation of MIF1 in this modulation [34].

Due to the great importance to public health of dogs in the cycle and transmission of *Leishmania sp.* for humans, it becomes necessary to study the alterations of lymph nodes associated with expression of cytokine MIF. Likewise, the literature has highlights that parasites utilize MIF as a tool in modulating the host immune response. As there are differences between the results in mice and no study has been done with *Leishmania infantum* in any specie, the study of MIF behavior in naturally infected dogs may contribute to understanding the complex pathogenesis of VL. Given the above, the present study aimed to evaluate the MIF in lymph nodes of dogs naturally infected with *Leishmania sp.* of asymptomatic and symptomatic groups, and compare these results with parasite load of each lymph node.

MATERIAL AND METHODS

The dogs investigated in this study were originated from the Zoonosis Control Center in Araçatuba, (São Paulo State, Brazil), a region that is endemic for VL [5-7,9,10]. Thirty-three *Leishmania infantum* – infected dogs were used, without preference for age, breed or gender. The animals were euthanized using an intravenous (IV) overdose of barbiturate, followed by IV administration of potassium chloride (decree number 51.838 of the Brazilian Ministry of Health and Resolution number 714, of June 20, 2002, of the Federal Veterinary Medicine Council). The necropsy of the dogs was performed immediately after their death. The control group consisted of five dogs from the routine of the Department of Veterinary Pathology Jaboticabal-SP, Brazil, and a non-endemic area for VL. Infected dogs and control dogs were selected, following confirmation or not of disease respectively, by RIFI and ELISA.

Fragments of peripheral lymph nodes (popliteal and subscapularis) and internal (mesenteric and iliac) were collected at necropsy, which were fixed in buffered formalin 10% solution (pH 7.2) and embedded in paraffin for analysis of morphological and immunohistochemical changes, in optical microscope.

Immunohistochemical technique used for parasitic load followed the protocol described by TAFURI et al. [34], with modifications [9]. After deparaffinization of sections, was performed antigen retrieval by heat to both antibodies, with 10 mM sodium citrate buffer solution (pH 6.0) for 30 minutes. Microwave oven (power of 720w) was used for MIF and water bath (Quimis, Q-codes 304-160) at 95ºC for parasite load. Blocking of nonspecific proteins was done with commercial product (Protein Block Serum-Free Dako Cytomation, cód. X0909) for 20 minutes. To block endogenous peroxidase a hydrogen peroxide (30vol, Merck) and 8% methyl alcohol solution was used. For MIF immunodetection (polyclonal antibody raised in rabbit, FL -115, Santa Cruz Biotechnology, SC 20121) was used at the dilution of 1:700, in a humid chamber at 4°C for 18 hours. For parasitic load, the primary antibody used was hyperimmune serum from a dog positive for VL (ELISA test). The secondary complex used was peroxidase-linked polymers (Advance kit, DakoCytomation, cod. K4068-1). Revelation was made with Diaminobenzidine chromogen (DAB, DakoCytomation, cód. K3468) and counter-staining with hematoxylin. In negative control reactions, the primary antibody was replaced by Tris-HCl buffer (pH 7.4).

For determination of immunostained cells density five microscopic high power fields (40x) were photographed and counting was performed in Micrometrics SE Premium image analysis software. Average of immunostained cells per lymph node was obtained for each animal.

Statistical analyses were done using the nonparametric Kruskal-Wallis test and Dunn's multiple comparison test, with comparisons between the groups of dogs for each lymph node. The Graphpad Prism statistical software (version 4.00, 2003) was used for all the analyses, and differences were taken to be significant when P < 0.05.

RESULTS

Peripheral lymph nodes showed most exuberant gross and microscopic lesions and greater parasite load. Popliteal lymph node of symptomatic animals showed diffuse granulomas, which caused distortion of architecture of this organ, as well as intense amounts of plasma cells in medullary cords. In asymptomatic animals, granulomas were smaller, with multifocal distribution and localized predominantly in the cortico-medullary transition, medullary cords and capsule of lymph node. Presence of

lymphocytes apoptosis from the paracortical area was observed in symptomatic and asymptomatic animals. Hemosiderosis was also observed in medullary sinusoidal macrophages in symptomatic dogs.

Identification of macrophages immunostained with amastigotes forms of *Leishmania sp.* was seen in the granulomas present in all regions of the lymph node, more frequently in medullary region (Figure 1A). The immunostaining occurred in the cytoplasm of macrophages of granulomas and fibroblasts capsule of lymph node.

MIF detection was performed only by the immunohistochemistry technique, where it can be observed that peripheral lymph nodes showed the highest parasitic load and MIF production, with higher median observed in the group of symptomatic dogs (Table 1). Popliteal lymph node showed the largest number of infected macrophages in both infected groups (Table 1). In subscapular lymph node parasite load was higher in the same group. Internal lymph nodes (mesenteric and iliac) had fewer parasitized macrophages. All lymph nodes of infected dogs differ (P< 0.05) from lymph nodes of control group for the number of parasitized macrophages (Table 1/Figure 2).

MIF immunostaining was observed in cytoplasm of macrophages, lymphocytes, plasma cells and capsular fibroblasts.
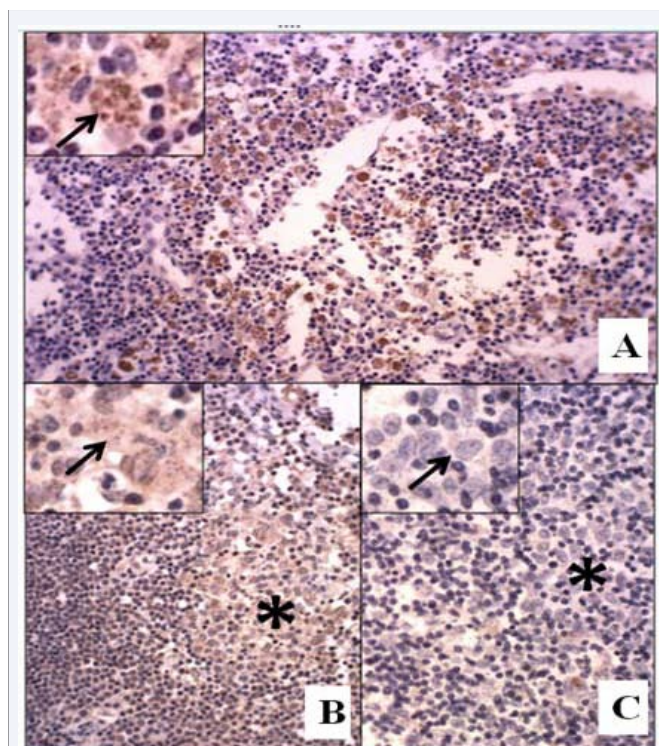
Figure 1 Photomicrograph of lymph node of dog with Visceral Leishmaniasis. (A) Notice the positive immunostaining for *Leishmania infantum* in cytoplasm of macrophages from medullary in popliteal lymph node (arrow/symptomatic group). (B) Note granuloma positive for MIF (*) in the same lymph node. In detail can be observed positive amastigotes forms for MIF (arrow). (C) In subscapular lymph node MIF immunostaining was poor (*). In detail notice negative macrophage for MIF (arrow/symptomatic dog). Peroxidase linked polymers complex (40x).

Intensive immunostaining was noted in granulomas positive for MIF, predominantly in symptomatic dogs and in popliteal lymph node (Figure 2B). Likewise, was observed parasite amastigotes forms immunostained (detail Figure 2B), present in the cytoplasm of macrophages, only in animals with high numbers of parasites such as symptomatic dogs. Intensity of immunostaining was lower or absent in macrophages of granulomas of subscapular lymph node (Figure 2C).

MIF immunodetection was observed in all lymph nodes of asymptomatic and symptomatic groups, which differed (P<0.05) only in the popliteal and mesenteric lymph nodes from control group (Table 1/Figure 3). In control group, all animals were negative for marking the parasite load and had low MIF immunostaining (Figures 2 and 3).

DISCUSSION

Lymphadenopathy is an important aspect of VL in dogs and findings of this study coincided with reports by other authors [2,3,9,10], as symptomatic dogs presented more exuberant lesions, such as reactivity lymphoid atrophy, diffuse to multifocal granulomas, macrophages with variable intracytoplasmic parasites load.

In this work, higher intensity of granulomatous inflammation and parasite load was showed by popliteal lymph node, mainly in symptomatic dogs, when compared to subscapularis lymph node. Moreira et al. [10], studying the same animals of the present study, found that lymphoid atrophy was increased in popliteal lymph node of symptomatic dogs. In another study, these authors related these differences to lymphocyte apoptosis. In this group

Table 1: Median of immunostained cells for parasite load and MIF in lymph nodes of dogs with Visceral Leishmaniasis.

Lymph nodes	Groups	Parasitic load	MIF
	A	10.3^{ab}	19.5^{ab}
Popliteal	S	29.2^{a}	46.2^{a}
	C	0.0^{b}	0.0^{b}
		P=0.001	P=0.0008
	A	2.3^{a}	15.8^{ns}
Subscapular	S	11.2^{a}	12.4^{ns}
	C	0.0^{b}	0.0^{ns}
		P=0.0001	P=0.0736
	A	1.4^{a}	21.0^{a}
Mesenteric	S	2.4^{a}	18.0^{a}
	C	0.0^{b}	0.0^{b}
		P=0.0008	P=0.0114
	A	1.6^{a}	18.4^{ns}
Iliac	S	2.4^{a}	22.2^{ns}
	C	0.0^{b}	0.0^{ns}
		P=0.0010	P=0.0691

Different lowercase letters in columns show significant differences (P <0.05) between groups per lymph node, by Dunn's Multiple Comparison test. Levels of significance of P <0.05 indicated a group effect for parasite load and MIF per lymph node, by Kruskal-Wallis tests. A = asymptomatic; S = symptomatic; C = control.
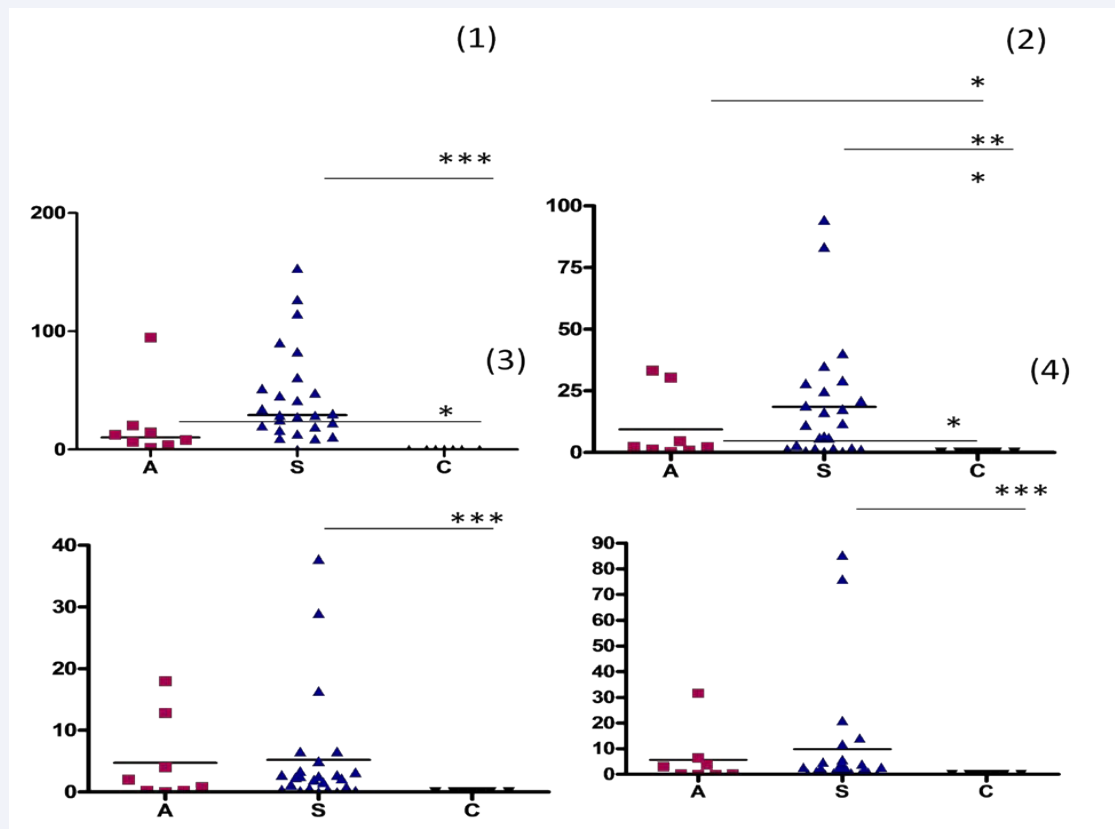
Figure 2 Median of parasitized macrophages in popliteal (1), pre-scapular (2), mesenteric (3) and iliac (4) lymph nodes of dogs with visceral leishmaniasis. Dunn's Multiple Comparison test. * = P <0.05 *** = P <0.001.
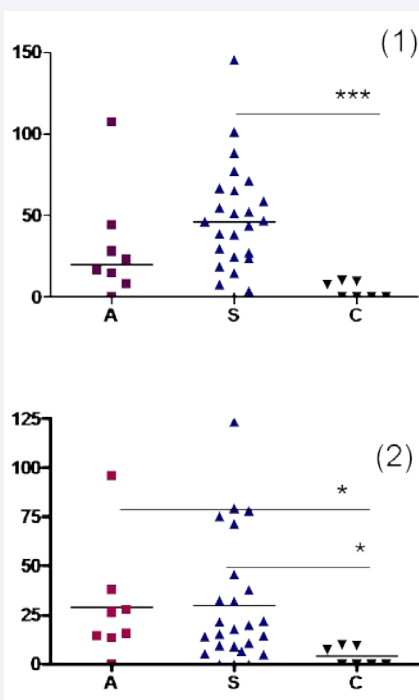
Figure 3 Median of immunostained cells for MIF in popliteal (1) and mesenteric (2) lymph nodes of dogs with Visceral Leishmaniasis. Dunn's Multiple Comparison test. * = P <0.05 *** = P <0.001.

the high density of apoptotic cells coincided with high parasite load. Subscapular lymph node presented lymphoid reactivity, although atrophy has also been observed in the same group. In asymptomatic dogs parasite load and severity of injuries were minor. Giunchetti et al. [3], also describe this kind of response in asymptomatic animals. Lima et al. [2], assessed that there is no relationship between clinical staging and parasite load with the intensity of the lesions in naturally infected dogs.

In the present study, internal lymph nodes (iliac, mesenteric) showed no significant (P>0.05) cellular responses that were important to characterize the systemic framework of VL. Peripheral lymph nodes are possibly more reactive due to the anatomical area that drains. Moreira et al. [10], pointed out that differences in reactivity between them could be explained by different phase of infection. In our study, even working with naturally infected dogs, where it was not possible to determine the exact phase of infection, we agree with these authors. Subscapular lymph node drains head skin, that showed up most affected by inflammatory reactivity and parasites density [10], which is the predilection area of the insect vector. Therefore, the subscapular lymph node would have initial contact with etiologic agent and would develop more severe response with chronic evolution. In popliteal lymph node, this contact would occur later. Animals with little apparent clinical signs it was verified discrete alterations in skin, already in symptomatic animals this drainage area was quite affected and popliteal lymph node lesions should indicate development of VL.

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Presence of macrophages was related to the formation of granulomatous reaction, regardless of clinical group. In animals with advanced symptoms (symptomatic group), this inflammatory reaction was intense and related with atrophy of spinal cord and highest amount of parasites in tissue. Moreira et al. [10], found that granulomas of popliteal lymph nodes were diffuse and lepromatous type, predominantly composed by macrophages linked to a few lymphocytes. In this type of granuloma describes the predominance of Th2-type cytokines [36]. In subscapular lymph node, Moreira et al. [10], noted the presence of greater numbers of lymphocytes interspersed with macrophages. By this fact, it can be inferred that subscapular lymph node was more efficient in containing parasite multiplication, when compared to popliteal, in the groups of infected dogs, by presenting lower density of parasitized macrophages (Table 1). Possibly this differential response profile could be related to the proportion of lymphocytes present in the granulomas of both lymph nodes [6,9,10]. Reis et al. [4], suggested a compartmentalized pattern of response in different host organs, which could be related to the proliferation or controlling of parasite growth in infected organs. Possibly this pattern was occurring in peripheral lymph nodes of this study.

Differences observed between peripheral lymph nodes could also be related to the microenvironment of cytokines in each lymph node, which could have pro or anti-parasite effect. Alves et al. [8], observed that Th2 cytokines (IL-4, IL-10 and TGF-β) in prescapular lymph nodes of symptomatic dogs, associated with high parasite load, led to increased susceptibility to infection by *Leishmania infantum*. Likewise, the anti-inflammatory cytokine IL-10 and TGF-β has been described in spleens of symptomatic and asymptomatic dogs infected by *Leishmania infantum*, confirming the predominance of Th2 profile in active disease, which is related to inefficient macrophages activation [28].

In evaluation of MIF presence, in *Leishmania infantum* naturally infected dogs, it was observed that this cytokine is significantly (P<0.05) present in popliteal and mesenteric lymph nodes of infected dogs. A key role of this cytokine is to keep the macrophage for a long time at the site of injury, preventing its apoptosis [15]. Thus, these cells become susceptible to parasite multiplication. In the present study, the largest number of immunostained cells for MIF occurred in the symptomatic group, coinciding with higher parasite load. Although MIF is described as a cytokine with proinflammatory activity [14], there are reports in the literature about its modulation by pathogens, targeting the immune escape. One of these ways of evading would be the protection of apoptosis in MIF induced macrophages [21]. In the case of parasitized macrophages, this protection would be essential for parasite survival and its systemic dissemination.

MIF immunostaining in amastigotes forms within macrophages suggests that *Leishmania* also produce this cytokine. This finding is consistent with studies of MIF orthologous forms described in *Leishmania major* [34]. This observation allows us to suggest that MIF could be used by protozoa to modulate host immune response, in view of the similarity of the cytokine produced by protozoa with mammalian cytokine. Richardson et al. [34], described a high homology between canine and parasitic MIF.

Satoskar et al. [31], conducted a study with MIF deficient mice, experimentally infected with *Leishmania major* and found that deficient mice were more susceptible to skin lesions caused by this protozoa. This study does not agree with ours results, considering that dogs with chronic disease and more exuberant lesions showed higher density of immunostained cells for MIF, reinforcing the hypothesis that MIF was favoring the development of the parasite in dogs with advanced disease. Probably part of MIF detected in lymph nodes of infected dogs in our study, could be from the parasite, as described by Richardson et al. [34], which describe a MIF produced specifically by amastigotes forms of *Leishmania major*, that could modulate the response of infected macrophages.

MIF presence in popliteal lymph node was higher than in subscapular (Table 1), which could be related to the ratio of lymphocytes present in granulomas of these lymph nodes and lymphoid atrophy in severe cases. Activated lymphocytes can produce MIF. Moreira et al. [10], reported that apoptosis was marked in symptomatic dogs and in popliteal lymph node. Considering that in these two cases parasite load was higher, it can be suggested that MIF produced in granulomas is induced by the parasite. Kamir et al. [15], observed inhibition of apoptosis of macrophages infected with *Leishmania major*, through an MIF ortholog produced by the parasite.

The proinflammatory cytokine MIF makes the connection between innate and adaptive immunity and can act on B lymphocytes, through its interaction with B lymphocytes receptors CD74 and CD44. This interaction inhibits apoptosis of these cells and promotes their proliferation. Perhaps the high density of plasma cells observed in this study could also be influenced by MIF. This cytokine protects macrophage and B lymphocyte from apoptosis, and can influence the cytokine profile of lymph node microenvironment. Elevated plasmocytosis is related to high titer of circulating nonspecific antibodies, further aggravating the clinical status of dogs, due to immune complexes deposition. Possibly, MIF is one of the tools that *Leishmania infantum* uses for its immune evasion, since this cytokine makes the connection between innate and adaptive immunity of host.

CONCLUSION

We conclude that MIF cytokines play an important role in the development of VL in dogs, due to their relation with the higher parasite load in the symptomatic group. This cytokine could contribute to the parasitic immune evasion, keeping the macrophages at the site of the lesion and, thus, favoring the multiplication and survival of the parasite, not only in the lymph node, but also in the infection of the organs susceptible to VL.

ETHICAL STANDARDS

The design for this study was approved by the Ethics and Animal Welfare Committee (CEUA nº. 0020676-08), of FCAV / UNESP, Jaboticabal, State of São Paulo, Brazil.

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