



Pro-inflammatory cytokines predominate in the brains of dogs with visceral leishmaniasis: A natural model of neuroinflammation during systemic parasitic infection

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ABSTRACT

Visceral leishmaniasis is a multisystemic zoonotic disease that can manifest with several symptoms, including neurological disorders. To investigate the pathogenesis of brain alterations occurring during visceral leishmaniasis infection, the expression of the cytokines IL-1 β , IL-6, IL-10, IL-12p40, IFN- γ , TGF- β and TNF- α and their correlations with peripheral parasite load were evaluated in the brains of dogs naturally infected with *Leishmania infantum*. IL-1 β , IFN- γ and TNF- α were noticeably up-regulated, and IL-10, TGF- β and IL-12p40 were down-regulated in the brains of infected dogs. Expression levels did not correlate with parasite load suggestive that the brain alterations are due to the host's immune response regardless of the phase of the disease. These data indicate the presence of a pro-inflammatory status in the nervous milieu of dogs with visceral leishmaniasis especially because IL-1 β and TNF- α are considered key factors for the initiation, maintenance and persistence of inflammation.

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1. Introduction

Leishmaniasis is a complex disease caused by more than 20 different species of protozoans of the *Leishmania* genus (Kinetoplastida, Trypanosomatidae). Leishmaniasis is transmitted by over 30 distinct phlebotomine sand flies and has four different types of clinical presentation: visceral leishmaniasis (VL), cutaneous leishmaniasis, muco-cutaneous leishmaniasis, and post-kala-azar dermal leishmaniasis (PKDL). Leishmaniasis is an important cause of human death, among the parasitic diseases it is

surpassed only by malaria (Chappuis et al., 2007). VL, also known as kala-azar, is an anthroponosis caused by the *Leishmania donovani* complex: *L. infantum* (Syn. *chagasi*) in the Americas and in the Mediterranean basin, and *L. donovani* in Asia and Africa (Baneth et al., 2008; Lukeš et al., 2007; Mauricio et al., 2000).

In addition to the skin, VL affects mainly the organs with cells of the mononuclear phagocyte system, such as the spleen, lymph nodes, liver, bone marrow. The typical histopathological finding is granulomatous inflammatory reaction associated with the presence of *Leishmania* amastigotes within macrophages (Alvar et al., 2004; Baneth et al., 2008). Although there are several reports of the systemic symptoms of VL, such as fever, anemia, weight loss, skin disease, and renal and ocular alterations (Alvar et al., 2004; Blavier et al., 2001; Ciaramella and Corona, 2003; Moreno et al., 1998), few studies have related the occurrence of injuries in the central nervous system (CNS).

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In humans, the involvement of the peripheral nervous system in VL is more usual than the involvement of the CNS (Snydman et al., 2006). Hashim et al. (1995) reported that 46% of patients with VL presented neurological symptoms, most of them describing the sensation of burning feet. In addition to peripheral neuropathy, there are reports of alterations similar to those in Guillain–Barré syndrome, cranial nerve dysfunction and meningitis (Snydman et al., 2006). Dogs with VL may present signs of generalized CNS involvement, with seizures, cranial nerve alterations, vestibular and cerebellar signs, motor incoordination, tetraparesis and tetraplegia, and myoclonia (Font et al., 2004; Ikeda et al., 2007; José-López et al., 2012; Melo et al., 2012). The main histopathological alterations in the brains of these dogs are meningitis and choroiditis, which are mainly secondary to antigen and immunoglobulin deposition and leukocyte infiltration (García-Alonso et al., 1996; Ikeda et al., 2007; Melo et al., 2009; Melo and Machado, 2009, 2011; Nieto et al., 1996; Viñuelas et al., 2001).

VL is an immunomediated disease, and studies have demonstrated a failure of the cellular response in symptomatic dogs that is characterized by a diminishment of the lymphoproliferative response against *Leishmania* antigens and a decrease in the number of CD4⁺ T lymphocytes (Pinelli et al., 1999; Solano-Gallego et al., 2000). In infected but clinically healthy dogs, the Th1 immune response prevails and is mediated by IL-2, IFN- γ and TNF- α . This is in contrast to symptomatic animals, in which cytokines such as IL-4, IL-10 and TGF- β mediate the Th2 response (Barbiéri, 2006; Reis et al., 2009; Solano-Gallego et al., 2011; Strauss-Ayali et al., 2005). Nevertheless, immune response polarization is not a rule during *Leishmania* infection due to the role of regulatory T cells (Treg), which suppress the immune response by means of cell-cell interactions and/or production of suppressor cytokines, such as IL-10 and TGF- β , which render Th0 the immune response against *Leishmania* (Belkaid et al., 2002; Campanelli et al., 2006; Gantt et al., 2003).

In view of the paucity of available data relating VL to the CNS and because the presence of cytokines in the nervous milieu may indicate an inflammatory environment, the aims of this study were to determine the gene expression of the pro-inflammatory cytokines IL-1 β , IL-6, IL-12p40, IFN- γ and TNF- α and the anti-inflammatory cytokines IL-10 and TGF- β in the brains of dogs naturally infected with VL and to determine the correlation between the brain cytokine profile and the peripheral parasite load.

2. Materials and methods

2.1. Animals

Twenty-five dogs, 13 male and 12 female, ranging in age from 1 to 6 years old that were referred to the Veterinary Teaching Hospital of UNESP, São Paulo State University in Araçatuba, São Paulo State, Brazil were included in this study. Nineteen naturally infected dogs, which were euthanized with the owners' permissions, in compliance with state law (São Paulo, 2006), as soon as the VL diagnosis was confirmed were included in the infected group, and

six uninfected healthy dogs, with no underlying condition and whose deaths had no sign of nervous involvement (e.g. trauma) were included in the control group. VL diagnosis was achieved using a routine ELISA (enzyme-linked immunosorbent assay) according to Lima et al. (2005). None of these animals were previously vaccinated against CVL. The animals were also negative for toxoplasmosis and neosporosis, as assessed by indirect immunofluorescence assays.

2.2. Sampling

Blood samples were collected, and the dogs were euthanized with an overdose of pentobarbital (Nembutal[®]) and potassium chloride. Necropsies were performed immediately after euthanasia, and macroscopic lesions were recorded. Brains were collected and separated into two hemispheres; one of which was placed in 10% buffered-formalin, and fragments of 0.5 cm³ from the thalamus, hippocampus, piriform/temporal cortex and periventricular white matter were collected from the other hemisphere and stored in RNAlater (Applied Biosystems, AM7020) and frozen at -80 °C. Fragments of 2 cm³ of spleen were also collected and immediately frozen at -80 °C.

2.3. Brain cytokine quantification by RT-qPCR

Total RNA was extracted from the brain samples stored in RNAlater weighing ca. 100 mg using the RiboPure kit (Applied Biosystems, AM1924) according to the manufacturer's protocol. After RNA extraction, any potential contaminating DNA was removed by incubation with DNase I (Applied Biosystems, AM1928) at 37 °C for 30 min. Next, RNA was quantified with a NanoDrop spectrophotometer (260/280 ratio between 2.0 and 2.3) and frozen at -80 °C until the reverse transcription reaction was performed. cDNA production was achieved using the RETROscript kit (Applied Biosystems, AM1710) with 1000 ng of RNA and oligo(dT) primers in a final volume of 20 μ L and cycled at 44 °C for 1 h and 92 °C for 10 min. Then, the cDNA was frozen at -20 °C until further analyses. Specific canine primers and hydrolysis probes for amplifying cytokines were selected based on the scientific literature (IL-1 β , IL-6, IL-10, IL-12p40, IFN- γ and TGF- β) or designed (TNF- α) using the on-line software Primer3Plus¹ (Untergasser et al., 2007). The hydrolysis probes were labeled with the reporter dye FAM at the 5' end, and with the quencher dye BHQ-1 at the 3' end (Table 1). qPCR was carried out in a real-time thermocycler (Bio-Rad, CFX96) using TaqMan Master Mix (Applied Biosystems, 4304437), 400 nM of each primer, 250 nM of the probe and 5 μ L of cDNA in a total volume of 25 μ L. The amplification conditions were as follows: 55 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min. For each cytokine, values of reaction efficiency, determination coefficients (r^2) and angular coefficients (slopes) were obtained from amplification of seven serial dilutions of a pool of cDNA. Quantification of gene expression was performed by the $2^{-\Delta\Delta Ct}$ method

¹ <http://www.primer3plus.com>.

Table 1

Primer (forward: F, reverse: R) and hydrolysis probe (P) sequences used for RT-qPCR cytokine quantification in the brains of dogs.

Target	Primers and probes	Sequence (5' → 3')	Product size (bp)	GenBank accession number	Reference
G3PDH	F R P	TCAACGGATTGGCCGTATTGG TGAAGGGGTCATTGATGGCG CAGGGCTGCTTTAACTCTGGCAAAGTGAA	90	AB022763	Peters et al. (2005)
IL-1 β	F R P	TCTCCCACCAGCTCTGTAACAA GCAGGGCTTCTTCAGCTTCTC CTGAGGCATTCGTGTCAGTCATTGTAGCTT	80	Z70047	Fujiwara et al. (2003)
IL-6	F R P	CTCTCCACAAGCGCCTTCTC TGAAGTGGCATCATCCTTGG TGGGGCTGCTCTGGTGATG	102	U12234	Peters et al. (2005)
IL-10	F R P	CGACCCAGACATCAAGAACC CACAGGGAAGAAATCGGTGA TCCCTGGGAGAGAAGCTCAAGACCC	101	U33843	Peters et al. (2005)
IL-12p40	F R P	CAGCAGAGAGGGTCAGAGTGG ACGACCTCGATGGGTAGGC TGGAGTGTCAAGAGGGCAGTGC	109	AF091134	Peters et al. (2005)
IFN- γ	F R P	TCAACCCCTTCTCGCCACT GCTGCCTACTTGGTCCCTGA CCCCACCCGAACCTTCTCT	113	AF126247	Peters et al. (2005)
TGF- β	F R P	CTGGAGTCGTGAGGCAGTG GCAGTGTGTATCTTTGCTGTCA TTTCGCCTCAGTGCCCACTG	96	L34956	Peters et al. (2005)
TNF- α	F R P	ACCCATGTGCTCCTCACC AGGGCTCTTGATGGCAGAGA CGCTTCGCCGTCTCTACCA	87	Z70046	–

according to Livak and Schmittgen (2001) using G3PDH (glyceraldehyde-3-phosphate dehydrogenase) as a reference gene. The results are expressed as relative gene expression, which indicates how many times (fold change) the gene expression of a cytokine is higher (up-regulated) or lower (down-regulated) in the infected dogs when compared with the control group.

2.4. Brain histopathology

Brain hemispheres were placed in 10% buffered-formalin and were kept in this fixative for a maximum of 24–48 h before being paraffin-embedded, sectioned (5 μ m) and stained with hematoxylin and eosin (HE) using routine protocols. The following brain areas were evaluated by light microscopy: cerebral cortex, thalamus, hippocampus, pons-medulla oblongata, cerebellum, and the ventricular choroid plexi and periventricular white matter.

2.5. Splenic parasite load determination by qPCR

Total DNA was extracted from spleen samples weighing ca. 25 mg using the DNeasy blood & tissue kit (Qiagen, 69506) according to the manufacturer's protocol. A standard curve was composed with *L. infantum* (Syn. *chagasi*) DNA (strain MHOM/BR/72/LD46) extracted from 1.6×10^7 promastigotes/mL using the phenol:chloroform method and divided into seven serial dilutions (1.6×10^5 until 1.6×10^{-1} promastigotes/mL). The DNA was then quantified with a NanoDrop spectrophotometer (260/280 ratio between 1.8 and 2.0) and

frozen at -20°C until further analyses. The selected primer pair (forward: 5'-CCTATTTTACCCAACCCCCAGT-3'; reverse: 5'-GGGTAGGGGCGTCTGCGAAA-3'; GenBank accession number Z35276) amplified a 116 bp fragment of the minicircle kinetoplast DNA (kDNA) of *Leishmania* (Ranasinghe et al., 2008). qPCR was carried out in a real-time thermocycler (Bio-Rad, CFX96) using SYBR green Master Mix (Sigma–Aldrich, S4438), 900 nM of each primer and 5 μ L of DNA in a total volume of 25 μ L. The amplification conditions were the following: 94°C for 2 min and 40 cycles of 94°C for 15 s and 60°C for 1 min. Then, the samples were submitted to a melt curve from 60°C to 95°C , with a 0.5°C increase every 5 s.

2.6. Statistical analysis

For statistical analysis, all values were log-transformed. For analyses involving comparisons between groups, the values were derived from the equation $2^{-\Delta\text{Ct}}$. Significant differences between groups were determined by Student's *t* test, and correlations were evaluated with Pearson's test. Statistical significance was accepted when $P < 0.05$, and changes were considered as trends when $0.05 < P < 0.1$. All statistical analyses were performed using Prism software (Prism 5, GraphPad)

2.7. Ethical issues

This study was approved by the institutional Ethics and Animal Welfare Committee (CEEA – Comissão de Ética e Experimentação Animal, UNESP, process #05/06).

Table 2

Reaction efficiency values (E), determination coefficients (r^2) and angular coefficients (slope) of the RT-qPCRs of each evaluated gene.

Target	E	r^2	Slope
G3PDH	99.8	0.956	-3.327
IL-1 β	99.9	0.976	-3.323
IL-6	96.5	0.983	-3.383
IL-10	99.9	0.981	-3.323
IL-12p40	98.5	0.971	-3.331
IFN- γ	99.1	0.969	-3.342
TGF- β	96.9	0.992	-3.399
TNF- α	97.2	0.989	-3.419

3. Results

3.1. Cytokine gene expression in the brain

With the selected primer pairs and hydrolysis probes, it was possible to quantify the gene expression of the pro-inflammatory cytokines IL-1 β , IL-6, IL-12p40, IFN- γ , and TNF- α , and the anti-inflammatory cytokines IL-10 and TGF- β . Values for reaction efficiency, slope and the r^2 of each evaluated gene are shown in Table 2. The levels of the gene expression of each cytokine in both experimental groups are shown in Table 3. Through the equation $2^{-\Delta\Delta Ct}$ it was possible to observe the pattern of the cytokines expression in the infected dogs, when compared with the control group (Fig. 1). In the brains of dogs with VL, IL-1 β expression was found to be significantly up-regulated by 8.04-fold, similarly to IFN- γ , by 5.85-fold, and to TNF- α , by 6.36-fold. The expressions of the anti-inflammatory cytokines were significantly down-regulated in the brains of dogs with VL: IL-10 (6.08-fold less) and TGF- β (5.25-fold

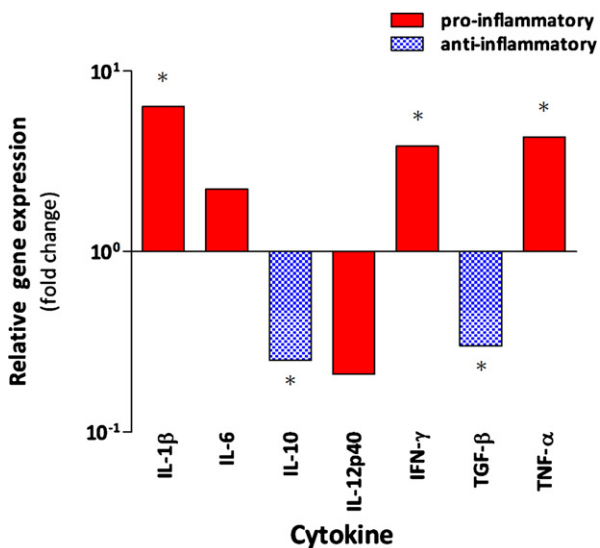


Fig. 1. Relative gene expression of cytokines in the brains of dogs with visceral leishmaniasis. The values were log-transformed and are expressed as fold changes. Positive values indicate up-regulation of gene expression and negative values indicate down-regulation of gene expression as compared to the control dogs. The normalization factor was the reference gene G3PDH according to the $2^{-\Delta\Delta Ct}$ method. * Indicates $P < 0.05$.

less). There was noticed a trend to the down-regulation of the pro-inflammatory cytokine IL-12p40 (6.81-fold less) in the brains of the infected dogs. Further, IL-6 expression was higher in the brains of dogs with VL (3.49-fold more) although with no significance.

To evaluate the interaction between different cytokines, log-transformed values were subjected to Pearson's correlation analyses. Among the pro-inflammatory cytokines, the expression of TNF- α had a strong positive correlation with IL-6 ($P = 0.0041$; $r = 0.693$; Fig. 2a) and moderate positive correlations with IL-1 β ($P = 0.0463$; $r = 0.489$; Fig. 2b) and IFN- γ ($P = 0.0101$; $r = 0.605$; Fig. 2c). IL-6 expression exhibited a trend toward a moderate positive correlation with IFN- γ ($P = 0.0997$; $r = 0.412$; Fig. 2d). In contrast, IL-6 gene expression was moderately negatively correlated with IL-10 ($P = 0.0142$; $r = -0.582$; Fig. 2e); specifically, up-regulation of IL-6 was related to down-regulation of IL-10.

3.2. Splenic parasite load

Through the standard curve composed of seven serial dilutions (10^5 – 10^{-1}) of *Leishmania* DNA, it was possible to quantify the amount of protozoan DNA in the spleens of all infected dogs. This number ranged from 4.44×10^{-1} to 8.31×10^5 parasites/25 mg of splenic tissue (mean 5.32×10^4). None of the control dogs presented positive amplification. Amplification reactions obtained an efficiency value of 100% with a determination coefficient of $r^2 = 0.968$ and a slope of -3.323 . The melt curve indicated the specificity of the primer pair selected, as only one peak was present at 83.5°C . To test whether the peripheral parasite load influenced cytokine gene expression in the brain, log-transformed values of splenic parasite load and cytokine gene expression were subjected to Pearson's correlation analyses. No correlations were noted between splenic parasite load and IL-1 β ($P = 0.3884$), IL-6 ($P = 0.1758$), IL-10 ($P = 0.8716$), IL-12p40 ($P = 0.6869$), IFN- γ ($P = 0.2606$), TGF- β ($P = 0.4029$) or TNF- α ($P = 0.1716$).

3.3. Brain histopathology

Histopathological analyses revealed inflammatory alterations in the brain, especially regarding leukocyte infiltration. Perivascular mononuclear cell infiltrates that ranged in intensity from mild to severe were detected in the leptomeninges, choroid plexus and periventricular areas. Perivascular mononuclear cell infiltration was also observed in the brain parenchyma, but with minimal to moderate intensity (Fig. 3a–d). Nervous cell alterations including the occurrence of satellitosis and neuronophagia were also observed. Glial cell alterations were more evident, particularly in periventricular areas, with focal and diffuse gliosis (Fig. 3e and f).

3.4. Macroscopic lesions

The main macroscopic alterations observed in the dogs during necropsic examination are reported in Table 4. The most prevalent findings were cutaneous lesions (seborrhea, periocular alopecia, generalized alopecia, nasal planum hyperkeratosis, pododermatitis), ocular lesions

Table 3

Cytokine gene expression in the brain of dogs. For each cytokine, individual values were normalized with the reference gene G3PDH using the equation $2^{-\Delta Ct}$. Values were then log-transformed and expressed as mean (\pm standard deviation).

Cytokine	Group		Relative expression ^a	P-value
	Infected	Control		
IL-1 β	-4.09 (\pm 0.47)	-4.90 (\pm 0.58)	↑	0.0020 [*]
IL-6	-4.44 (\pm 1.06)	-4.79 (\pm 0.89)	↑	0.4801
IL-10	-4.17 (\pm 0.55)	-3.57 (\pm 0.72)	↓	0.0383 [*]
IL-12p40	-4.45 (\pm 0.96)	-3.77 (\pm 0.91)	↓	0.0918
IFN- γ	-3.93 (\pm 0.79)	-4.94 (\pm 1.11)	↑	0.0238 [*]
TGF- β	-2.62 (\pm 0.41)	-2.10 (\pm 0.39)	↓	0.0106 [*]
TNF- α	-4.32 (\pm 0.74)	-5.24 (\pm 0.66)	↑	0.0174 [*]

^{*} Indicates $P < 0.05$.

^a Cytokine expression in the infected dogs in comparison with the control dogs. ↑: up-regulation; ↓: down-regulation.

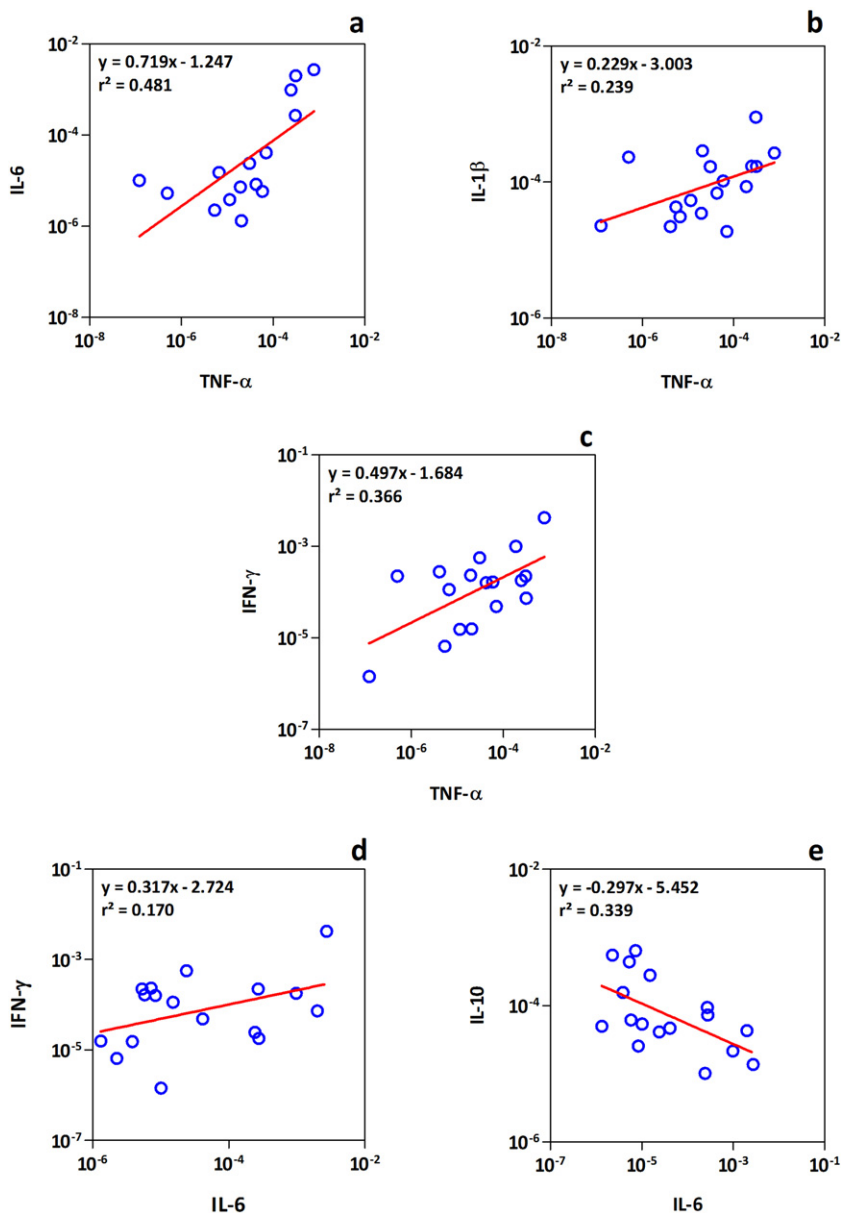


Fig. 2. Correlations between cytokine gene expression in the brains of dogs with visceral leishmaniasis. The scatter plots indicate the positive correlation between TNF- α and IL-6 (a; $P = 0.0041$), TNF- α and IL-1 β (b; $P = 0.0463$), TNF- α and IFN- γ (c; $P = 0.0101$), the trend toward a positive correlation between IL-6 and IFN- γ (d; $P = 0.0997$) and the negative correlation between IL-6 and IL-10 (e; $P = 0.0142$). The least squares equation and the coefficient of determination (r^2) are shown on the plots. All values were log-transformed.

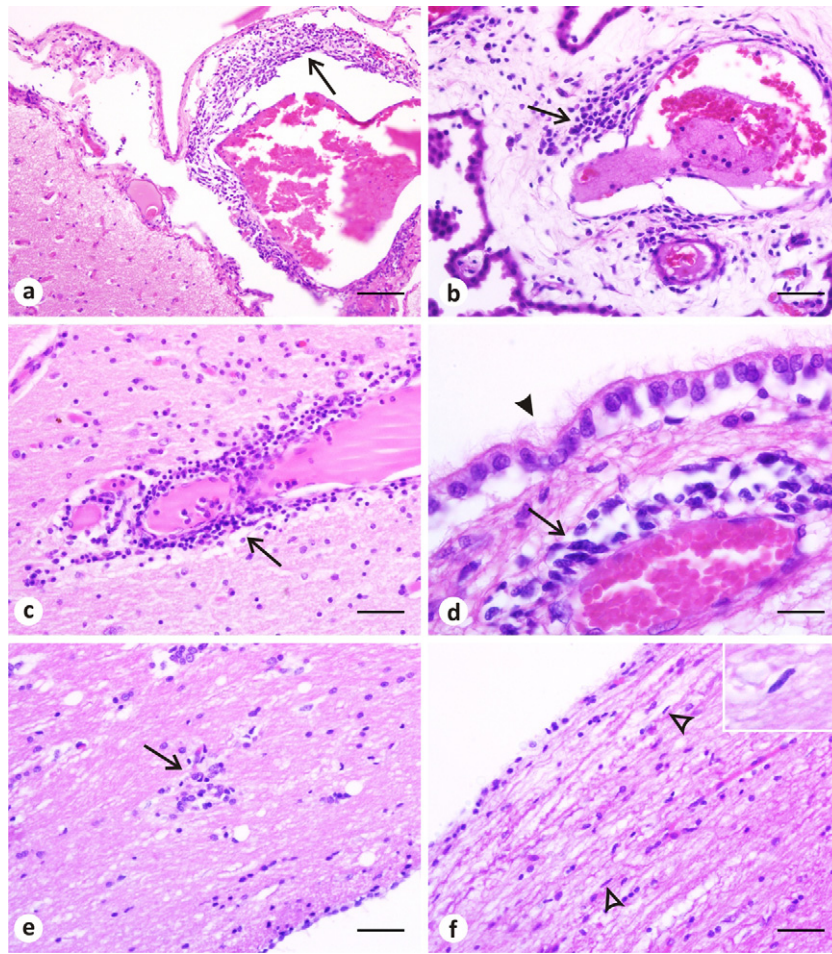


Fig. 3. Representative photomicrography of brain alterations in dogs with visceral leishmaniasis. (a) Intense perivascular lymphoplasmacytic infiltration (arrow) in cortical leptomenige. (b) Perivascular lymphoplasmacytic infiltration (arrow) in the choroid plexus from the lateral ventricle. (c) Mononuclear perivascular cuff (arrow) in the piriform cortex. (d) Mononuclear perivascular infiltration (arrow) in the subependymal area with no alteration of the ependymal layer (arrowhead). (e) Focal glial nodule (arrow) in the subependymal area. (f) Diffuse microgliosis in the subependymal area. Note the high number of microglial cells (arrowhead). *Inset*: high magnification of a microglial cell highlighting an elongated nucleus with a rod shape (rod cell). Hematoxylin and Eosin. Scale bar = 100 μm (a), 50 μm (b, c, e, f), 20 μm (d).

(conjunctivitis, blepharitis, ocular discharge), generalized lymphadenopathy and emaciation/cachexia. There were no signs of macroscopic brain lesions.

Table 4

Macroscopic alterations observed during the necropsic examination of the dogs.

Macroscopic alteration	Infected (n = 19)		Control (n = 6)	
	n	%	n	%
Brain alterations	–	–	–	–
Cutaneous lesions	11	57.9	–	–
Emaciation/cachexia	6	31.6	–	–
Evident splenic white pulp	3	15.8	–	–
Hepatosplenomegaly	4	21.1	–	–
Lymphadenopathy	11	57.9	–	–
Ocular lesion	9	47.4	–	–
Onycogryphosis	7	36.8	–	–
Other	3	15.8	–	–
No alteration	3	15.8	6	100.0

4. Discussion

The main histopathological lesions found in the brain of the dogs were related to leukocyte infiltration and glial cells activation. Meningitis and choroiditis are well described in dogs with VL (Nieto et al., 1996; Viñuelas et al., 2001; Melo et al., 2009; Melo and Machado, 2009). Vasculitis is a widespread condition found in dogs with VL primarily due to immune complex deposition with consequent complement activation; in the SNC this condition may lead to the occurrence of brain and spinal cord infarctions (Font et al., 2004; José-López et al., 2012). Similar inflammatory and ischemic lesions were also described in human and murine cerebral malaria (Penet et al., 2005; Turner, 1997), in feline toxoplasmosis (Dubey et al., 1996) and in the human coinfections toxoplasmosis/HIV (Ibebuikwe et al., 2012) and Chagas disease/HIV (Madalosso et al., 2004).

In order to associate the histopathological alterations with a “inflammatory status”, it was decided to determine the cytokine profile expressed in the brains of dogs with

VL. Consequently, it was possible to detect and quantify the expression of the pro-inflammatory cytokines IL-1 β , IL-6, IL-12p40, IFN- γ and TNF- α and the anti-inflammatory cytokines IL-10 and TGF- β in the brains of dogs by RT-qPCR.

In the brains dogs with VL, up-regulation of the expression of IL-1 β and TNF- α were noted, as well as a slight increased expression of IL-6. These are the main cytokines observed in the CNS during inflammation and are related to fever, defense against pathogens and induction of the production of other cytokines (de Vries et al., 1997). A positive correlation between the expression of the pro-inflammatory cytokines IL-1 β , IL-6, IFN- γ and TNF- α was also detected, which supports the notion of a relationship between the biosynthesis of these cytokines and illustrates the intricacy and redundancy of the immune response in the CNS.

More specifically, one key feature of the inflammatory process in the CNS is the integrity of the blood–brain barrier (BBB). IL-1 β is considered the most important cytokine acting in BBB breakdown, along with TNF- α and IL-6, which act by increasing the expression of adhesion molecules in the endothelium. This enables leukocyte traffic into the brain (Abbott et al., 2006; de Vries et al., 1996; Hickey, 1999; Quagliariello et al., 1991). Evidence supporting the action of IFN- γ across the BBB has also been reported. It has been shown that brain microvascular endothelial cells incubated with IFN- γ suffer morphological and organizational changes that enable permeability alterations and increase MHC class II expression (Huynh and Dorovini-Zis, 1993). Further, high levels of systemic IFN- γ has been described in VL, however, this increase was not related to parasite control, but with disease chronicity, probably due to a defective intracellular signaling and low expression of IFN- γ receptors (Ansari et al., 2006).

The integrity of the BBB is not only affected by factors that disrupt it but also by the lack of components that support its integrity. TGF- β is related to BBB integrity through the maintenance of the tight junctions between the endothelial cells (Dohgu et al., 2004) and by the continuous production by the BBB's pericytes (Dohgu et al., 2005). Further, regarding the role of pericytes, these cells are sensitive to TNF- α and release matrix metalloproteinase 9 (MMP-9), one important proteolytic enzyme related to BBB disruption (Takata et al., 2011). TNF- α was found up-regulated in the brain and because the presence of MMP-9 has already been described in cerebrospinal fluid (CSF) and in brains of dogs with VL (Machado et al., 2010; Marangoni et al., 2011), we hypothesize that *Leishmania* infection promotes a dysregulation of the BBB's selective permeability, with later leukocyte infiltration. MMP-9 was also up-regulated following an in vitro treatment with IL-1/TNF- α in choroid plexus epithelium cultures (Strazielle et al., 2003), an important structure through which lymphocytes migrate into the nervous milieu (Petito and Adkins, 2005).

The histopathological brain alterations in the dogs with VL might also be secondary to the action of pro-inflammatory cytokines, such as IFN- γ , which have previously been related to in vitro astrocyte proliferation and gliosis in the mouse brain (Yong et al., 1991), similar to what occurs in the brain of dogs with VL (Melo and

Machado, 2011). On the other hand, the lack of neuroprotective factors such as IL-10 and TGF- β may also contribute to brain lesions. IL-10 inhibits cytokine production and cytokine receptor expression in microglial cells (Sawada et al., 1999), and IL-10 knock-out mice were more susceptible and did not have spontaneous recovery in experimental autoimmune encephalomyelitis (Samoilova et al., 1998).

Regarding TGF- β , which is considered an anti-neurodegenerative agent, studies with knock-out mice revealed the ability of this cytokine to maintain neuronal integrity and survival, and regulate microglial activity (Brionne et al., 2003). The down-regulation of TGF- β gene expression provides further evidence for elucidation of the histopathological alterations observed, and corroborates the results from previous studies concerning microglial activation in the brain of the dogs with VL (Melo and Machado, 2011).

Surprisingly, the expression of the pro-inflammatory cytokine IL-12p40 tended to be diminished in the brains of dogs with VL. A possible explanation of this diminishment may be the negative feedback relationship with the action of other cytokines, such as TNF- α and type-I IFN, or even other inflammatory mediators, such as prostaglandin E2, components of the complement system, and immune complexes (Ma et al., 2000; Ma, 2001; McRae et al., 1998). Moreover, within the CNS, the main IL-12 producers are microglia, and interaction with other cells may interfere with its ability to produce this cytokine. In vitro, microglial cells stimulated with lipopolysaccharide (LPS)/IFN- γ produced high amounts of IL-12p40 and IL-12p70; however, in microglial and astroglial co-cultures, and in microglial cultures with added astroglial-conditioned medium, the detection of both of IL-12p40 and IL-12p70 presented significant decreases (Aloisi et al., 1997). Since IL-12 is a key Th1 polarizing cytokine (Watford et al., 2003) the diminishment of this cytokine may indicate a lack of ability to mount an effective immune response against *Leishmania* infection (Mattner et al., 1996), especially regarding the nervous milieu, but strongly suggests that leukocyte infiltration into the brain during VL is not dependent of this cytokine.

Among the main neurological symptoms reported in dogs with VL is the occurrence of seizures (Ikeda et al., 2007; Melo et al., 2012). Recently, inflammation and BBB disruption have been considered major causes of epileptogenesis (Choi and Koh, 2008; Vezzani and Granata, 2005), and the occurrence of spontaneous seizures has been associated with reactive gliosis and neuronal/astroglial degeneration (Borges et al., 2003; Choi and Koh, 2008; Vezzani and Granata, 2005). Furthermore, microglial expression of a variety of cytokines, such as IL-1, IL-2, IL-6, IFN- γ , TNF- α and GM-CSF has been related to astrogliosis; and because astrocytic hypertrophy deregulates glutamate absorption by astrocytes, astrogliosis may predispose to the occurrence of seizures (Hanisch, 2002; Choi and Koh, 2008).

To the authors' knowledge, this is the first study that evaluates the brain cytokine profile of dogs with VL. The brain cytokine profile is rather similar to comparative analyses with other protozoan diseases. Rats infected with *Trypanosoma brucei* exhibited high levels of IL-1 β and

TNF- α mRNA in the brain (Quan et al., 1999). In mouse toxoplasmic encephalitis, increased IL-1 β and also IL-10 (Schlüter et al., 1997) has been observed. Astrocytes infected with *Toxoplasma gondii* produced IL-1 β , IL-6 and GM-CSF, while microglia produced TNF- α and TGF- β concomitantly (Fischer et al., 1997). In murine cerebral malaria, increased IL-1 β , IFN- γ and TNF- α gene expression have been reported, but no alterations in IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 or GM-CSF have been described (Jennings et al., 1997). Further, cerebral-malaria-susceptible mice expressed lower levels of IL-10 in the brain (Kossodo et al., 1997), and in human cases of cerebral malaria, high immunohistochemical detection of TGF- β 1, 2 and 3 has been observed (Deininger et al., 2000). Despite the occurrence of an immune activation status in the CNS of patients with these diseases, the cytokine profiles are dissimilar, especially concerning the anti-inflammatory cytokines. This dissimilarity may be due to differences inherent to each pathogen and the absence of *Leishmania* in the nervous environment, unlike that which occurs with *Plasmodium*, *Toxoplasma* and *Trypanosoma*.

To date, there is not enough evidence of *Leishmania* entry into the CNS (Nieto et al., 1996; Viñuelas et al., 2001), and several studies failed the attempt to detect the parasite in the brain (Ikeda et al., 2007; Melo and Machado, 2009, 2011). Therefore, the inflammatory alterations in the brains of dogs with VL are considered to be a consequence of the peripheral immune stimuli and the immune complex deposition and circulating antigens (Garcia-Alonso et al., 1996; Ikeda et al., 2007).

When the BBB selective filtration is compromised, the passage of inflammatory mediators and immunoglobulins is allowed (Diamond et al., 2009). It is known that lymphocytes, macrophages, mast cells, as well as microglia, astrocytes, oligodendrocytes and also neurons possess receptors for the Fc portion of the immunoglobulins. The activation of these receptors initiates a series of biological responses including phagocytosis, degranulation and cytokine genes activation (Nimmerjahn and Ravetch, 2008; Okun et al., 2010). High titers of anti-*Leishmania* antibodies in the CSF have already been described in dogs with natural VL (Garcia-Alonso et al., 1996; Lima et al., 2003; Ikeda et al., 2007; Melo et al., 2009), and, therefore, the presence of circulating antibodies within the nervous milieu may contribute to the beginning and to the maintenance of an inflammatory process, as described in other immune and degenerative diseases of CNS (Seiwa et al., 2007; Deane et al., 2009).

The CNS response to peripheral stimuli is well described in experimental animals. Peripheral LPS administration in mice promotes IL-1 β , IL-6 and TNF- α gene expression in the spleen and brain with distinct expression patterns (Layé et al., 1994). Furthermore, in rats that received peritoneal LPS administration, up-regulation of IL-1 β gene expression in the brain was observed, and the first brain regions to present the expression of this cytokine were the choroid plexus, the circumventricular organs and the leptomeninges (Quan et al., 1998). Despite using LPS as an experimental model, these studies provide additional support for the hypothesis that peripheral stimuli, such as *Leishmania* infection, may promote

alterations in the nervous milieu and also provide support for the concept of immune response compartmentalization, in which different organs and tissues produce specific and distinct immune responses following the same stimulus (Alexandre-Pires et al., 2010; Reis et al., 2009). Cytokine profile patterns in dogs with VL have been described in the spleen, liver, lymph nodes, bone marrow, peripheral blood mononuclear cells (PBMC) and skin. In all these cases, dissimilarities were observed based on the organ/tissue studied and the clinical stage of the disease (Barbosa et al., 2011; Chamizo et al., 2005; Manna et al., 2006; Menezes-Souza et al., 2011; Panaro et al., 2009; Reis et al., 2009; Santos-Gomes et al., 2002).

In the data presented here, we observed an up-regulation of IL-1 β , IFN- γ and TNF- α gene expression and a down-regulation of IL-10 and TGF- β gene expression in the brains of dogs with VL. IL-12p40 presented a trend to down-regulation and IL-6 presented no significant difference. Altogether, these data indicate a pro-inflammatory environment in the CNS of dogs with VL and suggest that the brain alterations are related to the expressed cytokine profiles because cytokines such as IL-1 β and TNF- α are considered key factors for the initiation, maintenance and persistence of inflammation. The lack of correlations between cytokines and peripheral parasite load suggests that the brain alterations are due to the host's immune response regardless of the phase of the disease and corroborate other studies reporting T lymphocyte infiltration (Melo et al., 2009), glial activation (Melo and Machado, 2011), the presence of MMP-2 and MMP-9 in the CSF (Marangoni et al., 2011), and MMP-9 in the nervous tissue (Machado et al., 2010).

Conflict of interest statement

None of the authors of this paper have any financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this paper.

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