UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA VETERINÁRIA
CAMPUS DE ARAÇATUBA

LEISHMANIOSE VISCERAL E O SISTEMA NERVOSO CENTRAL: INFLAMAÇÃO NAS INFECÇÕES NATURAL CANINA E EXPERIMENTAL EM CAMUNDONGOS

Guilherme Dias de Melo
Médico Veterinário

ARAÇATUBA – SP
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Guilherme Dias de Melo

Orientador: Prof.ª Adjunto Gisele Fabrino Machado

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

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TÍTULO: Leishmaniose visceral e o sistema nervoso central: inflamação nas infecções natural canina e experimental em camundongos.

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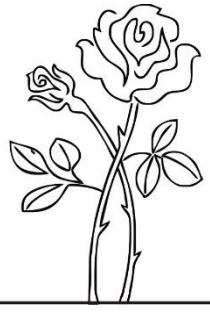
“Foi o tempo que perdeste com a tua rosa que a fez tão importante”

“C’est le temps que tu as perdu pour ta rose qui fait ta rose si importante”

Antoine de Saint-Exupéry

escritor francês (1900-1944)

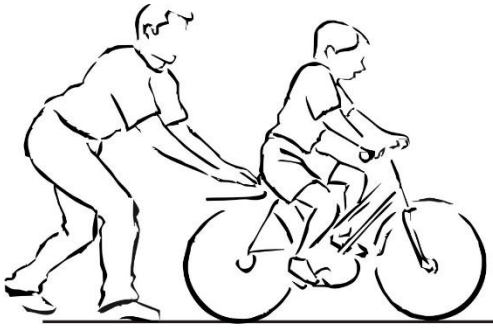


Ao meu pai Antonio,

cujo apoio foi constante ao longo do meu percurso,

mas que infelizmente não pôde estar presente na conclusão de tudo.

DEDICO



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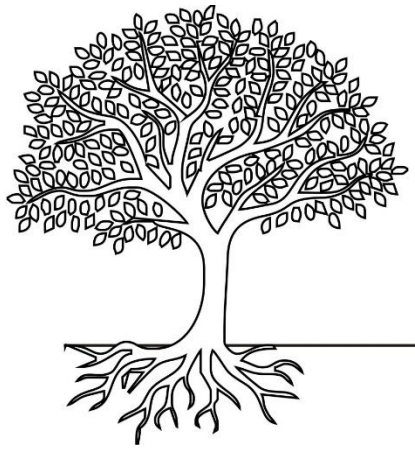

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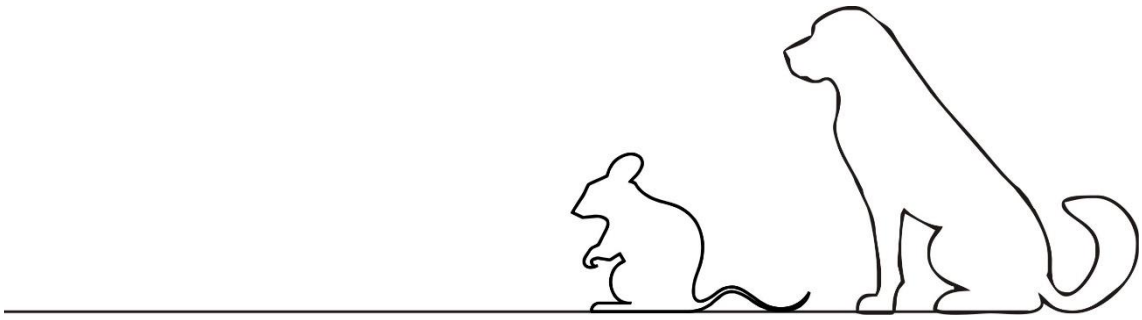

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LEISHMANIOSE VISCERAL E O SISTEMA NERVOSO CENTRAL: INFLAMAÇÃO NAS INFECÇÕES NATURAL CANINA E EXPERIMENTAL EM CAMUNDONGOS

RESUMO – A leishmaniose visceral é uma importante doença parasitária com distribuição mundial e que afeta homens e animais. Existem relatos de envolvimento encefálico durante a infecção, mas sua patogenia ainda não foi completamente elucidada. Com o presente estudo, objetivamos a avaliação de quimiocinas relacionadas com a resposta inflamatória no encéfalo e marcadores da integridade da barreira hematoencefálica em cães naturalmente infectados. Também foi estabelecido um modelo experimental murino que permite a avaliação da dinâmica da infecção, bem como localização do parasita e resposta inflamatória *in vivo*. Cães naturalmente infectados apresentaram um perfil específico de quimiocinas no encéfalo, altamente quimioatrativo para linfócitos T, além de evidente perda da integridade da barreira hematoencefálica, com elevado quociente de albumina e altas concentrações de anticorpos anti-*Leishmania* no líquido cefalorraquidiano. Utilizando inovadoras técnicas moleculares e de imagiologia *in vivo*, foi possível determinar a presença do parasita no encéfalo de camundongos Balb/c experimentalmente infectados, bem como sua localização intra-craniana. A inflamação no encéfalo, avaliada nos primeiros quatro meses pós-infecção, apresentou duas fases distintas, uma fase aguda nos primeiros quatorze dias, e uma fase de reinflamação após três meses de infecção. Conjuntamente, os dados desse estudo permitiram confirmar o envolvimento encefálico durante a leishmaniose visceral, e reforçam o fato de que o comprometimento encefálico tem sido negligenciado durante a leishmaniose visceral.

Palavras-chave: barreira hematoencefálica, encéfalo, imagiologia *in vivo*, modelos animais, quimiocinas, sistema imunológico

VISCERAL LEISHMANIASIS AND THE CENTRAL NERVOUS SYSTEM: INFLAMMATION IN THE CANINE NATURAL AND MICE EXPERIMENTAL INFECTIONS

SUMMARY – Visceral leishmaniasis is an important parasitic disease, with worldwide distributions that affects humans and animals. There are reports of brain involvement during the infection; however, its pathogenesis has not been completely elucidated. Therefore, with this study, we aimed the evaluation of the inflammatory response in the brain and the integrity of the blood-brain barrier in naturally infected dogs, and the establishment of an experimental mice model that could allow the evaluation of the infection dynamics, as well as the localization of the parasite and the inflammatory response *in vivo*. Naturally infected dogs presented a specific chemokine profile in the brain, highly attractive to T-lymphocytes, besides the evident blood-brain barrier disruption, with increased albumin quota and elevated concentrations of anti-*Leishmania* antibodies in the cerebrospinal fluid. Using molecular techniques and state-of-the-art *in vivo* imaging tools, we could determine the presence of parasites in the brain of experimentally infected Balb/c mice, as well as its intracranial localization. Brain inflammation, evaluated during four months post-infection, presented two distinct phases, one acute phase in the first fourteen days, and one phase of re-inflammation after three months of infection. Altogether, the data from this study allow us to confirm that the brain is affected during visceral leishmaniasis, and reinforce the fact that the brain involvement has been neglected during visceral leishmaniasis.

Keywords: brain, blood-brain barrier, chemokines, experimental animal models, immune system, *in vivo* imaging

CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

CONSIDERAÇÕES GERAIS

1.1 LEISHMANIOSE VISCERAL

A leishmaniose visceral (LV) é uma doença crônica causada por protozoários parasitas do complexo *Leishmania donovani* (Kinetoplastida, Trypanosomatidae), incluindo *L. donovani* e *L. infantum* (= *chagasi*). *L. infantum* está localizada sobretudo nas Américas e na bacia do Mediterrâneo, sendo o Brasil o país mais afetado, e *L. donovani* está presente na Ásia e na África principalmente em países como Bangladesh, Índia, Iraque, Etiópia e Sudão (BANETH et al., 2008; CHAPPUIS et al., 2007; LUKEŠ et al., 2007; WHO, 2013).

Os parasitas são transmitidos durante o repasto sanguíneo por mosquitos flebotomíneos dos gêneros *Phlebotomus* spp. e *Lutzomyia* spp., conhecidos como 'mosquito palha' ou '*sand fly*' (ALVAR et al., 2004; BANETH et al., 2008). Enquanto *L. infantum* apresenta caráter zoonótico, com o cão como principal reservatório da doença urbana, *L. donovani* é descrita classicamente por seu caráter antroponótico, todavia, o papel do cão na sua transmissão ainda não foi completamente descartado (BANETH et al., 2008; DEREURE et al., 2003; HASSAN et al., 2009).

O parasita se localiza em órgãos que contêm células do sistema fagocitário mononuclear, como fígado, baço, medula óssea, linfonodos, além da pele (ALVAR et al., 2004; BANETH et al., 2008). Cães infectados podem se manter assintomáticos por longos períodos ou rapidamente desenvolver os sintomas clássicos da doença, como lesões dermatológicas e oculares, insuficiência renal, anemia e diáteses hemorrágicas, caquexia, esplenomegalia e linfadenopatia generalizada (ALVAR et al., 2004; BLAVIER et al., 2001; CIARAMELLA; CORONA, 2003; MORENO et al., 1998; SOLANO-GALLEGO et al., 2011).

A progressão da doença está intimamente ligada ao padrão de resposta imune que o hospedeiro estabelece contra a infecção. Em animais infectados,

mas clinicamente saudáveis, a resposta do tipo Th1 (celular) prevalece, mediada por citocinas como IL-2, IFN-γ e TNF-α, ao passo que em animais sintomáticos há predomínio de citocinas como IL-4 e IL-10 mediando a resposta do tipo Th2 (humoral) (BARBIERI, 2006; REIS et al., 2009; SOLANO-GALLEGO et al., 2011; STRAUSS-AYALI et al., 2005). Entretanto um direcionamento específico Th1 ou Th2 não é soberano durante a leishmaniose, sobretudo devido à ação das células T reguladoras (Treg), que modulam a resposta imune por meio de interações célula-célula ou pela produção de citocinas como IL-10 e TGF-β (BELKAID et al., 2002; CAMPANELLI et al., 2006; GANTT et al., 2003).

1.1.1. Mediadores inflamatórios

Quimiocinas compõem uma superfamília de proteínas de baixo peso molecular (8-10 kDa) que atuam na resposta imune, sobretudo no que tange a ativação e o tráfego de leucócitos. A ação das quimiocinas é redundante e são consideradas 'promíscuas' em relação aos receptores, uma vez que diferentes quimiocinas podem se ligar a um mesmo receptor e também uma mesma quimiocina a diferentes receptores (BENDALL, 2005; MANTOVANI, 1999).

São subdivididas em quatro subfamílias, de acordo com a posição de seus resíduos de cisteína: CXC (ou α-quimiocinas), CC (ou β-quimiocinas), C e CX3C (MANTOVANI, 1999). Dentre as β-quimiocinas encontram-se as MCPs (proteínas quimioatraentes de monócitos) -1, -2, -3, e -5 (ou CCL2, CCL8, CCL7 e CCL12, respectivamente), as MIPs (proteínas inflamatórias de macrófagos) -1α e -1β (ou CCL3 e CCL4, respectivamente) e RANTES (expressa e secretada por células T normais e regulada após ativação; ou CCL5), que são altamente quimioatraentes para macrófagos e monócitos, diversas subpopulações de linfócitos, células dendríticas e células natural killer (NK) (BENDALL, 2005; RABIN, 2003).

Entre as α-quimiocinas encontra-se a CXCL-10 (proteína 10 induzida por IFN-γ ou IP-10), cuja principal função é regular a migração de células Th1

efetoras ao local da inflamação, durante a resposta imune adaptativa (BENDALL, 2005; MURPHY; 2003). Já a CX3CL1 (fractalquina) é, até o momento, a única quimiocina descrita na subfamília CX3C. Possui a particularidade de atuar como quimioatraente e também como molécula de adesão, uma vez que se encontra seja na forma solúvel, seja ancorada à membrana celular. É expressa por macrófagos, células dendríticas, neurônios e células endoteliais ativadas, e é quimioatraente sobretudo para linfócitos T, monócitos e células NK (BENDALL, 2005; RABIN, 2003).

As quimiocinas são produzidas por diversos tipos celulares ao passo que seus receptores são expressos na membrana celular de leucócitos. Sua produção é estimulada por diversas citocinas pró-inflamatórias como IL-1, IL-6, IFN-γ e TNF-α (BENDALL, 2005; MANTOVANI, 1999). Ademais, existem evidências de que quimiocinas podem estimular a síntese de outros mediadores inflamatórios, como os eicosanóides (PACHECO et al., 2007).

Os eicosanóides compõem uma grande variedade de metabólitos derivados do ácido araquidônico, e basicamente diferem entre si de acordo com a enzima que os originam. Os eicosanóides formados pela ação da enzima ciclo-oxigenase (COX) formam o grupo dos prostanóides, entre eles prostaglandinas, prostaciclinas e tromboxanos. A rota da enzima lipo-oxigenase (LOX) origina os leucotrienos, lipoxinas e os ácidos hidroxiperoxieicosatetraenoicos (HPETEs). Por último, há também a via do citocromo P450 ou epoxigenase, originando ácidos epoxieicosatrienóicos (EETs). Em especial, as enzimas COX-2 e 5-LOX dão origem, entre outros, à prostaglandina E2 (PGE2) e ao leucotrieno B4 (LTB4) respectivamente, dois importantes mediadores inflamatórios, envolvidos na resposta imune (DAUGSCHIES; JOACHIM, 2000; ENGBLOM et al., 2002).

Outro importante mediador inflamatório, que atua na defesa contra patógenos, é o óxido nítrico (NO). O NO é o único hormônio animal presente em estado gasoso, mas que possui alta solubilidade. Pode ser produzido por meio da ação de três enzimas: duas delas são constitutivas e dependentes de cálcio: a óxido nítrico sintase neuronal (nNOS) e a óxido nítrico sintase endotelial

(eNOS); já a óxido nítrico sintase induzível (iNOS) é independente de cálcio e produz NO somente na presença de estímulos inflamatórios (JAWOROWICZ et al., 1998; NORMAN; NORMAN, 2003).

Ainda, como um link entre imunidades inata e adaptativa, a IgG pode desenvolver um papel importante, precisamente por meio da interação com os receptores da porção Fc da IgG, os FcγRs. Divididos em quatro categorias principais, FcγRI (CD64), FcγRII (CD32), FcγRIII (CD16), e FcγRIV, atuam como ativadores da função celular, estimulando a fagocitose, a liberação de citocinas e a citotoxicidade celular dependente de anticorpo (ADCC), com exceção do FcγRIIB, que tem função inibitória (NIMMERJAHN et al., 2006; SIBÉRIL et al., 2007). Os receptores FcγRs se ligam tanto à IgG monomérica quanto a imunocomplexos, contudo a intensidade da ligação varia de acordo com a subclasse da IgG envolvida (SIBÉRIL et al., 2007). Os FcγRs são expressos sobretudo por células mielóides, como macrófagos, células dendríticas e neutrófilos, mas também em linfócitos e células NK, e no SNC, a expressão desses receptores em células microgliais, humanas e de camundongos, também já foi descrita (PERESS et al., 1993; SIBÉRIL et al., 2007).

1.2 SISTEMA NERVOSO CENTRAL

O tecido nervoso é composto primariamente por neurônios e células da glia, além das meninges e vasos sanguíneos. A glia, ou neuróglia, pode ser dividida em macróglia (astrócitos, oligodendrócitos e células ependimárias) e micróglia. As células da glia são células nervosas não neuronais que dão suporte e proteção aos neurônios; formam o neurópilo, que é composto por todo o tecido nervoso (glia, neuritos) excetuando-se os corpos celulares de neurônios (SUMMERS et al., 1995).

Um tipo específico de células ependimárias constitui o plexo coroide, estrutura composta por quatro partes, cada uma localizada em um ventrículo

cerebral. O plexo coroide é formado e se ancora na tela coroidea, região onde vasos da pia-máter entram em íntimo contato com o epêndima, e penetram o lúmen ventricular, formando um emaranhado de vasos revestidos por células ependimárias, que flutua livremente no líquido cefalorraquidiano (LCR) (BRINKER et al., 2014; DI TERLIZZI; PLATT, 2006).

1.2.1 Mecanismos de defesa no sistema nervoso central

Como mecanismos de defesa, o sistema nervoso central (SNC) conta inicialmente com a barreira física formada pela pele e pelo crânio e vértebras. Ademais, as meninges e o LCR proporcionam uma barreira física e também funcional, pelo fluxo do LCR no espaço subaracnóideo. Contudo, são as barreiras cerebrais, com características únicas e específicas do SNC, que formam uma importante estrutura de proteção, que previnem o contato de estruturas do SNC com componentes sanguíneos, com o intuito de manter o ambiente nervoso estável (BALLABH et al., 2004; GALEA et al., 2007).

A barreira hematoencefálica (BHE) propriamente dita é composta por células endoteliais especializadas que não apresentam fenestrações e que possuem junções do tipo oclusivas (*tight junctions* ou *zonula occludens*). É uma barreira entre a luz de vasos sanguíneos e o parênquima nervoso. Ainda, como defesa adicional, a BHE conta com a presença de pericitos, membrana basal, macrófagos perivasculares e pés terminais de astrócitos, que recobrem toda sua superfície e extensão (BALLABH et al., 2004; GALEA et al., 2007; SAUNDERS et al., 2008).

Em todo o SNC a BHE está presente, exceto nos órgãos circunventriculares (OCV), áreas adjacentes aos ventrículos cerebrais que possuem características neuroendócrinas e sensoriais, onde os capilares são fenestrados. O plexo coroide é por vezes considerado um OCV, por também conter capilares fenestrados, contudo, devido a características próprias, é

considerado separadamente na formação da barreira hematoliquórica (BHL) (SCHULZ; ENGELHARDT, 2005; WOLBURG; PAULUS, 2010).

A BHL está presente no plexo coroide e apresenta o mesmo princípio da BHE, ou seja, a unidade principal é composta por junções intercelulares do tipo oclusivas, contudo, essas junções não se encontram no endotélio, mas sim entre as células epiteliais. Ademais, com distribuição aleatória sobrejacente às células do epitélio estão presentes as células do epiplexo (ou células de Kolmer), consideradas células apresentadoras de antígeno residentes, além de células dendríticas e macrófagos no estroma (DI TERLIZZI; PLATT, 2006; JOHANSSON et al., 2008; WOLBURG; PAULUS, 2010).

O plexo coroide é considerado o principal produtor do LCR. Recentemente, foi proposto que o LCR circula por todo o SNC, não estando restrito aos ventrículos cerebrais e ao espaço subdural, mas inclusive no espaço perivascular (espaço de Virchow-Robin), onde pode ocorrer a drenagem, o *clearance* de moléculas e a interação com o sistema imune (BRINKER et al., 2014). Existem hipóteses de que a unidade funcional da BHE, a unidade capilar-astrócito, possa participar na produção ativa de liquido intersticial, que posteriormente se tornaria parte integrante do LCR (ABBOTT, 2004), de forma que o LCR possa refletir o estado geral do SNC e não somente alterações restritas ao plexo coroide e à BHL.

1.2.2 Inflamação no sistema nervoso central

O SNC em condições normais é classicamente considerado um local imunologicamente privilegiado, devido à ausência de circulação linfática, à baixa expressão celular de antígenos de histocompatibilidade, ao reduzido número de células dendríticas e à eficiência das barreiras cerebrais. Todavia, processos inflamatórios envolvendo o SNC fatalmente ocorrem (GALEA et al., 2007; SAUNDERS et al., 2008). Vários mediadores inflamatórios apresentam a capacidade de alterar a integridade da BHE, dentre eles bradicinina, histamina,

prostaglandinas e leucotrienos, citocinas, quimiocinas, óxido nítrico e radicais livres (ABBOTT et al., 2006). A investigação de falhas na integridade da BHE pode ser feita de diversas maneiras, como avaliação do extravasamento de corantes no tecido nervoso, detecção de proteínas de origem nervosa no soro, ou detecção de proteínas séricas no líquido cefalorraquidiano (LCR) e no tecido nervoso (GONÇALVES et al., 2008;).

Com a perda da integridade da BHE e com a presença de mediadores inflamatórios, ocorre o recrutamento de leucócitos para o SNC. São consideradas três as vias de entrada para leucócitos: (1) do sangue para o LCR via plexo coroide, (2) do sangue para o espaço subaracnóideo, (3) do sangue para o espaço perivascular no parênquima. Todas as vias envolvem a migração dos leucócitos através das barreiras cerebrais, contudo, para que os leucócitos consigam deixar o vaso sanguíneo e ultrapassar o endotélio é necessário que uma série de moléculas e receptores de ativação estejam presentes tanto nas células inflamatórias como no endotélio, incluindo moléculas de adesão e seus ligantes, quimiocinas e seus receptores (ENGELHARDT; RANSOHOFF, 2005; KIVISÄKK et al., 2003; RANSOHOFF et al., 2003;).

1.3 LEISHMANIOSE VISCERAL E O SISTEMA NERVOSO CENTRAL

Embora *Leishmania* spp. apresente tropismo para órgãos que contenham células do sistema fagocitário mononuclear, como fígado e baço, o parasita pode virtualmente se disseminar para todos os órgãos e tecidos, incluindo o sistema genital (DINIZ et al., 2005), músculos (GOMES et al., 2012) e o SNC (MÁRQUEZ et al., 2013).

Relatos do envolvimento clínico do SNC em casos de LV também existem em pacientes humanos e caninos (PETERSEN; GREENLEE, 2011). Em humanos, o envolvimento do sistema nervoso periférico é mais comum, contudo meningite, disfunção de nervos cranianos e parasitas no LCR já foram relatados

(HASHIM et al., 1995; PRASAD; SEN, 1995; SNYDMAN et al., 2006). Cães, ao contrário, podem apresentar sinais de envolvimento generalizado do SNC, com convulsões, disfunção de nervos cranianos, sinais cerebelo-vestibulares, incoordenação motora, paresia e mioclonia (FONT et al., 2004; IKEDA et al., 2007; JOSÉ-LÓPEZ et al., 2012). Todavia, especificamente no encéfalo, o parasita é raramente identificado, estando relacionado a relatos de casos esporádicos (MÁRQUEZ et al., 2013; NIETO et al., 1996; VIÑUELAS et al., 2001).

Mesmo na ausência do parasita, cães naturalmente infectados apresentam evidências de inflamação no encéfalo e desregulação da BHE, além de meningite e coroidite, deposição perivascular de imunoglobulinas, infiltração de leucócitos, ativação glial, enzimas que degradam componentes da matriz extracelular (MMP-2 e MMP-9) e um perfil de citocinas pró-inflamatório (GARCIA-ALONSO et al., 1996; IKEDA et al., 2007; JOSÉ-LÓPEZ et al., 2012; MELO; MACHADO, 2011; MELO et al., 2013).

1.4 OBJETIVOS

Objetivos gerais

- Estudar a infecção e resposta inflamatória no encéfalo de cães e camundongos com leishmaniose visceral.

Objetivos específicos

A. Em cães naturalmente infectados:

- Determinar a carga parasitária de *Leishmania* spp. no encéfalo por meio de qPCR e sua localização por meio de hibridização *in situ;*
- Determinar a expressão gênica das quimiocinas CCL-2, CCL-3, CCL-4, CCL-5, CCL-8, CXCL-10, CX3CL-1 e do receptor CX3CR-1 no encéfalo e no baço, bem como correlacionar com a carga parasitária e o estadiamento clínico;
- Avaliar a integridade da barreira hematoencefálica por meio da determinação do quociente de albumina, do IgG index e da concentração de anticorpos anti-*Leishmania* no líquido cefalorraquidiano;
- Determinar a localização tecidual de albumina, IgG e Fcγ-RI como forma de avaliação visual da integridade da barreira hematoencefálica;
- Determinar as concentrações séricas e liquóricas de prostaglandina E2, leucotrieno B4 e óxido nítrico por meio de ELISA.

B. Em camundongos experimentalmente infectados:

- Determinar a virulência do parasita transgênico que expressa o gene da luciferase no modelo animal avaliando a infecção e a resposta inflamatória no fígado e no baço por meio de ensaios de bioluminescência *in vivo* e RT-qPCR;
- Determinar a carga parasitária, a cinética da infecção e a localização dos parasitas no encéfalo por meio de ensaios de bioluminescência *in vivo*, RT-qPCR e isolamento de parasitas;
- Monitorar a cinética da resposta inflamatória no encéfalo por meio da determinação da expressão gênica de citocinas, quimiocinas e receptores de quimiocinas usando RT-qPCR;
- Determinar a atividade das enzimas metaloproteinases de matriz no encéfalo utilizando RT-qPCR e tomografia molecular fluorescente *in vivo;*
- Comparar os dados obtidos nesse modelo com dados previamente determinados em cães.

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CAPÍTULO 2 – INFECÇÃO POR *Leishmania* E NEUROINFLAMAÇÃO: PERFIL ESPECÍFICO DE QUIMIOCINAS E AUSÊNCIA DE PARASITAS NO ENCÉFALO DE CÃES NATURALMENTE INFECTADOS

Leishmania INFECTION AND NEUROINFLAMMATION: SPECIFIC CHEMOKINE PROFILE AND ABSENCE OF PARASITES IN THE BRAIN OF NATURALLY INFECTED DOGS

ABSTRACT – Visceral leishmaniasis is a chronic disease caused by parasitic protozoans from the *Leishmania donovani* complex. The parasite is mainly localized in organs with cells from the mononuclear phagocyte system, such as liver and spleen; nevertheless, it may spread everywhere. Since nervous involvement have already been described in canine visceral leishmaniasis, we aimed to detect the parasite in the brain of fifteen naturally infected dogs using *in situ* hybridization and qPCR. Further, since inflammatory cells are present in the brain of infected dogs, we evaluated the gene expression of selected chemokines by RT-qPCR in the brain, the organ of interest, and in the spleen, the target organ of the infection. By means of *in situ* hybridization, we were able to detect *Leishmania* DNA perivascular deposition in the choroid plexus in eleven dogs, associated to positive qPCR. No amastigotes were detected in the brain. The most common findings in the brain were mononuclear cells accumulation in the leptomeninges, choroid plexus and in perivascular cuffs, and up-regulation of CCL-3, CCL-4 and CCL-5, coherent with T lymphocytes accumulation. Further, a small portion of infected dogs (n=5) presented overexpression of CXCL-10, related to Th1 cells migration. On the other hand, the spleen presented up-regulation for CCL-2, CCL-3, CCL-4, CCL-5, CCL-8, CXCL-10 and CX3CL-1. This variation of chemokine patterns according to the organ corroborates not only the idea of immune compartmentalization during VL, but also that the brain is a pro-inflammatory environment, even in the absence of the parasite. Indeed, not the parasite itself, but rather its DNA seems to act as a trigger to promote brain inflammation during canine visceral leishmaniasis.

KEYWORDS: chemokine CCL3, chemokine CCL4, chemokine CCL5, central nervous system, *in situ* hybridization, visceral leishmaniasis

2.1 INTRODUCTION

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

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

Specifically in the brain, the parasite is not often detected (MÁRQUEZ et al., 2013; VIÑUELAS et al., 2001), however, inflammatory lesions even in the absence of the parasite are commonly observed, predominantly leptomeningitis and choroitis, with accumulation of mononuclear cells (IKEDA et al., 2007; NIETO et al., 1996; VIÑUELAS et al., 2001). For the occurrence of leukocytes migration from blood to the brain, chemokines are key molecules. They compose a superfamily of low molecular weight proteins (8-10 kDa), which act in the immune response, mainly activation and guidance of leukocyte traffic (chemotaxis) (BENDALL, 2005; MANTOVANI, 1999).

Chemokines are divided in four subfamilies, according to the position of cysteine residues: CXC (α-chemokines), CC (β-chemokines), C and CX3C (MANTOVANI, 1999; PEETERS et al., 2006). Among the β-chemokines there are MCPs (monocyte chemoattractant proteins) -1 and -2 (or CCL-2 and CCL-8,

respectively); MIPs (macrophage inflammatory proteins) -1α and -1β (or CCL-3 and CCL-4, respectively); and RANTES (regulated on activation, normal T cell expressed and secreted, or CCL-5), which are highly chemoattractive to monocytes/macrophages, several lymphocytes subsets, dendritic cells and NK cells (BENDALL, 2005; RABIN, 2003).

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

Limited studies focused on the neuropathogenesis of VL. We have previously observed the presence of inflammatory stimuli in the brain of infected dogs, such as glial activation, cytokines overexpression and matrix metalloproteinases enzymes (MELO; MACHADO, 2011; MELO et al., 2012; 2013) that could facilitate the accumulation of inflammatory cells, essentially T lymphocytes (MELO et al., 2009). Therefore, since the evidences of brain inflammation during canine VL are robust but the pathogenesis unclear, we aimed to evaluate the gene expression of CCL-2, CCL-3, CCL-4, CCL-5, CCL-8, CXCL-10, CX3CL-1 and the receptor CX3CR-1 in the brain of dogs naturally infected by *Leishmania* spp., comparing with the chemokine profile expressed in the spleen; and attempting to correlate the expression of the chemokines with the clinical stage and the parasite load, assessed by qPCR and *in situ* hybridization.

2.2 MATERIALS AND METHODS

2.2.1 Animals

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

2.2.2 Sampling

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

From the brain, we collected one hemisphere and stored in 10% buffered-formalin. After fixation, coronal sections were made and samples containing cerebral cortex, thalamus, hippocampus, pons-medulla oblongata, cerebellum, the ventricular choroid plexi and periventricular white matter were paraffin-embedded, sectioned (5 µm) and submitted to hematoxylin and eosin (HE) staining and to *in situ* hybridization. From the other hemisphere, unfixed, we collected a pool of fragments of 0.5 cm^3 from the thalamus, hippocampus, piriform/temporal cortex and periventricular white matter, stored in RNAlater (AM7020, Applied Biosystems, Foster City, CA, USA) for RNA extraction, or

directly frozen at −80 ◦C for DNA extraction. Regarding the spleen, we performed tissue smears, and we collected fragments in formalin, in RNAlater and to be directly frozen at -80°C.

2.2.3 Clinical staging

We performed the complete bloodwork of the animals, using routine methods to determine the serum concentrations of total protein, albumin, urea and creatinine. We determined the serum concentrations of anti-*Leishmania* antibodies using indirect ELISA (LIMA et al., 2005). Urinalysis and urinary protein/creatinine ratio (UPC;$\left\{\frac{urinary\ protein}{urinary\ creatinine}\right\}$) were also performed. The clinical staging was defined according to Solano-Gallego et al. (2011).

2.2.4 In situ hybridization (ISH) to detect Leishmania

We performed *in situ* hybridization in brain and spleen sections following Dinhopl et al. (2011). Briefly, slides containing tissue sections were dewaxed in xylene and hydrated in ethanol 100%, 70% 50% and distilled water, following by incubation with Proteinase K (S3004, Dako, Carpinteria, CA, USA) during 10 min and washing in distilled water, ethanol 96% and isopropanol for 5 min each. The slides were air-dried and frame seals (SLF-1201, Bio-Rad, Hercules, CA, USA) were attached. Then, we added 125 µL of hybridization mix containing 15 µL of distilled water; 25 µL of 20x SSC buffer; 62,5 µL of formamide 50%; 12,5 µL of dextran sulphate 50%; 2,5 µL of Denhardt's solution (D2532, Sigma-Aldrich, Saint Louis, MO, USA); 6,25 µL of herring sperm DNA (D7290, Sigma-Aldrich); and 1,25 µL of 3'-digoxigenin-conjugated probe (Eurofins MWG Operon, Huntsville, AL, USA). The probe, 5′-ACGGGGATGACACAATAGAGCTTCTCC-3′, in final concentration of 100 ng/mL, detects a segment of the 5.8S ribosomal RNA of *Leishmania* genus. The slides were incubated at 95°C for 6 min, immediately cooled in ice, and then incubated at 40°C for 14-16 hours in humid chamber. Afterwards, the slides were washed in 2x SSC, 1x SSC and 0.1x SSC

buffer for 5 min each, followed by incubation with the anti-digoxigenin antibody conjugated to alkaline phosphatase, diluted in TBS (1:200; 11093274910, Roche Diagnostics, Indianapolis, IN, USA) for 1 hour and then washed in TBS. Visualization was achieved using NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) (11681451001, Roche Diagnostics) for 1 hour in the dark. The reaction was stopped with TE buffer (pH 8.0) for 10 min followed by washing in distilled water. The slides were counterstained with Mayer's hematoxylin and mounted in aqueous medium (Faramount, S3025, Dako).

2.2.5 Leishmania DNA quantification

We extracted total DNA from tissue fragments (spleen and a pool of brain fragments) weighting ca. 25 mg using the DNeasy blood & tissue kit (69506, Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA was quantified with a NanoDrop spectrophotometer (260/280 ratio between 1.8 and 2.0). We performed qPCRs using CFX96^{TM} Real-Time System (Bio-Rad), SYBR Green PCR Master Mix (4309155, Applied Biosystems) and 900 nM of each primer (sense: 5'-CCTATTTTACACCAACCCCCAGT-3'; anti-sense: 5'-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 the following: 94°C for 2 min and 40 cycles of 94°C for 15 s and 60°C for 1 min. Then, the samples were submitted to a melt curve from 60°C to 95°C; with a 0.5°C increase every 5 s. We assessed the absolute quantification using a standard curve containing serial dilutions (from 10^{-1} to 10^{6} promastigotes) of *L. infantum* DNA (MHOM/BR/72/LD46). The lower limit of positivity (cut-off value) was established using the results obtained from spleens and brains of uninfected dogs.

2.2.6 Chemokines gene expression

We extract the RNA from brain and spleen samples stored in RNAlater using the RNeasy Mini kit (74104, Qiagen) following the manufacturer's instructions. Total RNA was quantified in a NanoDrop spectrophotometer (260/280 ratio between 2.0 and 2.3) and then submitted to genomic DNA elimination and reverse transcription using the QuantiTect Reverse Transcription kit (205314, Qiagen) following the manufacturer's instructions.

We performed qPCRs using CFX96^{TM} Real-Time System (Bio-Rad), TaqMan master mix (4304437, Applied Biosystems), and customized gene expression assays (4351372 and 4331182, Applied Biosystems): CCL-2 (Cf02671955_g1), CCL-3 (Cf02671956_m1), CCL-4 (Cf02622476_m1), CCL-5 (Cf02622325_m1), CCL-8 (Cf02622478_m1), CXCL10 (Cf02622528_m1), CX3CL1 (Cf02651336_m1) and CX3CR1 (Cf02695529_s1). The reference genes were RPL32 (ribosomal protein L32; Cf03986518_m1) and G3PDH (glyceraldehyde 3-phosphate dehydrogenase; Peeters et al., 2006). The amplification conditions were as follows: 55°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15 s and 60°C for 1 min. For each target, we obtained values of reaction efficiency from amplification of seven serial dilutions of a pool of cDNA). The relative gene expression was achieved using the REST® method (Pfaffl et al., 2002), which indicates how many times (fold change) the expression of a target gene is higher (up-regulated) or lower (down-regulated) in the infected group when compared with the control group.

2.2.6 Statistical analyses

Correlation was assessed by the Spearman test. Values of $P<0.05$ were considered statistically significant. Data were expressed as the median and the interquartile range. All statistical analyses were performed using Prism software (v6.05, GraphPad, La Jolla, CA, USA).

2.2.7 Ethical issue

All procedures were performed according to the Brazilian College of Animal Experimentation, and approved by the Institutional Ethics Committee (CEUA, FMVA-UNESP, process #2012-01093).

2.3 RESULTS

2.3.1 Clinical staging

The most common clinical sign was skin disease (53.3%, 8/15), including alopecia, nasal hyperkeratosis and seborrhea, splenomegaly (46.7%, 7/15), followed by lymphadenopathy (40%, 6/15), conjunctivitis (40%, 6/15), and ear pinna necrosis (40%, 6/15). Onycogryphosis and cachexia were observed in 26.7% (4/15) of the dogs.

Regarding laboratorial findings, 93.3% (14/15) of the infected dogs presented normocytic-normochromic anemia, 53.3% (8/15) presented lymphocytopenia and 66.7% (10/15) presented thrombocytopenia. All infected dogs presented hypoalbuminemia and reduced albumin/globulin ratio. Azotemia was evident in 26.7% (4/15) of the infected dogs, and 66.7% (8 out of 12 urine samples) presented increased UPC. All infected dogs presented positive concentrations of serum anti-*Leishmania* antibodies, 6.7% (1/15) with low intensity (from cut-off value 0.27 up to 0.4), 67.7% (10/15) with medium intensity (0.4 up to 0.81), and 26.7% (4/15) with high intensity (over 0.81).

Direct parasitological examinations in spleen smears were positive in 33.3% (5/15) of the infected dogs (Figure 1A); however, all the spleens of infected dogs were positive in qPCR. Therefore, we classified 20% (3/15) of the dogs in the stage I, 53.3% (8/15) in the stage II, 20% (3/15) in the stage III and 6.7% (1/15) of the dogs in the stage IV of the disease.

2.3.2 Brain histopathology

The most common histopathological findings in the brain of the infected dogs with VL were inflammatory changes (Figure 2), ranging from discrete to severe, including leptomeningitis (80.0%, 12/15), choroiditis (73.3%, 11/15), subventricular gliosis (73.3%, 11/15) and parenchymal mononuclear perivascular cuffs (53.3%, 8/15). Further, we also observed vascular congestion, microhemorrhages, satellitosis/neuronophagia, and glial nodules (Figure 2F). No association between clinical signs and brain histopathological alterations was observed. We did not detect in the parasite in the brain sections stained with HE, however, the parasites were clearly observed in spleen sections (Figure 1B).

2.3.3 Localization of Leishmania parasites in the brain

We did not detected *Leishmania* parasites within the brain of infected dogs using *in situ* hybridization. Nevertheless, we observed a perivascular positive staining in the choroid plexus of 78.6% of the dogs (11 out of 14 dogs where this structure were present in the sections) (Figure 3). In order to better describe this finding, we performed paired HE staining in the same sections containing positive ISH staining in the choroid plexi. In HE-stained tissues, there was no detectable parasite or parasitized cell in the choroid plexus, but inflammatory infiltrate and stroma thickening. Therefore, we classified this alteration as perivascular *Leishmania* DNA deposition. In the spleen, the control organ of the infection, we could easily identify the parasites by the dark-purple pigment deposition (Figure 1C).

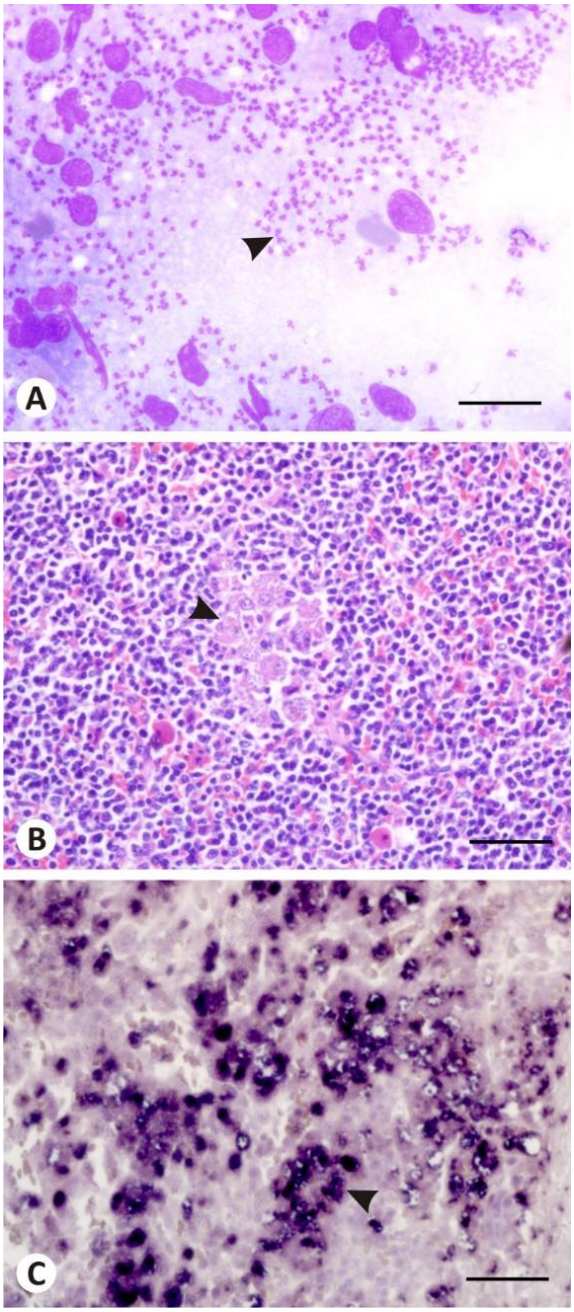

FIGURE 1 – Different protocols to visualize *Leishmania* in dogs with visceral leishmaniasis. (**A**) Cytologic preparation of the spleen exhibiting uncountable amastigotes forms of *Leishmania* (arrowhead). Quick Romanowsky-type stain, scale bar = 20 µm. (**B**) Spleen section presenting a cluster of parasitized macrophages (arrowhead). Hematoxylin and eosin, scale bar = 50 µm. (**C**) Spleen section submitted to *in situ* hybridization, where the localization of parasites is displayed by the deposition of dark-purple pigment (arrowhead). NBT/BCIP, scale bar = 50 µm.

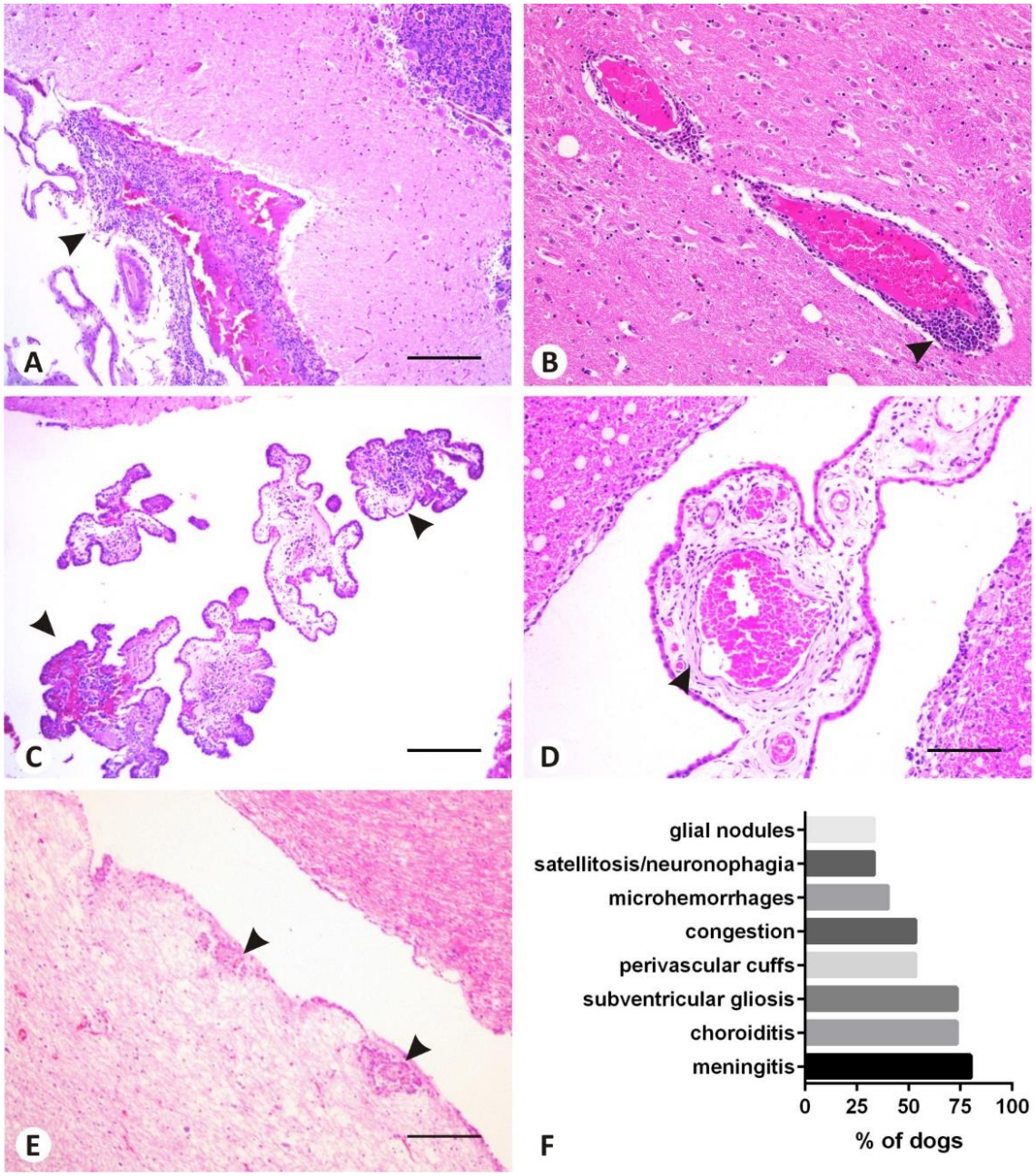

FIGURE 2 – Brain histopathological analysis in dogs with visceral leishmaniasis. (**A**) Cerebellar leptomeninge with intense mononuclear inflammatory infiltrate (arrowhead). (**B**) Mononuclear perivascular cuff in the thalamus (arrowhead). (**C**) Choroid plexus containing severe inflammatory infiltrate (arrowhead) and vascular congestion, leading to changes in its morphology. (**D**) Choroid plexus exhibiting mild inflammatory infiltrate and deposition of perivascular hyaline substance (arrowhead). (**E**) Subventricular gliosis (arrowhead) in the area of the lateral ventricle, in close contact with the ependymal lining. (**F**) Quantification of brain histopathological lesions in infected dogs (n=15). Hematoxylin and eosin, scale bar = 200 µm (A); 100 µm (B, C, E); 50 µm (D).

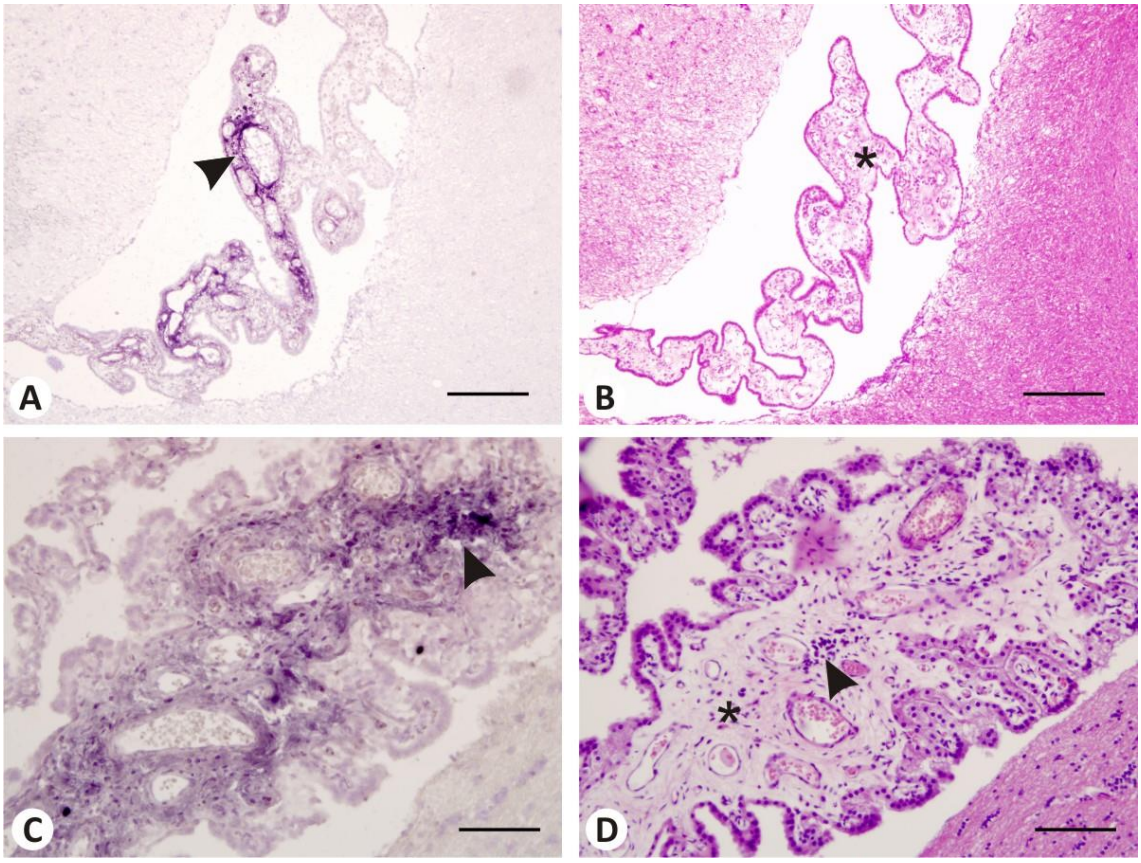

FIGURE 3 – *In situ* hybridization to detect *Leishmania* parasites in the brain of dogs with visceral leishmaniasis. The choroid plexus was the only structure presenting positive staining in the *in situ* hybridization analysis (arrowhead), but displaying only perivascular staining (**A, C**). In serial sections stained with hematoxylin and eosin (**B, D**), no parasite or parasitized cell was detected, but inflammatory infiltrate (arrowhead) and stroma thickening (*). NBT/BCIP, scale bar = 200 µm (A, B); 100 µm (C, D).

2.3.4 Leishmania DNA quantification in the brain

We were able to detect and quantify the parasite DNA in the brain of the infected dogs using qPCR. With large variability, the brain presented parasite loads ranging from $1.1x10^1$ to $6.6x10^3$ parasites/25mg of tissue (Figure 4). Nevertheless, only 53.3% (8/15) of the dogs presented values higher than the lower limit of positivity ($3.9x10^1$). There was no correlation regarding brain parasite load and the clinical stages. On the other hand, all the spleens of infected dogs were positives, ranging from $1.5x10^2$ to $7.2x10^5$ parasites/25 mg of tissue (cut-off value of $1.3x10^0$). Comparing brain and spleen, we noticed a positive correlation between parasite load in these organs (r=0.610; P=0.018).

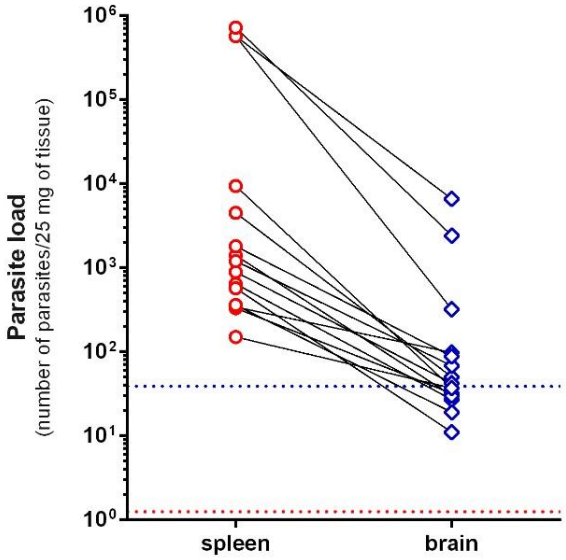

FIGURE 4 – Individual parasite load determination in the spleen and in the brain of dogs with visceral leishmaniasis. Black lines connect the spleen and the brain values of the same dog. The dotted lines represent the lower limit of positivity: spleen = red dotted line ($1.3x10^0$ parasites), brain = blue dotted line ($3.9x10^1$ parasites).

2.3.5 Chemokine gene expression in the brain and in the spleen of infected dogs

In order to evaluate the up- or down-regulation of selected chemokines gene expression in the brain and spleen of dogs with VL, we used the REST® method, which express how many times (fold changes) the target gene is more or less expressed in the infected dogs, compared to the control ones. Reaction efficiency values, determination coefficients and angular coefficients of each gene are shown in Table 1.

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

In the brain of infected dogs, only the gene expression of CCL-3 (19.9-fold), CCL-4 (16.2-fold) and CCL-5 (20.3-fold) were significantly up-regulated (Table 1; Figure 5), with strong positive correlation among them (Figure 6A-C). No correlation between the chemokines in the brain and clinical stage or parasite load was observed. As expected in a natural infection sampling, we observed important individual variability in all targets, nevertheless, different from the other targets, the distribution of CXCL-10 gene expression displayed a specific pattern in the infected dogs, with 10 dogs with expression values similar to the control dogs, and a small subpopulation (n=5) with evident higher gene expression. When evaluated separately, this subpopulation presented 83.8-fold more CXCL-10 expression than the controls (Figure 6D; P=0.002). Even in this particular case, we detected no correlation to the clinical stages, parasite load, or to other chemokines. CXCL-10 was correlated to CCL-2 in the spleen, and besides the absence of changes in the gene expression of CCL-2 in the brain, we observed a trend to positive correlation between CXCL-10 and CCL-2 in the brain (Figure 6E)

Table 1 – Reaction efficiency values (E), determination coefficients (r^2) and angular coefficients (slope) of the RT-qPCRs of each evaluated gene, and P-values regarding the relative gene expression of the target genes in the spleen and in the brain.

Target	E (%)	r^2	*slope*	P-value	
				spleen	brain
G3PDH	106,6	0,977	-3,173	-	-
RPL-32	100,2	0,994	-3,318	-	-
CCL-2	101,8	0,998	-3,279	0.000↑	0.337
CCL-3	99,1	0,999	-3,344	0.000↑	0.010↑
CCL-4	96,4	0,999	-3,412	0.002↑	0.007↑
CCL-5	101,7	0,992	-3,282	0.000↑	0.018↑
CCL-8	99,6	0,994	-3,330	0.000↑	0.353
CXCL-10	101,0	0,993	-3,299	0.000↑	0.388
CX3CL-1	99,4	0,995	-3,337	0.005↑	0.431
CX3CR-1	104,6	0,994	-3,217	0.168	0.700

↑ indicates significative up-regulation.

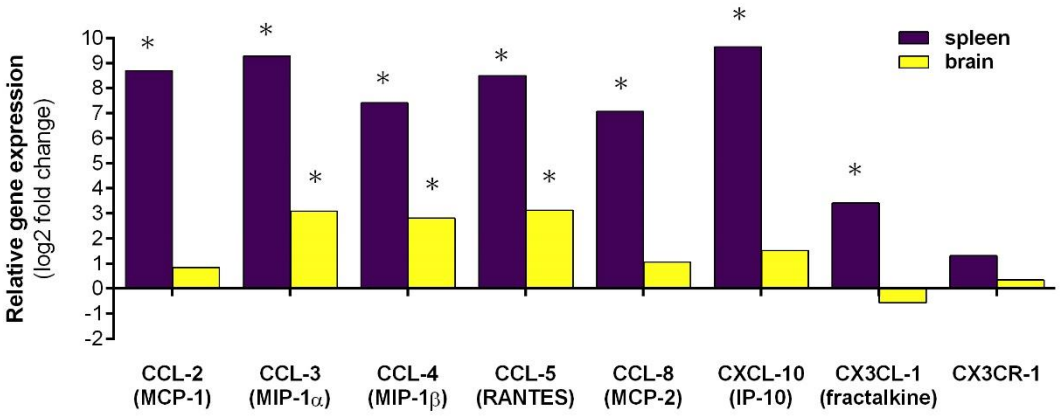

FIGURE 5 – Relative gene expression of chemokines in the spleen and in the brain of dogs with visceral leishmaniasis. The values are expressed as fold changes (log 2). Positive values indicate up-regulation and negative values indicate down-regulation, when compared to the control dogs. The normalization factor were the reference genes G3PDH and RPL-32. * indicates P<0.05.

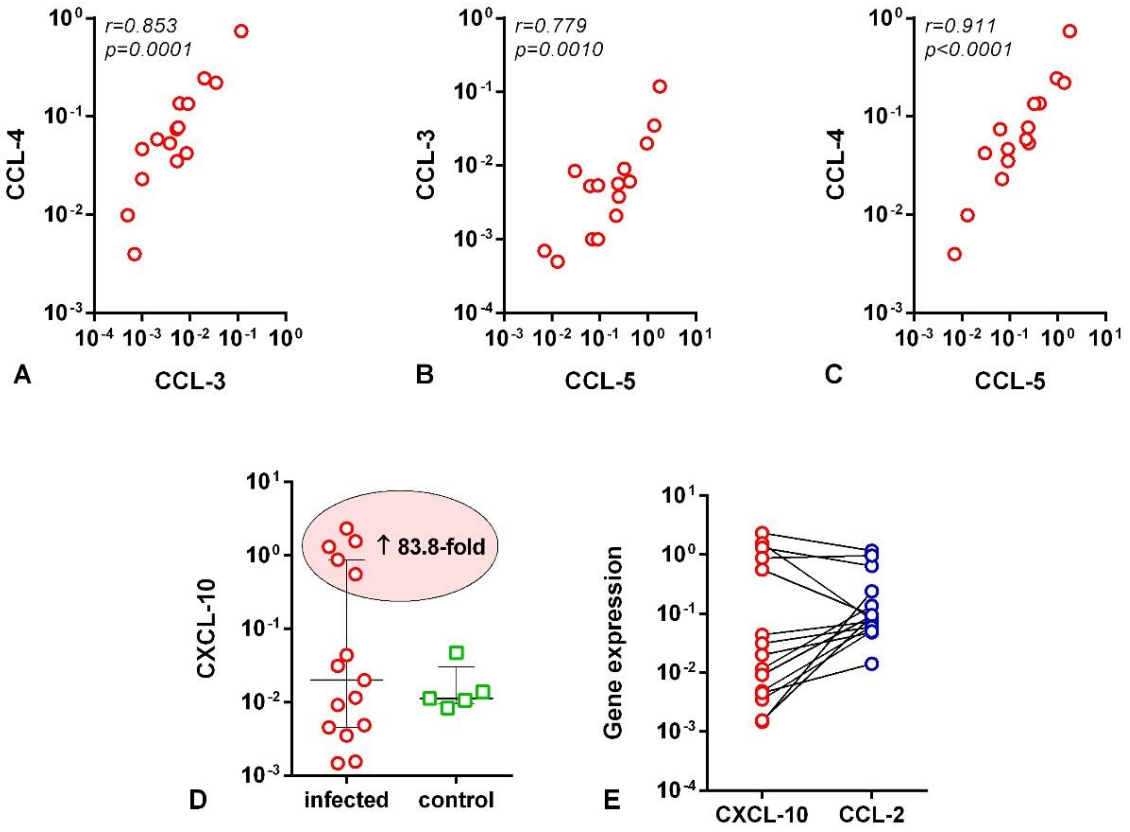

FIGURE 6 – Correlations between chemokines gene expression in the brain of dogs with visceral leishmaniasis. Correlations between CCL-3 and CCL-4 (A), CCL-3 and CCL-5 (B), CCL-4 and CCL-5 (C). The Spearman r and the p-values are shown on the plots. (D) Individual values of CXCL-10 gene expression in the brain of infected and control dogs, where it is possible to notice a subpopulation of 5 infected dogs with remarkable up-regulation (83.8-fold more). (E) Individual values of CXCL-10 and CCL-2 gene expression in the brain of infected dog, with a trend to positive correlation. Note that 3 out of the 5 infected dogs with the highest CXCL-10 expression also presented a trend to high CCL-2 expression.

2.4 DISCUSSION

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

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

Even though *in situ* hybridization reactions were effective to detect the parasite in the spleen, no parasite was observed in the brain. Nevertheless, we noticed perivascular staining exclusively at the choroid plexus, consistent with perivascular parasite DNA deposition. In oncology, a similar finding is named

Azzopardi phenomenon, which corresponds to DNA deposition around blood vessels in areas of tissue necrosis, since massive cell lysis releases large amounts of nucleic acids (PRITT; COOPER, 2003). To our knowledge, there is no description of Azzopardi phenomenon in infectious diseases; however, there are reports of free DNA fragments, smaller than 300 bp, circulating in biological fluids such as plasma, serum and urine. These fragments are called cell-free DNA, and they could be originated from the host cells death (self-DNA) or from microorganisms life cycle (replication, maturation and death) (DWIVEDI et al., 2012; GREEN et al., 2009), including *Leishmania* spp. (FRANCESCHI et al., 2007).

At histological examination, we did not detect parasites in the choroid plexus in the areas where *in situ* hybridization revealed perivascular *Leishmania* DNA deposition. The presence of parasite DNA in the brain was corroborated by qPCR, and even though positives, the average parasite load in the brain was low. Furthermore, the perivascular deposits in the choroid plexus were hyaline (Figure 2D) and not basophilic as expected for nucleic acids, nevertheless, small amounts of cell-free DNA, or DNA-containing immune complexes (ATKINS et al., 1972; FALANGOLA et al., 1994) may arrive to the choroid plexus stroma by vascular fenestrations and inflammatory endothelial lesions (WOLBURG; PAULUS, 2010), which could be enough to render positive a test based on DNA detection. High concentrations of circulating immune complexes have already been detected in VL, which has been correlated to glomerulonephritis and necrotizing vasculitis (ALVAR et al., 2004; BRANDONISIO et al., 1990).

The study of the immune response in the CNS during VL is infrequent and the evaluation of the chemokine profile in infected dogs is rare. Glial cells and even leukocytes within the inflammatory infiltrates may produce chemokines. We detected a specific pattern of chemokines expression, with up-regulation of CCL-3, CCL-4 and CCL-5 in the brain of infected dogs. CCL-5 is a well-characterized chemokine related to the recruiting and guidance of T lymphocytes towards the focus of inflammation. CCL-3 and CCL-4, despite being initially related to

macrophages and NK cell activation, they present a selective chemoattraction to CD8 and CD4 activated T lymphocytes, respectively (TAUB et al., 1993). Moreover, CCL-4 and CCL-5 promoted CD4 T lymphocytes adhesion to the endothelium activated by IFN-γ and TNF-α (QUANDT; DOROVINI-ZIS, 2004).

The interaction of these three chemokines, CCL-3, CCL-4 and CCL-5, is coherent with the predominance of T lymphocytes previously described in the brain of dogs with VL (MELO et al., 2009). In accordance, resistant mice presented low expression of CCL-3, CCL-4 and CCL-5, resulting in absence of CD8 T lymphocytes in the inflammatory infiltrate in a murine model of cerebral malaria (CLARK; PHILLIPS, 2011). The overexpression of CCL-5 by a recombinant rabies virus promoted severe blood-brain barrier disruption associated to chemokines production and inflammatory cells infiltration (ZHAO et al., 2009). Additionally, in murine toxoplasmic encephalitis, up-regulation of CCL-5 dependent on IFN-γ was described as protective, but when accompanied by up-regulation of CXCL-10 (WEN et al., 2010). IFN-γ-dependent CXCL10 was also essential to accumulation of T cells and parasites in the brain during experimental African trypanosomiasis (AMIN et al., 2009).

CXCL-10 presented an interesting pattern of expression in the infected dogs. Whereas 67.7% of the infected dogs presented no alterations for this chemokine, 33.3% (5/15) of them presented a remarkable overexpression of CXCL-10 in the brain, with no correlation with clinical stages or parasite load. The occurrence of up-regulation of CXCL-10, even in few dogs, is supported by up-regulation of IFN-γ, previously reported in the brain of dogs with VL (MELO et al., 2013). The production of CXCL-10 by astrocytes that have been previously activated by IFN-γ seems to be essential for the development of murine cerebral malaria (HUNT et al., 2014). IFN-γ also modulates CCL-2, and CXCL-10/CCL-2 have been implicated in systemic lupus erythematosus worsening (DOMINGUEZ-GUTIERREZ et al., 2014), and in brain inflammation during multiple sclerosis, with pro-inflammatory effects of CXCL-10 against anti-inflammatory effects of CCL-2 (SØRENSEN et al., 2001). The high variation of

CXCL-10 expression as well as the low average CCL-2 expression in the brain could be related to other individual factor not evaluated in this study, such as time of infection, similarly to what occurs in the spleen of experimentally infected dogs (STRAUS-AYALI et al., 2007).

CCL-8, CX3CL-1 and CX3CR-1 presented no alterations in the infected dogs; therefore, these chemokines apparently are not involved in the brain inflammation during VL. In the spleen, on the other hand, all chemokines presented up-regulation in the dogs with VL. Similarly, the spleens of naturally and experimentally infected dogs also presented overexpression of CCL-2, CCL-3, CCL-5 e CXCL-10 (STRAUSS-AYALI et al., 2007). Nascimento and colleagues (2013) detected up-regulation only for CXCL-10 in the spleen of naturally infected dogs, however, when compared asymptomatic and symptomatic dogs, CCL-2, CCL-5 and CXCL-10 were up-regulated and CCL-4 down-regulated. The liver of dogs with VL presented generalized decrease of chemokines expression (NASCIMENTO et al., 2013). CCL-2, CCL-4 and CCL-5 were overexpressed in the skin of dogs with VL, with positive correlation with the parasite load (MENEZES-SOUZA et al., 2011). In our study, none of the variables correlated with the clinical stage, however, all the dogs were symptomatic and we used a classification based on a four-point scale (SOLLANO-GALEGO et al., 2011), which could have dispersed the data, differently from the dichotomous classification asymptomatic-symptomatic.

This variation of chemokine patterns according to the organ corroborates not only the idea of immune compartmentalization during VL (REIS et al., 2009), providing a favorable environment to the accumulation of different cell populations, but also that the brain is a pro-inflammatory environment, propitious to a selective T lymphocytes accumulation, even in the absence of the parasite. Indeed, not the parasite itself, but rather its DNA seems to act as a trigger to brain inflammation, together with chronic systemic inflammation. Therefore, our data highlights the need to focus on brain involvement during VL, which has been neglected during the peripheral infection by *Leishmania* parasites.

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CAPÍTULO 3 – DESREGULAÇÃO DA BARREIRA HEMATOENCEFÁLICA DURANTE A LEISHMANIOSE VISCERAL CANINA

BLOOD-BRAIN BARRIER DISRUPTION DURING SPONTANEOUS CANINE VISCERAL LEISHMANIASIS

ABSTRACT – Visceral leishmaniasis is a complex disease caused by *Leishmania donovani* and *Leishmania infantum* with worldwide distribution. In dogs, besides the classical symptoms of the disease, there are descriptions of inflammatory alterations in the brain, neurological symptoms and in some rare cases, the presence of the parasite. Brain inflammation is a strict controlled process and since the brain counts on the efficiency of the blood-brain barrier (BBB), we aimed to assess BBB integrity in dogs with spontaneous visceral leishmaniasis. To this end, we evaluated markers in the cerebrospinal fluid (CSF) and in brain tissue related to BBB disruption and brain inflammation. Albumin quota revealed BBB breakdown in most of the dogs, corroborated by elevated concentrations of anti-*Leishmania* antibodies in the CSF along with negative IgG index. In the brain, albumin and IgG staining formed halos around blood vessels, classical visualization of BBB leakage. Soluble IgG was also detected in the choroid plexus and ependyma, and in these structures, IgG stained random resident cells (epithelial and ependymal cells, respectively), which could be related to excess of IgG in the CSF. IgG^+ cells and $Fc\gamma\text{-}RI^+$ cells were identified in the choroid plexus, ependymal area, and perivascular in the brain parenchyma. CSF cell count was elevated. Infected dogs also presented increased serum concentrations of leukotriene B4. Accordingly, we presented herein data that support the occurrence of BBB disruption in dogs with spontaneous visceral leishmaniasis, and state IgG as a key molecule capable to initiate and/or maintain the inflammatory stimuli in the nervous milieu, with the CSF acting as an important disseminator of inflammatory stimuli within the CNS.

KEYWORDS: albumin quota, central nervous system, cerebrospinal fluid, choroid plexus, Fc gamma receptors, immunoglobulin G

3.1 INTRODUCTION

Leishmaniasis is a complex disease caused by more than 20 species from the *Leishmania* genus transmitted by more than 30 species of sand flies, which present four different types of presentation: visceral leishmaniasis (VL), cutaneous leishmaniasis, muco-cutaneous leishmaniasis, and post-kala-azar dermal leishmaniasis (CHAPPUIS et al., 2007). *L. donovani* and *L. infantum* (syn=*chagasi*) are the species related to VL (BANETH et al., 2008; LUKEŠ et al., 2007).

In dogs, VL frequently causes a systemic disease, generally chronic and sometimes subclinical, presenting with irregular fever, anemia, cachexia, and skin, renal and ocular diseases (ALVAR et al., 2004). Despite common descriptions of systemic lesions, few data of brain lesions are reported. During VL in dogs, evidences of inflammatory alterations in the brain are described, such as meningitis, choroiditis, glial cells activation, T lymphocytes accumulation and pro-inflammatory cytokines, including, in some rare cases, the presence of the parasite (IKEDA et al., 2007; MÁRQUEZ et al., 2013; MELO; MACHADO, 2011; MELO et al., 2013; VIÑUELAS et al., 2001). Altogether, these alterations are supportive that the blood-brain barrier (BBB) may be damaged.

BBB is composed of specialized non-fenestrated endothelial cells that possess intercellular tight junctions. It is a barrier between blood vessels lumen and the neuropil. Additional defense mechanisms are present, such as pericytes, which share the basal membrane with the endothelial cells, perivascular macrophages and astrocytes end-feet, recovering all the extension of the BBB (BALLABH et al., 2004; GALEA et al., 2007; SAUNDERS et al., 2008). BBB is present in almost the entire central nervous system (CNS), except in the choroid plexus, where the blood-CSF barrier is located, and in the circumventricular organs, which are neuroendocrine and sensorial areas adjacent to the cerebral ventricles (SCHULZ; ENGELHARDT, 2005; WOLBURG; PAULUS, 2010).

Choroid plexus is considered the main producer of the cerebrospinal fluid (CSF). Recently, it was described that the CSF circulates throughout the CNS, being not restricted to the ventricles and subdural space, but including the perivascular space (Virchow-Robin space), where it may occur drainage, clearance of molecules and interaction with the immune system (BRINKER et al., 2014). There are hypothesis that the functional unity of the BBB, the capillary-astrocyte unity, may take part in the active production of interstitial fluid, the latter being component of the CSF (ABBOTT, 2004), in a way that the CSF may reflect the general state of the CNS, and not only changes in the choroid plexus and in the blood-CSF barrier.

Albumin in a serum protein present in minimal concentrations in the CSF, and it is largely used as a marker of BBB integrity (SORJONEN et al., 1987). Immunoglobulin G (IgG), besides being a further marker of BBB integrity, takes a role in the induction of immune response within the CNS, especially by interaction with the receptors Fcγ-R (NIMMERJAHN; RAVETCH, 2008; SIBÉRIL et al., 2007). The Fcγ-RI (also known as CD64) is an activator receptor, stimulating phagocytosis, cytokine release and antibody-dependent cellular cytotoxicity (ADCC). Fcγ-Rs are surface receptors present in monocytes/macrophages, dendritic cells and T lymphocytes, and within the CNS, they have already been identified in neurons, astrocytes, microglia and oligodendrocytes (OKUN et al., 2010). These receptors bind both monomeric IgG and immune complexes, and immune complexes have been implicated as causative of vascular permeability and increased leukocyte adhesion at the brain microvasculature (LISTER; HICKEY, 2006).

Further, other inflammatory molecules involved in the immune response that could act as BBB openers are the eicosanoids PGE2 and LTB4, and the nitric oxide (BLACK; HOFF, 1985; JAWOROWICZ et al., 1998). Eicosanoids are metabolites of the arachidonic acid involved in physiological and pathological responses, with differences according to the enzyme that originates them: cyclooxygenase 2 (COX-2) produces PGE2 whereas 5-lipoxygenase (5-LOX)

generates LTB4 (DAUGSCHIES; JOACHIM, 2000). Three different enzymes can produce nitric oxide, but under inflammatory stimuli, it is produced by the inducible nitric oxide synthase (iNOS) (JAWOROWICZ et al., 1998).

In view of the paucity of data regarding BBB in dogs, and due to evidences of BBB disruption during *Leishmania* infection, we aimed to evaluate BBB integrity measuring albumin and IgG concentrations in the CSF and localizing these molecules in brain tissue as markers of BBB permeability. We also aimed to detect the inflammatory mediators PGE2, LTB4 and nitric oxide and their producing enzymes in serum and CSF as well as the receptor Fcγ-RI in the brain of dogs with spontaneous VL.

3.2 MATERIALS AND METHODS

3.2.1 Animals and sampling

Fifteen naturally infected dogs, seven males and eight females, ranging in age from 1 to 4 years old were selected from the Zoonosis Control Center in the municipality of Araçatuba, São Paulo State, Brazil, which is an endemic area for VL. We collected peripheral blood (without anticoagulant), and cerebrospinal fluid samples by puncture of the cisterna magna. Dogs were then euthanized with owners' permission, according to the recommendations of the current VL control program (SÃO PAULO, 2006). VL diagnosis was achieved using an indirect ELISA (LIMA et al., 2005) and qPCR in spleen samples (MELO et al., 2013). We performed the necroscopic examinations immediately after euthanasia. We collected the brains and separated them in two hemispheres. One hemisphere was collected and stored in 10% buffered-formalin. After fixation, coronal sections were made and samples containing cerebral cortex, thalamus, hippocampus, pons-medulla oblongata, cerebellum, the ventricular choroid plexi and periventricular white matter were paraffin-embedded, sectioned (5 μm) and

submitted to immunohistochemical analyses. Five uninfected dogs which death was not related to brain disease were included as control.

3.2.2 Cerebrospinal fluid evaluation

To evaluate the integrity of the blood-brain barrier, we calculated the albumin quota (AQ) after routine determination of the albumin concentration in the serum and in the CSF (both expressed in mg/dL), using the formula $\left\{\frac{CSF\ albumin}{serum\ albumin} \times\ 100\right\}$ (SORJONEN, 1987), considering positives values higher than 0.64 (Gama et al., 2007). We used an indirect ELISA to determine the concentration of anti-*Leishmania* antibodies in the CSF (LIMA et al., 2003), and in order to determine the intrathecal production of IgG, we used the formula $\left\{\frac{CSF\ IgG}{serum\ IgG} \times \frac{serum\ albmin}{CSF\ albumin} \times 100\right\}$ after determination of serum and CSF IgG concentration using a commercial kit (20.011.00, BioTécnica, Varginha, MG, Brazil), with the cut-off value of 0.9 (MEJÌAS et al., 2008).

We also performed paired ELISA tests in CSF and in serum to detect the concentrations of PGE2 (KGE004B, R&D Systems, Cambridge, MA, USA), LTB4 (KGE006B, R&D Systems) and nitric oxide (nitrate/nitrite, KGE001, R&D Systems).

3.2.3 Cytology and immunofluorescence

We counted CSF cells in Neubauer chamber and analyzed using cytospin. We added 200 µL of CSF plus 10 µL of 22% (w/v) bovine serum albumin (BSA) into a cytoblock and cytocentrifuged at 150 × g for 5 min. The slides were air-dried and stained with a quick Romanowsky-type stain (Panótico rápido®, Laborclin, Pinhais, PR, Brazil) or fixed in 4% (w/v) paraformaldehyde for 15 min, washed in phosphate-buffered saline (PBS) for 5 min, air-dried and then frozen at -20°C. We collected paired spleen smears, fixed in paraformaldehyde and frozen at -20°C to serve as control.

Fixed CSF cells and spleen smears were submitted to immunofluorescence assays to detect the enzymes COX-2, 5-LOX and iNOS. Briefly, the slides were thawed and washed in PBS. Non-specific binding was blocked with 1% (w/v) BSA in PBS for 30 min. To detect COX-2, we used the polyclonal goat-anti-COX-2 (1:50, ab23672, Abcam, Cambridge, MA, USA), for 5-LOX detection, we used the polyclonal rabbit anti-5-LOX (1:50, ab39347, Abcam) and to detect iNOS, we used the polyclonal rabbit anti-iNOS (1:50, ab3523, Abcam). The primary antibodies were incubated for 14–16 hours at 4°C in a humidified chamber. Afterwards, we washed the slides in PBS and incubated with the following secondary antibodies: anti-goat IgG conjugated to the red fluorophore DyLight®550 (1:100, ab96928, Abcam) or anti-rabbit IgG conjugated to DyLight®550 (1:100, ab96884, Abcam) for 1 hour in the dark. The slides were then washed with PBS and the nuclei were stained with DAPI (4',6-diamidino-2-phenylindole, D9542, Sigma-Aldrich, Saint Louis, MO, USA) at final concentration of 1 µg/mL for 15 min, washed once more and mounted with coverslips.

3.2.4 Immunohistochemistry

Brain sections were submitted to immunohistochemical stain to detect and quantify IgG, albumin and Fcγ-RI. The sections were deparaffined and hydrated. The antigen retrieval was achieved by steam heating in 0.1M citrate buffer, pH 6.0 for 30 min. For inhibition of endogenous peroxidase, slides were incubated with 2% (v/v) hydrogen peroxide 30 vol. diluted in 50% (v/v) methanol for 30 min, and non-specific binding was blocked with 3% (w/v) non-fat dry milk in PBS for 30 min. For IgG detection, we used the rabbit polyclonal anti-dog IgG (1:100; A6792, Sigma-Aldrich), for albumin, the goat polyclonal anti-canine albumin (1:100; ab112986, Abcam), and for Fcγ-RI, the rabbit polyclonal anti-human Fcγ-RI (1:50; ab104273, Abcam), previously standardized in human tonsil and canine lymph node sections. The primary antibodies were applied for 14-16 hours at 4°C in a humidified chamber. Slides were washed in PBS, incubated with a biotinylated secondary antibody (LSAB+ Kit K0690, Dako, Carpinteria, CA, USA)

for 45 min at room temperature, washed once more with PBS, and incubated with streptavidin-horseradish peroxidase (HRP) complex (LSAB+ Kit K0690, Dako) for 45 min at room temperature. These steps were not performed for IgG and albumin since the primary antibodies were conjugated to HRP. The reaction was developed with DAB (3,3'-diaminobenzidine, K3468, Dako). The slides were then counterstained with Harris's hematoxylin, dehydrated, cleared, and mounted with coverslips. We examined the sections using light microscopy and the positive-stained area was measured by means of a computerized image-analysis software (Image-Pro Plus 6.1, Media Cybernetics, Rockville, MD, USA).

3.2.5 Statistical analyses

Significant differences between the infected and control groups were determined by Mann-Whitney test, and correlation was assessed by the Spearman test. Values of $P<0.05$ were considered statistically significant. Data were expressed as the median and the interquartile range. All statistical analyses were performed using the Prism software (v6.05, GraphPad, La Jolla, CA, USA).

3.2.6 Ethical issue

All procedures were realized according to the Brazilian College of Animal Experimentation, and approved by the Institutional Ethics Committee (CEUA, FMVA-UNESP, process #2012-01093).

3.3 RESULTS

3.3.1 Albumin and IgG in the cerebrospinal fluid as markers of blood brain barrier integrity

In the serum, all infected dogs presented hypoalbuminemia (Figure 1A). Regarding the CSF, all samples were clear and colorless. CSF albumin

concentration was elevated in 66.7% (10/15) of the infected dogs (Figure 1B). The same infected dogs (66.7%) presented values of albumin quote higher than cut-off (Figure 1C), related to BBB disruption.

Regarding IgG, 46.7% (7/15) of the infected dogs presented elevated anti-*Leishmania* antibody titers in the CSF, and two further animals (13.3%) presented antibody concentration near the cut-off value (Figure 1D). The IgG index was negative in all animals (Figure 1E), indicating that these antibodies proceeded from the serum, and consequently absence of intrathecal synthesis. A further evidence of the serum origin of the CSF antibodies was the positive correlation between anti-*Leishmania* antibody concentration in the CSF and in the serum ($r=0.782$, $P=0.0009$; Figure 1F).

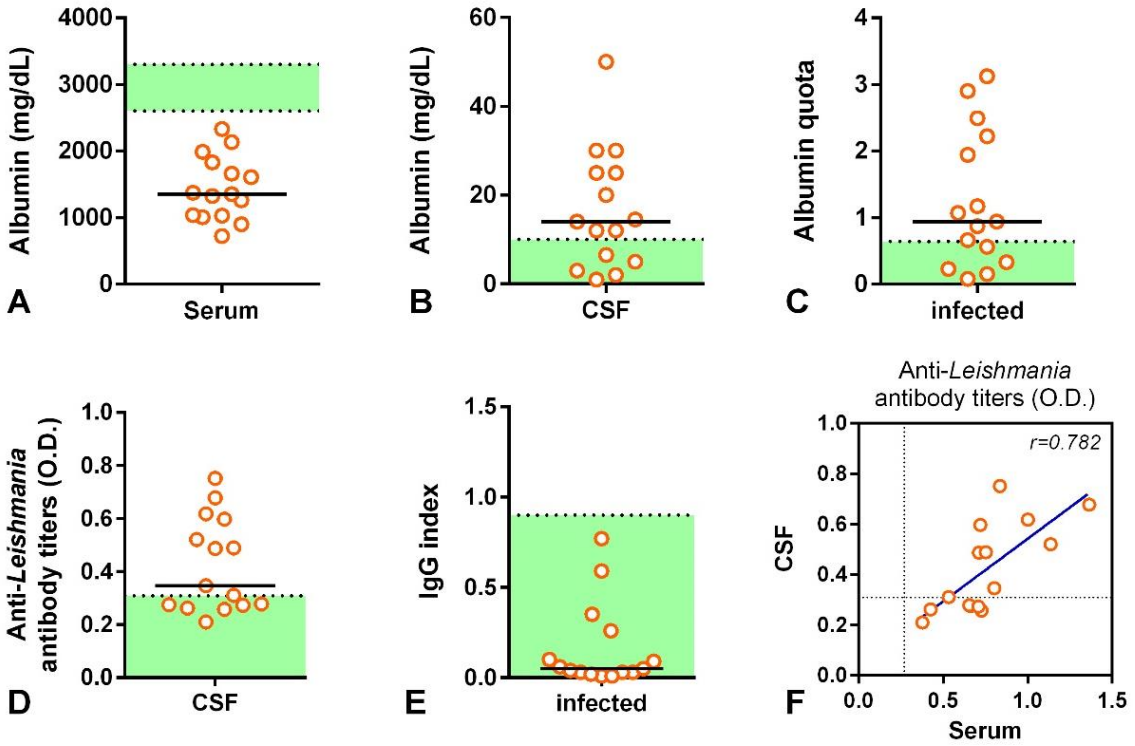

FIGURE 1 – Albumin, albumin quota and antibody concentration determination in the cerebrospinal fluid of dogs with visceral leishmaniasis. Albumin concentrations were determined in the serum (**A**) and in the CSF (**B**), and the albumin quota was stablished in the CSF of infected dogs (**C**). Concentration of specific anti-*Leishmania* antibodies (**D**) and IgG index determination (**E**) in the CSF of infected dogs. Horizontal line indicate the median. The green crosshatched areas indicate normal reference values. **F:** Correlation of anti-*Leishmania* antibodies in the CSF and in the serum. The dotted lines represent the cut-off values. O.D.: optical density.

3.3.2 Visualizing BBB disruption using IgG and albumin immunohistochemistry

IgG staining forming halos around brain parenchymal blood vessels is indicative of IgG leakage and consequently BBB disruption (Figure 2A). Occasionally, in the middle of the perivascular IgG staining, we detected positively stained mononuclear cells (Figure 2B) and glial cells (Figure 2C). Dogs with VL presented an important increase in perivascular IgG detection (P=0.0186), with median value of 1.1% of immunostained area, in comparison of control uninfected dogs (0.2%) (Figure 2D-G). In the same manner of IgG, we detected perivascular albumin around blood vessels (Figure 3A-B), revealing BBB opening in dogs with VL (P=0.0002), with a median stained value of 6.9% in the infected dogs and 0.2% in the control dogs (Figure 3C).

Compared to albumin, IgG was a more versatile marker, since IgG presented a widespread distribution within the brain, besides perivascular halos. We observed important accumulation of IgG in the choroid plexus (Figure 4). In this structure, due to the lack of tight junctions in the blood vessels (absence of BBB), IgG can be normally present in the choroid plexus stroma (Figure 4A), interfering with colorimetric analyses; therefore, we did not quantify IgG in this structure. Nevertheless, in the choroid plexus stroma of infected dogs, we noticed important IgG staining associated to connective tissue thickening (Figure 4B). The choroid plexus also harbored important quantities of IgG positive mononuclear cells (Figure 4C), possibly B lymphocytes/plasma cells. Further, we noticed the presence of epithelial cells positively stained for IgG, randomly distributed along the choroid plexus epithelium in 64.3% of the dogs (Figure 4D), even in the absence of inflammatory cells.

IgG also played a role at the ependymal region. Uninfected dogs presented no IgG staining at the subventricular/ependymal area (Figure 5A), while infected dogs presented a median stained area of 2.4%, significantly higher (P=0.0001; Figure 5B). In this area, infected dogs presented IgG staining in the border tissue under the ependymal layer (Figure 5C) and accumulation of IgG-

positive mononuclear cells (Figure 5D). Occasional ependymal cells randomly distributed throughout the ependymal lining were positive to IgG in 73.3% of the dogs (Figure 5D).

3.3.3 Fcγ-RI and a potential inflammatory role of IgG in the brain

Due to the intense detection of IgG in the brain of dogs with VL, and since this molecule is also involved in the immune response; we decided to analyze the presence of Fcγ-RI. At the ependymal surface, this receptor is expressed at basal levels in uninfected dogs (Figure 6A), but its production is increased during VL, mainly related to mononuclear cells (Figure 6B). Infected dogs presented a median immunostained area of 1.2%, higher (P=0.0192) than the 0.7% of immunostained area observed in the controls (Figure 6C). In a different pattern, when detected in the brain parenchyma, Fcγ-RI was identified in glial cells, possibly astrocytes (Figure 6D-E), or in mononuclear perivascular cuffs (Figure 6F). This receptor was also associated to mononuclear cells in the choroid plexus as well (Figure 6G).

3.3.4 Eicosanoids and oxide nitric

Since IgG and albumin are markers of BBB disruption, we decided to evaluate agents that could actively disrupt the BBB. Using commercial ELISA kits, we determined the paired serum and CSF concentrations of PGE2, LTB4 and NO. PGE2 presented no differences between infected and control dogs, neither in serum (P=0.8177) nor in CSF (0.1371), but it is a mediator presented in higher concentrations in the serum compared to CSF (P<0.0001; Figure 7A). LTB4 presented increased concentration in the serum of infected dogs (P=0.0397), but no changes were detected in the CSF (P=0.9189; Figure 7B). In a similar manner as in PGE2, concentrations of LTB4 were higher in serum than in CSF (P<0.0001). In an opposite way, NO concentrations were rather similar in serum (P=0.7584) and in CSF (P=0.5966) of both groups, with no differences

between them (P=0.8394; Figure 7C). Regarding the enzymes, COX-2 was identified in few single cells in spleen smears (Figure 7D). On the other hand, 5-LOX was intensely noticed in spleen smears, most of the times in the cytoplasm of parasitized cells (Figure 7E). Staining for iNOS was negative. None of these enzymes were detected in the cells from CSF.

3.3.5 CSF cell population

From the CSF samples of infected dogs submitted to cytological analyses, all of them presented pleocytosis (over 5 cells/μL), with predominance of monocytes and lymphocytes (Figure 7F). Cell population were highly variable, with a median value of 76.3 cells/μL, ranging from 8.9 to 256.7 cells/μL.

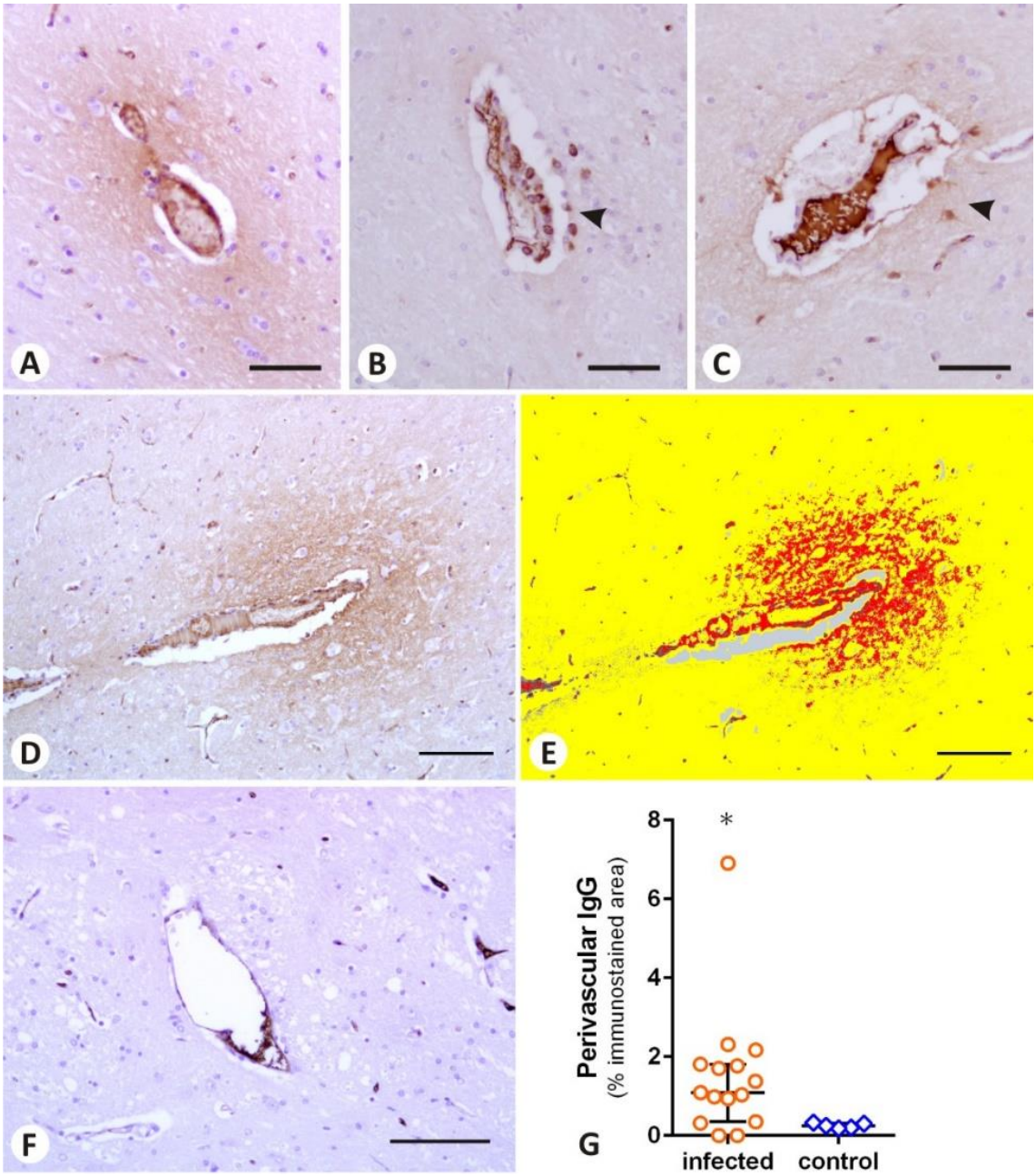

FIGURE 2 – Perivascular IgG in the brain of dogs with visceral leishmaniasis. Different staining patterns observed; (**A**) perivascular IgG at the interstitial space, (**B**) mononuclear perivascular cells (arrowhead), possibly B lymphocytes/plasma cells, and (**C**) perivascular glial cells (arrowhead). Perivascular IgG leakage forming halos (**D**) is indicative of BBB disruption and can be quantified by computerized image analyses (**E**). In the control uninfected dogs (**F**), IgG only stained serum, inside blood vessels. Immunoperoxidase; scale bar = 50 µm. (**G**) Quantification of perivascular IgG. Horizontal lines indicate the median and interquartile range. * indicates P<0.05.

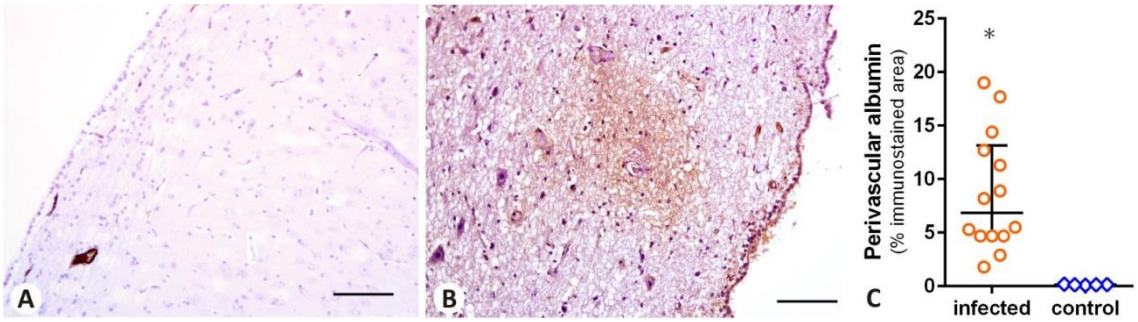

FIGURE 3 – Perivascular albumin in the brain of dogs with visceral leishmaniasis. (**A**) Uninfected control dogs presented albumin staining restrict to the interior of blood vessels, whereas infected dogs (**B**) presented intense staining in the interstitial space around and outside blood vessels. Immunoperoxidase; scale bar = 50 µm. (**C**) Quantification of perivascular albumin. Horizontal lines indicate the median and interquartile range. * indicates $P<0.05$.

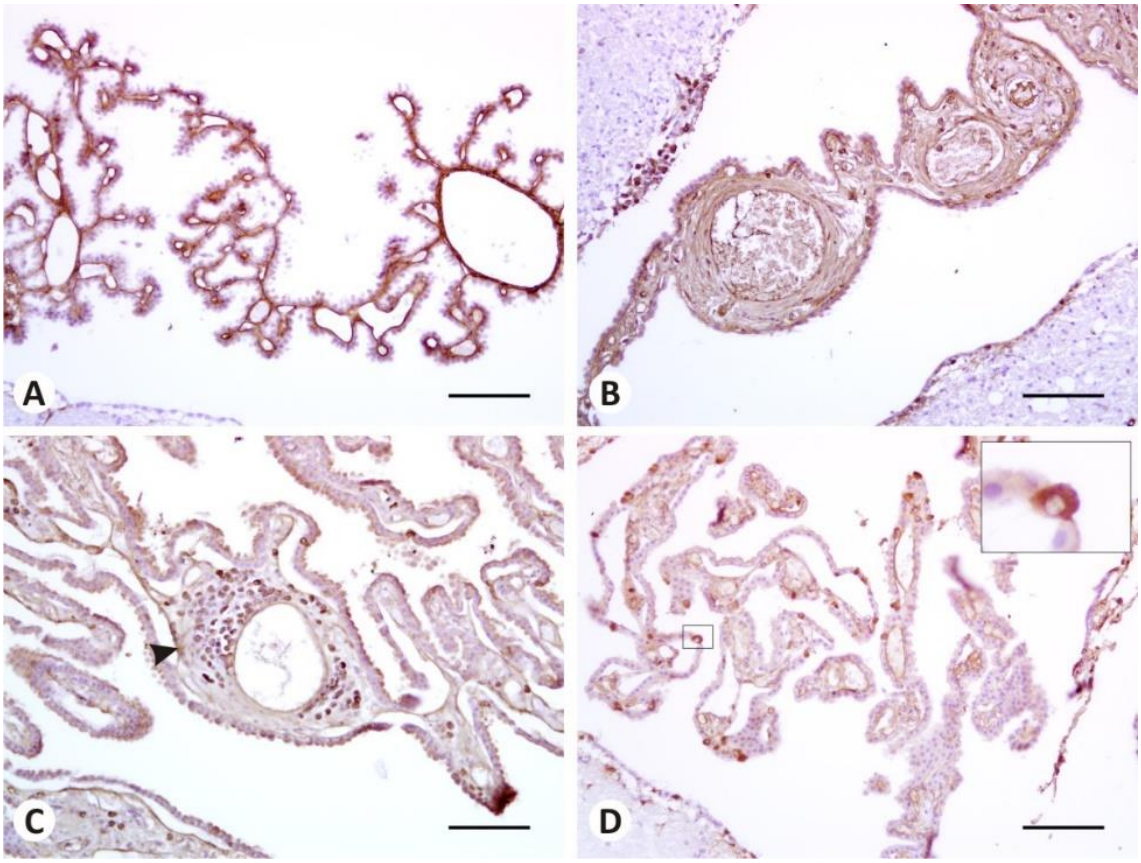

FIGURE 4 – IgG staining patterns in the choroid plexus of dogs with visceral leishmaniasis. (**A**) Choroid plexus of uninfected control dog present positive IgG staining in the stroma, but no morphological alterations are seen. Choroid plexus of infected dogs exhibiting (**B**) IgG staining associated to stroma thickening and (**C**) accumulation of IgG-positive mononuclear cells (arrowhead). (**D**) Morphologically normal choroid plexus exhibiting specific epithelial cells positively stained for IgG, randomly distributed along the choroid plexus epithelium (*inset*). Immunoperoxidase; scale bar = 50 µm.

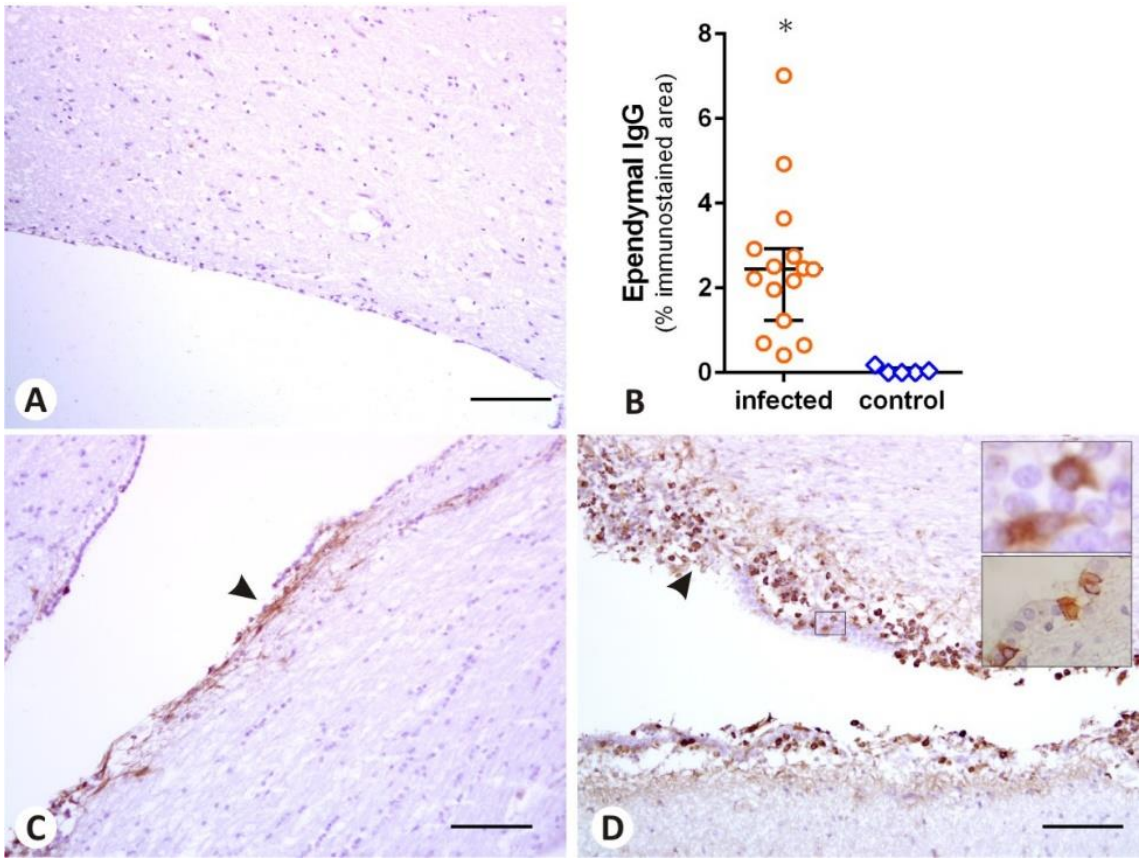

FIGURE 5 – IgG staining patterns in the ependymal area of dogs with visceral leishmaniasis. (**A**) Uninfected control dog presenting no IgG staining in the ependymal area. (**B**) Quantification of IgG at the subventricular/ependymal area. Horizontal lines indicate the median and interquartile range. * indicates P<0.05. (**C**) Soluble IgG (arrowhead) identified at the border of the brain tissue, just under the ependymal layer. (**D**) Intense accumulation of IgG mononuclear cells (arrowhead) under the ependymal layer, and presence of scattered ependymal cells positives to IgG (*inset*). Immunoperoxidase; scale bar = 50 µm.

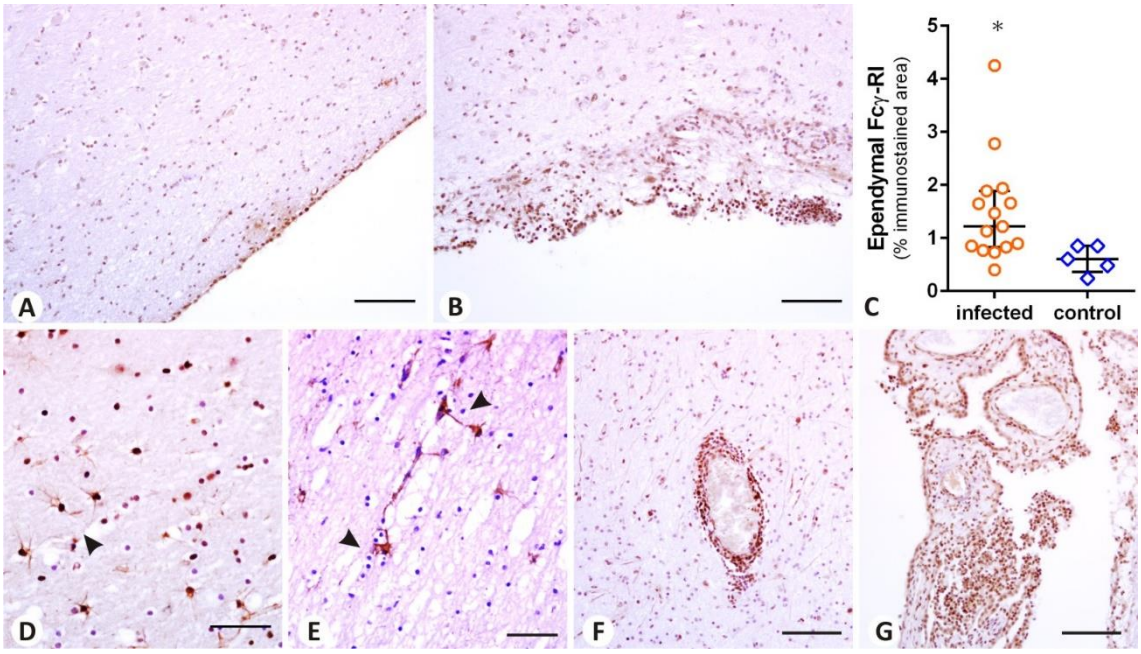

FIGURE 6 – Fcγ-RI staining patterns in the brain of dogs with visceral leishmaniasis. (**A**) Uninfected control dog exhibiting discrete Fcγ-RI at the ependymal surface. (**B**) Increased Fcγ-RI staining at the ependymal surface, among mononuclear cells accumulation. (**C**) Quantification of Fcγ-RI at the subventricular/ependymal area. Horizontal lines indicate the median and interquartile range. * indicates P<0.05. Within the brain parenchyma, positive Fcγ-RI staining in (**D**) protoplasmic astrocytes, in (**E**) perivascular fibrous astrocytes, and in (**F**) mononuclear perivascular cuffs. (**G**) Fcγ-RI positive mononuclear cells accumulation in the choroid plexus. Immunoperoxidase; scale bar = 50 μm (A, B, F, G); 20 μm (D, E).

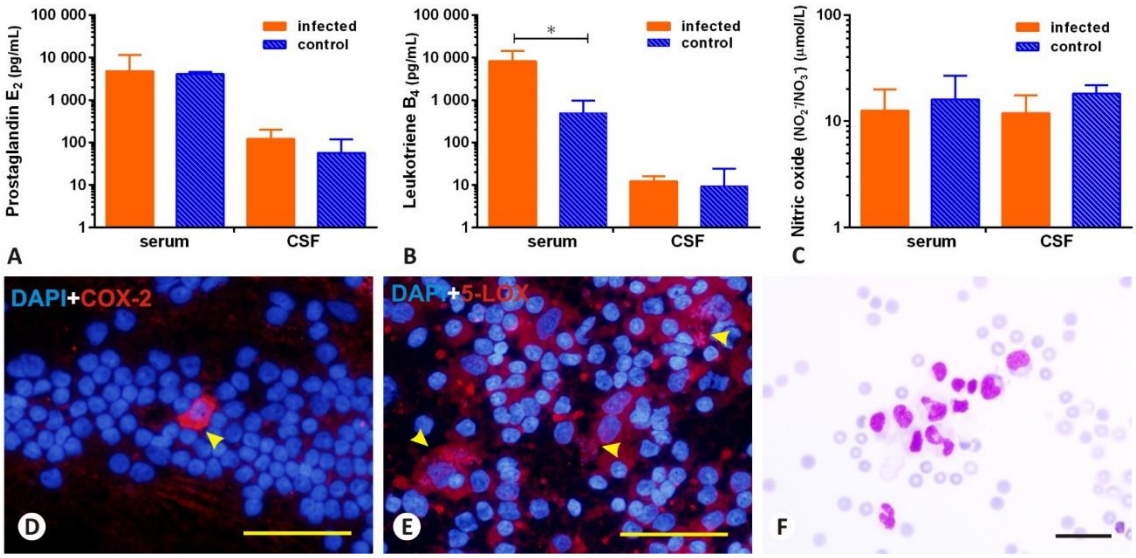

FIGURE 7 – Eicosanoids and oxide nitric in serum and CSF of dogs. Paired determination of prostaglandin E2 (**A**), leukotriene B4 (**B**) and nitric oxide (**C**) concentrations in serum and CSF of dogs infected with VL and uninfected control dogs. Bars indicate the median and interquartile range. * indicates P<0.05. (**D**) Single cell in a spleen smear from an infected dog with positive COX-2 staining (arrowhead). (**E**) Intense staining for the enzyme 5-LOX in a spleen smear, generally in parasitized cells (arrowhead). (**F**) Representative cytology of the CSF from an infected dog, exhibiting pleocytosis with monocytes and lymphocytes. Scale bar = 50 µm (D, E); 20 µm (F).

3.4 DISCUSSION

The blood-brain barrier is an important structure that helps giving the brain the immune privilege. An important marker of BBB function is albumin, by means of the albumin quota. This serum protein (~65 kDa) is produced predominantly in the liver and its presence in the CSF is indicative of serum proteins leakage into the CNS, and consequently loss of BBB integrity (SORJONEN, 1987). By the albumin quota, we detected an expressive number of infected dogs with evident BBB disruption. In dogs, inflammatory, infectious or neoplastic brain diseases tend to elevate the values of albumin quota (BEHR et al., 2006; GAMA et al., 2007), and during VL, we have previously detected other evidences of BBB breakdown, such as overexpression of IL-1β and TNF-α, activity of matrix metalloproteinases, and T-cell accumulation in the brain (MELO et al., 2009; 2012; 2013).

An additional protein that could be related to BBB dysfunction is IgG (~150 kDa), detected in the CSF, choroid plexus and brain parenchyma of dogs with VL. We observed elevated concentrations of anti-*Leishmania* antibodies, but with a negative IgG index, which indicates that there was no intrathecal antibody synthesis (MEJÍAS et al., 2008), and that the CSF IgG was originated from peripheral blood. Indeed, perivascular IgG staining, in format of halos, is associated to IgG leakage from blood; and in this case, it may be even absorbed by perivascular astrocytes or neurons (RIGAU et al., 2007). It has also been demonstrated that IgG binding to astrocytes modifies the expression of aquaporin-4 and promotes BBB dysfunction during neuromyelitis optica (VINCENT et al., 2008).

IgG staining may also occur in epithelial cells from the choroid plexus, related to transcellular transport from blood to CSF (SIMARD et al., 2011); in a similar manner that occurs in the immature brain (SAUNDERS et al., 2012). In dogs with VL, we observed positive IgG staining in scattered epithelial cell along the choroid plexus epithelium, in the edge of the brain tissue just under the

ependyma, and in random ependymal cells. These findings could be related to the excess of IgG in the CSF, and in the latter case, as occurs in the immature brain, ependymal cells are able to capture exceeding proteins from the CSF, with more intense activity after inflammatory stimuli (SAUNDERS et al., 2012).

The choroid plexus is involved in the communication between periphery and the CNS. It rapidly responds to peripheral injuries and acts as a source of leukocytes (PETITO; ADKINS, 2005). Considered as the main route for leukocyte migration into the brain, cells arriving to the choroid plexus can reach the CSF and then the brain through ependyma, but the opposite way is true, when cells originated in the brain parenchyma reach the CSF through the ependyma (KLEINE; BENES, 2006; PETITO; ADKINS, 2005). Once in the CSF, leukocytes could spread to everywhere in the CNS. Herein we detected important amounts of IgG^+ inflammatory cells, related mainly to B-lymphocytes and plasma cells, corroborating a previous study of our research group where we have detected significant numbers of B-lymphocytes (using the CD79α antibody) in the brain of infected dogs, especially in the choroid plexus (MELO et al., 2009);

Additionally, the choroid plexus may be site of antibodies and immune complexes deposition (FALANGOLA et al., 1994). The occurrence of elevated concentrations of circulating immune complexes has already been described during VL (BRANDONISIO et al., 1994), which has been correlated to glomerulonephritis and necrotizing vasculitis (ALVAR et al., 2004). IgG and immune complex deposition in the choroid plexus may act as a trigger to inflammation. Consistent with the remarkable presence of IgG in the CNS of dogs with VL, we noticed increased populations of cells with the IgG receptor Fcγ-RI, mainly in astrocytes and mononuclear cells within the inflammatory infiltrate.

In a murine model of transient BBB disruption induced by adrenalin, increased expression of Fcγ-RI in astrocytes and microglia has been described, associated to an anti-inflammatory response mediated by IL-10 (LI et al., 2008). Conversely, the expression of IL-10 in the brain of dogs with VL was down-regulated (MELO et al., 2013), with pro-inflammatory consequences, possibly

related to a prolonged BBB disruption. Additionally, microglial activation via Fcγ-Rs may stimulate the production of the chemokines in human and murine *in vitro* models (SONG et al., 2002).

BBB disruption may be due to interaction with other mediators. 5-LOX and LTB4 are related to parasite control for different *Leishmania* species (LEFÈVRE et al., 2013; REINER; MALEMUD, 1984; SEREZANI et al., 2006). Despite not being detected in the CSF, LTB4 concentrations were elevated in the serum of infected dogs. Serum LTB4 may play a role in BBB disruption, as observed in rats with cerebral ischemia (JUAN et al., 2009). On the other hand, PGE2 and nitric oxide are apparently not involved in the pathogenesis of brain lesions during VL.

Despite the efficiency of the BBB, this is not an insurmountable barrier. Several pathogens have been identified as capable of crossing the BBB, such as *Borrelia burgdoferi*, *Cryptococcus neoformans, Neisseria meningitidis, Toxoplasma gondi* and *Trypanosoma brucei* (CHARLIER et al., 2005; GRAB et al., 2005; LACHENMAIER et al., 2011; MASOCHA et al., 2007; NASSIF et al., 2002), however, in several diseases, brain lesions occur due to the presence of inflammatory cells and not a pathogen, including human multiple sclerosis and neuromyelitis optica, and canine granulomatous meningoencephalomyelitis and necrotizing meningoencephalitis (MINAGAR; ALEXANDER, 2003; TALARICO; SCHATZBERG, 2010; VINCENT et al., 2008). Once leukocytes have crossed the BBB, inflammation can occurs.

Accordingly, we presented herein data that support the occurrence of BBB disruption in dogs with spontaneous VL, and IgG as a key molecule to initiate or maintain the inflammatory stimuli in the nervous milieu. The accumulation of mononuclear inflammatory cells (herein B lymphocytes/plasma cells) in the choroid plexus and in the ependyma states this structures as important pathways of leukocyte migration and the close contact with the CSF suggests that the CSF is an important disseminator of inflammatory stimuli within the CNS. In this manner, this study is pioneer in the investigation of BBB integrity in dogs with VL

and points out that the peripheral infection by *Leishmania* is able to produce brain alterations.

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CAPÍTULO 4 – NEUROPATOGENIA DA LEISHMANIOSE VISCERAL EXPERIMENTAL: INVESTIGAÇÃO DA MIGRAÇÃO DO PARASITA E DA RESPOSTA IMUNE EM CAMUNDONGO

NEUROPATHOGENESIS OF EXPERIMENTAL VISCERAL LEISHMANIASIS: INVESTIGATION OF PARASITE MIGRATION AND IMMUNE RESPONSE IN A MICE MODEL

ABSTRACT – Visceral leishmaniasis is a zoonotic disease caused by the parasitic protozoan *Leishmania*. In both humans and dogs, neurological involvement during the infection are reported; nevertheless, the pathogenesis of the brain lesions is still not fully elucidated. Therefore, we aimed to establish an experimental mice model to study this disease focusing on the dynamics of the infection and inflammatory response in the brain. To this end, Balb/c mice were infected with a strain of *Leishmania donovani* expressing the gene of the firefly luciferase and analyzed during 120 days. The liver and the spleen presented high parasite load and inflammatory mediators, attesting the parasite virulence. Using *in vivo* bioluminescence tools, we detected and quantified the parasite load in the brain as soon as 3 days post-infection and throughout the duration of the study, confirmed by RT-qPCR and parasite isolation. Regarding the inflammatory mediators, the brain presented a two-phased inflammation pattern: the first one from day 3 to day 14 p.i. with remarkable overexpression of IL-10 associated to IFN-γ, CXCL-10/CXCR-3, CCL-7/CCR-1 and CCR-2, MMP-2 and MMP-9; and the re-inflammation phase after 90 days p.i., presenting up-regulation of IFN-γ, IL-1β, TNF-α, CCL-12/CCR-2, CXCL-10/CXCR-3 and MMP-9. The present study was pioneer to demonstrate the dynamics of brain involvement during *Leishmania* infection, and since our experimental model shares some inflammatory mediators observed in human and canine spontaneous visceral leishmaniasis, we could argue that the inflammatory environment observed in the brain of infected mice resembles what happens in humans and dogs.

KEYWORDS: bioluminescence measurements, central nervous system, experimental animal models, *in vivo* imaging, inflammation, *Leishmania donovani*

4.1 INTRODUCTION

Leishmaniasis is a neglected disease caused by different protozoans species of the *Leishmania* genus (Kinetoplastida, Trypanosomatidae) (CHAPPUIS et al., 2007). Visceral leishmaniasis (VL) is both zoonosis and anthroponosis, caused by parasites from the *Leishmania donovani* complex: *L. infantum* (syn. *chagasi*), localized mainly in the Americas and in the Mediterranean basin, with dogs representing the main urban reservoir of the disease; and *L. donovani*, present in Asia and Africa, where the role of the dogs in the transmission of the disease is still not fully elucidated (BANETH et al., 2008; DEREURE et al., 2003; HASSAN et al., 2009; MAURICIO et al., 2000).

VL affects organs with cells of the mononuclear phagocyte system, such as liver, spleen, bone marrow and lymph nodes, in addition to the skin (ALVAR et al., 2004; BANETH et al., 2008). Although the existence of several reports concerning systemic symptoms of VL, such as fever, anemia, weight loss, skin lesions, renal disease, ocular alterations (ALVAR et al., 2004; BLAVIER et al., 2001; CIARAMELLA; CORONA, 2003; MORENO et al., 1998), a limited number of studies have related the occurrence of injuries in the central nervous system (CNS).

In humans, the involvement of the peripheral nervous system in VL is more usual (HASHIM et al., 1995; SNYDMAN et al., 2006); however, meningitis and parasites in the cerebrospinal fluid have been sporadic detected (PRASAD; SEN, 1995). Dogs with VL may present signs of generalized CNS involvement, with seizures, cranial nerve alterations, vestibular and cerebellar signs, motor incoordination, paresis and myoclonia (FONT et al., 2004; IKEDA et al., 2007; JOSÉ-LÓPEZ et al., 2012). Nevertheless, the presence of the *Leishmania* parasite within the CNS of dogs is rarely described, usually reported as single cases (MÁRQUEZ et al., 2013; NIETO et al., 1996; VIÑUELAS et al., 2001), at the same time as other studies failed the attempt to detect the parasite in the brain (IKEDA et al., 2007; MELO; MACHADO, 2011).

Even in the absence of the parasite, dogs with spontaneous VL present evidences of brain inflammation and blood-brain barrier disruption, with meningitis and choroiditis, immunoglobulin deposition, leukocyte infiltration, glial cell activation, matrix metalloproteinases (MMPs) -2 and -9, and a pro-inflammatory cytokine profile (GARCIA-ALONSO et al., 1996; IKEDA et al., 2007; MELO; MACHADO, 2011; MELO et al., 2013; NIETO et al., 1996; VIÑUELAS et al., 2001).

Consequently, in view of the paucity of evidences about the presence of the *Leishmania* parasite within the CNS, a laboratory animal model must be established to study the neuropathogenesis of VL, especially to detect the actual parasite migration towards the nervous milieu. Thus, we propose a mice model to analyze the infection dynamics in the brain and to quantify the parasite load in living animals. Further, the proposed model is pioneer to study brain lesions during VL since it allows the evaluation of both infection and inflammatory response in the same animals, widening the possibilities of understanding the neuropathogenesis of VL. Moreover, the development of this experimental model would provide additional data to understand the alterations previously reported in dogs with spontaneous VL.

Therefore, using bioluminescent parasites and *in vivo* imaging tools, the aim of this study was to evaluate the presence and the kinetics of parasite migration into the central nervous system, comparing with the peripheral targets of the parasite (liver, spleen, bone marrow), as well as to monitor the inflammatory response in these organs, evaluating the gene expression of pro and anti-inflammatory cytokines, chemokines, chemokines receptors and enzymes of the matrix metalloproteinases family in a mouse model of *Leishmania donovani* infection.

4.2 MATERIALS AND METHODS

4.2.1 Animals and parasites

Six-week-old female BALB/c mice were purchased from Janvier Laboratories (Le Genest-Saint-Isle, France), and handled under specific pathogen-free conditions according to the institutional guidelines of the Central Animal Facility at Institute Pasteur (Paris, France). A fully virulent *Leishmania donovani* strain (LD1S/MHOM/SD/00-strain 1S) expressing the firefly luciferase gene (DE LA LLAVE et al., 2011; GOYARD et al., *submitted*) was used in this study.

4.2.2 Infection and in vivo imaging

Short-term cultures of *L. donovani* promastigotes were obtained from splenic amastigotes isolated from infected hamsters. Then parasites were expanded until infectious metacyclic promastigotes from 9 days stationary phase cultures. Parasites were centrifuged for 5 min at 20°C and 1300 × g and enriched metacyclic promastigotes were collected from supernatant by centrifugation at 3350 × g for 10 min. Mice were infected with $5x10^7$ promastigotes in 150 µL of PBS by intraperitoneal route. Before each sampling, we evaluated the infection using *in vivo* bioluminescence assays (DE LA LLAVE et al., 2011). Briefly, at different time points following *Leishmania* inoculation D-luciferin (the luciferase substrate) was injected intraperitoneally at 500 mg/kg; the animals were anaesthetized in a 2.5% isoflurane atmosphere (Aerane®, Baxter SA, Maurepas, France) and placed in the imaging chamber of the IVIS™ Spectrum (PerkinElmer, Walthan, MA, USA). 2D-bioluminescence images were captured and total photon emission was determined in a defined region of interest (ROI) and expressed in photons/s using the Living Image software (PerkinElmer). At specific time points, we also performed a 3D-micro–computed tomography using the IVIS™ Spectrum CT (PerkinElmer) to provide the anatomy localization of the bioluminescence signals.

4.2.3 Sampling

Following the 2D-bioluminescence analyses, three representative mice, selected based on the bioluminescence values in the spleen (de la Llave et al., 2011), were euthanized and biological samples were collected at specific post infection (p.i.) time points, according to the following schema. At each time point, one uninfected mouse was collected as control.

sampling	**acute phase**						**chronic phase**		
days p.i.	3	5	7	14	21	30	60	90	120
months p.i.	1						2	3	4

The entire brain was collected, including the leptomeninges, separated in two hemispheres and subsequently divided in two samples: (1) cortex, including the cerebral cortex and the hippocampus; and (2) base, including the brainstem and the diencephalon (Figure 1). We collected the liver, the spleen and the femoral bone marrow as representative samples of the systemic infection.

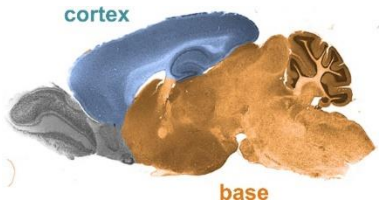

FIGURE 1 – Graphical illustration of the encephalic areas used in this study. Sagittal view of a mouse brain exhibiting the cortex (blue), including cerebral cortex and hippocampus; and the base (orange), including the brainstem and the diencephalon.

4.2.4 Parasite isolation from brain tissue

At different time points post-inoculation, paired cortex and base of each infected mice was collected in 1 mL of supplemented M199 medium and homogenized using the Precellys® 24 System (Bertin Technologies, Saint-Quentin-en-Yvelines, France). The volume of the homogenate was completed with supplemented M199 medium to 5 mL and centrifuged for 5 min at 150 × g. 500 µL of the supernatant was added into a 24-well plate and serial dilutions (10- and 100-fold) were made. The plates were incubated at 26°C up to 14 days and observed in light microscopy to detect promastigote forms.

4.2.5 RNA isolation and transcriptional analyses by quantitative PCR

At different time points following *Leishmania* infection, mice were sacrificed. Livers and spleens were removed, disrupted and lysed in 5 and 3 mL of Trizol® (Invitrogen, Paisley, UK), respectively, using tubes M and the gentleMACS™ dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). Cortex and base of the brains were collected and homogenized in 1 mL of Trizol® using the Precellys® 24 System (Bertin Technologies). The bone marrow was flushed out from the femur using a syringe filled with PBS; the cells were pelleted by centrifugation for 5 min at 5000 rpm and 4°C, and homogenized in 1 mL of Trizol®. Due to volume limitation, we collected a total pool volume of 250 µL of blood in heparin only at days 3, 5 and 30 p.i., and homogenized in 750 µL of Trizol®. RNA isolation was performed on the clear upper aqueous layer with the RNeasy Plus Mini kit (74134, Qiagen, Courtaboeuf, France) according the manufacturer's instructions. Evaluation of RNA quality was performed by optical density measurement using the NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and their integrity was assessed using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) that allowed the calculation of an RNA integrity (RNAi) number (Schroeder et al., 2006). Total RNAs were reverse transcribed to first strand cDNA using random hexamers (11034731001, Roche Diagnostics, Meylan, France), a set of dNTPs (10297-018,

Invitrogen) and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT, 28025013, Invitrogen).

PCR were performed in a final volume of 11 µl per reaction in white 384-well PCR plates (Thermo Scientific) using a thermocycler (7900HT fast real time PCR system, Applied Biosystems, Villebon-sur-Yvette France). Briefly, 2 µl of cDNA (20ng) was added to 9 µl of a master mix containing 5 µl of QuantiTect SYBR Green Kit (Qiagen) and 4 µl of nuclease-free water with primers at a final concentration of 1 µM (Table 1). The amplification conditions were as follows: 95°C for 15 min, 45 cycles of 95°C for 10 s, 54°C for 25 s and 72°C for 30 s; followed by a melt curve, from 60°C to 95°C. The mice gene targets were selected for quantifying host inflammatory mediators transcripts in liver, spleen and brain (cortex and base) (Table 1). For normalization calculations, candidate control genes were tested with the geNorm algorithm (VANDESOMPELE et al., 2002). The pairs *ldha* (lactate dehydrogenase A)/*l19* (ribosomal protein L19) and tbp (TATA box-binding protein)/rpIIe (RNA polymerase E) were selected as the most stable reference genes for the livers and spleens of mice, respectively. The reference genes for the cortex were ldha/tbp, and l19/rpIIe for the base. The fold change in the expression was calculated as the n-fold change in expression in the infected organs compared to the uninfected organs. The relative expression software tool (*REST©-MCS)* was used for determining group wise comparison and statistical analysis of relative expression results in real-time PCR (PFAFFL et al., 2002).

4.2.6 Experimental procedure to quantify Leishmania

Serial 10-fold dilutions of parasites were added either to livers (from 10^8 to 10^3), spleens (from 10^8 to 10^3), bone marrows (from 10^6 to 10^1), and brains (from 10^5 to 10^0) recovered from naïve mice. Total RNAs were further extracted and processed for RT-PCR as described above. The *Leishmania* gene target (*ssrRNA*) was selected for quantifying the number of parasites as previously described on murine cDNAs (DE LA LLAVE et al., 2011). A linear regression for

each standard curve was determined (quantification of *Leishmania* parasites against the relative expression of *ssrRNA* values).

Table 1 – Primers sequences used for RT-qPCR and reaction efficiency values (E) for each target.

Target	Sense (5' – 3')	Anti-sense (5' – 3')	E
Leishmania ssrRNA	CCATGTCGGATTTGGT	CGAAACGGTAGCCTAGAG	2.04
CCL-2 (MCP-1)	CAAGAAACGCAAGCGG	ACGGGATCTGAAAGACG	1.76
CCL-3 (MIP-1α)	ACCACTGCCCTTGCTGTTC	TCTGCCGGTTTCTCTTAGTCAG	1.95
CCL-4 (MIP-1β)	CCAATGGGCTCTGACC	ACTCCAAGTCACTCATGT	2.13
CCL-5 (RANTES)	GCTGCCCTCACCATCATCC	GTATTCTTGAACCCACTTCTTCTCTG	1.82
CCL-7 (MCP-3)	CCAATGCATCCACATGCTGC	GCTTCCCAGGGACACCGAC	2.11
CCL-12 (MCP-5)	AGTCCTCAGGTATTGGC	ACTTCTCCTTGGGGTCA	2.17
CCR-1	CATTTCCCCTACAAGAGC	CAAATATCAGACGCACGG	2.00
CCR-2	AGCACTTAGACCAGGC	CCAACCGAGACCTCTT	1.93
CCR-5	CTAGACCAGGCCATGC	CCTGTGGATCGGGTAT	1.97
CX3CL-1 (fractalkine)	CCTCACTAAAAATGGTGGCAAG	ATGTCAGCCGCCTCAAAAC	1.81
CX3CR-1	CAACCCCTTTATCTACGC	GCTTGTGTAGTGAGTGAAAC	2.04
CXCL-10 (IP-10)	GCAACTGCATCCATATCG	GGATTCAGACATCTCTGCT	1.89
CXCR-3	CAGCCTGAACTTTGACAGAACC	GCCGAAAACCCACTGGAC	1.95
IFN-γ	CTTCTTCAGCAACAGCAAGG	TGAGCTCATTGAATGCTTGG	1.83
IL-1β	AGGCAGGCAGTATCAC	CACACCAGCAGGTTATC	1.84
IL-2	AGGAACCTGAAACTCCC	AGTCCACCACAGTTGC	2.04
IL-4	GGAGCCATATCCACGG	AAGCCCTACAGACGAG	1.88
IL-6	ACAACGATGATGCACTT	CTTGGTCCTTAGCCACT	2.00
IL-10	CTGGACAACATACTGCTAACCGAC	ATTCATTCATGGCCTTGTAGACACC	1.99
IL-12p35	GGCCACCCTTGCCCTCCTA	GGGCAGGCAGCTCCCTCTT	1.91
TGF-β	GCGGACTACTATGCTAAAGA	GTAACGCCAGGAATTGT	1.84
TNF-α	CATCAGTTCTATGGCCC	GTGAGGAGCACGTAGT	1.92
MMP-2	CGCTCAGATCCGTGGTGA	CGCCAAATAAACCGGTCCTT	2.19
MMP-9	AAAACCTCCAACCTCACGGA	GCGGTACAAGTATGCCTCTGC	2.12
L19	TACTGCCAATGCTCGG	AACACATTCCCTTTGACC	1.93
LDHA	AACCCTCAAGGACCAG	CAAGCTCATCCGCCAA	2.21
RPIIE	AAGATCCGCAAGACGA	GGGAAGAACACAAACATCTG	2.04
TBP	CCTATGACCCCTATCACT	GTCCGTGGCTCTCTTAT	1.86

4.2.7 In vivo activity of matrix metalloproteinases enzymes

We evaluated the activity of the matrix metalloproteinases enzymes by quantitative fluorescence molecular tomography (FMT). At 90 days p.i., five infected mice and five uninfected ones were injected intravenously with 2 nmol of MMPsense 680 (NEV10126, PerkinElmer), a fluorescent agent activated by matrix metalloproteinases, including MMP-2, -3, -9 and -13. Then, 30 hours after injection, the animals were anaesthetized in a 2.5% isoflurane atmosphere (Aerane®) and placed in the imaging chamber of the FMT 2500™ system (VisEn Medical, Bedford, MA, USA), where 3D-fluorescence data were acquired. The collected data were reconstructed using the TruQuant software (PerkinElmer), tridimensional regions of interest (ROIs) were delimited for each organ (brain, liver and spleen) and the fluorescence values were automatically converted in pmols according to internal standards, after applying a threshold equal to 2-times the mean fluorescence value (nM) detected in the control uninfected mice (VASQUEZ et al., 2011).

4.2.8 Ethical issue

All procedures were approved by the Institutional Ethics Committee (Comité d'éthique de l'Institut Pasteur, process #2013-0047).

4.3 RESULTS

4.3.1 Parasite virulence and infection establishment

Since liver, spleen and bone marrow are considered target organs of *Leishmania* infection, we analyzed in our experimental model parasite implantation and inflammatory mediators in these organs in order to investigate parasite virulence and the establishment of the infection.

In both the livers and spleens, the first bioluminescent signals were detected at 3 day p.i., then rose slightly to reach a peak at day 30 pi (Figure 2A-

B). After this, the bioluminescence signal declined steadily with a significant decrease at 120 days pi. To determine the absolute number of *Leishmania* in tissues, parasite transcripts were quantified by RT-qPCR. A high linearity was observed over the range of template parasite numbers added for each tissue (data not shown). The *L. donovani* transcript abundance and bioluminescence kinetics recorded from the liver and the spleen had a similar profile (Figure 2A, B), indicating a good correlation between real time RT-qPCR and bioluminescence (DE LA LLAVE et al., 2011).

The femoral bone marrow was not suitable for *in vivo* bioluminescence evaluation probably due to the low parasite burden in this tissue or associated to important barriers for light diffusion such as bones and muscles. Nevertheless, we were able determining the parasite load by RT-qPCR (Figure 2C). In the femoral bone marrow, two different phases of parasite load were observed; during the first phase, the parasite load rose sharply over the first 14 days p.i., and then decreased slightly to reach a plateau between days 30 and 60. After this first phase, the parasite burden increased gradually and reached a plateau 90 days pi. In the blood, parasite burden reach $2.7x10^{1}$ parasites/µL at day 3 p.i. This phase was followed by a rapid decrease with a load of $4.9x10^{0}$ parasites/µL at day 5 p.i. Lastly, at day 30 p.i., the parasite load was extremely low, with $2.4x10^{-1}$ parasites/µL.

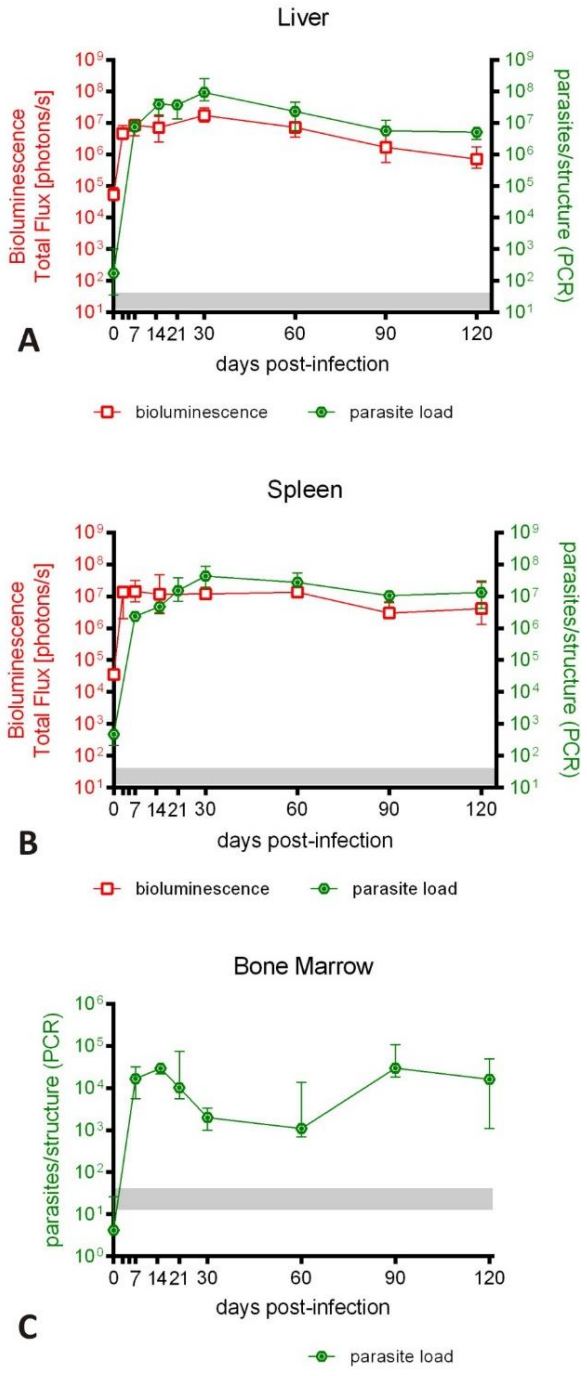

FIGURE 2 – *In vivo* (bioluminescence) and *ex vivo* (PCR) determination of the parasite load in BALB/c mice infected with *Leishmania donovani*. AB: Follow-up of the infection in the liver and in the spleen using bioluminescence (left y-axis) and RT-qPCR (right y-axis) at different days post infection. **C:** Monitoring of the parasite load in the femoral bone marrow by RT-qPCR. The grey areas correspond to background signals in PCR.

4.3.2 Pro and anti-inflammatory response in the liver and spleen of L. donovani-infected mice

Transcriptional analysis revealed complex patterns of regulation modulation pro and anti-inflammatory factors in both livers and spleen. Regarding the inflammatory response, the liver seemed to be the main target organ in this experimental model (Figure 3A). The gene expression of the pro-inflammatory cytokines IL-1β, IFN-γ and TNF-α were remarkably up-regulated throughout the time. Interestingly, the expression of the anti-inflammatory cytokine IL-10 was also up-regulated overtime, with a peak at day 14 p.i. An up-regulation of multiple pro-inflammatory chemokine was observed mainly in the liver. The expression of CCL-3, CCL-4, CCL-5 and CXCL-10 were highly up-regulated. At day 21 p.i., we noticed peaks of several mediators, including -1β, IL-2, IFN-γ and CCL-4, and it was the only time point with significant increasing in the gene expression of IL-4, IL-6, IL-12p35. In the spleen, in the other hand, we detected no important changes in the gene expression of inflammatory mediators before 21 days p.i., except for IFN-γ, up-regulated in all time points, with the highest expression at day 120 p.i. (Figure 3B). The inflammatory involvement of the spleen apparently becomes important after day 30 p.i., with a down-regulation of IL-2, along with up-regulation, with different intensities, of IL-1β, IL-6, CCL-3, CCL-4, CCL-5 and CXCL-10. Changes for the anti-inflammatory IL-10 and TGF-β were discrete and sporadic.

We also evaluated the activity of MMPs that could account for leucocytes invasiveness thus potentially increasing the population of monocytes harboring live amastigotes in the liver and the spleen. The transcript abundance of MMP2 was not significantly increased during the infectious process. Nevertheless, we noticed MMP-9 up-regulation in both organs, with significant values after day 14 p.i. for the liver, and at days 14, 30, 60, 90 and 120 p.i. for the spleen (Figure 3C). By means of FMT, we detected increased MMPs activity (including MMP-2, -3, -9 and -13) in the liver all infected mice (Figure 3D) and in the spleen of 4 out of 5 infected mice (Figure 3E). Additionally, with the tridimensional reconstruction, we could clearly detected the fluorescent signals from the liver and from the spleen

in the same image acquisition (Figure 3F). Altogether these transcriptional modifications could favor a local inflammation that could be critical for the further expansion of parasite load the in both liver and spleen. Altogether, these results clearly indicated the ability of our strain to implant and expand in tissues. Thus, this result constitutes a key-pre-requisite analysis for further relevant *in vivo* investigations, in particular in the brain

4.3.3 Parasite implantation and kinetics of infection in the brain

The first bioluminescent signals and transcripts of the parasites were detected as soon as 3 days p.i. as well (Figure 4A). The parasite load monitored by RT-qPCR separately in the cortex and in the base showed an important variability among samples, but do not revealed significant variation throughout the time. By contrast, the bioluminescence signals, presented minimal individual variation, and kept stably overtime. In order to verify the exact localization of the 2D bioluminescence signal detected through the cranium (Figure 4B), we performed a 3D bioluminescence assay combined with a micro–computed tomography, and consequently strongly suggest the bioluminescent parasites were localized within the cranial cavity (Figure 4C).

Following the parasite load detection in the brain using bioluminescence and RT-qPCR, attempts to isolate living parasites from the brain were performed. We successfully isolated parasites from both cortex (Figure 5A) and base (Figure 5B) of the brain, at different time points. Regarding the time points, the isolation of parasites were more frequent within the first month of infection. Concerning the regions, we noticed more positive cultures obtained from the base of the brain than from the cortex, with a peak of detection at day 21 p.i. All the parasites isolated from the brain presented morphology and motility equivalent to a standard promastigote culture (Figure 5C). We also obtained parasite positive cultures isolated from brain tissue from PBS-perfused animals (data not shown).

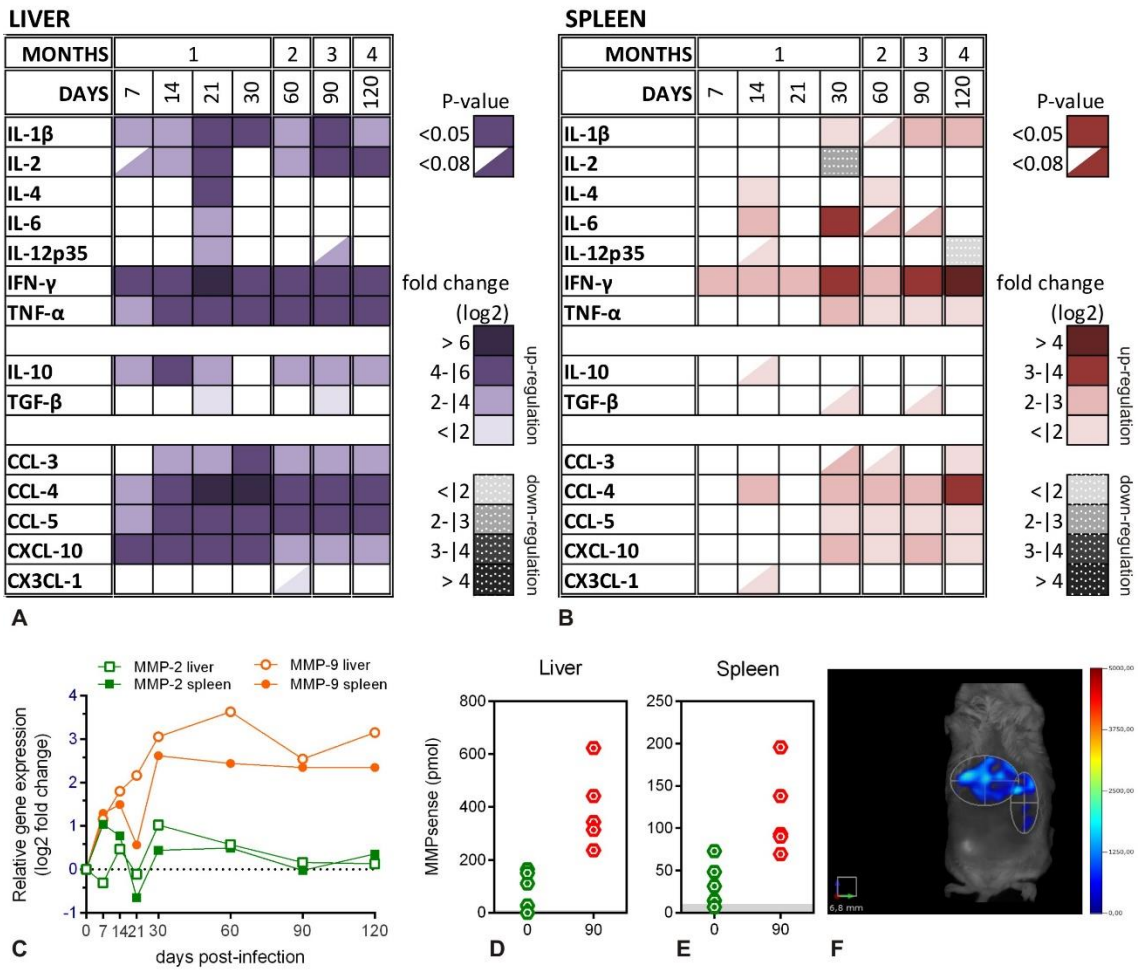

FIGURE 3 – Inflammatory response assessment in the liver and in the spleen of BALB/c mice infected with *Leishmania donovani*. (**AB**) Relative gene expression of pro- and anti-inflammatory cytokines, and chemokines in the liver (A) and spleen (B). The results are expressed as fold change (up or down-regulation) for each time point post infection. Full-colored squares represent significant fold changes (P<0.05) and half-colored squares represent statistical trend (0.05<P<0.08). (**C**) Relative gene expression of MMP-2 and MMP-9 in the liver and in the spleen of infected mice. Significant up-regulation was detected for MMP-9 after 14 days p.i. for in the liver and at day 14 and after 30 days p.i. in the spleen. (**DE**) *In vivo* detection of MMPs activity (including MMP-2, -3, -9 and -13) in the liver (B) and spleen (C) of mice at day 90 p.i. (n=5) by quantitative fluorescence tomography (FMT) using the activatable fluorescent agent MMPsense 680®. The grey area correspond to background signals. (**F**) Tridimensional reconstruction of FMT from a representative infected mouse showing a ventral view, with selected region of interests (ROI) delimitating the areas of the liver and the spleen.

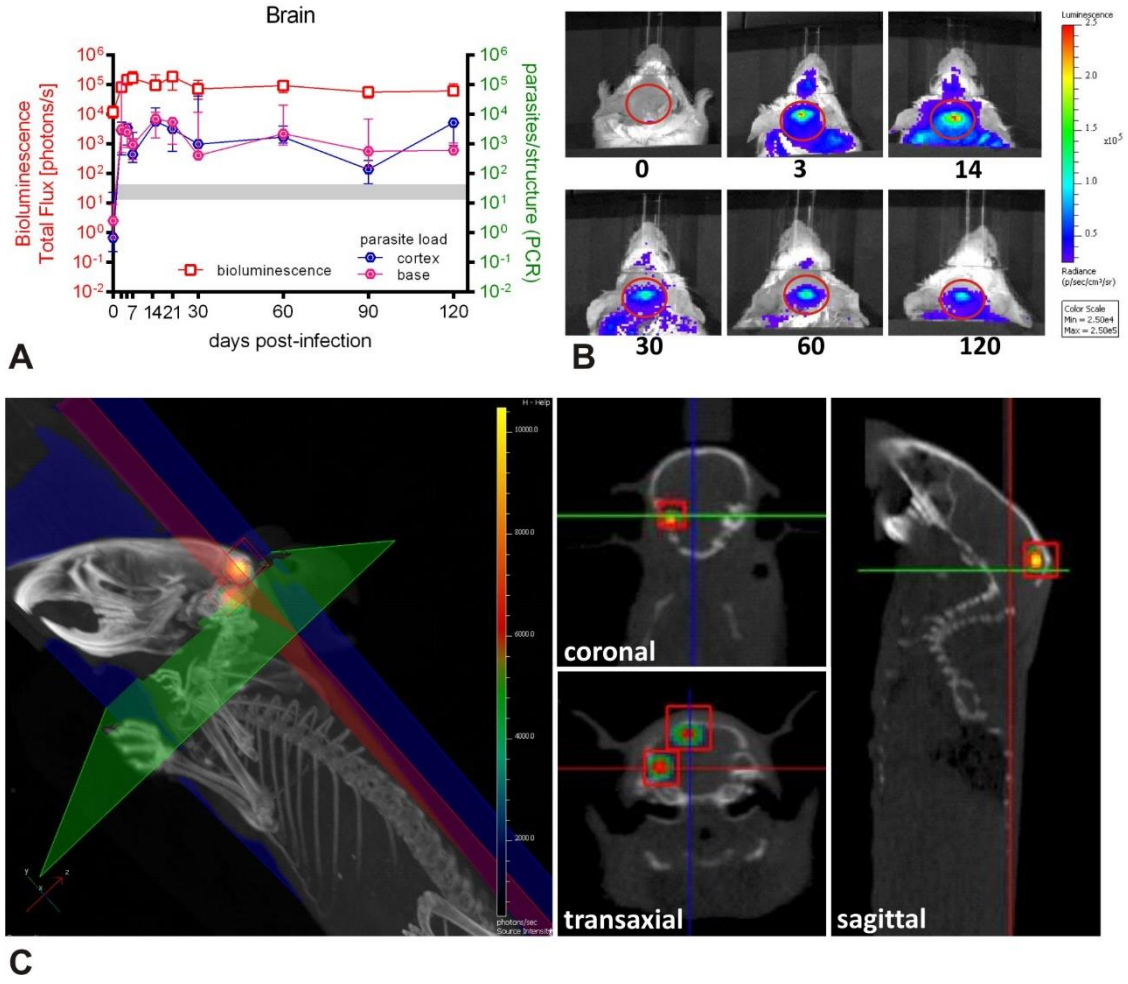

FIGURE 4 – *In vivo* (bioluminescence) and *ex vivo* (qPCR) determination of the parasite load in BALB/c mice infected with *Leishmania donovani*. (**A**) Kinetics of the parasite load in the brain detected by bioluminescence (left y-axis) and in the cortex and base detected by qPCR (right y-axis). The grey areas correspond to background signals in PCR. (**B**) Representative images illustrating the 2D-bioluminescence analysis of the parasite detection in the brain at specific time points post infection. The regions of interest (ROIs) corresponding to the brain are represented as red circles. (**C**) *In vivo* tridimensional micro-computed tomography to localize the bioluminescence signals within the brain. Representative tridimensional reconstruction of an infected mouse skeleton at 120 days post infection exhibiting the x-axis (blue), y-axis (green) and z-axis (red), and two bioluminescent foci (red cubes). The cross-sectional images exhibit the coronal (z-axis), transaxial (y-axis) and sagittal (x-axis) views and allow the localization of both bioluminescent foci within the cranial cavity (red squares).

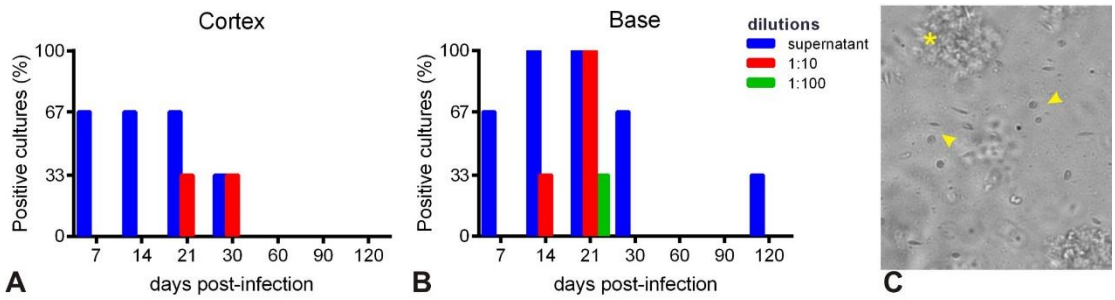

FIGURE 5 – Parasite isolation from brain tissue of BALB/c mice infected with *Leishmania donovani*. AB: Percentage of positive parasite cultures from the cortex (A) and from the base (B) of infected BALB/c mice brains, according to different dilutions and time points (n=3/time point). **C:** Representative image of a parasite culture from a cortex at day 7 post infection, where it is possible to observe *Leishmania donovani* promastigotes with different morphologies (arrowhead) surrounded by cellular debris (*).

4.3.4 Pro and anti-Inflammatory response in the brain of L. donovani-infected mice

Our analyses revealed complex patterns of transcriptional modulations pro-inflammatory factors indicating two phases of inflammation in both cortex and base. The first phase (day 3 to day 14 p.i.) corresponded to the acute phase; the second phase (named re-inflammation phase) was characterized by a second increase of inflammatory mediators by days 90 p.i. (Figure 6). The comparison of the levels of transcripts seemed to indicate that the cortex was more affected by inflammation than the base.

Regarding cytokines, the transcript abundance of IFN-γ, was significantly up-regulated from day 3 p.i. in the cortex. A slight increase of IL-1β was also noted at day 14 p.i. No changes were detected in the base in the first phase. On the other hand, in both cortex and base IL-10 transcripts were highly expressed during the first phase reaching a peak at day 5 p.i. A second peak was also observed at day 14 p.i. At the re-inflammation phase, we noticed no changes in

IL-10, and up-regulation of IL-1β, IL-2 and TNF-α in both cortex and base. Interestingly, during this phase, an increase of IL-6 concomitantly with a down-regulation of IL-4 was only observed in the base.

The up regulation of multiple pro-inflammatory chemokines and chemokines receptors in the cortex and the base was also observed during acute and re-inflammation phases. The expression of chemokines were significantly up-regulated, especially CCL-7, with a peak at day 5 p.i., CCL-12 and CXCL-10, along with up-regulation of the chemokine receptors, notably CCR-1, CCR-2 and CXCR-3. The chemokine CXCL-10 was the only target detected with increased gene expression at time points between the two phases. Among all the up-regulated chemokines and chemokine receptors, CXCL-10/CXCR-3 and CCL-12/CCR-2 were the most noteworthy.

4.3.5 Transcript abundance and activity of MMPs in the brain of L. donovani-infected mice

By means of RT-qPCR, we also checked the gene expression of the matrix metalloproteinases enzymes MMP-2 and MMP-9. Two phases were observed in the cortex of infected mice. The first phase (day 0 to day 14 p.i.) was characterized by the rapid increase of both MMP-2 and MMP-9 transcripts followed by a rapid decrease up day 30 p.i. The second phase (day 30 to day 90 p.i.) was delineated by a novel up-regulation. On the contrary, for the base of the brain, the gene expression of these enzymes presented no changes (Figure 7A). Since MMPs are produced in an inactive latent form, we further evaluated the actual activity of these enzymes *in vivo*. Using a 3D fluorescence molecular tomography (FMT) method and an activatable fluorescent agent, which is activated by MMPs, including MMP-2, MMP-3, MMP-9 and MMP-13, we detected remarkable MMPs activity in 3 out of 5 infected mice brains (Figure 7B). This technique was sensitive enough to allow the anatomic localization, making possible the delimitation of regions of interest (Figure 7C-D).

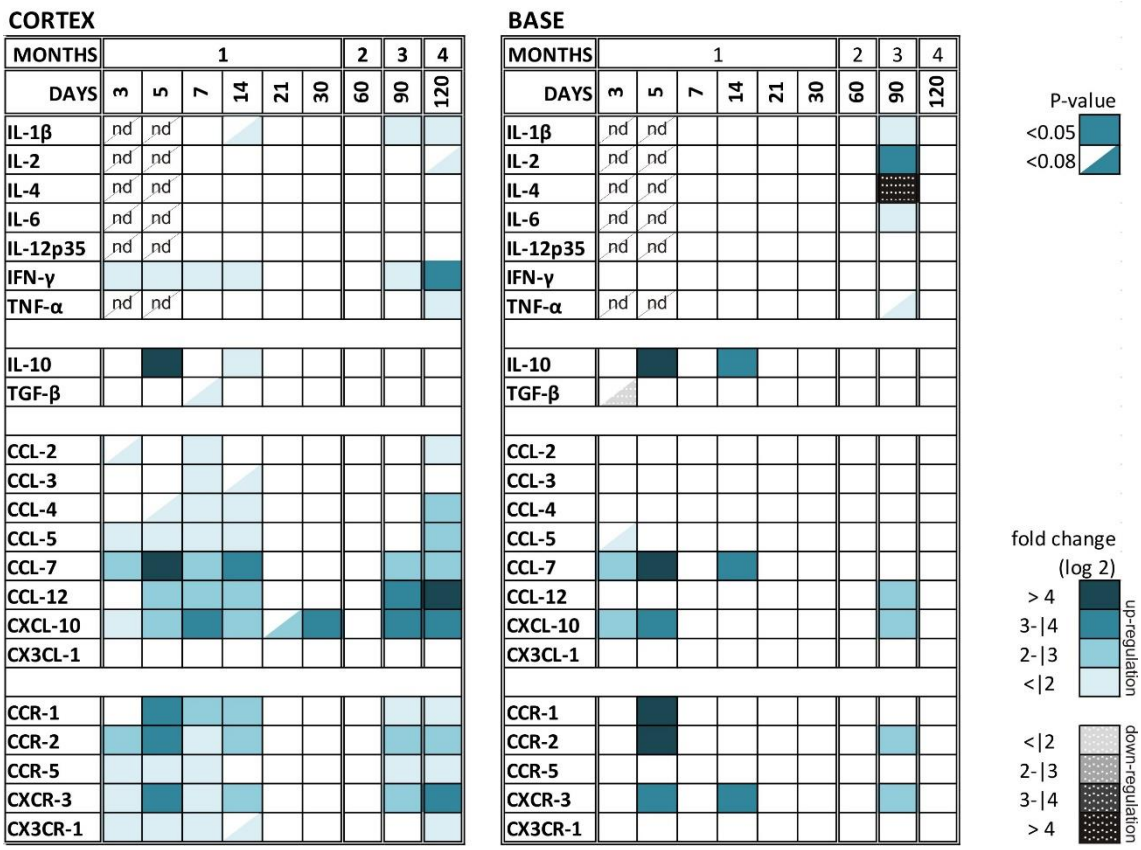

FIGURE 6 – Relative gene expression of pro- and anti-inflammatory cytokines, chemokines and chemokine receptors in the cortex and in the base of BALB/c mice brains infected with *Leishmania donovani*. The results are expressed as fold change (up or down-regulation) for each time point post infection. Full-colored squares represent significant fold changes ($P<0.05$) and half-colored squares represent statistical trend ($0.05<P<0.08$). nd: data points not performed (not determined).

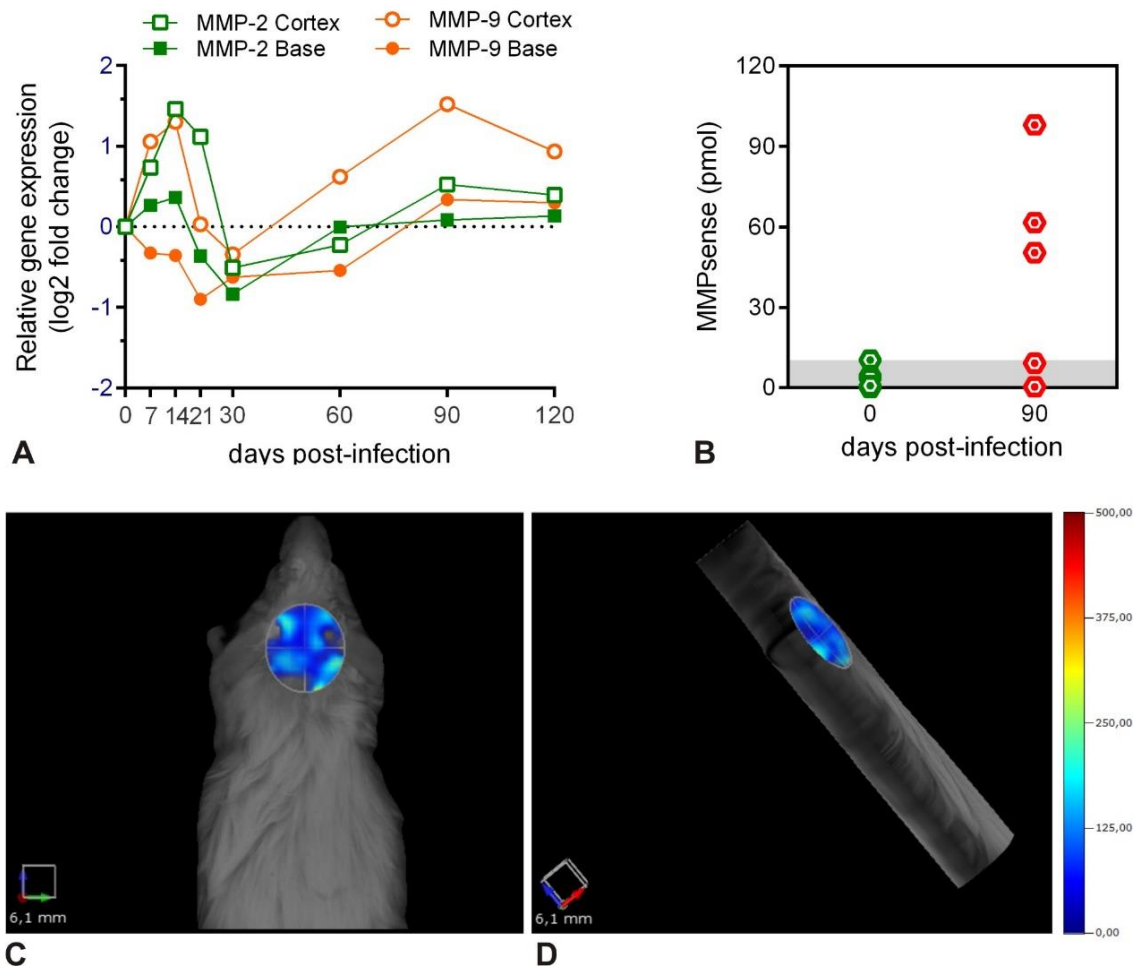

FIGURE 7 – Transcript abundance and activity of matrix metalloproteinases (MMPs) enzymes in the brain of BALB/c mice infected with *Leishmania donovani*. A: Relative gene expression of MMP-2 and MMP-9 in the cortex and in the base of infected mice. Significant up-regulation was detected only in the cortex, at days 7 and 14 p.i. for MMP-2, and days 7, 14, 90 and 120 p.i. for MMP-9. **B:** *In vivo* detection of MMPs activity (including MMP-2, -3, -9 and -13) in the brain of mice at day 90 p.i. (n=5) by quantitative fluorescence tomography (FMT) using the activatable fluorescent agent MMPsense 680®. The grey area correspond to background signals. **CD:** Tridimensional reconstruction of fluorescent tomography from a representative infected mouse exhibiting dorsal (C) and lateral views (D), with a selected region of interest (ROI) delimitating the area of the brain, representative of 50.35 pmoles of MMPsense.

4.4 DISCUSSION

During leishmaniasis, *in vivo* bioluminescence assays have been used to detect parasites in the skin, in the liver and in the spleen (DE LA LLAVE et al., 2011; LANG et al., 2009; MICHEL et al., 2011), but no studies focusing on the brain have been performed. In the brain, this technique have already been used with other pathogens, such as *Trypanosoma vivax*, *Toxoplasma gondii* and Herpes simplex virus (D'ARCHIVIO et al., 2013; LUKER et al., 2002; VYAS et al., 2007). Herein, using 2D-bioluminescence assays, we were able to detect and quantify the presence of parasites in the brain. Further, combining bioluminescence with a micro-computed tomography, we could clearly localize the parasites within the cranial cavity. However, differently from the liver and the spleen, which exhibited concordant values for parasite load in bioluminescence and in RT-qPCR, in the brain, there was a gap regarding the data obtained from these two techniques, with higher bioluminescence values. One reason for this inconsistence may be the presence of bioluminescent parasites in the bone marrow of cranial bones, since besides the femur; we were able to isolate living parasites from bone marrow of vertebrae and cranial vault (data not shown). Nevertheless, we detected significant parasite loads in the brain in all time points.

One important bias that must be considered when analyzing the presence of parasites within the brain is blood contamination. In order to minimize this error, we also performed parasite isolation from the brain of PBS-perfused animals, and RT-qPCR on blood samples. PBS-perfused brains generated positive parasite cultures. Further, it was estimated that the cerebral blood volume in mice is around 50 µL/g of brain tissue (MODAK et al., 1978). Considering that the mice brains in our study presented a mean weight of 0.4 g, we could estimate their cerebral blood volume as 20 µL. By means of RT-qPCR, we detected $5.4x10^2$ parasites/20 µL of blood at day 3 p.i., which could explain the arrival of the parasites in the brain, but not brain positivity at day 3 p.i., since it contained 80-fold more parasites ($4.2x10^4$ parasites = $2.9x10^3$ in the cortex plus $3.9x10^4$ in the base). The drastic decrease of peripheral blood parasitemia noticed at days 5

and 30 p.i. confirm our hypothesis, since during this period the parasite burden in the brain remained at the same level.

The parasite load in the brain presented an interesting profile: the number of parasites was estimated between 900 and 12,000 parasites, not elevated compared to liver and spleen, but the parasite burden remained rather constant throughout time. Despite this stability, the parasite isolation from the brain were more successful within the first 30 days p.i., with more positive cases in the base than in the cortex. Further, the brain presented a two-phase inflammatory response: an acute phase from 3 to 14 days p.i.; and a re-inflammation phase starting at 90 days p.i. In the brain, the acute inflammation phase may be consequence of the direct presence of the parasite in the CNS. IFN-γ, a key mediator during VL, was up-regulated, along with several chemokines and chemokine receptors, with CCL-7/CCR-2 being the most elevated. In the brain, CCL-7 produced by astrocytes is related to the normal recruitment of perivascular macrophages, and it has been implicated with the early entrance of HIV-infected cells into the brain (RENNER et al., 2011).

However, with the overexpression of IL-10, the pro-inflammatory stimuli may be suppressed. It is known that IL-10 is a potent anti-inflammatory cytokine that promotes down-regulation of pro-inflammatory cytokines (CSUKA et al., 1999), and in a pro-inflammatory milieu, IL-10 is able to produce chemokine decoy receptors in monocytes and dendritic cells (D'AMICO et al., 2000). IL-10 may be the responsible for parasite persistence and suppression of the inflammatory mediators, since it was described that predominance of IL-10, and not absence of IFN-γ, was key to disease progression (MURPHY et al., 2001). Further, intralesional parasites are thought to present a semi-quiescent state, with slow growth rate, which allows long-term expansion and persistence (BELKAID et al., 2000; KLOEHN et al., 2015; SANGÜEZA; CARDENAS, 1981).

Attempts to visualize and localize the parasites and inflammatory lesions in the brain of infected Balb/c mice were made by histology and immunohistochemistry, nevertheless no alterations were seen in any time post-infection (data not shown), probably due to the low number of parasites randomly

distributed throughout the brain. The duration of the two inflammatory phases in the brain could be connected to the two phases of parasite load observed in the bone marrow, differently from a progressive growing pattern (COTTERELL et al., 2000). The first inflammation phase in the brain is thought to be related to the activation of innate immune sensors, such as the Toll-like receptors, since these receptors, especially TLR-2 and TLR-9, have been implicated in the neuropathology of canine VL (MELO et al., 2014). The re-inflammation phase in the brain may be linked to the chronic, persistent peripheral inflammation associated to the delayed involvement of the spleen, which started to exhibit up-regulation of certain inflammatory mediators after 30 days p.i. In the brain, the re-inflammation phase was characterized with no changes of IL-10 and up-regulation of the pro-inflammatory cytokines IFN-γ, IL-1β, IL-6 and TNF-α with known involvement in brain inflammation and BBB disruption (ABBOTT et al., 2006). The chemokines CCL-4, CCL-5, CCL7 and the receptors CCR-1 and CCR-3 were up-regulated only in the cortex, but CCL-12/CCR-2 and CXCL-10/CXCR-3 were the most up-regulated targets in both cortex and base.

Since in the vertebrate host *Leishmania* is an intracellular parasite, its arrival in the brain is thought to occur via the 'Trojan horse' mechanism, inside leukocytes (LACHENMAIER et al., 2011). Sporadic detection of parasites and parasitized cells in the choroid plexus, meninges and cerebrospinal fluid of humans and dogs have already been reported (PRASAD; SEN, 1995; NIETO et al., 1996; VIÑUELAS et al., 2001). Therefore, three pathways could be possible: (1) direct invasion of the brain parenchyma through the blood-brain barrier, (2) via the choroid plexus, and/or (3) passage across meningeal blood vessels (RANSOHOFF et al., 2003). The more likely cell to carry *Leishmania* towards the brain are CCR-2+ monocytes, via CCL-2/CCL-7/CCL-12 signaling (TSOU et al., 2007). The bone marrow could act as a reservoir of parasitized cells, liberating them in the bloodstream in response to specific stimuli, and then infected cells may cross the BBB, similarly as with some viruses (ALEXAKI; WIGDAHL, 2008; MINTON et al., 1994); and under inflammatory conditions, the replacement of

meningeal and perivascular monocytes by hematogenous monocytes is enhanced (LASSMANN et al., 1993).

Whereas, in our model, the spleen presented a delayed inflammatory response, the liver displayed a mixed inflammatory response (high levels of both pro- and anti-inflammatory cytokines), with overexpression of IL-10, IFN-γ and TNF-α throughout the evaluated post-infection time. Recently, the concept of liver-brain inflammation axis has become focus of researches, since studies have proven that chronic liver diseases are connected with changes in CNS activity, namely behavioral changes (D'MELLO; SWAIN, 2011). Peripheral TNF-α was related to microglial activation with subsequent production of CCL-2 and attraction of monocytes towards the brain via CCR-2 in a model of liver inflammatory injury (D'MELLO et al., 2009).

CCL-12, similar to CCL-2, is potently attractive for the inflammatory subset of monocytes/macrophages, involved in the host response to pathogens via activation of CCR-2 (SARAFI et al., 1997). In a model of West Nile encephalitis, CCR-2 was related to macrophage accumulation in the CNS, but not by stimulating the traffic of cells through the BBB, performed by CCR-5 (GLASS et al., 2005), but by selectively inducing monocytosis (LIM et al., 2010). CXCR-3, on the other hand, is expressed in neuronal and glial cells (CARTIER et al., 2005), it is further expressed in T lymphocytes, and it acts as an indicator of cells that have the ability to cross the blood-brain barrier (CALLAHAN et al., 2004). The system CXCL-10/CXCR-3 was related to CD8 T lymphocytes migration into the brain during viral encephalitis (KLEIN et al., 2005; WUEST; CARR, 2008) and in the pathogenesis of cerebral malaria (CAMPANELLA et al., 2008).

Further evidences of brain inflammation was the detection of MMPs, enzymes related to blood-brain barrier disruption and leukocyte migration (ROSENBERG, 2002). The cortex presented up-regulation of MMP-2 and MMP-9 in the acute phase, and MMP-9 in the re-inflammation phase. Human immunodeficiency virus-type 1 (HIV-1) possess a surface glycoprotein, named gp120, which induces MMP-2 activity in transgenic mouse brains (MARSHALL et al., 1998). In a comparable approach, *Leishmania* also possess a surface

glycoprotein, named gp63 or leishmanolysin, produced mainly in the promastigote extracellular form (MCGWIRE et al., 2003). This could explain the early overexpression of MMP-2s in the acute brain inflammation phase. Similarly, in cerebral malaria, MMP-2 and MMP-9 were overexpressed 8 days p.i., with evidences of MMP-9 activation (VAN DEN STEEN et al., 2006). MMP-9 is considered a hallmark of inflammation, since it acts also in cytokines and chemokines processing (OPDENAKKER et al., 2001) and its novel up-regulation at day 90 p.i. was connected to MMPs activity in the brain, as assessed by *in vivo* fluorescence tomography, therefore corroborating the occurrence of the re-inflammation phase in the brain of infected mice. In this case, probably not related to the promastigote gp63, but rather due to chronic systemic inflammation (SANKOWSKI et al., 2015).

Even though golden Syrian hamsters are considered the best animal model for VL (LORIA-CERVERA; ANDRADE-NARVAEZ, 2014), we demonstrated herein that *Leishmania donovani* is able to infect Balb/c mice and to persist in the tissues, and that the infected organs present distinct inflammatory responses against the infection. More specifically, the present study was pioneer to demonstrate brain involvement during infection, with detection of viable parasites throughout the study, from day 3 to day 120 p.i. The brain presented a dual-phase and compartmentalized inflammatory response, with a profile of mediators that differed from those observed in liver and spleen: the first phase more likely due to the parasite itself and the second phase related to chronic systemic inflammation.

Consequently, this study was crucial to include the brain in the list of organs affected by the parasite, and opened up the need of further studies to better understand the neuropathogenesis of VL, focusing on the inflammatory cell populations and on the mechanisms underlying the parasite arrival into the central nervous system. Finally, since our experimental model shares some inflammatory mediators observed in the liver and the spleen with human and canine spontaneous VL; therefore, we could argue that the inflammatory

environment and parasite detection observed in the brain of infected mice resembles what happens in humans and in dogs.

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CAPÍTULO 5 – IMPLICAÇÃO

IMPLICAÇÃO

O encéfalo é atingido durante a leishmaniose visceral.

Essa é a principal implicação do presente estudo. De fato, desde o ano de 2006 este grupo de pesquisa busca compreender a relação entre a infecção periférica por *Leishmania* spp. e as lesões observadas no encéfalo. Dessa maneira, a presente pesquisa é continuação de estudos anteriores onde houve detecção de alterações inflamatórias no plexo coroide, infiltração de linfócitos T, atividade das enzimas metaloproteinases de matriz MMP-2 e MMP-9 no soro, líquido cefalorraquidiano e tecido nervoso, ativação de astrócitos e da micróglia, aumento da expressão gênica de citocinas pró-inflamatórias e de receptores do tipo Toll, além da detecção do DNA do parasita no tecido nervoso.

Avaliando globalmente os dados apresentados nessa tese, as seguintes asserções podem ser concebidas, no que tange a leishmaniose visceral canina:

- A barreira hematoencefálica encontra-se desregulada.
- O encéfalo possui um perfil específico de expressão gênica de quimiocinas, altamente seletivo para linfócitos T.
- Existem elevadas concentrações de imunoglobulina G no líquido cefalorraquidiano, resultando na presença dessa molécula no citoplasma de células do epêndima e do epitélio do plexo coroide.
- A imunoglobulina G atua na resposta inflamatória no ambiente nervoso por meio de interação com seu receptor Fcγ-RI.
- A resposta inflamatória no encéfalo é compartimentalizada, ou seja, a resposta inflamatória no encéfalo apresenta características individuais e específicas, diferente do que ocorre na periferia, como no baço.
- Não há relação entre inflamação observada no encéfalo e a manifestação clínica clássica da doença (estadiamento clínico).

Por conseguinte, de maneira coerente com as asserções acima, as seguintes proposições podem ser determinadas, ainda em relação à leishmaniose visceral canina:

- O DNA do parasita no plexo coroide, e não necessariamente o parasita íntegro, pode ser responsável pela iniciação do estímulo inflamatório no encéfalo.
- O plexo coroide parece ser importante porta de entrada e o líquido cefalorraquidiano o difusor de estímulos inflamatórios dentro do ambiente nervoso.

Ademais, a avaliação comparada entre os resultados obtidos na infecção natural de cães e na infecção experimental em camundongos dá suporte para as seguintes conjecturas:

- O parasita pode penetrar o sistema nervoso central, mas a resposta inflamatória não é necessariamente dependente da sua presença.
- A inflamação sistêmica crônica pode influenciar a inflamação no encéfalo.
- A inflamação no encéfalo de camundongo, avaliada nos primeiros quatro meses pós-infecção, é dinâmica e apresenta duas fases distintas. Eventuais diferenças observadas no encéfalo de cães e de camundongos podem estar ligadas à impossibilidade do estabelecimento do tempo de infecção nos cães.

Com isso, os resultados contidos nessa tese corroboram os dados anteriores, reforçam o fato de que o comprometimento encefálico tem sido negligenciado durante a leishmaniose visceral, e abrem novas perspectivas de estudo do envolvimento do encéfalo nessa enfermidade, sobretudo no tocante aos mecanismos e estímulos da migração de células pela barreira hematoencefálica, a determinação do perfil de citocinas, quimiocinas e receptores de quimiocinas principalmente relacionado aos linfócitos

B/plasmócitos, a detecção de imunocomplexos circulantes contendo DNA de *Leishmania*, a caracterização dos depósitos perivasculares no plexo coroide, além da persistência na busca pelo parasita no sistema nervoso central e eventuais mecanismos de neuroinvasão.

O modelo murino aqui descrito, desenvolvido para estudo do processo inflamatório no encéfalo, é pioneiro e torna-se então essencial para a avaliação dos itens supracitados, uma vez que se mostrou válido, produzindo alterações semelhantes às observadas na infecção natural canina. A produção de cepas de *Leishmania infantum* de origem humana e canina que expressam o gene da luciferase se encontra em fase final de testes, e brevemente será possível avaliar comparativamente a resposta imune produzida por *L. donovani* e por *L. infantum*. De qualquer maneira, tal modelo permite a determinação, de maneira dinâmica, da carga parasitária e da localização do parasita *in vivo*, e reduz drasticamente o número de animais necessários para obtenção de resultados fidedignos, cumprindo os preceitos de ética e bem-estar animal.

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