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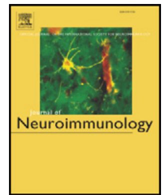

# *Leishmania* infection and neuroinflammation: Specific chemokine profile and absence of parasites in the brain of naturally-infected dogs

Guilherme D. Melo [a,1], José Eduardo S. Silva [a], Fernanda G. Grano [a], Milena S. Souza [b], Gisele F. Machado [a,*]

[a] Faculdade de Medicina Veterinária, UNESP – Univ Estadual Paulista, Laboratório de Patologia Aplicada (LApap), Araçatuba, São Paulo, Brazil
[b] Faculdade de Medicina Veterinária, UNESP – Univ Estadual Paulista, Araçatuba, São Paulo, Brazil

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ABSTRACT

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 leptomeningitis 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 superfamily 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., 2006). 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

* Corresponding author at: Departamento de Clínica, Cirurgia e Reprodução Animal, Faculdade de Medicina Veterinária, Universidade Estadual Paulista "Júlio de Mesquita Filho" (UNESP), Rua Clóvis Pestana, 793, Araçatuba, São Paulo CEP 16050-680, Brazil.
*E-mail addresses:* gdiasdem@pasteur.fr (G.D. Melo), giselem@fmva.unesp.br (G.F. Machado).
[1] Present address: Laboratoire des Processus Infectieux à Trypanosomatidés, Département Infection et Epidemiologie, Institut Pasteur, Paris, France.

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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 (Melo and Machado, 2011; Melo et al., 2013) that could facilitate the accumulation of inflammatory cells, essentially T lymphocytes (Melo 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 necroscopic 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, unfixed, we collected a pool of fragments of 0.5 $cm^3$ from the thalamus, hippocampus, piriform/temporal cortex and periventricular white matter, stored in RNAlater (AM7020, Applied Biosystems, Foster City, CA, USA) for RNA extraction, or directly frozen at −80 °C for DNA extraction. Regarding the spleen, we performed tissue smears, and we collected fragments in formalin, in RNAlater and to be directly frozen at −80 °C.

### 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 anti-*Leishmania* antibodies using indirect ELISA (Lima et al., 2005). Urinalysis and urinary protein/creatinine ratio (UPC; $\{\frac{\text{urinary protein}}{\text{urinary creatinine}}\}$) 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 buffer; 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 (Eurofins MWG Operon, Huntsville, AL, USA). The probe, 5′-ACGGGGATGACACAATAGAGCTTC TCC-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′-CCTATTTTACACCAACCCCCAGT-3′; anti-sense: 5′-GGGTAGGGGCGTTC TGCGAAA-3′) which amplify a 116 bp fragment of the minicircle kinetoplast DNA (kDNA) of *Leishmania* spp. (Ranasinghe 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 $10^{-1}$ to $10^6$ 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

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