Leishmania infection and neuroinflammation: Specific chemokine profile and absence of parasites in the brain of naturally-infected dogs

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A B S T R A C T

Visceral leishmaniasis is a chronic disease caused by Leishmania infantum. We aimed to detect the parasite in the brain of fifteen naturally-infected dogs using in situ hybridization and immunohistochemistry, and the gene expression of selected chemokines by RT-qPCR. We detected no parasite in the brain, but perivascular deposition of parasite DNA and IgG in the choroid plexus. We noticed up-regulation of CCL-3, CCL-4 and CCL-5, coherent with T lymphocyte accumulation, stating the brain as a pro-inflammatory environment. Indeed, not necessarily the parasite itself, but rather its DNA seems to act as a trigger to promote brain inflammation during visceral leishmaniasis.

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1. Introduction

Visceral leishmaniasis (VL) is a chronic disease caused by parasitic protozoans from the Leishmania donovani complex, namely L. (L.) donovani and Leishmania (L.) infantum (syn = chagasi), which belong to the family Trypanosomatidae. Dogs are considered the main urban reservoir of this neglected disease, which presents worldwide distribution, and a zoonotic importance in Brazil and in the Mediterranean basin (Baneth et al., 2008; Chappuis et al., 2007).

Infected dogs present with different patterns of immune response against the parasite, with an effective cellular activation or a deleterious humoral response (Barbiéri, 2006). Infected dogs may keep asymptomatic for long periods or quickly develop the classical symptoms of the disease such as skin and ocular diseases, renal failure, anemia, cachexia and generalized lymphadenopathy (Alvar et al., 2004). Despite the predilection for the liver, spleen and bone marrow, the parasite could virtually spread everywhere, including the genital system (Diniz et al., 2005), muscles (Gomes et al., 2012), and the central nervous system (Márquez et al., 2013).

Specifically in the brain, the parasite is not often detected (Márquez et al., 2013; Viñuelas et al., 2001), however, inflammatory lesions even in the absence of the parasite are commonly observed, predominantly leptomenigitis and choroitis, with accumulation of mononuclear cells (Ikeda et al., 2007; Nieto et al., 1996; Viñuelas et al., 2001). For the occurrence of leukocyte migration from blood to the brain, chemokines are key molecules. They compose a superfamilly of low molecular weight proteins (8–10 kDa), which act in the immune response, mainly activation and guidance of leukocyte traffic (chemotaxis) (Bendall, 2005; Mantovani, 1999).

Chemokines are divided in four subfamilies, according to the position of cysteine residues: CXC (α-chemokines), CC (β-chemokines), C and CX3C (Mantovani, 1999; Peeters et al., 2008). Among the β-chemokines there are MCPs (monocyte chemoattractant proteins) – 1 and –2 (or CCL-2 and CCL-8, respectively); MIPs (macrophage inflammatory proteins) -1α and -1β (or CCL-3 and CCL-4, respectively); and RANTES (regulated on activation, normal T cell expressed and secreted, or CCL-5), which are highly chemoattractive to monocytes/macrophages, several lymphocytes subsets, dendritic cells and NK cells (Bendall, 2005; Rabin, 2003).

A major representative of α-chemokines is CXCL-10 (interferon gamma-induced protein 10, or IP-10), which main function is to regulate effector Th1 cell migration to the site of inflammation during adaptive immune response (Bendall, 2005; Murphy, 2003). Further, CX3CL-1 (fractalkine) is the only chemokine belonging to the CX3C subfamily. It acts as a chemoattractant and as an adhesion molecule, since it is present in both soluble and membrane-anchored forms. CX3CL-1 is
expressed by macrophages, dendritic cells, neurons and activated endothelial cells, and it is chemoattractive principally to T lymphocytes and NK cells (Bendall, 2005; Maeda et al., 2012). In the brain, neurons express elevated concentrations of CX3CL-1, which acts in microglial modulation by interaction with the receptor CX3CR-1, expressed by microglia (Hanisch and Kettenmann, 2007).

Limited studies focused on the neuropathogenesis of VL. We have previously observed the presence of inflammatory stimuli in the brain of infected dogs, such as glial activation, cytokine overexpression and matrix metalloproteinase enzymes (Meló and Machado, 2011; Meló et al., 2013) that could facilitate the accumulation of inflammatory cells, essentially T lymphocytes (Meló et al., 2009). Therefore, since the evidences of brain inflammation during canine VL are robust but the pathogenesis is unclear, we aimed to evaluate T lymphocyte populations and the gene expression of CCL-2, CCL-3, CCL-4, CCL-5, CCL-8, CXCL-10, CX3CL-1 and the receptor CX3CR-1 in the brain of dogs naturally infected by Leishmania spp., comparing with the chemokine profile expressed in the spleen; and attempting to correlate the expression of the chemokines with the clinical stage and the presence of the parasite in the brain, assessed by qPCR, in situ hybridization and immunohistochemistry.

2. Materials and methods

2.1. Animals

Twenty dogs were included in this study. Fifteen dogs proceeding from the Zoonosis Control Center in the municipality of Araçatuba, São Paulo State, Brazil, were selected as soon as VL diagnosis was achieved by serology (DPP and ELISA, Bio-Manguinhos/Fiocruz, Manguinhos, RJ, Brazil). The age ranged from 1 to 4 years old, 7 males and 8 females. Five uninfected dogs which death was not related to brain disease (i.e. trauma) were included as control.

2.2. Sampling

The dogs were euthanized with the owners’ permission according to the recommendations of the current VL control program (São Paulo, 2006), using sodium thiopental and potassium chloride. We collected peripheral blood samples in tubes with and without EDTA, and urine samples by cystocentesis; however, the bladder was empty in three animals. Afterwards, we performed necropsy examinations to evaluate macroscopic alterations and to collect samples of brain and spleen. The brain, representative of the central nervous system (CNS), was considered our organ of interest, while the spleen, representative of the periphery, was considered the target organ of the infection.

From the brain, we collected one hemisphere and stored in 10% buffered-formalin. After fixation, coronal sections were made and samples containing cerebral cortex, thalamus, hippocampus, pons–medulla oblongata, cerebellum, the ventricular choroid plexi and periventricular white matter were paraffin-embedded, sectioned (5 μm) and submitted to hematoxylin and eosin (HE) staining, in situ hybridization and immunohistochemistry. From the other hemisphere, we performed neuroscopic examinations to evaluate macroscopic alterations and to collect samples of brain and spleen. The brain, representative of the central nervous system (CNS), was considered our organ of interest, while the spleen, representative of the periphery, was considered the target organ of the infection.

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2.3. Clinical staging

We performed the complete bloodwork of the animals, using routine methods to determine the serum concentrations of total protein, albumin, urea and creatinine. We determined the serum concentrations of α-2-macroglobulin and urinary protein/creatinine ratio (UPC = urinary protein/creatinine) were also performed. The clinical staging was defined according to Solano-Gallego et al. (2011).

2.4. In situ hybridization (ISH) to detect Leishmania

We performed in situ hybridization in brain and spleen sections following Dinhopl et al. (2011). Briefly, slides containing tissue sections were dewaxed in xylene and hydrated in ethanol at 100%, 70%, and 50% and distilled water, followed by incubation with Proteinase K (S3004, Dako, Carpinteria, CA, USA) during 10 min and subsequent washing in distilled water, in ethanol 96% and in isopropanol for 5 min each. The slides were air-dried and frame seals (SLF-1201, Bio-Rad, Hercules, CA, USA) were attached. Then, we added 125 μL of hybridization mix containing 15 μL of distilled water; 25 μL of 20× SSC; 62.5 μL of formamide 50%; 12.5 μL of dextran sulfate 50%; 2.5 μL of Denhardt’s solution (D2532, Sigma-Aldrich, Saint Louis, MO, USA); 6.25 μL of herring sperm DNA (D7290, Sigma-Aldrich); and 1.25 μL of 3′-digoxigenin-conjugated probe (Eurofin W2G Operon, Huntsville, AL, USA). The probe, 5′-ACCGGGATGACACAGATAGCTTTCAGCCAC-3′, in final concentration of 100 ng/mL, detects a segment of the 5.8S ribosomal RNA of Leishmania genus. The slides were incubated at 95 °C for 6 min, immediately cooled in ice, and then incubated at 40 °C for 14–16 h in humid chamber. Afterwards, the slides were washed in 2× SSC, 1× SSC and 0.1× SSC buffer for 5 min each, followed by incubation with the anti-digoxigenin antibody conjugated to alkaline phosphatase, diluted in TBS (1:200; 11093274910, Roche Diagnostics, Indianapolis, IN, USA) for 1 h and then washed in TBS. Visualization was achieved using NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) (11681451001, Roche Diagnostics) for 1 h in the dark. The reaction was stopped with TE buffer (pH 8.0) for 10 min followed by washing in distilled water. The slides were counterstained with Mayer’s hematoxylin and mounted in aqueous medium (Faramount, S3025, Dako). Parasites were identified by a dark purple signal (Supplemental Fig. 1).

2.5. Leishmania DNA quantification

We extracted total DNA from tissue fragments (spleen and a pool of brain fragments) weighting ca. 25 mg using the DNeasy blood & tissue kit (69506, Qiagen, Hilden, Germany) according to the manufacturer’s protocol. The DNA was quantified with a NanoDrop spectrophotometer (260/280 ratio between 1.8 and 2.0). We performed qPCRs using CFX96™ Real-time System (Bio-Rad), SYBR Green PCR Master Mix (4309155, Applied Biosystems) and 900 nM of each primer (sense: 5′-CTATTTTTACACACCCCGT-3′; anti-sense: 5′-GGTAGGGCCGTTCGCGAAA-3′) which amplify a 116 bp fragment of the minicircle kinetoplast DNA (kDNA) of Leishmania spp. (Ransinghe et al., 2008), in a total volume of 25 μL. 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. We assessed the absolute quantification using a standard curve containing serial dilutions (from 10−3 to 106 promastigotes) of L. infantum DNA (MHOM/BR/72/LD46). The lower limit of positivity (cut-off value) was established using the results obtained from spleens and brains of uninfected dogs.

2.6. Chemokine gene expression

We extract the RNA from brain and spleen samples stored in RNAlater using the RNeasy Mini kit (74104, Qiagen) following the manufacturer’s instructions. Total RNA was quantified in a NanoDrop spectrophotometer (260/280 ratio between 2.0 and 2.3) and then submitted to genomic DNA elimination and reverse transcription using
Table 1
Panel of antibodies used in the immunohistochemical analyses in the brain of dogs with visceral leishmaniasis.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Specificity</th>
<th>Dilution (1:100)</th>
<th>Pre-treatment</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyclonal rabbit anti-CD3</td>
<td>T cells</td>
<td>1:200</td>
<td>Citrate 10 mM pH 6.0 in steamer for 30 min</td>
<td>Dako, A0452</td>
</tr>
<tr>
<td>Monoclonal mouse anti-CD4 clone 1F6</td>
<td>CD4+ cells</td>
<td>1:100</td>
<td>EDTA 1 mM pH 8.0 in steamer for 30 min</td>
<td>Novocastra, NCL-L-CD4-1F6</td>
</tr>
<tr>
<td>Monoclonal mouse anti-CD8 clone 1A5</td>
<td>CD8+ cells</td>
<td>1:100</td>
<td>Citrate 10 mM pH 6.0 in steamer for 30 min</td>
<td>Novocastra, NCL-CD8-295</td>
</tr>
<tr>
<td>Polyclonal rabbit anti-dog IgG</td>
<td>IgG</td>
<td>1:100</td>
<td>Citrate 10 mM pH 6.0 in steamer for 30 min</td>
<td>Sigma-Aldrich, A6792</td>
</tr>
<tr>
<td>Heterologous hyperimmune canine serum</td>
<td>Leishmania sp.</td>
<td>1:100</td>
<td></td>
<td>–</td>
</tr>
</tbody>
</table>

* According to Tafuri et al. (2004).
where this structure was present in the sections) (Fig. 2A–C). In order to better describe this finding, we performed paired HE and anti-IgG immunohistochemical analyses in the same sections containing positive ISH staining in the choroid plexus. In HE-stained tissues, there was no detectable parasite or parasitized cell, but inflammatory infiltrate and stroma thickening (Fig. 2B). Immunohistochemistry revealed intense IgG deposition in the choroid plexus’ stroma (Fig. 2D). Therefore, we classified this alteration as perivascular Leishmania DNA/IgG deposition.

3.4. Leishmania DNA quantification in the brain

We were able to detect and quantify the parasite DNA in the brain of the infected dogs using qPCR. With large variability, the brain presented parasite loads ranging from $1.1 \times 10^1$ to $6.6 \times 10^3$ parasites/25 mg of tissue (Fig. 3). Nevertheless, only 53.3% (8/15) of the dogs presented values higher than the lower limit of positivity ($3.9 \times 10^1$). There was no correlation regarding brain parasite load and the clinical stages. On the other hand, all the spleens of infected dogs were positives, ranging from $1.5 \times 10^2$ to $7.2 \times 10^5$ parasites/25 mg of tissue (cut-off value of $1.3 \times 10^5$). Comparing brain and spleen, we noticed a positive correlation between parasite load in these organs ($r = 0.610; P = 0.018$).

3.5. Chemokine gene expression in the brain and in the spleen of infected dogs

In order to evaluate the up- or down-regulation of selected chemokine gene expression in the brain and spleen of dogs with VL, we used the REST® method, which expresses how many times (fold changes) the target gene is more or less expressed in the infected dogs, compared with the control ones. Reaction efficiency values, determination coefficients and angular coefficients of each gene are shown in Table 2.

All chemokines were up-regulated in the spleen of infected dogs (Table 2; 4). No changes were observed for the receptor CX3CR-1. No correlation between the clinical stage and parasite load with chemokine expression was noticed, however, in the spleen; CCL-3, CCL-4, CCL-5 and CCL-8 were positively correlated among them, as well as CCL-2 with CCL-8 and CXCL-10.

In the brain of infected dogs, only the gene expression of CCL-3 (19.9-fold), CCL-4 (16.2-fold) and CCL-5 (20.3-fold) was significantly
up-regulated (Table 2; Fig. 4), with strong positive correlation among them (Fig. 5A-C). No correlation between the chemokines in the brain and clinical stage or parasite load was observed. As expected in a natural infection sampling, we observed important individual variability in all targets, nevertheless, different from the other targets, the distribution of CXCL-10 gene expression displayed a specific pattern in the infected dogs, with 10 dogs with expression values similar to the control dogs, and a small subpopulation (n = 5; #GD3, #GD4, #GD5, #GD9, #GD10) with evident higher gene expression. When evaluated separately, this subpopulation presented 83.8-fold more CXCL-10 expression than the controls (Fig. 5D; P = 0.002). Even in this particular case, we detected no correlation to the clinical stages, parasite load, and inflammatory status or to other chemokines. CXCL-10 was correlated to CCL-2 in the spleen, and besides the absence of changes in the gene expression of CCL-2 in the brain, we observed a trend to positive correlation between CXCL-10 and CCL-2 in the brain (Fig. 5E).

3.6. T lymphocytes in the brain

CD3+ cells were the main component of the inflammatory infiltrate in the brain of the infected dogs, located in the leptomeninges, subependymal area, choroidplexus and in the parenchymal perivascular cuffs (Fig. 6A-C). Infected dogs presented a median value of 0.57% (IQR 0.73) whereas control dogs presented solely 0.04% (IQR 0.04).

Table 2

<table>
<thead>
<tr>
<th>Target</th>
<th>Efficiency (%)</th>
<th>$r^2$</th>
<th>Slope</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spleen</td>
<td>Brain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G3PDH</td>
<td>106.6</td>
<td>0.997</td>
<td>-3.173</td>
<td>0.001</td>
</tr>
<tr>
<td>RPL-32</td>
<td>100.2</td>
<td>0.994</td>
<td>-3.311</td>
<td>0.001</td>
</tr>
<tr>
<td>CCL-2</td>
<td>101.8</td>
<td>0.998</td>
<td>-3.279</td>
<td>0.001</td>
</tr>
<tr>
<td>CCL-3</td>
<td>99.1</td>
<td>0.999</td>
<td>-3.344</td>
<td>0.001</td>
</tr>
<tr>
<td>CCL-4</td>
<td>96.4</td>
<td>0.999</td>
<td>-3.412</td>
<td>0.001</td>
</tr>
<tr>
<td>CCL-5</td>
<td>101.7</td>
<td>0.997</td>
<td>-3.282</td>
<td>0.001</td>
</tr>
<tr>
<td>CCL-8</td>
<td>99.6</td>
<td>0.994</td>
<td>-3.330</td>
<td>0.001</td>
</tr>
<tr>
<td>CXCL-10</td>
<td>101.0</td>
<td>0.993</td>
<td>-3.299</td>
<td>0.001</td>
</tr>
<tr>
<td>CX3CR-1</td>
<td>99.4</td>
<td>0.995</td>
<td>-3.337</td>
<td>0.001</td>
</tr>
<tr>
<td>CX3CR-1</td>
<td>104.6</td>
<td>0.994</td>
<td>-3.217</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* indicates significant up-regulation.
0.02) of immunostained area (P = 0.0002). CD4 and CD8 were rarely detected, only in the infected dogs (Fig. 6D).

4. Discussion

We observed the presence of inflammation, chemokine expression and parasite DNA in the brain of dogs with VL. The sample population included dogs belonging to the four clinical stages of the classification proposed by Solano-Gallego et al. (2011). We chose the spleen as the control organ of the infection since it is considered one of the parasite’s targets and it is the focus of inflammation during the disease. The cytohistopathological findings in this organ support this statement, as they were coherent with the classical lesions previously reported (Lima et al., 2012). Regarding the brain, we observed important inflammatory alterations, with different intensities, ranging from discrete to intense, corroborating previous studies from our research group and others (Márquez et al., 2013; Melo et al., 2013; Viñuelas et al., 2001); consequently, we can confirm that the brain is also affected during VL, even in the absence of neurological symptoms. Nevertheless, its pathogenesis is still unclear.

One possible stimulus to trigger the inflammatory response in the brain would be the presence of the parasite itself. Infection by other members of the Trypanosomatidae family leads to parasite accumulation in the brain. Trypanosoma brucei invades the CNS via the choroid plexus and the circumventricular organs (Masocha et al., 2007). Trypanosoma evansi was present in the brain of naturally-infected horses (Rodrigues et al., 2009) as well as Trypanosoma cruzi in immunodeficient humans (Javier et al., 1998). Leishmania on the other hand has been sporadically noticed in the CNS, using either histology or immunohistochemistry (Márquez et al., 2013; Nieto et al., 1996; Viñuelas et al., 2001).

Even though in situ hybridization reactions were effective to detect the parasite in the spleen, no parasite was observed in the brain. Nevertheless, we noticed perivascular staining exclusively at the choroid plexus, consistent with perivascular parasite DNA deposition. In oncology, a similar finding is named Azzopardi phenomenon, which corresponds to DNA deposition around blood vessels in areas of tissue necrosis, since massive cell lysis releases large amounts of nucleic acids (Pritt and Cooper, 2003). To our knowledge, there is no description of Azzopardi phenomenon in infectious diseases; however, there are reports of free DNA fragments, smaller than 300 bp, circulating in biological fluids such as plasma, serum and urine. These fragments are called cell-free DNA, and they could be originated from the host cells’ death (self-DNA) or from microorganisms’ life cycle (replication, maturation and death) (Dwivedi et al., 2012; Green et al., 2009), including Leishmania spp. (Francesci et al., 2007).

At histological examination, we did not detect parasites in the choroid plexus in the areas where in situ hybridization revealed perivascular Leishmania DNA deposition. The presence of parasite DNA in the brain was corroborated by qPCR, and even though positive, the average parasite load in the brain was low. Furthermore, the perivascular deposits in the choroid plexus were hyaline, consistent with IgG deposition, and not basophilic as expected for nucleic acids. Nevertheless, DNA-containing immune complexes or even small amounts of cell-free DNA (Atkins et al., 1972; Falangola et al., 1995)
may arrive to the choroid plexus stroma by vascular fenestrations and inflammatory endothelial lesions (Wolburg and Paulus, 2010), which could be enough to render positive a test based on DNA detection and to initiate inflammation. High concentrations of circulating immune complexes have already been detected in VL, which has been correlated to glomerulonephritis and necrotizing vasculitis (Alvar et al., 2004; Brandonisio et al., 1990). The parasite DNA could also trigger inflammation by activation of Toll-like receptors, probably TLR-9, which binds DNA and that have already been detected in the choroid plexus of dogs with VL (Melo et al., 2014).

The first report of Leishmania infection in the choroid plexus in dogs was published by Nieto et al. (1996). Thereafter, histological alterations were described in the choroid plexus of dogs with VL, but with no detectable parasites (Pumarola et al., 1991; Ikeda et al., 2007; Melo and Machado, 2009). Since we have been noticing alterations in the choroid plexus and brain of dogs with VL with no detectable parasites within the CNS (Melo and Machado, 2009, 2011; Melo et al., 2009, 2013, 2014), we can propose two hypotheses to brain inflammation during VL: (1) parasite-dependent, where the parasites enter the CNS, stimulate inactivation and may be latter destroyed, rendering difficult their detection, and (2) parasite-independent, where peripheral stimuli (parasite antigens/DNA, inflammatory mediators) reach the CNS and trigger inflammation. In one way or another, brain inflammation occurs in dogs with VL.

The study of the immune response in the CNS during VL is infrequent and the evaluation of the chemokine profile in infected dogs is rare. Gial cells and even leukocytes within the inflammatory infiltrates may produce chemokines. We detected a specific pattern of chemokine expression, with up-regulation of CCL-3, CCL-4 and CCL-5 in the brain of infected dogs. CCL-5 is a well-characterized chemokine related to the recruiting and guidance of T lymphocytes towards the focus of inflammation. CCL-3 and CCL-4, despite being initially related to macrophages and NK cell activation, they present a selective chemotraction to CD8 and CD4 activated T lymphocytes, respectively (Taub et al., 1993). Moreover, CCL-4 and CCL-5 promoted CD4 T lymphocyte adhesion to the endothelium activated by IFN-γ and TNF-α (Quandt and Dorovini-Zis, 2004).

The interaction of these three chemokines, CCL-3, CCL-4 and CCL-5, is coherent with the predominance of T lymphocytes previously described in the brain of dogs with VL (Melo et al., 2009). In accordance, resistant mice presented low expression of CCL-3, CCL-4 and CCL-5, resulting in absence of CD8 T lymphocytes in the inflammatory infiltrate in a murine model of cerebral malaria (Clark and Phillips, 2011). The overexpression of CCL-5 by a recombinant rabies virus promoted severe blood–brain barrier disruption associated with chemokine production and inflammatory cell infiltration (Zhao et al., 2009). Despite immunohistochemistry being not the ideal technique to characterize lymphocyte subsets, we have shown here that the main inflammatory cells in the brain are CD3 + T lymphocytes, and along with a minimal detection of CD4 or CD8, these cells might belong to the CD3+/CD4−/CD8− cell subset, nevertheless, an extensive study of the T cell subpopulations in the brain of dogs with VL using flow cytometry is required. Recently, different subsets of T lymphocytes gained focus on parasitic diseases, especially the double negative (DN) T cells, which express neither CD4 nor CD8, including CD4+/CD8− DN T cells, γδ DN T cells, NK T cells (D’Acquisto and Crompton, 2011). In cases of human cutaneous leishmaniasis, elevated numbers of circulating CD4+ DN T cells are described, associated with elevated production of IFN-γ and TNF-α and high levels of the activation markers CD69 and CD56 (Antonelli et al., 2006; Gollob et al., 2008). Further, the association between NK T cells and immune response during VL has already been studied in the liver of mice infected with L. donovani (Ampray et al., 2004).

Regarding other protozoan diseases in the brain, NK T cells have been related to IFN-γ production and recruitment of T lymphocytes via CXCL-10/CXCR-3 during cerebral malaria (Hansen et al., 2003; Hunt et al., 2014). Additionally, in murine toxoplasmosis encephalitis, up-regulation of CCL-5 dependent on IFN-γ was described as protective, but when accompanied by up-regulation of CXCL-10 (Wen et al., 2010). IFN-γ-dependent CXCL10 was also essential to accumulation of T cells.

![Image of immunohistochemical characterization of the lymphocytic infiltrate in the brain of dogs with visceral leishmaniasis.](https://example.com/image1.png)

**Fig. 6.** Immunohistochemical characterization of the lymphocytic infiltrate in the brain of dogs with visceral leishmaniasis. The inflammatory infiltrate is composed chiefly by CD3+ T lymphocytes (arrowhead), located in cortical leptomeninges (A), subependymal area (B), and in parenchymal perivascular cuffs (C). Immunoperoxidase, scale bar = 50 μm. (D) Quantification of CD3+, CD4+ and CD8+ cells in the brain of dogs. Data are expressed as the percentage of the immunostained area in relation to the total evaluated area (814.554.4 μm²). Columns represent the median value of the groups.
and parasites in the brain during experimental African trypanosomiasis (Amin et al., 2009). CXCL-10 presented an interesting pattern of expression in the infected dogs. Whereas 67.7% of the infected dogs presented no alterations for this chemokine, 33.3% (5/15) of them presented a remarkable overexpression of CXCL-10 in the brain, with no correlation with clinical stages, parasite load or brain inflammatory infiltrate. The occurrence of up-regulation of CXCL-10, even in few dogs, is supported by up-regulation of IFN-γ, previously reported in the brain of dogs with VL (Melo et al., 2013). The high variation of CXCL-10 expression as well as the low average CCL-2 expression in the brain could be related to other individual factor not evaluated in this study, such as specific T cell subsets or time of infection, similar to what occurs in the spleen of experimentally infected dogs (Strauss-Ayali et al., 2007).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at, http://dx.doi.org/10.1016/j.ejneuro.2015.10.004. These data include the Google map of the most important areas described in this article.

References


