Veterinary Parasitology 253 (2018) 30–38

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

Toll-like receptors and cytokines in the brain and in spleen of dogs with visceral leishmaniosis

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ARTICLE INFO

Keywords:
Central nervous system
Gene expression
Leishmania infantum
Neuroinflammation
q-PCR

ABSTRACT

Visceral leishmaniosis (VL) is a multisystem disease that affects domestic dogs and can have several clinical manifestations, including some rare reports of neurological clinical signs, or it may remain asymptomatic, depending on the individual immune response against the *Leishmania* parasite. VL involves immune system sensors, such as the Toll-like receptors (TLRs), that are related to innate immunity and inflammation. Previously, we have reported the presence of brain inflammation in infected dogs. Here, we investigated the gene expression profile of TLRs 1–10 in the brain and the spleen of infected dogs, along with the production of proinflammatory cytokines (TNF-α, IFN-γ, IL-1β and IL-6) with the aim of explaining the origin of brain inflammation. The gene expression of TLRs has varied according to the tissue evaluated. In the brain, TLR-4 was only up-regulated in a small subpopulation of infected dogs, while in the spleen, we detected an increase in TLR-5 and TLR-9 transcripts, as well as a reduction in TLRs 2-4 and TLR-10. All cytokines except IL-6 were detected in infected dogs. Moreover, we detected *Leishmania* DNA in all infected dogs in both tissues evaluated. In the histopathological analysis, we observed a predominance of lymphoplasmacytic infiltrate, mainly in leptomeninges and choroid plexuses, ranging from mild to intense. This study provides the first insight into the TLRs profile in the brain and the spleen during canine VL and provides support to confirm the involvement of sensors of the innate immune system sensors against L. infantum parasites.

1. Introduction

Visceral leishmaniosis (VL) is an multisystemic disease caused by the *Leishmania infantum* (*L. chagasi*) protozoan (Mauricio et al., 2000). Domestic dogs can present with several systemic manifestations (Garcia-Alonso et al., 1996; Ciaramella et al., 1997; Blavier et al., 2001; Reis et al., 2009), including some rare reports of neurological clinical signs (Font et al., 2004; Ikeda et al., 2007; José-lópez et al., 2012; Melo et al., 2012; Gianuzzi et al., 2017), or the infection may remain asymptomatic.

Neurological clinical signs include walking in circles, seizures, paresis, tetraplegia, head tilt, motor incoordination, intention tremor, nystagmus, strabismus and myoclonia (Font et al., 2004; Ikeda et al., 2007; José-lópez et al., 2012; Márquez et al., 2013; Gianuzzi et al., 2017). In addition to neurological clinical signs, brain inflammation has also been reported in dogs with VL, where meningitis and choroiditis are the usual histopathological findings (Nieto et al., 1996; Viñuelas et al., 2001; Melo et al., 2009, 2013, 2015a; Grano et al., 2016; Gianuzzi et al., 2017). Considering these findings, the focus of our research group in recent years has been to investigate the question about why infected dogs present neurological clinical signs and brain inflammation. We have previously suggested two hypotheses. One is that parasite migration from macrophage-rich infected tissues to the brain stimulates inflammation. However, the presence of the *Leishmania* parasite in the brain is still unclear, although we have detected its DNA in this compartment (Grano et al., 2014; Melo et al., 2015a). The second hypothesis is that peripheral stimuli, such as inflammatory mediators, can reach the central nervous system (CNS), developing local inflammation (Melo et al., 2015a).

Pattern recognition receptors (PRRs) are an important group of innate immune receptors that recognize pathogen-associated molecular patterns (PAMPs), which are conserved structures present in fungi, viruses, bacteria and protozoans (reviewed by Ospelt and Gay, 2010) and recognize damage-associated molecular patterns (DAMPs), which

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https://doi.org/10.1016/j.vetpar.2018.02.030
Received 16 September 2017; Received in revised form 14 February 2018; Accepted 15 February 2018
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are host-derived proteins (Miyake, 2007; Piccinini and Midwood, 2010) in chronic inflammation, triggering a cascade of immune reactions (Fukata et al., 2009).

TLRs are PRRs that act on the recognition of microbial structures and induce innate and adaptive immune responses (Tuon et al., 2008). Different TLRs detect distinct molecules from viruses, bacteria, fungi and parasites (Kawai and Akira, 2011). Signaling activation through TLRs causes the production of chemokines, inflammatory cytokines, adhesion molecules and co-stimulatory molecules (Ospelt and Gay, 2010). Gene expression of cytokines and chemokines has already been characterized during canine VL in parasite-infected tissues (Panaro et al., 2009; Barbosa et al., 2011; Melo et al., 2015a), as well as in the CNS (Melo et al., 2013; 2015a), where there was an increase in the transcripts of proinflammatory cytokines such as TNF-α, IFN-γ and IL-1β in the brain (Melo et al., 2013).

Concerning TLRs, there are only a few study reports in canine VL. Most studies have focused on TLRs 2, 4 and 9, especially in peripheral blood mononuclear cells (PBMCs) (Melo et al., 2014a), jejunum and colon (Figueiredo et al., 2013), skin (Esteve et al., 2015; Hosein et al., 2015), liver (Hosein et al., 2015), spleen, lymph nodes (Melo et al., 2014b; Hosein et al., 2015) and brain (Melo et al., 2014b).

Studies evaluating the immune response in the CNS of dogs with leishmaniosis are scarce. Despite the growing number of studies about PRRs in recent years, the Toll-like receptor profile in the brain, or even in the spleen, remains to be characterized in order to elucidate which receptors might be related to the recognition of the *Leishmania* parasite or its antigens in the brain during canine VL. Therefore, the aim of this study was to determine the gene expression of TLR 1–10 and the production of proinflammatory cytokines (TNF-α, IFN-γ, IL-1β and IL-6) in two target compartments of our previous studies: 1) CNS, which is represented here by the brain, the main focus of our studies, and 2) spleen, which is considered an organ bearing the bulk of parasite burden, representing the peripheral system, in which we investigated previously and detected an up-regulation of TLR-2 (Melo et al., 2014b).

2. Material and methods

2.1. Animals

Twenty-one dogs, 13 males and 8 females, ranging in age from 1 to 4 years old, were selected from the Zoonosis Control Center in the municipality of Araçatuba, São Paulo State, Brazil and included in this study. Seventeen naturally infected dogs by the *Leishmania* parasite were euthanized with the owner's permission, in compliance with state law (Brasil, 2008), as soon as the VL diagnosis was confirmed. All infected dogs were symptomatic, but they did not present a history of neurological signs. Four uninfected healthy dogs, without underlying conditions or nervous involvement at the time of death, were included in the control group. VL diagnosis was achieved using serology analysis (DPP and ELISA, Bio-Manguinhos/Fiocruz, Manguinhos, RJ, Brazil) and parasitological analysis (popliteal lymph node fine-needle aspiration). All dogs in the study were negative for other infectious agents that can affect the CNS, such as *Neospora caninum* and *Toxoplasma gondii* (indirect immunofluorescence = RIFI), and *Ehrlichia* spp. and *Babesia* spp. (enzyme-linked immunosorbent assay = ELISA).

2.2. Sampling

Dogs were anesthetized with pentobarbital (Hypnol®). Peripheral blood samples were collected in tubes with and without EDTA for hemogram and biochemical analysis, and dogs were euthanized with potassium chloride. Afterwards, we performed necroscopic examinations to evaluate macroscopic alterations and to collect brain and spleen samples. Brains were sagitally sliced and fragments of 0.5 cm^3 were pooled from the thalamus, hippocampus, piriform/temporal cortex and periventricular white matter, and 0.5 cm^3 fragments of the spleen were stored in RNAlater (AM7020; Applied Biosystems, Austin, TX, USA) and frozen at −80 °C or they were directly frozen. The other brain hemisphere was placed in 10% buffered formalin and paraffin embedded. Fragments were sectioned (5 μm) and submitted to hematoxylin and eosin (HE) staining for histopathological analysis.

The brain inflammation was evaluated according to the intensity of the inflammatory infiltrate through the use of a ponderal index divided into four grades (grade 0–3), in accordance with Grano et al. (2016): Grade 0 (no inflammation observed); Grade 1, mild inflammation (slight inflammatory cell infiltrate mainly in the choroid plexuses and leptomeninges); Grade 2, moderate inflammation (moderate inflammatory cell infiltrate mainly in the choroid plexuses, and leptomeninges, along with the presence of some perivascular lymphocytes in the brain tissue); and Grade 3, severe inflammation (remarkable inflammatory cell infiltrate mainly in the choroid plexuses and leptomeninges along with intense perivascular infiltration in the brain tissue).

Regarding the histopatologycal analysis in the spleen, the main objective of this study was more specifically determine the TLR profile in the brain and correlate TLRs with brain inflammation, which we have reported previously. During the project, we decided to compare TLRs from the brain and spleen, as the spleen is one of the most affected organs during the disease, and the spleen is the organ that we have analyzed in other studies (Melo et al., 2014a, b, 2015a). Therefore, we did not collect samples from the spleen for histopathological analysis.

2.3. Clinical staging

We determined the serum concentrations of total protein, albumin, urea and creatinine, along with the serum concentrations of anti-*Leishmania* antibodies with indirect ELISA (Lima et al., 2005). The clinical staging of the animals was made according to Solano-Gallego et al. (2011).

2.4. Leishmania DNA quantification using qPCR

Total DNA extraction was performed from brain and spleen samples with the DNeasy blood & tissue kit (69504, Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was quantified using a NanoDrop spectrophotometer (260/280 ratio between 1.8 and 2.0). qPCRs were performed using the Eppendorf Mastercycler® RealPlex^2, SYBR Green PCR Master Mix (4309155, Applied Biosystems) and 900 nM of each primer (sense: 5′-CCTATTTTACACCAACCCCCAGT-3′; anti-sense: 5′-GGGTAGGGGCGTTCTGCGAAA-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 as follows: 94 °C for 2 min and 40 cycles of 94 °C for 15 s and 60 °C for 1 min. Then, samples were submitted to a melting curve from 60 °C to 95 °C; increasing 0.5 °C every 5 s. The absolute quantification was evaluated using a standard curve with serial dilutions (from 10^{-1} to 10^8 promastigotes) of L. infantum DNA.

2.5. Evaluation of Gene expression by RT-qPCR

Total RNA was extracted from the samples stored in RNAlater using the RNeasy® Lipid Tissue Mini Kit (74804, Qiagen) for brain samples and the RNeasy® Mini kit (74104, Qiagen) for spleen, according to the manufacturer's protocol. RNA was quantified with a NanoDrop spectrophotometer (260/280 ratio between 2.0 and 2.3) and then submitted to genomic DNA elimination (RNase-Free DNase Set: 79254; Qiagen), and cDNA production was achieved using the RT^2 First Strand Kit (330404; Qiagen). Specific canine primers and hydrolysis probes (5'-FAM – 3'-BHQ-1) were selected based on the scientific literature (TLR-6, TLR-8 and G3PDH) or gene expression assays were purchased from a commercial source (all the other genes evaluated) (Table 1). qPCR was carried out in a real-time thermocycler (Eppendorf Mastercycler®

Table 1
Primers (forward: F, reverse: R) and hydrolysis probe (P) sequences used for RT-qPCR.

Target	Primers and probes	Sequence (5'–3')	Product size (pb)	GenBank accession number	Reference
TLR-1		ASSAY ID: Cf03811563_s1	100	NM_001146143.1	Life Technologies Foster City, CA, USA (4351372)
TLR-2		ASSAY ID: Cf02625049_s1	69	NM_001005264.3	Life Technologies Foster City, CA, USA (4351372)
TLR-3		ASSAY ID: Cf04419557_m1	63	–	Life Technologies Foster City, CA, USA (4331182)
TLR-4		ASSAY ID: Cf02622203_g1	120	NM_001002950.1	Life Technologies Foster City, CA, USA (4331182)
TLR-5		ASSAY ID: Cf04245132_s1	100	NM_001197176.1	Life Technologies Foster City, CA, USA (4351372)
TLR-6	F	TCAAGCATTTAGACCTCTCATTCA	109	EU551147.1	Mercier et al. (2012)
	R	CCGTAACTTTGTAGCACTTAAACCT			
	P	TGCCCATCTGTAAGGAATTTGGCA			
TLR-7		ASSAY ID: Cf02710573_s1	124	NM_001048124.2	Life Technologies Foster City, CA, USA (4351372)
TLR8	F	TCAGCTACAATGCACACTACTTCC	138	XM_005641119.1	Mercier et al. (2012)
	R	ACGCTTCTCAGGTCTTGCTC			
	P	TCCTAGGCGGTGCGTCACCC			
TLR-9		ASSAY ID: Cf02717353_g1	136	NM_001002998.1	Life Technologies Foster City, CA, USA (4351372)
TLR-10		ASSAY ID: Cf04181843_s1	100	NM_001173127.1	Life Technologies Foster City, CA, USA (4351372)
RPL32	–	ASSAY ID: Cf03986518_m1		customized assay	Life Technologies Foster City, CA, USA (4351372)
G3PDH	F	TCAACGGATTTGGCCGTATTGG	90	AB022763	Peters et al. (2005)
	R	TGAAGGGGTCATTGATGGCG			
	P	CAGGGCTGCTTTTAACTCTGGCAAAGTGAA			

RealPlex^2) using Taqman® Universal Master Mix (4326708, Applied Biosystems). For TLR-6 and TLR-8 we used a concentration of 400 nM of each primer and 250 nM of the probe and for G3PDH (glyceraldehyde-3-phosphate dehydrogenase) we used 400 nM of each primer and 200 nM of the probe. Each reaction was performed with 5 μL of cDNA at a final concentration of 100 ng. The amplification conditions were as follows: 50 °C for 2 min, 95 °C for 10 min and 45 cycles of 95 °C for 15 s and 60 °C for 1 min. For each target gene, we obtained values of reaction efficiency from amplification of six serial dilutions of a pool of cDNA. Normalization of RT-qPCR data was performed using the reference genes G3PDH and RPL32 (ribosomal protein L32).

2.6. Cytokine quantification by capture ELISA

2.6.1. Brain and spleen extracts

Extracts were obtained to quantify cytokines by the capture ELISA technique. For this, 60 mg of brain or spleen fragments was added to 1 ml suspensions of complete RPMI-1640 (Sigma, USA), pH 7.2, kept on ice and homogenized in a Tissue Ruptor (Qiagen, Germany) for approximately 5 min. Then, the resulting homogenate was centrifuged at 10,000 × *g* for 15 min at 4 °C, and the supernatant was immediately stored at −80 °C.

2.6.2. Cytokine quantification

TNF-α, IFN-γ, IL-1β and IL-6 concentrations were determined in the brain and spleen supernatants of the dogs of this study by means of capture ELISA using the Duo SET® Canine TNF-α (Cat. Number: DY1507), Duo SET® Canine IFN-γ (Cat. Number: 781B), and Duo SET® Canine IL-1β (Cat. Number: DY3747) (R & D System, Minneapolis, USA) commercial reagents. Procedures were performed in accordance with the manufacturer's instructions. For IL-6, we used an anti-canine monoclonal antibody (mAb) produced in mice (Cat. Number: MAB16091, R & D System, USA) and biotinynated anti-canine polyclonal antibody produced in goats (Cat. Number: BAF1609, R & D System, USA). Plates with 96 wells (Corning, USA) were sensitized with 2 μg/ml of mAb and with 1 μg/ml of detection antibody. Recombinant canine IL-6 (Cat. Number: 1609-CL, R & D System, USA) was used to generate standard curves. The test was developed with 3,3',5,5'-tetramethylbenzedine – TMB (Sigma, USA). The plates were read using a spectrophotometer (Spectra Count, Packard Bio Science Company, USA) at 450 nm. All samples were measured in duplicate. The detection limit was 1.95 pg/mL for TNF-α in the brain, 1.95 pg/mL for TNF-α in the spleen, 62.50 pg/mL for IFN-γ in the brain, 125.00 pg/mL for IFN-γ in the spleen, 0.97 pg/mL for IL-1β in the brain, 7.81 pg/mL for IL-1β in the spleen, 39.06 pg/mL for IL-6 in the brain, and 78.12 pg/mL for IL-6 in the spleen.

2.7. Statistical analysis

For statistical analysis, all values were log-transformed. For cytokine analysis, we used a four-parameter logistic (4-PL) curve fit. Significant differences between groups were determined by the Mann-Whitney test or Wilcoxon test for paired samples. Correlations were evaluated with the Spearman correlation coefficient. The Friedman test, followed by the Dunn test, was used to evaluate the intensities of brain inflammation. All analyses were performed using Prism software (Prism 6, GraphPad). Statistical significance was considered when $P < 0.05$.

2.8. Ethical issues

This study was approved by the institutional Ethics and Animal Welfare Committee (CEEA - Comissão de Ética e Experimentação Animal, UNESP, process FOA 00633-2016).

3. Results

3.1. Clinical staging

We found in the macroscopic examination that 70.58% (12/17) of the infected dogs presented cachexia or weight loss, followed by 64.70% (11/17) with skin disease, including alopecia, nasal hyperkeratosis, ulcers and seborrhea, 35.29% (6/17) with splenomegaly, 35.29% (6/17) with conjunctivitis and onychogryphosis, and 29.41% (5/17) with lymphadenopathy.

Concerning the laboratory findings, we observed that 88.23% (15/17) of the infected dogs presented anemia, 52.94% (9/17) presented lymphocytopenia and 29.41% (5/17) presented thrombocytopenia. All infected dogs presented hypoalbuminemia. Azotemia was evident in 35.29% (6/17) of the infected dogs. All infected dogs presented positive concentrations of serum anti-*Leishmania* antibodies through ELISA, except two dogs that were serology negative; 17.64% (3/17) presented low concentrations of antibodies (from cut-off value 0.27 up to 0.4), 35.3% (6/17) presented medium concentrations (0.4 up to 0.81), and 35.3% (6/17) presented high concentrations (over 0.81).

Both parasitological examinations in aspirate from lymph nodes aspirates and qPCR were positive in all dogs. Therefore, we classified 29.41% (5/17) of the dogs as stage I, 52.94% (9/17) as stage II, 11.76% (2/17) as stage III and 5.88% (1/17) of the dogs as the stage IV of the disease.

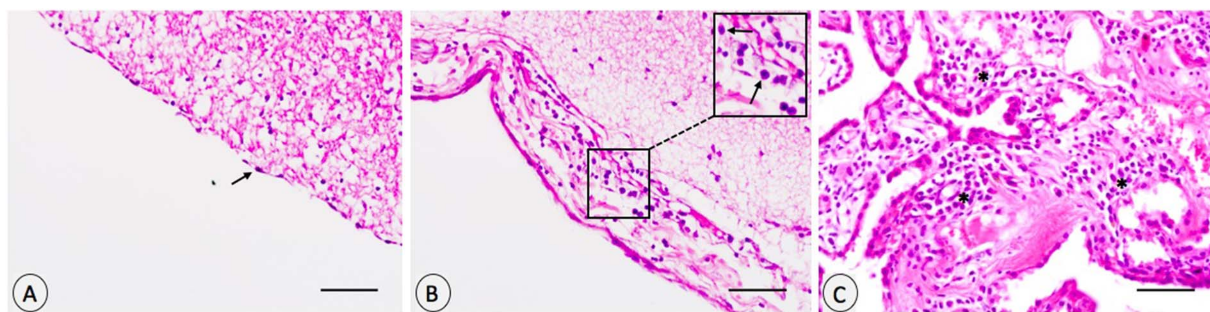

Fig. 1. Representative photomicrography of brain alterations in canine visceral leishmaniosis. (A) Mild inflammatory cell infiltrate (arrow) in the subependymal area (Grade 1). (B) Moderate inflammatory cell infiltrate (arrows) in leptomeninges (Grade 2). (C) Intense inflammatory cell infiltrate (asterisk) in the choroid plexus from the fourth ventricle (Grade 3). Inset: mononuclear cells enlarged. Hematoxylin and eosin. Scale bar = 50 µm.

3.2. Brain histopathological analysis

Histopathological analysis was performed in the brains of infected and healthy dogs. For the healthy dogs, we detected no brain lesions in the histopathological evaluation. In the infected dogs, we observed mononuclear cell infiltrates, especially composed of lymphocytes and plasma cells in the three brain areas. Leptomeninges and choroid plexuses presented the highest concentration of inflammatory cells when compared with the subependymal area ($P < 0.0001$), ranging from mild to intense inflammation. Fig. 1 shows representative photomicrographs of inflammatory infiltrates in brain areas according to the intensity of inflammation.

In leptomeninges, 21.1% of the dogs (n = 4) presented mild infiltration of inflammatory cells, 36.8% (n = 7) presented moderate inflammatory infiltrate, and 42.1% (n = 8) presented intense inflammatory infiltrate. In the choroid plexuses, 5.3% (n = 1) of the dogs presented no inflammatory infiltrate, 15.8% (n = 3) presented mild intensity, 47.4% (n = 9) showed moderate inflammatory infiltrate and 31.6% (n = 6) presented intense inflammation. In the subependymal area, 42.1% (n = 8) of the dogs presented with absence of inflammation, 36.8% (n = 7) showed mild inflammatory infiltrate and 21.1% (n = 4) presented moderate intensity of inflammatory cells (Fig. 2). In the study presented here, we did not detect amastigotes of the *Leishmania* parasite in the H-E stained brain samples. To evaluate if the intensity of inflammation could vary according to different clinical stages of the disease, we performed a correlation test; however, no correlation was found between these two parameters.

3.3. Parasite load

The presence of *Leishmania* kDNA was detected in all spleen samples from infected dogs. For the brain, *Leishmania* kDNA was detected in all samples except those from four dogs. The standard curve allowed us to quantify the parasite load in both tissues. Amplification reactions obtained an efficiency value of 95.9% with a determination coefficient of $r^2 = 0.998$ and a slope of -3.511.

The detection range was from 30 to 2,260.000 parasites/10 mg of spleen and 18 (n = 4) to 1.380 parasites/10 mg of brain (Fig. 3). No amplification was observed in the samples used as the negative control for both tissues. The number of parasites in the spleen was significantly higher when compared to the brain ($P < 0.0001$). However, there was no correlation in the parasite load between both tissues. An absence of correlation was also verified between parasite load and the intensity of brain inflammation.

3.4. TLR Gene expression in the brain and spleen of infected dogs

To evaluate the up- or down-regulation of TLR gene expression in the brains and spleens of dogs with VL, we used the $2^{-\Delta\Delta Ct}$ method according to Livak and Schmittgen (2001) to quantify gene expression using G3PDH and RPL32 as reference genes. The fold change represents by how many times the target gene is more or less expressed in the infected dogs, compared to the control dogs.

In the spleen, we detected an up-regulation of TLR-5 (6.91-fold) and TLR-9 (11.92-fold) in infected dogs, along with a down-regulation of TLR-2 (0.35-fold), TLR-3 (0.23-fold), TLR-4 (0.35-fold) and TLR-10 (0.26-fold). Nevertheless, no correlation between the clinical stage or

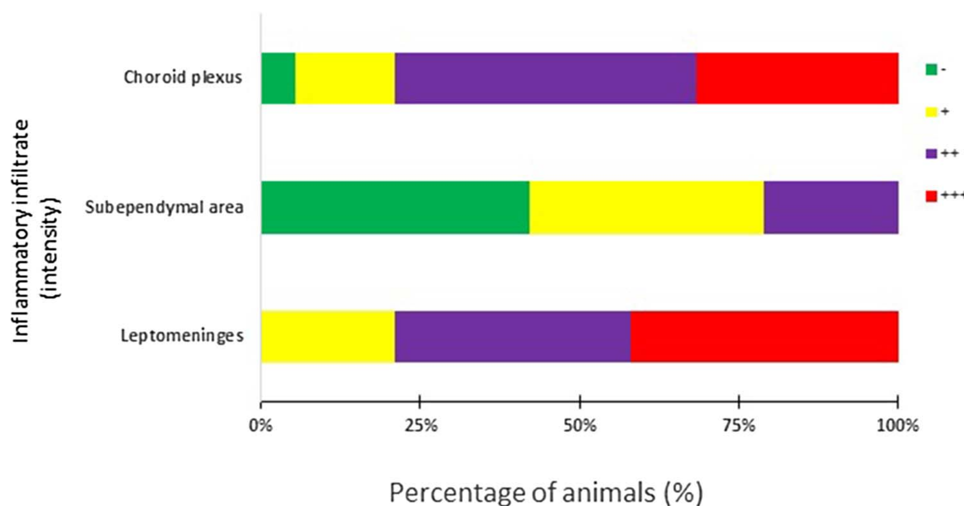

Fig. 2. Percentage (%) of dogs according to the intensity of inflammation in the choroid plexus, subependymal area and leptomeninges. −: absence of inflammatory infiltrate; +: mild inflammatory infiltrate; + +: moderate inflammatory infiltrate; + + +: intense inflammatory infiltrate.

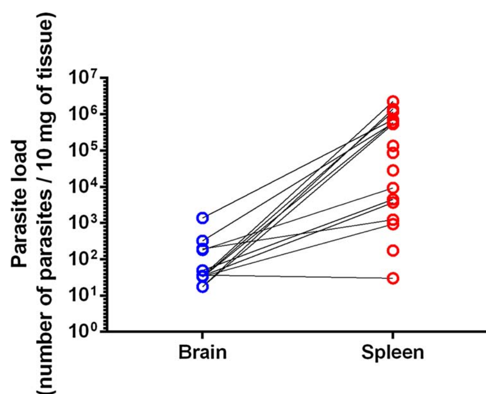

Fig. 3. Individual parasite load determination in the brain and in the spleen of dogs with visceral leishmaniosis. Black lines connect the brain and the spleen values for the same dog.

parasite load and TLRs was observed. For the brain, we did not observe any changes in TLR gene expression (Fig. 4).

As we expected in the distribution of samples from natural infection, we observed important individual variability in all targets evaluated. Nevertheless, different from the other target genes, the distribution of TLR-4 gene expression showed a specific pattern in the brain from infected dogs, with 13 dogs having expression values similar to those of the control dogs and a small subpopulation (n = 4) with more evident gene expression. When we evaluated this subpopulation separately, it presented 20.13-fold more TLR-4 expression than the controls (Fig. 5; $P < 0.05$). This subpopulation included dogs at several stages, i.e., stage II (n = 2), III (n = 1) and IV (n = 1). Even in the particular case of these dogs, we detected no correlation among the clinical stages with the parasite load or TLR-4.

3.5. Cytokine quantification

All cytokines were detected in infected and healthy dogs in both tissues evaluated (Fig. 6), except for IL-6, which was absent in all dogs. IL-1β was detected at higher concentrations in the spleen from infected dogs, while TNF-α was found at lower levels in the brain of these dogs. For the other proteins, there was no significant difference between the infected and control groups.

Moreover, there was no correlation between the clinical stage or TLR expression and cytokine production for both tissues. The absence of correlation was also verified between the splenic parasite load and the cytokines in the spleen. However, in the brain, there was a moderate negative correlation between the parasite load and IL-1β ($r = -0.72$; $P < 0.01$) and TNF-α ($r = -0.53$; $P < 0.05$) levels.

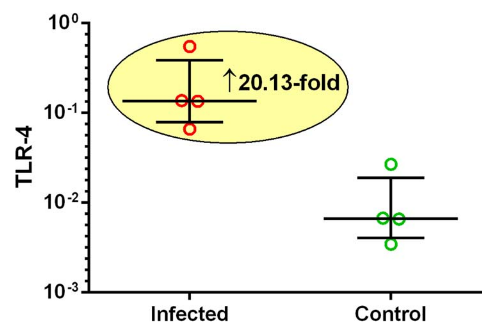

Fig. 5. Individual values of a subpopulation of four infected dogs with remarkable up-regulation (20.13-fold more) of TLR-4 gene expression in the brains when compared with the control group. Horizontal lines represent the median and interquartile range values.

4. Discussion

We detected brain inflammation, as well as TLR gene expression and *Leishmania* DNA in the brain and spleens of dogs with VL. The population of dogs evaluated here included animals belonging to the four clinical stages of the classification proposed by Solano-Gallego et al. (2011). However, there was no correlation between the clinical stage or parasite load and TLR expression. An absence of correlation was also observed between the clinical classification and the intensity of brain inflammation. These data indicate that brain inflammation is not related to the clinical staging of infected dogs. Thus, dogs in different clinical stages of the disease can present the same intensity of brain inflammation. Our findings support another study performed with infected dogs presenting neurological clinical signs, which there was absence of correlation between the severity of the animal clinical condition and the intensity of histopathological brain alterations (Ikeda et al., 2007).

Similarly, to previous reports, brain inflammation ranged from mild to intense in the present study (Viñuelas et al., 2001; Márquez et al., 2013; Melo et al., 2013; Grano et al., 2016; Gianuzzi et al., 2017; Oliveira et al., 2017). The presence of lymphocytes and plasma cells observed in the brains from the infected dogs in our study is probably related to blood-brain barrier breakdown (BBB), described previously during canine VL (Melo et al., 2015b), which could allow the entrance of cells and inflammatory mediators to the cerebral parenchyma, contributing to brain inflammation. Observation of neuroinflammation here confirms the brain as an affected organ during canine VL, even in the absence of whole parasite detection in H-E stained sections or of neurological clinical signs. Indeed, there are a limited number of cases reporting the presence of *Leishmania* in the CNS. The amastigote form of the parasite was previously identified in meninges (Viñuelas et al., 2001) and choroid plexus (Nieto et al., 1996) using H-E staining. In addition, *Leishmania* was also detected by immunohistochemistry in the

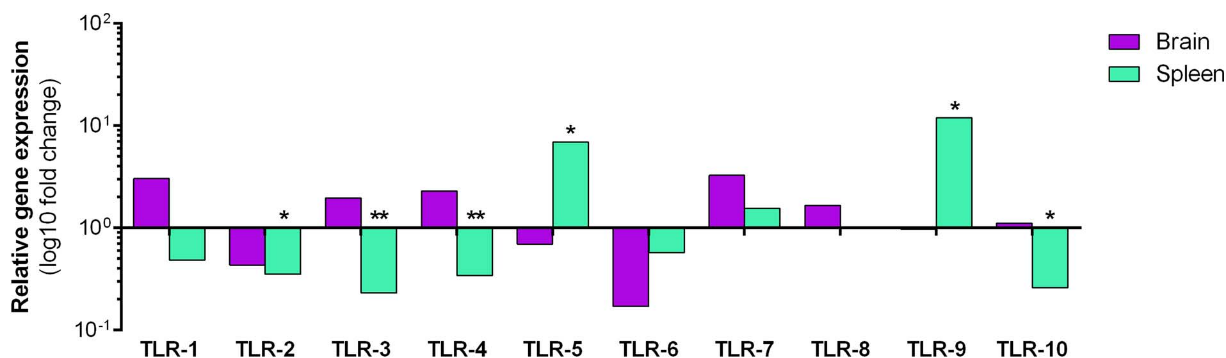

Fig. 4. Relative gene expression of Toll-like receptors in the brain and in the spleen of dogs with visceral leishmaniosis. The values are expressed as fold changes (log 10). Positive values indicate up-regulation and negative values indicate down-regulation when compared to the control dogs. The normalization factor was the reference gene G3PDH and RPL32. * indicates $P < 0.05$; ** indicates $P < 0.01$.

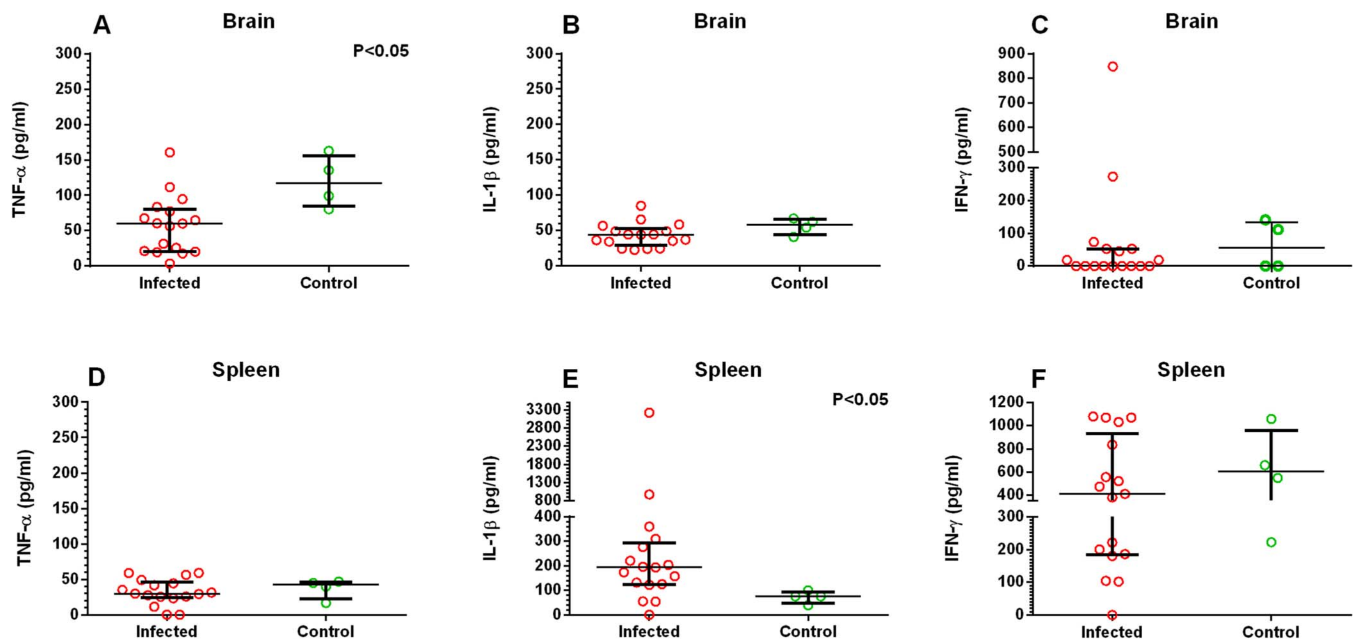

Fig. 6. Individual values of cytokines in naturally infected and healthy dogs. TNF-α, IL-1β and IFN-γ levels in the brain (A–C) and spleen (D–F). Horizontal lines represent the median and interquartile range values.

CNS (Oliveira et al., 2017), including the spinal cord, brain parenchyma and choroid plexus of one chronically infected dog (Márquez et al., 2013). Although amastigotes were not detected by means of H-E staining, *Leishmania* DNA was determined in brain samples. Unfortunately, immunohistochemistry was not performed, and this is a limiting factor of this study.

Whole parasites were not detected in the histopathological analysis of the brain, but we detected its DNA. The same was previously verified by our group in other dogs with VL (Melo et al., 2015a; Grano et al., 2016). Perhaps this can be occurring due to the use of a staining technique of low sensitivity. Moreover, the lack of correlation between parasite load and brain inflammation intensity suggests that the development of an inflammatory nervous milieu could occur through other mechanisms and not by direct stimulation by the presence of parasite DNA. One possible mechanism would be the presence of peripheral inflammatory mediators and cells stimulating the CNS. Evidence of blood-cerebrospinal fluid barrier breakdown has been reported during canine VL, in which T lymphocytes from the blood would somehow stimulate the development of brain inflammation (Grano et al., 2016). Another type of peripheral stimulation in the CNS could be through exosomes. Recently, exosomes were reported to have a role in *Leishmania* infection. These vesicles can support the transfer of parasite proteins to the cytosol of host cells (Silverman et al., 2010) and appear to contribute to pathogenesis by delivering protein virulence factors to macrophages (Lambertz et al., 2015). In addition, it is also possible that inflammation can originate locally in the brain. The production of inflammatory mediators, such as cytokines and chemokines in the brain, has been reported in dogs with VL (Melo et al., 2013; 2015a), confirming the potential of the brain in the development of a local immune response.

This study provides the first insight into the expression profile of TLR transcripts in the brain, along with the spleen during canine VL. We observed an up-regulation of TLR-9 in the spleens of infected dogs. Activation of TLR-9 occurs by the recognition of microbial DNA and by immune complexes containing DNA (Ospelt and Gay, 2010). An accumulation of immune complexes containing DNA usually can be found in autoimmune diseases (Tian et al., 2007), and the presence of anti-double-stranded DNA antibodies has already been reported previously in human VL (Sakkas et al., 2008; Liberopoulos et al., 2013). In agreement, we have here detected *Leishmania* DNA in the spleen, which could explain the effect on TLR-9 in this tissue and showing evidence that this immune sensor has a role in detecting the parasite in this tissue. In another study with canine VL, increased frequency of TLR-9 was associated with a lower parasite load in jejunum samples, suggesting the role of TLR-9 in the parasite clearance (Figueiredo et al., 2013). On the other hand, although we detected *Leishmania* DNA in the brain, TLR-9 gene expression was unchanged in this tissue, which is agreement with our previous results in infected dogs (Melo et al., 2014b). This suggests that the immune response is compartmentalized, and other receptors can be related to immune vigilance in the CNS.

An up-regulation in TLR-5 transcripts was also verified in the spleens from infected dogs. The known ligand for TLR5 is a bacterial flagellin (Singh et al., 2012). Therefore, the role of TLR-5 in directly promoting an immune response against Leishmania parasites or even if it plays roles through its interaction with other TLRs is still unknown and necessitates further investigation.

Regarding TLR-4, only a subpopulation of infected dogs presented up-regulation in the brain compared to the control group. Another detailed study concerning to TLR-4 would be necessary to evaluate the true role of this receptor in canine VL and its participation in the neuropathogenesis of the disease. In a previous study performed by our research group, we found that TLR-4 transcript was unchanged in the brain and in the spleen, but it was up-regulated in lymph nodes (Melo et al., 2014b), although we detected here down-regulation of the TLR-4 transcript in the spleen. The TLR-4 transcript was also unchanged in the blood during canine VL (Montserrat-Sangrà et al., 2016). The most important known TLR-4 ligand is LPS from gram-negative bacteria (Ospelt and Gay, 2010). Thus, it is unclear which other ligands could be interacting with TLR-4 during VL.

A slightly increased expression of TLR-7 and TLR-8 was observed in the brain and the spleen of dogs with VL, along with TLR-3 in the brain, although this increase was not statistically significant. TLR-3 was reported to contribute to the recognition of the *Leishmania* parasite (Flandin et al., 2006). This receptor, like TLR-7, TLR-8, and TLR-9, is found inside intracellular endosomal membranes and is able to recognize double-stranded RNA (dsRNA), triggering the NF-κB cascade and IFN-γ production (Alexopoulou et al., 2001), while TLR-7 and TLR-8 share the same ligand, recognizing single-stranded RNA (ssRNA) from viruses or endogenous sources (Heil et al., 2004). dsRNA is not present in the Leishmania parasite, but many protozoan parasites carry

endosymbiotic dsRNA viruses (Ogg et al., 2003). *Leishmania* RNA virus (LRV) exists in several species of *Leishmania*, including *L. infantum* (Hajjaran et al., 2016), confirming the potential of TLR-3 in having a role in VL. In lymph nodes, the down-regulation of TLR-3 transcripts has been associated with the establishment of disease (Hosein et al., 2015). Moreover, TLR3 was described to be needed for nitric oxide production and parasite phagocytosis (Flandin et al., 2006). Therefore, the lack of TLR-3 observed here in the spleen of infected dogs could be related to susceptibility to *Leishmania* infection.

A significant reduction in TLR-2 and TLR-10 transcripts was observed in the spleens of infected dogs. Furthermore, a slightly reduced expression of TLR-1 and TLR-6 was observed in the spleen, along with TLR-2 and TLR-6 in the brain, although these reductions were not statistically significant. Therefore, gene expression of transcripts was similarly reduced among TLR-1, TLR-2, TLR-6 and TLR-10 in the spleen, as well as TLR-2 and TLR-6 in the brain. TLR-1 and TLR-10 were slightly elevated in the brain. This can occur because the receptors act synchronously among them. TLRs have been reported to act as dimers in the recognition of PAMPs (Ospelt and Gay, 2010). For example, TLR-2 signaling occurs through the formation of heterodimers with TLR-1, TLR-6 or TLR-10 (Takeuchi et al., 2002; Ospelt and Gay, 2010). TLR-1 can signal through dimerization with TLR-2 (Wyllie et al., 2000; Takeuchi et al., 2002), TLR-6 with TLR-1 or TLR-2 (Hornung et al., 2002) and TLR-10 with TLR-1 or TLR-2 (Hasan et al., 2005). Thus, study of the TLR profile is essential because the immune response against *Leishmania* parasites may involve interactions among multiple receptors.

In our study, TLR-2 transcripts were unchanged in the brains of infected dogs. TLR-2 has already been found unchanged in the brain during canine VL, but an up-regulation of TLR-2 and TLR-9 transcripts was observed particularly in the choroid plexuses from infected dogs (Melo et al., 2014b). Here, we evaluated a pool of samples from several brain areas but not from choroid plexuses. We observed that all TLRs evaluated were unchanged in the brain, except TLR-4, which was elevated in a small subset of animals. Therefore, we suggest the possibility that the choroid plexuses structure is related to the activation of inflammatory pathways related to TLRs in the CNS because it was the structure that presented the most severe inflammation intensity in this study, along with leptomeninges.

We found a reduction in TLR-2 transcripts in the spleens of infected dogs. A reduction in TLR-2 and TLR-4 was previously detected in infected dogs, in macrophages from mononuclear cells and PBMCs, respectively (Melo et al., 2014a). On the other hand, TLR-2 was found to be widely expressed in the spleens of dogs with VL (Melo et al., 2014b). This difference in spleen transcripts can occur due to variability in the individual immune response. In addition, high amounts of TLR-2 and TLR-4 were detected in the spleen during experimental infection with L. chagasi, mainly in the acute phase of the disease, presenting a correlation with high parasitism and high levels of cytokines and nitric oxide (Cezário et al., 2011). In fact, TLR-2 was also up-regulated in the blood of dogs with moderate to severe leishmaniosis at diagnosis but it decreased with clinical improvement during treatment (Montserrat-Sangrà et al., 2016).

In our study, we observed meningitis and choroiditis in infected dogs, showing evidence of blood brain barrier breakdown. IL-1β is the main cytokine involved in BBB breakdown, in addition to TNF-α and IL-6. These cytokines have a role in increasing the expression of adhesion molecules in the endothelium, allowing leukocyte trafficking into the brain (Quagliarello et al., 1991; De Vries et al., 1996; Hickey, 1999; Abbott et al., 2006). The presence of T lymphocytes has already been reported in the CNS of infected dogs (Melo et al., 2009; Grano et al., 2016). Here, IL-1β was detected in similar amounts in the brains of infected and healthy dogs, while IL-6 was not detected in this tissue. These data provide evidence that these cytokines are not related to brain inflammation or active disease.

BBB integrity in dogs with VL can be affected not only by substances that disrupt it but also by a deficiency of structures that support its integrity, such as the tight junctions between the endothelial cells and BBB pericytes (Melo et al., 2013). Pericytes are sensitive to TNF-α and release matrix metalloproteinase 9 (MMP-9), a proteolytic enzyme related to BBB breakdown (Takata et al., 2011). MMP-9 has already been reported in the cerebrospinal fluid as well as in the brains of infected dogs (Machado et al., 2010). We observed more TNF-α in the brains of healthy dogs. Reduced TNF-α production may be due to the action of TNF-α on target cells. Its action occurs through interaction with membrane-bound receptors. After being exposed to TNF-α, these cells may reduce their responsiveness to this cytokine by releasing these receptors to the circulation (De Beaux and Fearon et al., 1996), where they can bind to TNF-α, making its measurement by ELISA difficult. We have previously reported the brain's potential to produce TNF-α through the detection of an increase in TNF-α transcripts in the brains of infected dogs (Melo et al., 2013). Therefore, we cannot discard TNF-α as a substance related to BBB breakdown in dogs with VL.

On the other hand, we detected TNF-α in the spleens of infected dogs; however, it did not differ from control dogs. The lack of a relationship between TNF-α and active disease was previously reported in the sera (Lima et al., 2007). In contrast, TNF-α has been found at higher concentrations in the spleen, liver (De F Michelin et al., 2011) and lymph nodes (Alves et al., 2009; Venturin et al., 2016) from infected dogs compared to healthy dogs.

For IFN-γ, the levels were similar in the brains and spleens of infected and healthy dogs. In another study, it was verified that decreased intracellular survival of *L. infantum* in canine macrophages is associated with increased production of IFN-γ and TNF-α, as well as decreased production of IL-10 (Turchetti et al., 2014). Another study supports these findings, where infected dogs lacking *L. infantum*-specific IFN-γ production in stimulated whole blood had a high blood parasitemia and severe clinical disease (Solano-Gallego et al., 2016). Therefore, IFN-γ seems to have a protective role during canine VL, and the lack of more elevated levels of this cytokine in infected dogs, as observed, can be related to their susceptibility to the disease and brain involvement.

5. Conclusions

Our data provide support to explain the involvement of innate immune sensors in the immune response against L. infantum. The data presented herein provide important evidence that TLR-5 and TLR-9 play a role in splenic innate immunity during canine VL and that TLR-4 might be related to the pathogenesis of brain lesions, although the number of brain samples that presented up-regulation of TLR-4 was low, and future investigations should be performed. Concerning cytokines, IFN-γ may be related to the resistance to systemic infection and to brain commitment, while TNF-α might be related to BBB breakdown, contributing to brain inflammation. Moreover, we suggest that the brain inflammation observed here might be triggered by other inflammatory pathways, perhaps through other innate immune receptors, in addition to TLRs, or it might originate in another brain area, such as the as choroid plexus.

Conflict of interest statement

The authors of this work do not have any financial, personal or other relationship with organizations or people that could inadequately influence the content of this paper.

Acknowledgments

F.G. Grano was supported by a Ph.D.'s degree scholarship from the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP, grant number 2013/25498-1). The research project was supported by FAPESP, grant number 2016/02384-9 and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), grant number

420970/2016-9.

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