



Research paper

T lymphocyte immunophenotypes in the cerebrospinal fluid of dogs with visceral leishmaniasis



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ABSTRACT

Visceral leishmaniasis (VL) is a disease causing several clinical manifestations in dogs, including neurological disorders. Nevertheless, there are few studies related to the evaluation of the brain alterations during VL. Evidences of the involvement of cerebral barriers in infected dogs was reported, including the presence of brain inflammatory infiltrate, with a predominance of CD3+ T cells. Therefore, the aim of this study was to determine the immunophenotypes of T lymphocytes in the cerebrospinal fluid (CSF), as well as in peripheral blood, and to correlate with brain alterations in dogs with VL. We detected elevated percentages of double negative (DN) and double positive (DP) T cells in the CSF, with a predominance of TCR $\alpha\beta$. In the histopathological analysis, we observed a predominance of lymphoplasmacytic infiltrate, mainly in leptomeninges, ranging from mild to intense, and we observed a positive correlation between the intensity of inflammation in the subependymal area and the DN T cells of the CSF. Thus, the DN T cells seem be acting as villains of the immune system through pro-inflammatory mechanisms. Further, the proportion of the different population of CSF T cells did not differ from those observed in the blood, which provides us with more evidence of blood-CSF barrier breakdown. Together, the results provide more explanation to the inflammation observed in the brain of dogs with VL, which the DN T cells contribute to the origin and progression of the neurological disease. This study provides insight into the immunophenotypes of T lymphocytes in the CSF during canine visceral leishmaniasis.

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1. Introduction

Visceral leishmaniasis (VL) is an anthroponosis caused by *Leishmania infantum* (*Syn L. chagasi*; Maurício et al., 2000). In Brazil, dogs are the main hosts of the parasite and can present several clinical manifestations, from subclinical to systemic disorders.

Chronically infected dogs may rarely develop neurological clinical disorders; nevertheless, the pathogenesis of the cerebral form of the VL has still not been elucidated. Infected dogs may present tetraplegy, generalised seizures, walking in circles, vestibular and cerebellar signs, myoclonia and motor incoordination (Font et al., 2004; Ikeda et al., 2007; José-López et al., 2012).

Brain lesions have been described in some reports; the main findings related to canine visceral leishmaniasis were meningitis and choroiditis (Nieto et al., 1996; Viñuelas et al., 2001; Melo et al., 2009, 2013; Melo and Machado, 2011) and the deposition of antigens and immunoglobulins in the central nervous system (CNS) (García-Alonso et al., 1996; Melo et al., 2015a). Multiple brain infarcts were also described in two infected dogs (José-López et al., 2012).

A limited number of reports have pointed to parasite detection together with lesion descriptions. Parasite migration to the meninges (Viñuelas et al., 2001), choroid plexus (Nieto et al., 1996; Márquez et al., 2013) and brain parenchyma (Márquez et al., 2013) was described in a chronically infected dog. Recently, we have detected the presence of *L. infantum* DNA in brain areas (Grano et al., 2014) and up-regulation of the gene expression of some toll-like

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receptors (Melo et al., 2014) within the brain of naturally infected dogs.

The choroid plexi (CP) in the blood-CSF (cerebrospinal fluid) barrier and can prevent the entrance of toxic molecules and drugs into the CNS (Liddelow, 2015). Several bacteria, parasites and viruses such as *Neisseria meningitidis*, *Streptococcus suis*, *Trypanosoma brucei*, Sendai virus, Measles virus, Coxsackievirus B3 (CVB3), AIDS (HIV-1) Echovirus 30 (EV30) and leukaemia (HTLV-1) viruses present tropism for the CP (Levine, 1987; Strazielle and Ghersi-Egea, 2000; Schwerk et al., 2015). Thereby, these structures can act as an entry route for the *Leishmania* parasite within the brain. As the brain is a site that is inaccessible for many diagnostic procedures and since there is a proximity of the CSF to brain lesions, the CSF can act as a predictive tool and reflect alterations in the CNS (Duque et al., 2002).

In a previous study performed by our research group, it was observed that dogs with VL presented brain inflammatory infiltrate with a predominance of CD3+ T lymphocytes (Melo et al., 2009), but a low percentage of CD4+ and CD8+ T lymphocytes (Melo et al., 2015b). This suggests that another subset of T cells in the brain might be involved in the triggering of brain inflammation. Furthermore, the leukocyte populations of the CSF differ from those of the blood in healthy dogs, requiring a deeper evaluation of both compartments (Duque et al., 2002).

There are papers describing T lymphocytes immunophenotypes in dogs, especially in the blood (Tipold et al., 1998; Itoh et al., 2009; Alexandre-Pires et al., 2010; Watabe et al., 2011; Lima et al., 2012), although there are only a few conflicting papers describing the function of double-negative (CD4– CD8– = DN) and double-positive (CD4+ CD8+ = DP) T cells. In dogs, DP T cells present an activated phenotype and may have unrecognised functions in *in vivo* immunity and infection, as well as in inflammatory diseases such as allergy, chronic infection, autoimmunity or cancer (Buttlar et al., 2015). TCR $\alpha\beta$ +DN T cells have been connected to autoimmune conditions and present a pro-inflammatory profile. These cells were reported to be increased in several autoimmune/inflammatory disorders (Blesing et al., 2001; Crispín et al., 2008; Alunno et al., 2013), produce pro-inflammatory cytokines (Crispín et al., 2008), and on the other hand also have regulatory properties (D'Acquisto and Crompton, 2011). With regard to DN cells, there are only a few papers reporting their function, which appear to be conflicting. The DN T cells have potential for a pathogenic role during autoimmunity, acting in the development of disease, as well as for homeostatic role in suppressing excessive immune responses that are deleterious to the host (D'Acquisto and Crompton, 2011). Nevertheless, there are no studies reporting the presence of these cells in the CSF of dogs infected by *Leishmania*.

Therefore, in view of the paucity of data regarding brain inflammation during VL, and in view of the possible role of T lymphocytes during infection, the aim of this study was to determine the immunophenotypes of T lymphocytes in the CSF of infected dogs and to compare with the same cell populations in peripheral blood. Brain lesions were also evaluated in order to correlate with the T lymphocyte populations in the CSF.

2. Material and methods

2.1. Animals

Seventeen naturally infected dogs, eleven male and six female, ranging in age from 1 to 8 years-old, were selected from the Veterinary Teaching Hospital of UNESP, São Paulo State University and from the Zoonosis Control Centre in the municipality of Araçatuba, São Paulo State, Brazil, which is an area endemic for VL. VL diagnosis was achieved using a routine ELISA (enzyme-linked immunosor-

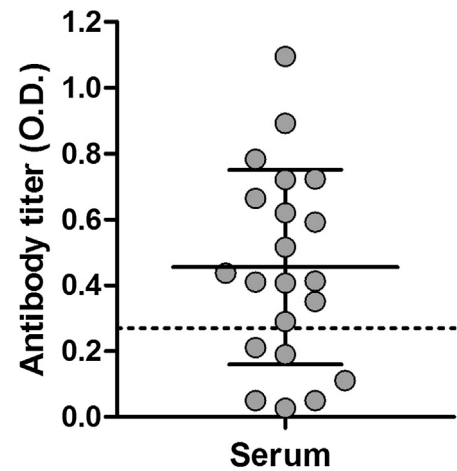


Fig. 1. Scatter plot presenting serum reactivity (IgG) against *Leishmania* antigens evaluated by ELISA. Anti-*L. infantum* anti-antibody titers were determined by optical density (O.D.; absorbance at 492 nm). Horizontal lines indicate the mean and SD. The dotted lines represent the lower limit of positivity (cut-off): 0.270.

bent assay) according to Lima et al. (2005). The cut-off point was determined previously with serum from 38 healthy dogs from a non-endemic area, using as reference the mean added by 3 times the standard deviation obtained for the group. The samples were analyzed in duplicate and a blank well (PBS + 0.05% Tween 20[®] solution) was included in all plates. The ELISA results were confirmed by popliteal lymph node fine-needle aspiration. Of the infected dogs, 64.7% (n = 11) presented anti-*L. infantum* IgG antibody titres higher than the cut-off value in sera, ranging from 0.289 to 1.095 (Fig. 1). Moreover, we detected *Leishmania* amastigotes in all of the dogs through popliteal lymph node fine-needle aspiration.

Dogs were euthanised with the owners' permission, in compliance with state law (São Paulo et al., 2006). None of the animals were previously vaccinated against VL. All animals were symptomatic, with at least three clinical signs. 82.35% (n = 14) of the dogs presented dermatological alterations (seborrhea, generalised alopecia, nasal hyperkeratosis and hypotrichosis), 76.47% (n = 13) presented generalised lymphadenopathy, 52.94% (n = 9) presented onychogryphosis, 52.94% (n = 9) presented cachexia, 29.41% (n = 5) presented ophthalmic alterations (conjunctivitis and ocular discharge) and 11.76% (n = 2) presented temporalis muscle atrophy. Nevertheless, they did not present a history of neurological signs and were also serologically negative for toxoplasmosis and neosporosis, as assessed by indirect immunofluorescence assays.

2.2. Sampling

The dogs were anaesthetised with pentobarbital (Hypnol[®]). Peripheral blood samples (4 ml) were obtained from the cephalic vein into tubes with sodium heparin and 5–6 ml of CSF was collected from the cerebello-medullary cistern. Following that, the animals were euthanised with potassium chloride. Necropsies were performed immediately after euthanasia. The brain was collected and separated into two hemispheres, one of which was placed in 10% buffered-formalin. After fixation, fragments of some areas (hippocampus, diencephalon, lateral ventricle choroid plexus, mid-brain, brainstem, cerebellum and fourth ventricle choroid plexus) were embedded in paraffin and submitted to histological procedures and haematoxylin-eosin (H–E) staining.

The inflammatory lymphoplasmacytic infiltrate was evaluated according to intensity using a ponderal index divided into four grades: Grade 0 (absence of inflammation); Grade 1, mild inflammation (slight inflammatory cell infiltrate mainly at the lep-

tomeninges and choroid plexus); Grade 2, moderate inflammation (moderate inflammatory cell infiltrate mainly at the leptomeninges and choroid plexus, but with the presence of some perivascular lymphocytes in the brain tissue); and Grade 3, intense inflammation (remarkable inflammatory cell infiltrate mainly at the leptomeninges and choroid plexus plus intense perivascular infiltration in the brain tissue).

2.3. Flow cytometry analysis

Peripheral blood samples were submitted to the separation of mononuclear cells on a Ficoll-Paque™ gradient (Amersham Biosciences, Piscataway, NJ, USA), in accordance with the manufacturer's recommendations. After this, the red blood cells were lysed by adding 5 ml of lysis buffer (0.16 M NH₄Cl and 0.17 M Tris) to the isolated cells and incubating at 4 °C for 10 min. The cells were washed three times adding 30 ml of PBS (10 min at 400 × g) at 20 °C. CSF was centrifuged at 400 × g for 10 min. The pellets were transferred into a 1.5 ml microcentrifuge tube and resuspended in 1 ml of PBS. The centrifugation was repeated three times. Each sample was individually processed.

The cellular concentration of blood and CSF was adjusted individually to 10⁵ cells in 100 μl of phosphate-buffered saline solution for each dog sample. The cells were incubated for 60 min at 4 °C with monoclonal anti-dog antibodies: FITC-labelled anti-CD3 (clone CA17.2A12), RPE-labelled anti-CD4 (clone YKIX302.9), Alexa Fluor® 647-labelled anti-CD8 (clone YCATE55.9) (Serotec, UK), anti-TCRαβ (clone CA15.8G7) and anti-TCRγδ (clone CA20.8H1) (UC Davis, USA). Following this, secondary antibodies conjugated to FITC (TCRαβ) or RPE (TCRγδ) were used (Abcam, UK). Isotype controls (Serotec, UK) were used to delimit the negative populations of the stained cells. Following antibody incubation, washing was performed with PBS (pH 7.0) and the cells were resuspended in fixation buffer (10% formaldehyde).

To analyse the different immunostained populations in a Guava Easy-CyteMini system by using CytoSoft software, 10,000 events were acquired in accordance with the recommendations in the Guava PCA User's Guide and respective package inserts. The values obtained for healthy dogs from the literature, according to Tipold et al. (1998), Duque et al. (2002), Itoh et al. (2009), Alexandre-Pires et al. (2010), Watabe et al. (2011) and Lima et al. (2012), were used as reference values for this study.

2.4. Statistical analysis

The statistical evaluation between blood and CSF was performed by the Wilcoxon test. T lymphocyte subset analysis in CSF and blood and inflammation intensity analysis in the brain were performed using the Friedman test, followed by Dunn's test. The Spearman test was performed to correlate the blood and CSF cells with the intensity of brain lesions. Statistical significance was accepted when $P < 0.05$. All statistical analyses were performed using Prism software (Prism 6, GraphPad).

2.5. Ethical issues

This study was approved by the institutional Ethics and Animal Welfare Committee (CEEA – Comissão de Ética e Experimentação Animal, UNESP, process #01463-2012).

3. Results

3.1. Immunophenotyping of T lymphocytes

The quantification of T lymphocytes was carried out according to the fluorescence of CD3+ cells. The CD4+, CD8+, DN and DP cells

subsets were identified and quantified within the CD3+ T lymphocytes population (Fig. 2), while TCRs were quantified in relation to the total count of mononuclear cells.

When we compared the CD3+ T cells percentage between CSF and blood (Fig. 3A) we observed that there was no statistical difference, or for all of the subtypes of T cells evaluated, that were present in similar proportions in these compartments ($P > 0.05$). A predominance of TCRαβ lymphocytes in both, blood and CSF was observed ($P < 0.0001$), whereas the TCRγδ cells were found at a lower percentage (Figs. 3B and C). Moreover, the predominant T lymphocytes subsets in the CSF were the DN cells (Fig. 3D), although there was no difference between DN and DP cells in this compartment. Furthermore, there was no difference between the percentage of CD4+ and CD8+ cells and between CD4+ and DP cells in the CSF. In both the blood and CSF, it was verified that CD8+ T cells were lower when compared to the DN T lymphocytes and there was no difference between the percentage of CD4+, CD8+ and DP cells or CD4+, DP and DN cells in the blood (Fig. 3E). Moreover, the mean percentages of all T cells populations were lower in relation to the reference values, except for DP and DN percentages, that were higher in the blood (Table 1).

3.2. Evaluation of the inflammatory infiltrate in the brain

Histopathological analysis revealed mononuclear cells infiltrate, especially composed of lymphocytes, plasma cells and few histiocytes in the choroid plexus and leptomeninges and lymphocytes and plasma cells in periventricular areas, with inflammation intensity ranging from mild to intense. The leptomeninges were the site with the highest concentration of inflammatory cells, differing from the other areas evaluated, and there was no difference in the intensity of the inflammatory infiltrate between the choroid plexus and subependymal area. Fig. 4 shows the representative photomicrographs of inflammatory infiltrate in brain areas, according to the intensity of inflammation.

In leptomeninges, 58.8% of the dogs (n = 10) presented moderate infiltration of inflammatory cells, 35.3% (n = 6) presented mild inflammatory infiltrate and 5.9% (n = 1) presented severe inflammatory infiltrate. In the subependymal area, 64.7% (n = 11) of the animals had mild inflammatory infiltrate, 11.8% (n = 2) presented moderate intensity and 23.5% (n = 4) showed no inflammatory infiltrate. In the choroid plexus, 35.3% (n = 6) of the dogs presented mild inflammatory infiltrate, 17.6% (n = 3) showed moderate inflammatory infiltrate, 5.9% (n = 1) presented severe intensity of inflammatory cells and 41.2% (n = 7) showed no inflammatory infiltrate. In the study presented here, we did not observe amastigotes of *Leishmania* in the H-E stained nervous tissue samples.

3.3. Correlation of the T lymphocytes immunophenotypes with the intensity of brain inflammation

There was no correlation between the investigated immunophenotypes of T lymphocytes of the CSF and the intensity of inflammation in the leptomeninges. However, in the choroid plexus, a weak negative correlation was observed between DP cells from the CSF and the intensity of inflammation (Fig. 5A). The other cell types did not present any correlation. Also, in the subependymal area, there was a moderate negative correlation of the inflammatory infiltrate intensity with DP and CD8+ cells (Fig. 5B and D), and weak negative correlation with CD4+ (Fig. 5C) of the CSF. Still, for the DN cells, moderate positive correlation with inflammation was observed (Fig. 5E).

Correlation of the amount of blood mononuclear cells with inflammation intensity in the brain was also performed, but there were no correlations between these parameters.

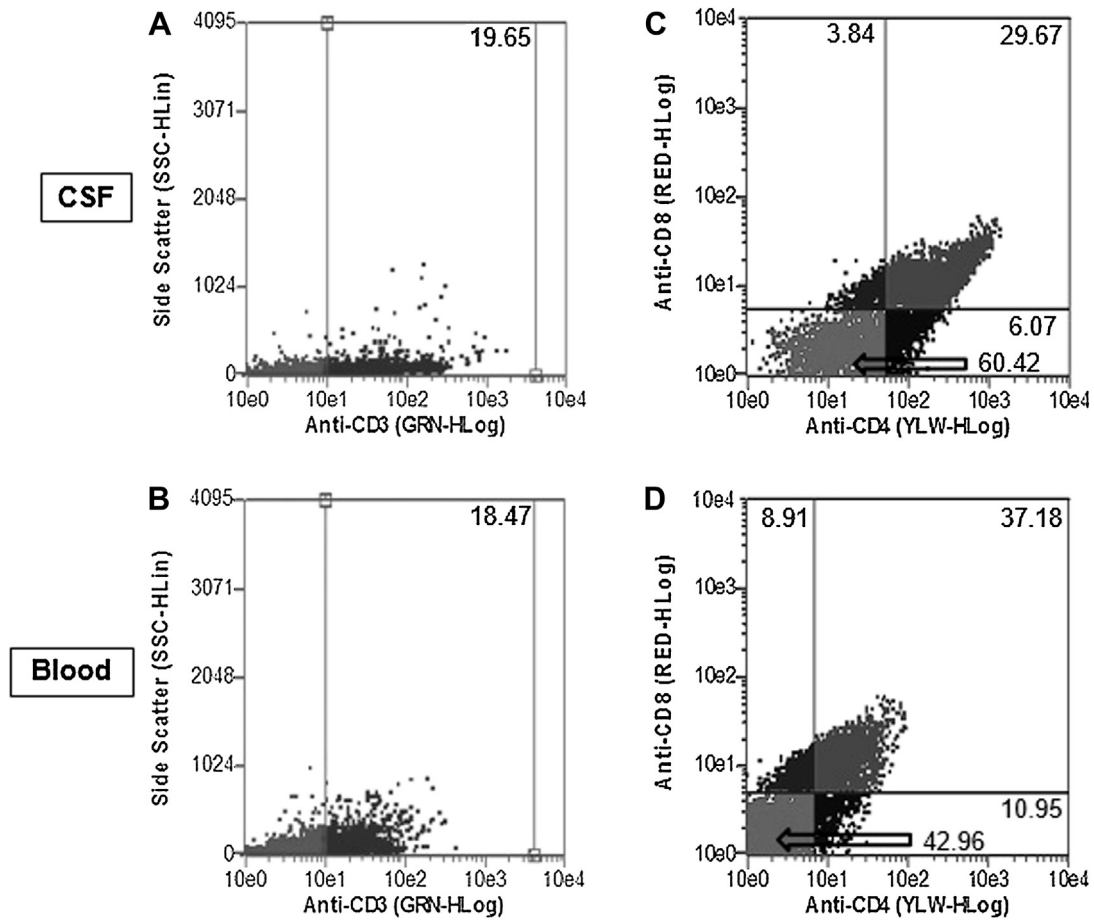


Fig. 2. Flow cytometry analysis: fluorescence plot for gated T lymphocytes from *Leishmania* infected dogs (Anti-CD3 monoclonal antibody FITC). Percentage inside right quadrant correspond to lymphocyte CD3 positive cells antibodies in the cerebrospinal fluid (CSF) (A) and in peripheral blood mononuclear cells (PBMC) (B); representative fluorescence plot for gated CD3+ T lymphocytes stained for CD4+ and CD8+ antibodies in the CSF (C) and in PBMC (D). Percentage inside upper left quadrant corresponds to CD8+ T cells; lower right quadrant corresponds to CD4+ T cells; upper right quadrant corresponds to double-positive T cells (double stained); and lower left quadrant corresponds to double-negative T cells.

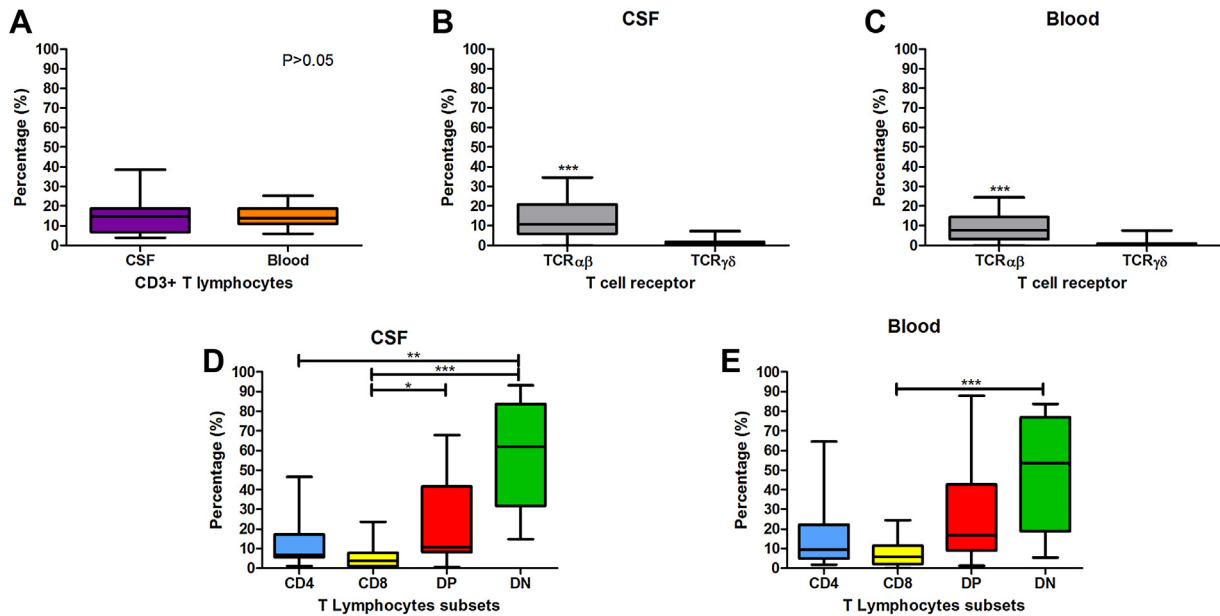


Fig. 3. Boxplot of the immunophenotypic profile of T lymphocytes in canine visceral leishmaniasis. (A) CD3+ T lymphocytes in cerebrospinal fluid (CSF) and blood; (B) T cell receptor in the CSF; (C) T cell receptor in the blood; (D) T lymphocytes subsets in the CSF; (E) T lymphocytes subsets in the blood; Results are expressed as in percentage. Significant difference by Dunn's multiple comparison test is indicated by * ($P < 0.05$), ** ($P < 0.01$) and *** ($P < 0.001$).

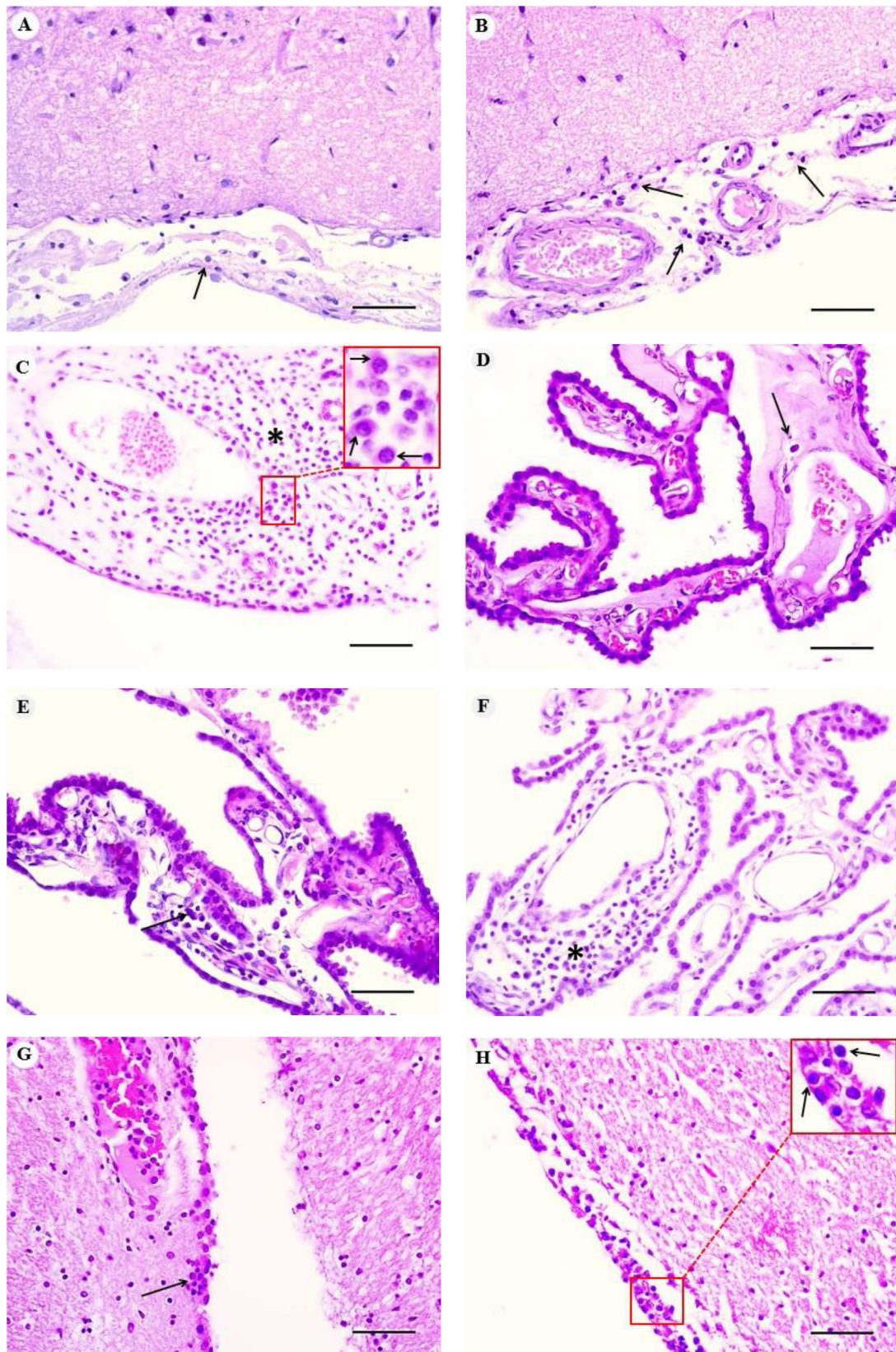
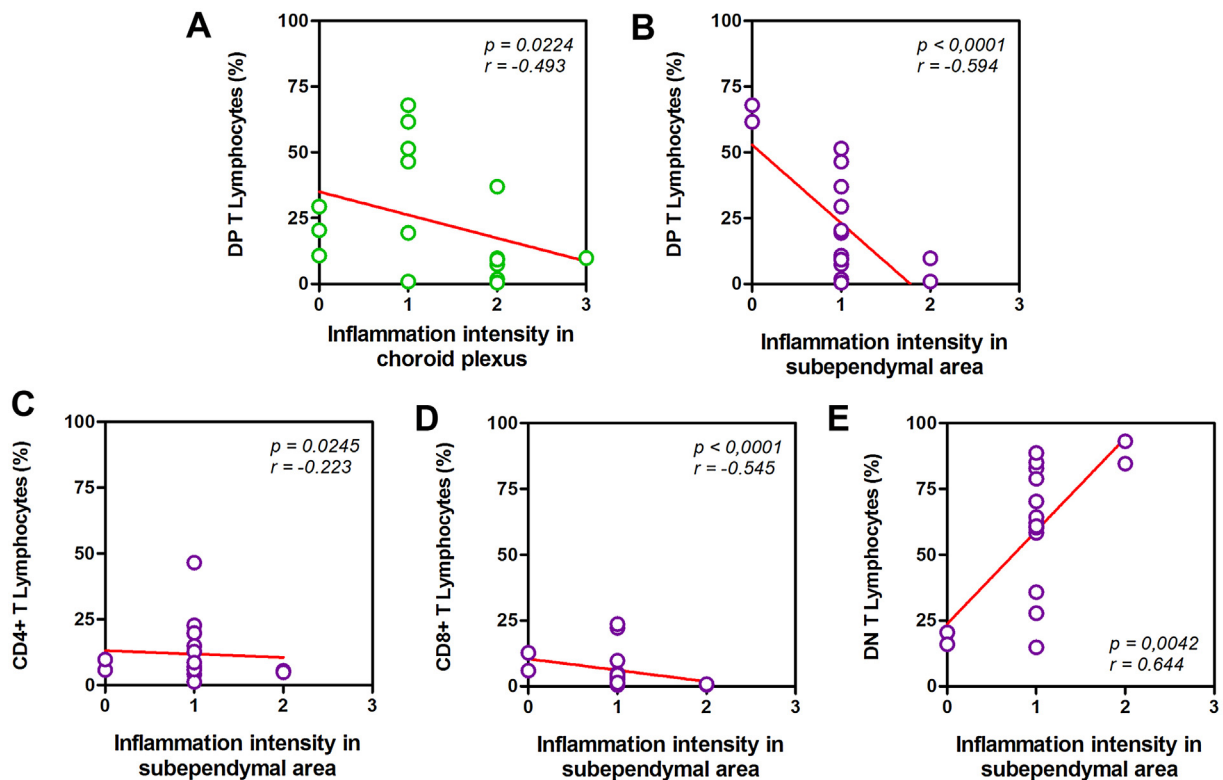


Fig. 4. Representative photomicrography of brain alterations during the canine visceral leishmaniasis. (A) Mild lymphoplasmacytic infiltration (arrow) in cortical leptomeninge (Grade 1). (B) Moderate lymphoplasmacytic infiltration (arrows) in cortical leptomeninge (Grade 2). (C) Intense lymphoplasmacytic infiltration (asterisk) in cortical leptomeninge (Grade 3). (D) Mild lymphoplasmacytic infiltration (arrow) in the choroid plexus from the lateral ventricle (Grade 1). (E) Moderate lymphoplasmacytic infiltration (arrow) in the choroid plexus from the lateral ventricle (Grade 2). (F) Intense lymphoplasmacytic infiltration (asterisk) in the choroid plexus from the lateral ventricle (Grade 3). (G) Mild lymphoplasmacytic infiltration (arrow) in the subependymal area (Grade 1). (H) Moderate lymphoplasmacytic infiltration in the subependymal area (Grade 2). *Inset:* mononuclear cells amplified. Hematoxylin and Eosin. Scale bar = 50 μ m.

Table 1Lymphocyte subsets of cerebrospinal fluid (CSF) and peripheral blood in infected dogs and in healthy dogs in percentage (%) (mean \pm standard deviation).

Populations	CSF		Blood	
	Infected dogs	Reference values	Infected dogs	Reference values
CD3+	15.1 \pm 9.1	53.0 \pm 11.0 ^a	14.8 \pm 5.2	68.0 \pm 10.0 ^a
CD4+	11.8 \pm 10.9	28.6 \pm 12.4 ^d	13.4 \pm 18.0	70.0 ^b
CD8+	6.1 \pm 7.1	34.0 \pm 8.0 ^a	8.2 \pm 7.9	80.6 \pm 5.0 ^c
DN	59.0 \pm 26.6	22.0 \pm 13.6 ^d	49.2 \pm 27.3	39.1 \pm 10.7 ^e
DP	23.1 \pm 21.9	25.0 \pm 19.0 ^a	26.1 \pm 23.6	40.1 \pm 5.6 ^f
TCR $\alpha\beta$	13.6 \pm 10.8	–	9.3 \pm 7.1	22.2 \pm 2.9 ^f
TCR $\gamma\delta$	1.4 \pm 2.1	–	0.9 \pm 1.9	25.7 ^{b,g}
		4.5 \pm 6.0 ^a		1.5 ^{b,g}
				66.0 \pm 11.0 ^a
				3.2 \pm 2.8 ^a

^a Tipold et al. (1998).^b Alexandre-Pires et al. (2010).^c Lima et al. (2012).^d Duque et al. (2002).^e Watabe et al. (2011).^f Itoh et al. (2009).^g Representative values from one dog.**Fig. 5.** Correlation of the intensity of brain inflammation with the DP cells of the choroid plexus (A), and with the DP (B), CD4+ (C), CD8+ (D) and DN (E) cells in the subependymal area.

4. Discussion

We observed a low number of CD3+ T lymphocytes in the CSF, as well as CD4+ and CD8+ T single positive (SP) lymphocytes, indicating that these cells are reduced during the canine VL when compared to the values for healthy dogs (Tipold et al., 1998; Duque et al., 2002). The reduction of T cell number may occur in the CSF, as well as in others sites, such as the spleen and peripheral blood, due to the high level of apoptosis in these cells (Lima et al., 2012; Perosso et al., 2014), or T cells exhaustion, a state of T cell dysfunction

that arises during many chronic infections and cancer (Wherry, 2011).

Although there was a reduction in the total amount of T cells, we verified a predominance of DN and DP T cells in the CSF. During normal T-lymphocyte development in the thymus, DN cells originate the DP cells, which differentiate to mature CD4+ or CD8+ SP cells (D'Acquisto and Cromptom, 2011). The low number of SP cells observed in the infected dogs of our study may be due to acquisition of the DP phenotype outside of the thymus or due to the liberation of immature cells into the bloodstream. The CD4+ and CD8+ cells can act as progenitors of DP cells, as described previously in dogs

(Bismarck et al., 2014), as well as in swine (Saalmuller et al., 2002) and humans (Blue et al., 1986; Nascimbeni et al., 2004; Parel and Chizzolini, 2004; Schenkel et al., 2010). The extrathymic origin of DP T cells was reported in several other species, such as mice, rats, chickens, monkeys, swine and humans, and there appears to be no predilection of localisation, as reviewed by Overgaard et al. (2015).

We observed that there was a predominance of lymphocytes expressing TCR $\alpha\beta$ in the CSF, which also occurs in healthy dogs (Tipold et al., 1998). This phenotype was reported predominantly in the tissues of healthy dogs, as intestinal lamina propria (German et al., 1999), respiratory tract and in the alveolar interstitium of dogs, while TCR $\gamma\delta$ cells were absent or present in a very low number (Peeters et al., 2005). Thereby, the $\gamma\delta$ T cells seem to represent a small population of cells, which is consistent with the results obtained. The recruitment of $\gamma\delta$ T cells during inflammation has been reported during canine atopic dermatitis (Olivry et al., 1997) and in the inflamed uteruses of bitches affected by pyometra (Bartoskova et al., 2012). Until now, these cells seem to not be related to any brain inflammation in dogs, although a recent review by Malik et al. (2016) showed the presence of $\gamma\delta$ T cells in the brain of humans with multiple sclerosis, but their function during disease activity is not clearly understood.

Moreover, we have observed that all immunophenotypes of the T lymphocytes analysed were present in similar proportions when comparing the CSF and peripheral blood, with no difference between these two compartments. In contrast, in healthy dogs, there is a higher proportion of CD4+ cells in the blood than in the CSF (Duque et al., 2002). This difference might occur because the CD4+ cells can have an important role in the immune response in healthy dogs and they are fundamental for the development of an effective immune response, and a low number of these cells appears to contribute to parasite replication, indicating a poor prognosis of disease progression (Leal et al., 2014).

The high variability of the obtained results in this study may be because of the dogs were naturally infected. In a previous research we observed that brain inflammation may occur in symptomatic or asymptomatic dogs (Melo et al., 2009). It is possible that this variability was reduced if we had evaluated the parameters necessary to separate the dogs into groups, according to the clinical score, as established by Solano-Gallego et al. (2011).

Furthermore, we have previously reported disruption of the blood-brain barrier (BBB) in other dogs with visceral leishmaniasis (Melo et al., 2015a) and findings by our research group have supported the hypothesis that a breakdown of the blood-CSF barrier is also occurring (Melo et al., 2009; Machado et al., 2010; Marangoni et al., 2011). There is also evidence of alterations in the selective filtering of the blood CSF-barrier, caused by peripheral inflammatory processes; Lima et al. (2003) observed a strong correlation of antibody titres in the sera and in the CSF of dogs with VL, proposing the passage of these antibodies and antigens from the blood to the CSF compartment. Since cells of the blood and CSF of this study are present in similar proportions, breakdown of the blood-CSF barrier might be allowing the passage of cells from the blood to the CSF, which CP may act as a source of T cells for the CSF or to the adjacent brain areas (Petito and Adkins, 2005).

Although Nieto et al. (1996) and Viñuelas et al. (2001) found the presence of amastigotes of *L. infantum* in the meninges and choroid plexus, we did not observe the presence of the parasite in the studied brain areas. Nevertheless, all dogs in this study showed mild to intense mononuclear cell infiltration in all three brain areas evaluated, but mainly in the leptomeninges. Meningitis and choroiditis are common findings during canine VL (Nieto et al., 1996; Viñuelas et al., 2001; Melo et al., 2009, 2013; Melo and Machado, 2009; Márquez et al., 2013). In order to explain the origin of the brain inflammatory cells, it was decided to search for corre-

lation between the T lymphocyte populations present in the CSF and the histopathological alterations.

The negative correlation of the intensity of inflammation in the subependymal area with the DP and SP cells, as well as the negative correlation observed in the choroid plexus for the DP T cells of the CSF, show that these cells are present in higher amounts in dogs with a mild intensity of inflammation, and probably stimulate anti-inflammatory mechanisms in the initial phase of brain inflammation. The higher level of SP cells has been associated with the protection and control of infection in the canine VL (Pinelli et al., 1995; Reis et al., 2006; Coura-Vital et al., 2011; Leal et al., 2014). DP cells acting against the infection was reported by Alexandre-Pires et al. (2010) in dogs with VL, who detected a reduction of these cells in the bone marrow of treated dogs and suggested that they might be retained in other organs, inhibiting the replication of parasites. Moreover, data from Bismarck et al. (2012) link the phenotype of canine CD4+ CD8+ T cells to activated effector/memory cells. Canine DP cells are present in lower amount, but are more heterogeneous than porcine ones, consisting of a small proportion of CD8 cells expressing the β chain in addition to the α chain, similarly to human CD4+ CD8+ T cells (Bismarck et al., 2012); however, as canine DP T cells appear to be unique compared to other species, more investigation is necessary to determine their possible role in the canine immune response (Buttlar et al., 2015), particularly when considering the brain milieu.

On the other hand, DN T cells seem to act as villains of the immune system in the CSF of dogs in this study by promoting inflammation, considering the positive correlation observed between the intensity of inflammation in the subependymal area and the proportion of DN T cells of the CSF. These cells produce inflammatory cytokines, such as IL-17 and IFN- γ (Cowley et al., 2010; Crispín et al., 2008) and have been reported to play a key role in various models of infection (D'Acquisto and Cromptom, 2011). Upregulation of the gene expression of IFN- γ in the brain, as well as other inflammatory cytokines have been detected in a previous study performed by our research group (Melo et al., 2013), indicating that the brain has potential to produce these cytokines locally, contributing to the inflammation. Alexandre-Pires et al. (2010) also reported the frequencies of DN T cells in the peripheral blood of dogs with VL, but in a low frequency in asymptomatic animals. They suggested that the presence of *Leishmania* induces a down-regulation of these cells. This hypothesis is in agreement with our results, where we have found a high frequency of these cells in the brain, along with the absence of the parasite.

Furthermore, we have observed no correlation between the population of T cells in the CSF with the intensity of inflammation in the leptomeninges. It is possible that the inflammation observed in leptomeninges has different phenotypic composition. Previous works characterised the presence of T lymphocytes, B lymphocytes, phagocytic cells, plasm cells and polymorphonuclear cells in the inflammatory meningeal infiltrate during canine VL (Viñuelas et al., 2001; Melo et al., 2009).

5. Conclusions

In the data presented here, we describe insight into the immunophenotypes of T lymphocytes in the CSF and, particularly, about the possible role of DN and DP T cells in coordinate the brain inflammation during VL. We verified that infected dogs present similar proportions of T cells in both the blood and CSF, which may be related to the breakdown of the brain and CSF barriers. Moreover, the higher number of DN T cells in dogs with more severe brain inflammation indicates that these cells somehow are contributing to the brain lesions. Further, we suggest that the levels of

DN and DP T cells in the CSF may be indicative of the stage of brain inflammation.

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.

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