UNIVERSIDADE ESTADUAL PAULISTA
“JÚLIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA VETERINÁRIA
CÂMPUS DE ARAÇATUBA

INVESTIGAÇÃO DO PERFIL DE EXPRESSÃO GÊNICA DE RECEPTORES TIPO TOLL E CITOCINAS INFLAMATÓRIAS NO ENCÉFALO E NO BAÇO DE CÃES COM LEISHMANIOSE VISCERAL

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ARAÇATUBA – SP
2017

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Orientadora: Prof.ª Adjunto Gisele Fabrino Machado

Tese apresentada à Faculdade de Medicina Veterinária – Unesp, Câmpus de Araçatuba, como parte das exigências para a obtenção do título de Doutora em Ciência Animal (Fisiopatologia Médica e Cirúrgica).

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TÍTULO: Investigação do perfil de expressão gênica de receptores tipo toll e citocinas inflamatórias no encéfalo e no baço de cães com leishmaniose visceral

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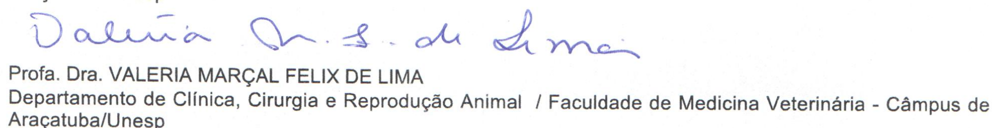

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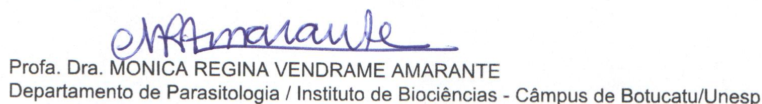

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“Alice: How long is forever?

White Rabbit: Sometimes, just one second.”

" Alice: Quanto tempo dura o eterno?

Coelho Branco: Às vezes apenas um segundo.”

(Lewis Carrol, 1832-1898)

Dedico

À minha família:

Mamãe Rosana e papai Antonio;

Irmãs mais do que queridas Mariana e Carolina.

À Hanny e Mel, minhas cachorrinhas.

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SUMÁRIO

Página

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS **19**
1 LEISHMANIOSE VISCERAL CANINA **19**
2 RECEPTORES TIPO TOLL EM CÃES COM LV **21**
3 RECEPTORES TIPO TOLL E CITOCINAS NO SNC **24**
4 OBJETIVOS **26**
4.1 Objetivos Gerais **26**
4.2 Objetivos Específicos **26**
5 REFERÊNCIAS **27**
CAPÍTULO 2 – TOLL-LIKE RECEPTORS AND CYTOKINES IN THE BRAIN AND SPLEEN OF DOGS WITH VISCERAL LEISHMANIASIS **39**
SUMMARY **39**
1 INTRODUCTION **40**
2 MATERIAL AND METHODS **42**
2.1 Animals **42**
2.2 Sampling **43**
2.3 Clinical Staging **44**
2.4 *Leishmania* DNA Quantification using qPCR **44**
2.5 Evaluation of Gene Expression by RT-qPCR **45**
2.6 Cytokines Quantification by Capture ELISA **47**
2.6.1 Brain and spleen extracts **47**
2.6.2 Cytokines quantification **47**
2.7 Statistical Analysis **48**
2.8 Ethical Issues **48**
3 RESULTS **49**
3.1 Clinical Staging **49**
3.2 Brain Histopathological Analysis **49**
3.3 Parasite Load **51**

3.4 TLR Gene Expression in the Brain and Spleen of Infected dogs **52**
3.5 Cytokines Quantification **54**
4 DISCUSSION **55**
5 CONCLUSION **62**
6 REFERENCES **62**
CAPÍTULO 3 – IMPLICAÇÃO **74**
REFERÊNCIAS **75**

LISTA DE ABREVIATURAS E SÍMBOLOS

°C =	grau Celsius
cm^3 =	centímetro cúbico
nM =	nanomolar
mg =	miligrama
µm =	micrômetro
µl =	microlitro
pg/ml =	picrograma/mililitro
% =	porcentagem
α =	alpha
β =	beta
γ =	gama
ALRs =	AIM-like receptors
BHE =	barreira hematoencefálica
BHL =	barreira hematoliquórica
cDNA =	DNA complementar
CTLs =	C-type lectins
DNA =	ácido desoxirribonucleico
dsRNA =	RNA de dupla fita
ssRNA =	RNA de fita simples
kDNA =	DNA do cinetoplasto
DAMP =	padrões moleculares associados aos danos
ELISA =	ensaio de imunoabsorção enzimática
GIPLs =	glicoinositol-fosfolipídeos
G3PDH =	glyceraldehyde-3-phosphate dehydrogenase
H-E =	hematoxilina-eosina
IFN =	interferon
IL =	interleucina
LPG =	lipofosfoglicanos

LRV =	Virus de RNA de *Leishmania*
LV =	leishmaniose visceral
Min. =	minutos
MMP-9 =	matrix metalloproteinase 9
MyD88 =	molécula adaptadora fator de diferenciação mileoide 88
NFκB =	fator nuclear kappa B
NLR =	nucleotide-binding leucine-rich repeat-containing receptors
PAMP =	padrões moleculares associados aos patógenos
PBMC =	células mononucleares do sangue periférico
PRR =	receptored de reconhecimento padrão
pH =	potencial hidrogeniônico
RASGRP3 =	Ras guanine nucleotide-releasing protein 3
RNA =	ácido ribonucleico
RLRs =	RIG-like receptors
RPL32 =	ribosomal protein L32
RT =	reverse transcription
VL =	visceral leishmaniasis
SNC =	sistema nervoso central
Th1 =	linfócito T auxiliar (*helper*) tipo 1
Th2 =	linfócito T auxiliar (*helper*) tipo 2
TLR =	receptor tipo Toll
TNF =	fator de necrose tumoral

LISTA DE FIGURAS

Página

FIGURE 1 - Representative photomicrography of brain alterations during the canine visceral leishmaniasis. (A) Moderate inflammatory cells infiltrate (arrows) in leptomeninge (Grade 2). (B) Intense inflammatory cells infiltrate (asterisk and arrows) in the choroid plexus from the fourth ventricle (Grade 3). (C) Mild inflammatory cells infiltrate (arrow) in the subependymal area (Grade 1). Inset: mononuclear cells amplified. Hematoxylin and Eosin. Scale bar = 50µm...**50**

FIGURE 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..**51**

FIGURE 3 - Individual parasite load determination in the brain and in the spleen of dogs with visceral leishmaniasis. Black lines connect the brain and the spleen values of the same dog..**52**

FIGURE 4 - Relative gene expression of Toll-like receptors in the brain and in the spleen of dogs with visceral leishmaniasis. 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*..**53**

FIGURE 5 - Individual values of TLR-4 gene expression in the brain of infected

and control dogs, where it is possible to notice a subpopulation of 4 infected dogs with remarkable up-regulation (20.13-fold more). Horizontal lines represent the median and the interquartile range values..**54**

FIGURE 6 - Individual values of cytokines in naturally infected and healthy dogs. TNF-α, IL-1β and IFN-γ levels in brain (A, B, C) and in spleen (D, E, F). Horizontal lines represent the median and the interquartile range values. **55**

LISTA DE TABELAS

Página

Table 1 - Gene expression assay and primers and hydrolysis probe used for RT-qPCR……………..**46**

INVESTIGAÇÃO DO PERFIL DE EXPRESSÃO GÊNICA DE RECEPTORES TIPO TOLL E CITOCINAS INFLAMATÓRIAS NO ENCÉFALO E NO BAÇO DE CÃES COM LEISHMANIOSE VISCERAL

RESUMO - A leishmaniose visceral (LV) é uma doença parasitária que apresenta distribuição mundial e que pode afetar homens e animais, sendo que o cão é considerado o principal hospedeiro da doença. Cães infectados pelo parasito *Leishmania* podem apresentar-se assintomáticos ou com desordens generalizadas, incluindo alterações neurológicas. Existem alguns relatos do acometimento do encéfalo durante a infecção, mas a neuropatogenia da doença não foi completamente elucidada. Há evidências do comprometimento das barreiras encefálicas e da presença do DNA do parasito no encéfalo. Os receptores tipo Toll (TLRs) são sensores do sistema imune inato capazes de detectar padrões moleculares associados aos patógenos (PAMPs), desencadeando uma resposta inflamatórias com produção de diversos mediadores inflamatórios, incluindo citocinas. Desta forma, o objetivo deste estudo foi avaliar o perfil de expressão gênica dos Tolls 1-10, assim como a produção de citocinas pró-inflamatórias TNF-α, IFN-γ, IL-1β e IL-6 no encéfalo e no baço de cães com leishmaniose visceral. No baço houve aumento de expressão gênica de TLR-5 e TLR-9, enquanto no encéfalo houve aumento de TLR-4 em uma pequena população de cães infectados. Em relação às citocinas, todas as citocinas foram detectadas nos dois tecidos avaliados, com excessão de IL-6. Nos cães infectados, TNF-α e IL-1β estavam presentes em maiores concentrações no encéfalo e no baço, respectivamente. Este estudo fornece suporte para explicar o envolvimento de TLRs na LV e nossos dados confirmam o envolvimento encefálico durante a doença.

Palavras-Chave: inflamação, *Leishmania infantum*, sistema nervoso central

INVESTIGATION OF THE GENE EXPRESSION PROFILE OF TOLL-LIKE RECEPTORS AND INFLAMMATORY CYTOKINES IN THE BRAIN AND IN THE SPLEEN OF DOGS WITH VISCERAL LEISHMANIASIS

SUMMARY - Visceral leishmaniasis (VL) is a parasitic disease that presents world distribution, affecting humans and animals. Dogs are considered the main hosts of the disease. Infected dogs with the *Leishmania* parasite can be asymptomatic or present generalized disorders, including neurological alterations. There are some reports of brain commitment during infection. Nevertheless, neuropathogenesis of VL is not completely elucidated. There are evidences of brain barriers breakdown and of the presence of *Leishmania* DNA in the brain. Toll-like receptors (TLRs) are innate immune sensors capable of detecting pathogen-associated molecular patterns (PAMPs), trigger an inflammatory response with production of several inflammatory mediators, including cytokines. Therefore, the aim of this study was to evaluate gene expression profile of TLRs1-10, along with the production of proinflammatory cytokines in both brain and spleen in dogs with VL. In spleen there was an up-regulation of TLR-5 and TLR-9 while in the brain there was up-regulation of TLR-4 in a few number of infected animals. Regarding cytokines, all cytokines were detected in both tissues, except IL-6. In the infected dogs, TNF-α and IL-1β were present at higher concentrations in the brain and spleen, respectively. This study provides support to explain the involvement of TLRs in VL and our data confirm the brain as an affected organ in this disease.

Keywords: central nervous system, inflammation, *Leishmania infantum*

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1 LEISHMANIOSE VISCERAL CANINA

A leishmaniose é uma doença causada por mais de 20 espécies do protozoário do gênero *Leishmania* (família Trypanosomatidae, ordem Kinetoplastida), que são transmitidas por cerca de 30 espécies de mosquitos flebotomíneos e é classificada em duas principais formas: leishmaniose cutânea e leishmaniose visceral (LV). A LV é uma antropozoonose causada pelo parasito *Leishmania donovani* na Ásia e na África e por *Leishmania infantum (= L. chagasi)* na região do Mediterrâneo e nas Américas, incluindo o Brasil (ALVAR et al., 2004; BANETH et al., 2008; LUKEŠ et al., 2007; MAURICIO et al., 2000).

No Brasil, o cão é considerado o principal hospedeiro urbano do parasito *L. infantum.* A apresentação clínica da LV pode variar de cães assintomáticos a manifestações crônicas e sistêmicas, caracterizadas por anemia, perda progressiva de peso, anorexia, letargia, dermatopatias, hepatomegalia, esplenomegalia, lesões oculares, alterações renais e desordens do sistema cardiovascular e respiratório (BLAVIER et al., 2001; CIARAMELLA et al., 1997; GRANO et al., 2016; MARTÍNEZ-HERNÁNDEZ et al., 2017; REIS et al., 2009; SOLANO-GALLEGO et al., 2011).

Embora haja diversos relatos de lesões sistêmicas em cães com LV (KOUTINAS; KOUTINAS, 2014), existe um número limitado de estudos descrevendo as alterações no sistema nervoso central (SNC). De fato, não é comum a presença de sinais clínicos neurológicos em cães com LV. De uma população de 215 cães de Araçatuba-SP, avaliada no período de janeiro a novembro do ano de 1999, apenas 4% apresentaram sinais clínicos neurológicos, caracterizados por paraparesia e paraplegia (FEITOSA et al., 2000). Em um estudo mais recente, casos de cães provenientes de uma área da Itália foram avaliados retrospectivamente no período de 2010 a 2015. De 54 cães

com leishmaniose, 10 cães apresentaram sinais clínicos neurológicos, como andar em círculos, *head tilt*, ausência de alguns reflexos neurólogicos no exame físico, paresia, deficits de propriocepção, dor cervical, tetraparesia, estrabismo (GIANNUZZI et al., 2017)

Além dos sinais clínicos neurológicos em cães com LV, foram descritos a deposição de antígenos e imunoglobulinas (GARCÍA-ALONSO et al., 1996) e existem relatos da detecção do parasito no SNC (OLIVEIRA et al., 2017) em meninges (VIÑUELAS et al., 2001), plexo coroide (MÁRQUEZ et a., 2013; NIETO et al., 1996), medula espinhal e parênquima cerebral (MARQUÉZ et al., 2013). Além disso, a detecção do DNA de *Leishmania* também foi relatada no encéfalo (CARDINOT et al., 2016; GRANO et al., 2014; MELO et al., 2015a; OLIVEIRA et al., 2017) e, particularmente, no líquido cefalorraquidiano (GIANNUZZI et al., 2017; OLIVEIRA et al., 2017) e na medula espinhal (OLIVEIRA et al., 2017) de cães infectados. Por outro lado, outros autores não detectaram a presença do parasito íntegro no encéfalo (GRANO et al., 2016; IKEDA et al., 2007; MELO; MACHADO, 2009; 2011; MELO et al., 2015a).

O encéfalo é considerado um órgão imunoprivilegiado, o que dificulta a entrada do parasito, de células inflamatórias e de proteínas neste tecido. Em condições normais, ele é protegido da corrente sanguínea por meio de algumas barreiras. A barreira hemato-encefálica (BHE), localizada no endotélio dos vasos sanguíneos cerebrais é composta por células endoteliais que apresentam junções intercelulares do tipo oclusivas (“tight junctions”), com ausência de fenestrações intercelulares (PERSIDSKY et al., 2006; SAUNDERS et al., 2008; WOLBURG; LIPPOLDT, 2002). Os pericitos, a membrana basal, os pedículos dos astrócitos e alguns terminais axônicos são outros componentes que formam a BHE (SAUNDERS et al., 2012). Outra barreira presente no SNC é a barreira hemato-liquórica (BHL), que está presente no epitélio do plexo coroide (DZIEGIELEWSKA et al., 2001).

O comprometimento da BHE já foi relatada em cães com LV (MELO et al., 2015b), o que possibilitaria a entrada de algumas substâncias e células no SNC. A presença de enzimas do grupo das metaloproteinases da matriz, a deposição

de antígenos e imunoglobulinas, assim como a presença de infiltrado de células inflamatórias no plexo coroide, área periventricular e leptomeninges e a ativação de células da glia já foram relatados em cães infectados. Além disso, meningite, coroidite, ativação das células gliais e alterações degenerativas de neurônios também já foram descritas em cães com VL (GARCÍA-ALONSO et al., 1996; GRANO et al., 2016; IKEDA et al., 2007; MACHADO et al., 2010; MELO; MACHADO, 2011; MELO et al., 2009, 2013, 2015a).

Em estudo anterior nós propusemos duas hipóteses para explicar a neuroinflamação em cães com LV. A primeira seria diretamente relacionada à entrada do parasito no encéfalo, o que estimularia o desenvolvimento da inflamação local. Posteriormente, o parasito poderia ser destruído, por mecanismos ainda desconhecidos, o que dificultaria sua detecção nesse tecido. A segunda hipótese estaria relacionada aos estímulos periféricos, como mediadores inflamatórios, que poderiam chegar ao SNC, desencadeando a inflamação (MELO et al., 2015a)

2 RECEPTORES TIPO TOLL EM CÃES COM LV

O sistema imune inato apresenta um mecanismo de detecção específico para microorganismos por meio de receptores de reconhecimento padrão (PRRs), que reconhecem padrões moleculares associados a patógenos (PAMPs) (MEDZHITOV, 2007). Esses receptores podem ser expressos na superfície celular, em compartimentos intracelulares, ou secretados no sangue e fluidos teciduais (MEDZHITOV et al., 1997). As principais funções dos PRRs incluem opsonização, ativação do complemento e coagulação, fagocitose, ativação de mecanismo de sinalização pró-inflamação e indução da apoptose (JANEWAY; MEDZITHOV, 2002).

Os receptores tipo Toll (TLRs) são PRRs que atuam no reconhecimento de estruturas microbianas e induzem resposta adaptativa e inflamatória (TUON

et al., 2008). Cada um dos TLRs detectam moléculas distintas de vírus, bactérias, fungos e parasitos (KAWAI; AKIRA, 2011). Até hoje, foram identificados 13 TLRs distintos nos mamíferos em geral, que apresentam especificidade para diferentes patógenos e que estimulam a produção de diferentes citocinas (JANSSENS; BEYAERT, 2003). Como revisado por Faria et al. (2012), os TLRs podem ser divididos em extracelulares (TLR 1, 2, 4, 5, 6, 10 e 11) ou intracelulares (TLRs 3, 7, 8, 9 e 13), reconhecendo grupos específicos de ligantes na superfície celular ou no compartimento endossomal, respectivamente.

O parasito *L. infantum* é um patógeno intracelular capaz de estimular o sistema imune inato por meio de diferentes PAMPs, dependendo de seu estágio no ciclo de vida. Glicoinositol-fosfolipídeos (GIPLs), lipofosfoglicanos (LPG), glicoproteína gp63 e o DNA do protozoário são potenciais moléculas de *Leishmania* que se ligam em alguns TLRs de hospedeiros vertebrados (TUON et al., 2008). Além disso, TLRs também podem ser estimulados por proteínas derivadas do hospedeiro, como proteínas do choque térmico ("heat shock proteins") e fragmentos de fibronectina (AKIRA et al., 2001).

Após o reconhecimento dos PAMPs, TLRs sinalizam ativando cascata que leva à fosforilação de IκB, liberando NF-κB (DIDONATO et al., 1996; GHOSH; KARIN, 2002). A ativação de NFκB é mediada por moléculas adaptadoras, sendo a mais comum denominada de MyD88. Um outro mecanismo seria o recrutamento de TRIF, que transmite sinalização levando à ativação de IRF3 ou pode também induzir à ativação de NFκB. TLR-3 é o único TLR que atua exclusivamente pelo mecanismo de ativação de TRIF, enquanto os TLRs 1, 2, 5, 6, 7, 8, 9 e 10 são restritos à via de ativação de MyD88 e o TLR-4 atua por ambos mecanismos (OSPELT; GAY, 2010; SHASTRI et al., 2013).

A ativação do fator de transcrição NF-κB induz a produção de citocinas inflamatórias, como o TNF-α, interferon tipo 1, interleucina 12 (IL-12), quimiocinas, moléculas de adesão e moléculas co-estimulatórias (GALLEGO et al., 2011; MEDZHITOV, 2001). A produção de citocinas e quimiocinas já foi descrita na LV canina em diversos órgãos, incluindo o SNC (BARBOSA et al.,

2011; MELO el al., 2013; 2015a; PANARO et al., 2009; SANTOS-GOMES et al., 2002).

Até o presente, há poucos estudos na literatura direcionados à investigação dos TLRs na LV canina, sendo que a maioria dos estudos focam os TLRs 2, 4 e 9, especialmente em células mononucleares do sangue periférico (MELO et al., 2014a), jejuno e cólon (FIGUEIREDO et al., 2013), pele, fígado (HOSEIN et al., 2015), baço, linfonodos (HOSEIN et al., 2015; MELO et al., 2014b) e encéfalo (MELO et al., 2014b). Outros estudos também foram realizados com *L. donovani* e *L. major* (FLANDIN et al., 2006; KROPF et al., 2004; KUMAR et al., 2017), sendo que Flandin et al. (2006) verificaram que o TLR-3 também apresenta uma papel no reconhecimento do parasito.

Os TLRs são descritos por existirem e atuarem em dímeros no reconhecimento de PAMPs, conforme revisão feita por Ospelt e Gay (2010). Por exemplo, a sinalização do TLR-2 ocorre por meio da formação de heterodímeros com o TLR-1, TLR-6 ou TLR-10. O TLR-5 pode atuar como homodímero ou pode formar heterodímero com o TLR-4. Não está esclarecido se os TLR-3 e TLR7–9 também requerem multimerização para ativar a cadeia de transdução. Para TLR-9, existem evidências de efeito alostérico (LATZ et al., 2007). Deste modo, o estudo do perfil dos receptores tipo Toll é necessário, já que a resposta imune contra o parasito *Leishmania* pode envolver a cooperação entre múltiplos receptores.

O TLR-2 é um dos receptores com maior número de ligantes. Na formação do heterodímero de TLR-1/2, os dois TLRs atuam em conjunto no reconhecimento de lipopeptídeos triacetilados, presentes principalmente em bactérias como *Mycobacterium tuberculosis* or *Borrelia burgdorferi* (ALEXOPOULOU et al., 2002; TAKEUCHI et al., 2002;). O complexo TLR6/2 reconhece lipopeptídeos diacetilados (OZINSKY et al., 2000). Além disso, outras substâncias como ácido lipoteicoico e peptidoglicanos, assim como o zimosan, presentes em bactérias e micobactérias, respectivamente, foram relatados por também induzir a ativação de TLR-2 (OZINSKY et al., 2000; SCHWANDNER et al., 1999; UNDERHILL et al., 1999). A ativação de TLR-2 já foi relatada em

infecções parasitárias como *Trypanosoma cruzi* or *Schistosoma mansoni* por meio do reconhecimento de proteínas dos parasitos (CAMPOS et al., 2001; OUAISSI et al., 2002; VAN DER KLEIJ et al., 2002).

3 RECEPTORES TIPO TOLL E CITOCINAS NO SNC

No SNC de humanos e camundongos, a microglia expressa mRNA para os TLR 1-9 (OLSON; MILLER, 2004), enquanto oligodendrócitos expressam mRNA para TLRs 2 e 3 (BSIBSI et al., 2002). Os neurônios têm sido relatados por expressarem TLRs intracelulares, como o TLR-3, TLR-7, TLR-8 e TLR-9 (WADACHI; HARGREAVES, 2006; TANG et al., 2007). Já os astrócitos expressam um perfil de TLR limitado, considerando que não são células clássicas do sistema imune, baseado em sua origem ectodérmica, mas se necessário, contribuem para a inflamação (RAJAGOPAL et al., 2007).

Em projeto recente nós avaliamos a expressão gênica dos TLRs 2, 4 e 9 no SNC (encéfalo e plexo coroide) e também no sistema periférico (linfonodos e baço) de cães com LV. Neste trabalho observamos que a expressão gênica desses receptores variou de acordo com o tipo de tecido avaliado, mostrando que a resposta immune na LV é compartimentalizada. Particularmente no encéfalo, foi detectada tendência de aumento de TLR-2 e, no plexo coroide, houve aumento de TLR-2 e TLR-9 em cães infectados, indicando um papel desses TLRs na neuropatogenia da doença (MELO et al., 2014b).

As vias de sinalização por meio de TLRs resultam na produção de citocinas inflamatórias, quimiocinas, moléculas de adesão e moléculas co-estimulatórias (OSPELT; GAY, 2010). As células da micróglia são o centro da regulação inata da resposta inflamatória do SNC e os TLRs controlam a geração da maioria dos mediadores inflamatórios produzidos pela ativação da micróglia (STREIT et al., 2005). Após ativação induzida pelos PAMps, a microglia tem capacidade de estimular a produção de citocinas pró-inflamatórias, como IL-6 e

TNF-α (LEHNARDT, 2010; SUH et al., 2009). Mesmo se a BHE não for comprometida, a microglia pode ser ativada em infecções sistêmicas, o que sugere que PAMPs são capazes de cruzar a BHE ou de ativar a microglia em órgãos circunventriculares. Além disso, macrófagos e citocinas do sistema imune periférico também são capazes de cruzar a BHE, interceptando patógenos invasores e também microglias ativadas (BAKER et al., 2010).

A estimulação de migróglia de camundongos com TLRs agonistas levaram à secreção de citocinas incluindo IFN-α, IFN-β, IL-1β, IL-6, IL-10, IL-12, IL-18, TNF-α e óxido nítrico (OLSON; MILLER, 2004). Jack et al. (2005) em estudo com ativação da micróglia verificaram que TLR-3 induziu intensa resposta pró-inflamatória, com a secreção de citocinas como IL-12, TNF-α, IL-6, IL-10 e IFN-β, enquanto em resposta mediada por TLR-2 houve secreção de IL-6 e IL-1β, sugerindo que a resposta imune inata pode variar no SNC de acordo com os receptores envolvidos.

Na leishmaniose, as células do sistema fagocitário mononuclear infectadas pelo parasito agem como células apresentadoras de antígeno, ativando linfócitos T CD4+, o que resulta em resposta imune do tipo Th1 (“T helper 1”) ou Th2 (“T helper 2”). Cães infectados pelo parasito e clinicamente saudáveis apresentam predomínio de resposta imune do tipo Th1. Esta resposta é mediada pelas citocinas IL-2, IFN-γ e TNF-α, ao contrário do observado em cães infectados sintomáticos, em que há resposta do tipo Th2, principalmente pela produção de IL-4, IL-10 e TGF-β (BARBIÉRI, 2006; REIS et al., 2009; SOLANO-GALLEGO et al., 2011; STRAUSS-AYALI et al., 2005).

Em estudo anterior foram avaliadas algumas citocinas com atividade pró e anti-inflamatórias no encéfalo de cães com LV por meio de RT-qPCR. Os resultados mostraram maior expressão gênica de citocinas pró-inflamatórias, como IL-1β, IL-6, IFN-γ e TNF-α (MELO et al., 2013), o que sugere que a produção dessas citocinas no SNC poderia contribuir para explicar as lesões inflamatórias previamente relatadas na literatura.

Frente à necessidade de investigar os mecanismos envolvidos na ocorrência do processo inflamatório encefálico observado previamente em cães

com leishmaniose visceral, bem como de esclarecer a patogenia da doença no SNC, propomos avaliar os sensores do sistema imune inato relacionados ao reconhecimento de antígenos do parasito, por meio da investigação do perfil de TLRs, bem como avaliar a produção de citocinas pró-inflamatórias no encéfalo de cães infectados naturalmente por *L. infantum.*

4 OBJETIVOS

4.1 Objetivos Gerais

Investigar o perfil gênico de receptores tipo Toll e produção de citocinas no SNC (representado pelo encéfalo) e na periferia (representada aqui pelo baço) em cães com leishmaniose visceral.

4.2 Objetivos Específicos

Os objetivos deste estudo foram:

- Avaliar a expressão gênica dos TLRs 1, 2, 3, 4, 5, 6, 7, 8, 9 e 10 no encéfalo e no baço de cães com LV.
- Detectar e quantificar as citocinas: IL-1β, IL-6, TNF-α e IFN-γ no encéfalo e no baço de cães com LV.
- Avaliar a carga parasitária de *Leishmania spp.* no encéfalo e no baço de cães com LV.
- Correlacionar o perfil de receptores tipo Toll com a carga parasitária de *Leishmania* no encéfalo e no baço.
- Correlacionar o perfil de receptores tipo Toll com o estagiamento clínico dos animais.

- Correlacionar os receptores tipo Toll e a produção de citocinas no encéfalo e no baço.
- Caracterizar as lesões inflamatórias encefálicas em cães com LV.
- Correlacionar a intensidade das lesões inflamatórias encefálicas com a expressão dos receptores tipo Toll, com a produção de citocinas e com o estagiamento clínico dos animais.

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CAPÍTULO 2 – TOLL-LIKE RECEPTORS AND CYTOKINES IN THE BRAIN AND IN THE SPLEEN OF DOGS WITH VISCERAL LEISHMANIASIS

CAPÍTULO 2 - TOLL-LIKE RECEPTORS AND CYTOKINES IN THE BRAIN AND IN THE SPLEEN OF DOGS WITH VISCERAL LEISHMANIASIS

SUMMARY - Visceral leishmaniasis (VL) is a multisystem disease that affects domestic dogs, and can have several clinical manifestations, including some rare reports of neurological clinical signs or may remain asymptomatic, depending on the individual immune response against the *Leishmania* parasite. It involves immune system sensors, 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 have investigated the gene expression profile of TLRs1-10 in the brain and in the spleen from infected dogs, along with the production of proinflammatory cytokines (TNF-α, IFN-γ, IL-1β and IL-6) with the aim to explain the origin of brain inflammation. Gene expression of TLRs has varied according to the tissue evaluated. In the brain, there was only up-regulation of TLR-4 in a small subpopulation of infected dogs while in the spleen we have detected an increase in TLR-5 and TLR-9 transcripts, as well as a reduction in TLRs 2-4 and TLR-10. Regarding cytokines, all cytokines were detected in infected dogs, except IL-6. Moreover, we have 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 plexus, ranging from mild to intense. This study provides the first insight about TLRs profile in the brain and in the spleen during canine VL and provides support to explain the involvement of these sensors of the innate immune system against *L. infantum* parasite.

Keywords: central nervous system, DNA, inflammation, *Leishmania infantum*, real-time polymerase chain reaction

1 INTRODUCTION

Visceral leishmaniasis (VL) is an anthropozoonosis caused by the *Leishmania infantum* (=*L. chagasi*) protozoan (MAURICIO et al., 2000). Domestic dogs can present with several systemic manifestations (BLAVIER et al., 2001; CIARAMELLA et al., 1997; GARCÍA-ALONSO et al., 1996; REIS et al., 2009), including some rare reports of neurological clinical signs (FONT et al., 2004; GIANUZZI et al., 2017; IKEDA et al., 2007; JOSÉ-LÓPEZ et al., 2012; MELO et al., 2012) or may remain asymptomatic.

Neurological clinical signs include walking in circles, seizures, tetraplegy, vestibular and cerebellar syndrome, head tilt, motor incoordination and myoclonia (FONT et al., 2004; GIANUZZI et al., 2017; IKEDA et al., 2007; JOSÉ-LÓPEZ et al., 2012; MÁRQUÉZ et al., 2013). In addition to neurological clinical signs, brain inflammation was also reported in dogs with VL, where meningitis and choroiditis are the usual histopathological findings found in infected dogs (GIANUZZI et al., 2017; GRANO et al., 2016; NIETO et al., 1996; VIÑUELAS et al., 2001; MELO et al., 2009, 2013, 2015a). Considering these findings, the focus of our research group in last years has been to investigate the intriguing question about why infected dogs present neurological clinical signs and brain inflammation. We have previously suggested two hypotheses. One would be that parasite migration from peripheral organs to the brain stimulates inflammation. However, the presence of the *Leishmania* parasite in the brain remains yet to be elucidated, although we have detected its DNA in this compartment (GRANO et al., 2014; MELO et al., 2015a). The second hypothesis would be 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; GAY, 2010) and also recognize damage-

associated molecular patterns (DAMPs) in the chronic inflammation, which are host-derived proteins (MIYAKE, 2007; PICCINI; MIDWOOD, 2010), triggering a cascade of immune reactions (FUKATA et al., 2009). The main functions of PRRs are related to opsonization, phagocytosis, activation of complement, coagulation cascades and proinflammation signaling pathways, as well as induction of apoptosis (JANEWAY; MEDZITHOV, 2002).

There are several sub-families of PRRs, such as: nucleotide-binding leucine-rich repeat-containing receptors (NLRs), Toll-like receptors (TLRs), RIG-like receptors (RLRs), C-type lectins (CTLs), and AIM-like receptors (ALRs) (MOTTA et al., 2015). PRRs may be present in cell surface (some TLRs), acting as extracellular sentinels or intracellularly (NLRs, RLRs, some TLRs), which are activated after some internal stimuli (KIGERL et al., 2014).

TLRs are PRRs that act on the recognition of microbial structures and induce innate and adaptive response (TUON et al., 2008). Different TLRs detect distinct molecules of viruses, bacteria, fungi and parasites (KAWAI; AKIRA, 2011). Thirteen distinct TLRs have been identified in mammals in general, which present specificity for different pathogens and stimulate the production of several cytokines (JANSSENS; BEYERT, 2003). Ten TLRs have been identified in humans (TLRs 1-10) and 12 in mice (TLRs 1-9 and 11-13) (KAISHO; AKIRA, 2006). TLRs can be divised into extracelular (TLRs 1, 2, 4, 5, 6, 10 and 11) or intracellular (TLRs 3, 7, 8, 9 and 13), recognizing specific groups of ligands in the cell surface or in the endosomal compartment (eg. DNA), respectively (FARIA et al., 2012). The activation of signaling through TLRs causes the production of chemokines, inflammatory cytokines, adhesion molecules and co-stimulatory molecules (OSPELT; GAY, 2010). Gene expression of cytokines and chemokines has already been characterized during canine VL, in peripheral organs (BARBOSA et al., 2011; MELO et al., 2015a; PANARO et al., 2009), as well as in the CNS (MELO et al., 2013, 2015a).

Concerning canine VL, there are only a few number of authors studying TLRs. Most part of the studies have focused on TLRs 2, 4 and 9, especially in peripheral blood mononuclear cells (PBMC) (MELO et al., 2014a), jejunum and

colon (FIGUEIREDO et al., 2013), skin, liver (HOSEIN et al., 2015), spleen, lymph nodes (HOSEIN et al., 2015; MELO et al., 2014b) and brain (MELO et al., 2014b).

Studies evaluating the immune response in the CNS is not frequent in dogs with visceral leishmaniasis. Despite the growing number of studies about PRRs in last years, Toll-like receptors 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 debris in the brain during canine VL. Therefore, the aim of this study was to determine gene expression of TLR1-10 in two compartments: CNS, which is represented here by brain, target organ of our previous studies, and spleen, considered an organ bearing the bulk of parasites burden, representing the peripheral system.

2 MATERIAL AND METHODS

2.1 Animals

Twenty-one dogs, 13 males and 8 females, ranging in age from 1 to 4 years old, that were selected from the Zoonosis Control Center in the municipality of Araçatuba, São Paulo State, Brazil, were included in this study. Seventeen naturally infected dogs by the *Leishmania* parasite were euthanized with the owner's permission, in compliance with state law (SÃO PAULO, 2006), as soon as the VL diagnosis was confirmed. These dogs were included in the infected group, where all the infected dogs were symptomatic, but they did not present a history of neurological signs. Four uninfected healthy dogs, without underlying conditions or 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 by parasitological analysis (popliteal lymph node fine-needle aspiration). All dogs in the study were negative

for other infeccious agents that can affect the CNS, such as *Neospora caninum* and *Toxoplasma gondii* (indirect immunofluorescence = RIFI), and *Erlichia 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 have performed necroscopic examinations to evaluate macroscopic alterations and to collect brain and spleen samples. Brains were sagitally sliced and fragments of $0.5cm^3$ were pooled from thalamus, hippocampus, piriform/temporal cortex and periventricular white matter, and fragments of $0.5\ cm^3$ 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 (5μm) 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 to 3), in accordance with Grano et al. (2016), which: Grade 0 (no inflammation observed); Grade 1, mild inflammation (slight inflammatory cell infiltrate mainly in the choroid plexi and leptomeninges); Grade 2, moderate inflammation (moderate inflammatory cell infiltrate mainly in the choroid plexi, 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 plexi and leptomeninges along with intense perivascular infiltration in the brain).

2.3 Clinical Staging

We have 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 was performed using 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. Following that, 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® $RealPlex^2$) using Taqman® Universal Master Mix (4326708, Applied Biosystems). For TLR-6 and TLR-8 we have used a concentration of 400 nM of each primer and 250 nM of the probe and for G3PDH (glyceraldehyde-3-phosphate dehydrogenase) we have used 400 nM of each primer and 200 nM of the probe. Each reaction was performed with 5 µL of cDNA in a final concentration of 100ng. 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 have 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).

Table 1 - Gene expression assay and primers and hydrolysis probe used for RT-qPCR

Target	**Product size (pb)**	**GenBank accession number**	**Reference**
TLR-1	100	NM_001146143.1	Life Technologies Foster City, CA, USA (4351372); ASSAY ID: Cf03811563_s1
TLR-2	69	NM_001005264.3	Life Technologies Foster City, CA, USA (4351372); ASSAY ID: Cf02625049_s1
TLR-3	63	-	Life Technologies Foster City, CA, USA (4331182); ASSAY ID: Cf04419557_m1
TLR-4	120	NM_001002950.1	Life Technologies Foster City, CA, USA (4331182); ASSAY ID: Cf02622203_g1
TLR-5	100	NM_001197176.1	Life Technologies Foster City, CA, USA (4351372); ASSAY ID: Cf04245132_s1
TLR-6	109	EU551147.1	Mercier et al., 2012
TLR-7	124	NM_001048124.2	Life Technologies Foster City, CA, USA (4351372); ASSAY ID: Cf02710573_s1
TLR8	138	XM_005641119.1	Mercier et al., 2012
TLR-9	136	NM_001002998.1	Life Technologies Foster City, CA, USA (4351372); ASSAY ID: Cf02717353_g1
TLR-10	100	NM_001173127.1	Life Technologies Foster City, CA, USA (4351372); ASSAY ID: Cf04181843_s1
RPL32		customized assay	Life Technologies Foster City, CA, USA (4351372); ASSAY ID: Cf03986518_m1
G3PDH	90	AB022763	Peters et al., 2005

2.6. Cytokines 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 from brain or spleen fragments was added to 1 ml suspensions of complete RPMI-1640 (Sigma, USA), pH 7.2, were kept in ice and homogeneized in a tissue ruptor (Qiagen, Germany) for approximately 5 minutes. Following that, the resulting homogenate was centrifugated at 10,000 × g for 15 min at 4ºC and the supernatant was immediately stored at -80ºC.

2.6.2 Cytokines quantification

TNF-α, IFN-γ, IL-1β and IL-6 concentrations were determined in the supernatants from the brain and spleen of the dogs of this study by means of capture ELISA using the commercial reagent Duo SET® Canine TNF-α, Duo SET® Canine IFN-γ and Duo SET® Canine IL-1β (R & D System, Minneapolis, USA). Procedures were performed in accordance with the manufacturer's instructions. For IL-6 we have used the anti-canine monoclonal antibody (mAb) produced in mice (Cat. Number: MAB16091, R & D System, USA) and biotininated anticanine polyclonal antibody produced in goat (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, respectively. 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, 78.12 pg/mL for IL-6 in the spleen.

2.7 Statistical Analysis

For statistical analysis, all values were log-transformed. For cytokines analysis we have used a four parameter logistic (4-PL) curve-fit. Significant differences between groups were determined by Mann-Whitney test or Wilcoxon test for paired samples. Correlations were evaluated with the Spearman correlation coefficient. Friedman test, followed by Dunn test, was used to evaluate the intensities of brain inflammation. All analysis were performed using Prism software (Prism 6, GraphPad). Statistical significance was accepted 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 have 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, splenomegaly, 35.29% (6/17) with conjunctivitis and onycogryphosis, and 29.41% (5/17) with lymphadenopathy.

Concerning the laboratorial findings, we have 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 negative in the serology; 17.64% (3/17) presented low concentration of antibodies (from cut-off value 0.27 up to 0.4), 35.3% (6/17) presented medium concentration (0.4 up to 0.81), and 35.3 % (6/17) presented high concentration (over 0.81).

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

3.2 Brain Histopathological Analysis

In the brain histopathological analysis, we have detected mononuclear cells infiltrate, especially composed of lymphocytes and plasm cells in the three

brain areas. Leptomeninges and choroid plexus presented the highest concentration of inflammatory cells when compared with the subependymal area (*P<0.0001*), ranging from mild to intense inflammation. Figure 1 shows the representative photomicrographs of inflammatory infiltrate in brain areas, according to the intensity of inflammation.

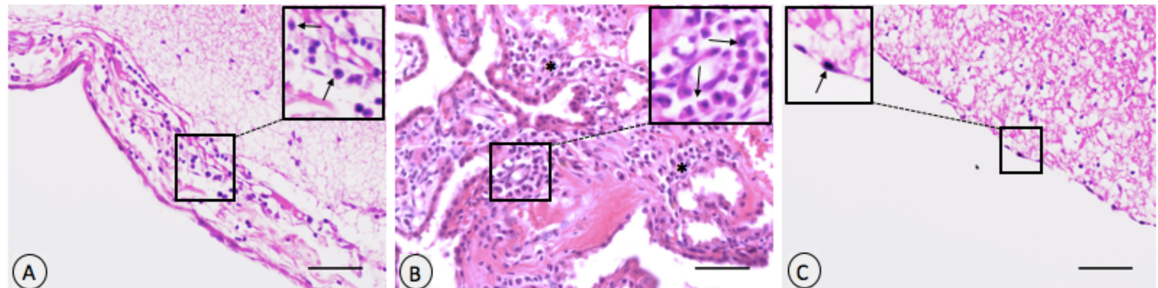

FIGURE 1 - Representative photomicrography of brain alterations during the canine visceral leishmaniasis. (A) Moderate inflammatory cells infiltrate (arrows) in leptomeninge (Grade 2). (B) Intense inflammatory cells infiltrate (asterisk and arrows) in the choroid plexus from the fourth ventricle (Grade 3). (C) Mild inflammatory cells infiltrate (arrow) in the subependymal area (Grade 1). Inset: mononuclear cells amplified. Hematoxylin and Eosin. Scale bar = 50μm.

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 plexus, 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 (Figure 2). In the study presented here, we did not detect amastigotes of the *Leishmania* parasite in the H-E stained brain samples. Furthermore, in order to evaluate if the intensity of inflammation could range among dogs belonging to different clinical stages, we have done correlation test. However, there was no correlation between these two parameters.

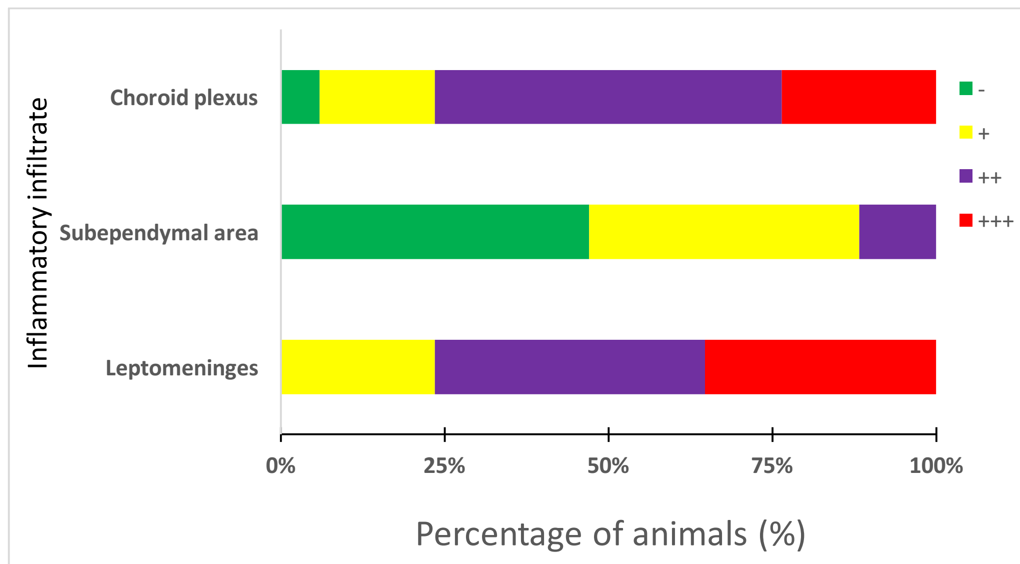

FIGURE 2 - Percentage (%) of dogs according to the intensity of inflammation in the choroid plexus, subependymal area and leptomeninges. -: absence of inflammatory infiltrate; +: mild inflammatory infiltrate; ++: moderate inflammatory infiltrate; +++: intense inflammatory infiltrate.

3.3 Parasite Load

The presence of the *Leishmania* kDNA was detected in all spleen and all infected dogs. For the brain, *Leishmania* kDNA was detected in all samples, except 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 r2= 0.998 and a slope of −3.511.

The detection range was from 1.52 to 226.000 parasites/10mg of spleen and 0 (n=4) to 1.380 parasites/10mg of brain (Figure 3). No amplification was observed in the samples used as the negative control. The number of parasites in the spleen was significantly higher than the brain (*P<0.0001*). However, there was no correlation in the parasite load between both tissues. Absence of correlation was also verified between parasite load and the intensity of brain inflammation.

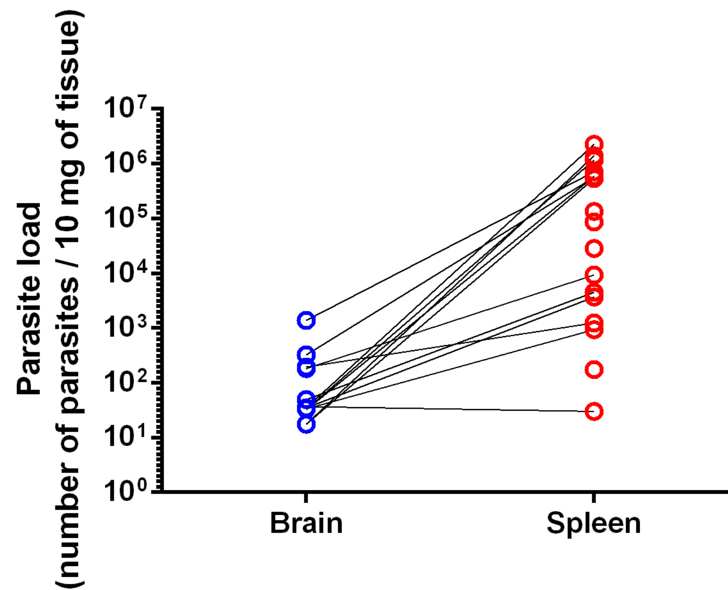

FIGURE 3 - Individual parasite load determination in the brain and in the spleen of dogs with visceral leishmaniasis. Black lines connect the brain and the spleen values of the same dog.

3.4 TLR Gene Expression in the Brain and Spleen of Infected Dogs

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

In the spleen we have 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 parasite load and TLRs was observed. For the brain, we did not observe any changes in the TLRs gene expression (Figure 4).

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

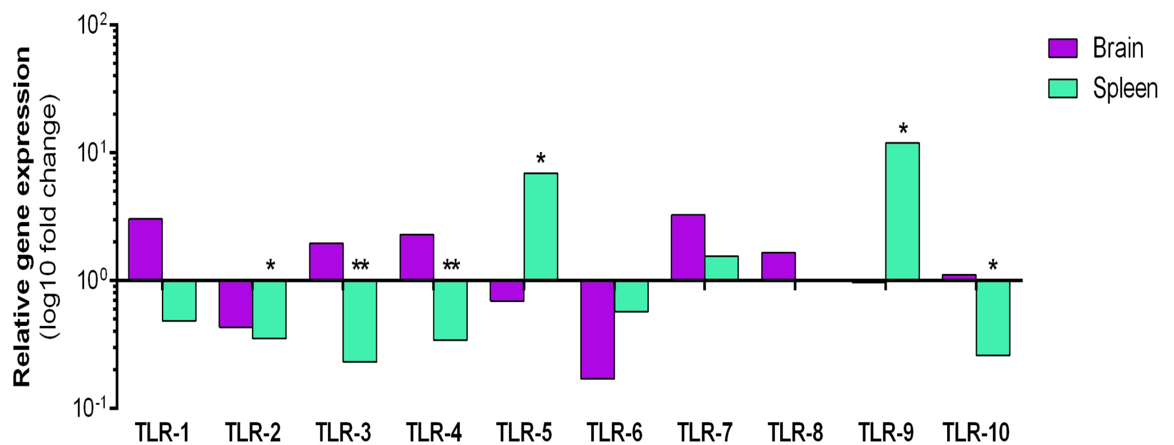

FIGURE 4 - Relative gene expression of Toll-like receptors in the brain and in the spleen of dogs with visceral leishmaniasis. The values are expressed as fold changes (log 10). Positive values indicate up-regulation and negative values indicate down-regulation, when compared to the control dogs. The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

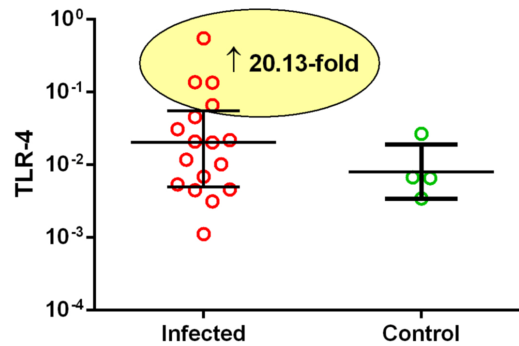

FIGURE 5 - Individual values of TLR-4 gene expression in the brain of infected and control dogs, where it is possible to notice a subpopulation of 4 infected dogs with remarkable up-regulation (20.13-fold more). Horizontal lines represent the median and the interquartile range values.

3.5 Cytokines Quantification

All cytokines were detected in infected and healthy dogs in both tissues evaluated (Figure 6), except IL-6 production, that 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 statistical difference between infected and control groups.

Moreover, there was no correlation between the clinical stage or TLRs expression and the cytokines production for both tissues. 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 of the parasite load and IL-1β ($r = -0.72$; *$P<0.01$*) or TNF-α ($r = -0.53$; *$P<0.05$*) levels.

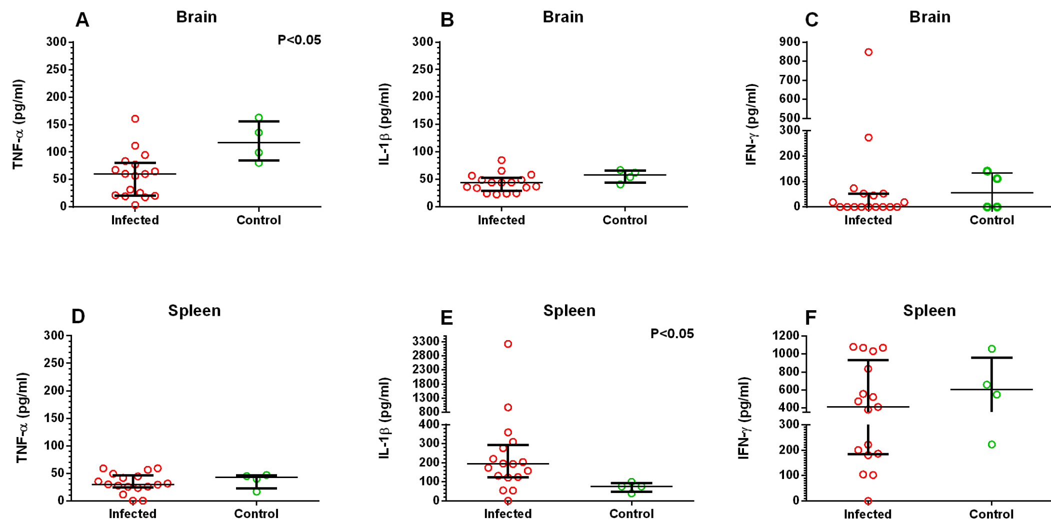

FIGURE 6 - Individual values of cytokines in naturally infected and healthy dogs. TNF-α, IL-1β and IFN-γ levels in brain (A, B, C) and in spleen (D, E, F). Horizontal lines represent the median and the interquartile range values.

4 DISCUSSION

We observed brain inflammation, as well as TLRs gene expression and *Leishmania* DNA in the brain and in the spleen 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 TLRs expression. Absence of correlation was also observed between the clinical classification and the intensity of brain inflammation. This data indicates that the brain inflammation is not related to the clinical staging of infected dogs. Thus, dogs in an early or chronic stage of the disease can present the same kind of brain inflammation. In other study which the authors evaluated infected dogs presenting neurological clinical signs, they also did not detect correlation between the severity of the animal clinical stage and the intensities of brain inflammation (IKEDA et al.,

2007).

Brain inflammation ranged from mild to intense, corroborating with previous studies (GIANUZZI et al., 2017; GRANO et al., 2016; MÁRQUEZ et al., 2013; MELO et al., 2013; OLIVEIRA et al., 2017; VIÑUELAS et al., 2001). Presence of lymphocytes and plasm cells observed in the brain from the infected dogs of our study is probably related to the 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 an affected organ during canine VL, even in the absence of detection of the whole parasite or of neurological clinical signs. Indeed, there is a limited number of cases reporting the presence of *Leishmania* in the CNS. The amastigote form of the parasite was identified in meninges (VIÑUELAS et al., 2001) and choroid plexus (NIETO et al., 1996), but *Leishmania* was also detected in the CNS by immunohistochemistry (OLIVEIRA et al., 2017), including spinal cord, brain parenchyma and choroid plexus of one chronically infected dog (MARQUÉZ et al., 2013).

Here, we did not detect the whole parasite in the brain but we have detected its DNA. The lack of correlation between parasite load and intensity of brain inflammation suggests that the development of an inflammatory nervous milieu could occur through other mechanisms, and not by direct stimulation by the presence of the whole parasite or its 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, which T lymphocytes from the blood would be somehow stimulating the development of the brain inflammation somehow (GRANO et al., 2016). 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 the inflammation can be originated locally in the

brain. 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 about the expression profile of TLR transcripts in the brain, along with the spleen during canine VL. We have observed an up-regulation of TLR-9 in the spleen of infected dogs. Activation of TLR-9 occurs by the recognition of microbial DNA and also by immune complexes containing DNA (OSPELT; GAY, 2010). Accumulation of immune complexes containing DNA can be found usually in autoimmune diseases (TIAN et al., 2007) and the presence of anti-double-stranded DNA antibodies have already been reported previously in human VL (LIBEROPOULOS et al., 2013; SAKKAS et al., 2008). We have detected *Leishmania* DNA in the spleen, which could explain the effect in TLR-9 in this tissue, showing evidence that this immune sensor has a role in detecting the parasite in this tissue. On the other hand, although we have detected *Leishmania* DNA in the brain, TLR-9 gene expression was observed unchanged in this tissue, which is agreement with our previous results in infected dogs (MELO et al., 2014a). This suggests that the immune response is compartmentalized and other receptors can be related to immunovigilance in the CNS.

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

Regarding TLR-4, only a subpopulation of infected dogs presented up-regulation in the brain when compared to the control group, showing a role of this immune receptor during canine VL. On the other hand, in a previous study performed by our research group, we have 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., 2014a), although we have detected here down-regulation of TLR-

4 transcript in the spleen. TLR-4 transcript was also detected unchanged in the blood during canine VL (MONTSERRAT-SANGRÀ et al., 2016). The most important TLR-4 ligand known is LPS from Gram-negative bactéria (OSPELT; GAY, 2010). However, it is not known if the *Leishmania* parasite has the ability to synthetize LPS. Therefore, we suggest that other molecular patterns found on *Leishmania* might be interacting with TLR-4 and activating the host response against the parasite, especially in this subpopulation of dogs observed in this study.

A slightly increased expression of TLR-7 and TLR-8 was observed in the brain and in the spleen of dogs with VL, along with TLR-3 in the brain, although this increase was no statiscally 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 cascade of NF-κB and the production of IFN-γ (ALEXOPOULOU et al., 2001), while TLR-7 and TLR-8 share the same ligand, recognizing single stranded RNA (ssRNA) from viruses or from endogenous sources (HEIL et al., 2004). Ds RNA 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, a 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 of transcripts of TLR-2 and TLR-10 was observed in the spleen of infected dogs. Moreover, 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 no 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 or slightly elevated in the brain (TLR-1 and TLR-10). This can be occurring because the receptors are acting synchronously among them. TLRs have been reported to act as dimers in the recognition of PAMPs (OSPELT; GAY, 2010). For example, TLR-2 signaling occurs through the formation of heterodimers with TLR-1, TLR-6 or TLR-10 (OSPELT; GAY, 2010; TAKEUCHI et al., 2002). TLR-1 can signalize dimerizing with TLR-2 (TAKEUCHI et al., 2002; WYLLIE et al., 2000) and TLR-6 with TLR-1 or TLR-2 (HORNUNG et al., 2002). Thus, the study of the TLR profile is essential since the immune response against *Leishmania* parasite may involve the interaction among multiple receptors.

In our study, TLR-2 transcripts were found unchanged in the brain 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 plexus (CP) from infected dogs (MELO et al., 2014a). Here, we have evaluated a pool of samples from several brain areas, but not from CP. We have observed that all TLRs evaluated were unchanged in the brain, except TLR-4 that was elevated in a small subset of animals. Therefore, we suggest that CP might be the structure related to the activation of inflammatory pathways related to TLRs in the CNS, even because it was one of the structutes that presented the most severe intensity of inflammation in this study, along with leptomeninges.

Regarding the spleen, we have found a reduction of TLR-2 transcripts in infected dogs. A reduction of TLR-2 and also of TLR-4 was detected in infected dogs previously, in macrophages from mononuclear cells and PBMC, respectively (MELO et al., 2014b). On the other hand, TLR-2 was found to be widely expressed in the spleen in dogs with VL (MELO et al., 2014a). This difference in the spleen transcripts can occur due to the variability in the individual immune response. Thus, it is possible that their dogs can be in different clinical stage than the dogs of this study. 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 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 leishmaniasis at diagnosis but it was reduced with clinical improvement during treatment (MONTSERRAT-SANGRÀ et al., 2016).

In our study we have observed meningitis and choroiditis in infected dogs, showing evidence of BBB breakdown. IL-1β is the main cytokine involved in BBB breakdown, as well as TNF-α and IL-6. These cytokines have a role in increasing the expression of adhesion molecules in the endothelium, allowing possible the leukocyte traffic into the brain (ABBOTT et al., 2006; DE VRIES et al., 1996; HICKEY, 1999; QUAGLIARELLO et al., 1991). 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 amount in the brain of infected and healthy dogs while IL-6 was not detected in this tissue. These data provides evidence that these cytokines are not related to brain inflammation.

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's perycites (MELO et al., 2013). Perycites 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 brain of infected dogs (MACHADO et al., 2010). We have observed here a higher amount of TNF-α in the brain 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; FEARON, 1996), where they can bind to TNF-α, difficulting its measurement by ELISA. We have reported previously the brain's potential to produce TNF-α through the detection of an increase in TNF-α transcripts in the brain of infected dogs (MELO et al., 2013). Therefore, we can not discard TNF-α as a substance related to BBB breakdown in dogs with VL.

On the other hand, we have detected TNF-α in the spleen of infected dogs, however it did not differ from control dogs. The lack of relation between TNF-α and the active disease was reported previously 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 than in healthy dogs.

For IFN-γ, levels were similar in brain and spleen of infected and healthy dogs. In other 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). Other 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 elevated levels of this cytokine in infected dogs, as observed here, can be related to their susceptibility to the disease.

In regards to IL-6, this cytokine was not detected in the brain or spleen of infected and control dogs in this study, although an up-regulation of IL-6 expression has been reported in dogs with VL (MELO et al., 2013). Our data indicates that IL-6 is not related to the active disease. Recently, RasGRP3 (Ras guanine nucleotide-releasing protein 3) was found to have a regulatory role, limiting the production of TLR-triggered proinflammatory cytokines, specially IL-6, in human and murine macrophages (TANG et al., 2014). RASGRP3 gene was found in infected dogs (BATISTA et al., 2016) and its presence might explain the absence of IL-6 observed in our study.

5 CONCLUSION

Our data provides support to explain the involvement of innate immune sensors in the immune response against *L. infantum*. Data presented herein provides important evidence that TLR-5 and TLR-9 play a role during canine VL in the spleen and that TLR-4 can be related to neuropathogenesis of the disease. Furthermore, we suggest that the brain inflammation observed here might be also triggered by other inflammatory pathways, perhaps through other innate immune receptors, in addition to TLRs, or it might be originated in other brain areas not evaluated in this study.

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CAPÍTULO 3 - IMPLICAÇÃO

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Desde o ano de 2011 venho desenvolvendo projetos de pesquisa estudando a relação entre a infecção periférica (principalmente em baço) e o SNC, tentando explicar a origem das lesões encefálicas. Dessa forma, o presente estudo complementa os estudos anteriores, no qual nosso grupo tem detectado infiltrado inflamatório no encéfalo composto predominantemente por células T, aumento da expressão gênica de citocinas pró-inflamatórias e de quimiocinas, presença do DNA do parasito em diversas áreas encefálicas e disfunção da barreira hematoencefálica em cães naturalmente infectados.

Avaliando os resultados obtidos nessa tese, é possível fazer as seguintes afirmações:

- A expressão gênica de TLRs é compartimentalizada, variando entre encéfalo e baço.
- TLR-5 and TLR-9 apresentam um papel no baço de cães com LV e TLR-4 pode estar relacionado a neuropatogenia da doença.
- O DNA do parasito, e não o parasito íntegro, foi detectado no encéfalo e no baço dos cães naturalmente infectados. No entanto, maior carga parasitária foi observada no órgão periférico.
- Não há correlação entre os receptores tipo Toll e a carga parasitária, tanto para o encéfalo, como para o baço.
- Não há correlação entre os receptores tipo Toll e os sinais clínicos da doença (estadiamento clínico).
- A produção de todas as citocinas, exceto IL-6, foi observada no encéfalo e também no baço dos cães infectados e também dos cães saudáveis.
- IL-1β está relacionada à doença ativa, ao menos no órgão periférico estudado aqui.
- TNF-α está diminuída em cães infectados, mas isso não descarta o seu possível papel na disfunção da BHE.

- Não há correlação entre a produção de citocinas e a carga parasitária, tanto para o encéfalo, como para o baço.
- Não há correlação entre a produção de citocinas e os receptores tipo Toll, tanto para o encéfalo, como para o baço.
- Foi observada inflamação encefálica, principalmente nas leptomeninges e no plexo coroide.
- A resposta inflamatória observada no encéfalo não é dependente da presença do parasito íntegro.
- Não há correlação entre a intensidade de inflamação encefálica e os sinais clínicos da doença (estadiamento clínico).
- Não há correlação entre a intensidade de inflamação encefálica e a expressão gênica dos receptores tipo Toll ou produção de citocinas no encéfalo.
- Nós sugerimos que outras áreas do SNC (além das áreas avaliadas neste estudo) poderiam estar relacionada à ativação dos TLRs e neuroinflamação.

Com isso, os resultados apresentados nessa tese reforçam o comprometimento do encéfalo na LV canina e fornecem evidência para explicar o envolvimento de sensores do sistema imune inato na infecção por *L. infantum.* Mais estudos são essenciais para avaliar não somente o papel dos TLRs mais conhecidos, como TLR-2, TLR-4 e TLR-9, mas também dos outros TLRs, cujo papel na infecção por *Leishmania* in vivo necessita ainda ser elucidado. Além disso, sugerimos a realização de estudos avaliando os possíveis ligantes do parasito *Leishmania* com esses receptores.

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