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EFEITO REGULADOR DA PGE_2 NA PRODUÇÃO DE TNF-α E IL-17 NA ATIVIDADE MICROBICIDA NA LEISHMANIOSE CANINA

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Tese apresentada à Faculdade de Medicina Veterinária de Araçatuba da Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP, como parte dos requisitos para a obtenção do título de Doutora em Ciência Animal (Área de Medicina Veterinária Preventiva e Produção Animal).

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RESUMO

A leishmaniose canina (CanL) é causada pelo parasito intracelular *Leishmania infantum.* Devido ao alto parasitismo na pele o cão é considerado o principal reservatório urbano da *L. Infantum.* A prostaglandina-E_2 (PGE_2) possui propriedades reguladoras potentes do sistema imunológico e pode se ligar aos receptores EP1, EP2 e EP4 que geram ativação celular ou EP3 que gera inibição de resposta celular. Na CanL o papel regulador da PGE_2 ainda não foi estudado, por isso, os parâmetros foram avaliados em cultura de leucócitos esplênicos de cães com CanL. Avaliando o nível de PGE_2, seus receptores, e seu efeito modulador sobre a PGE_2 na atividade da arginase, NO_2, citocinas IL-10, IL-17 e TNF-α e carga. Para isso utilizamos seus agonistas, antagonista e inibidor. Nossos resultados mostraram que a expressão do receptor EP2 diminuiu nos leucócitos esplênicos dos cães com CanL quando comparado aos cães saudáveis. Observamos que o NO_2 diminuiu quando tratados com os agonistas dos receptores de PGE_2 (EP1/EP2/EP3), antagonista dos receptores de PGE_2 (AH-6809) e inibidor de COX-2 (NS-398). A concentração das citocinas TNF-α e a IL-17 diminuíram quando tratadas com agonista do receptor de PGE_2 (EP2), e quando estimuladas com a PGE_2. A carga parasitária diminuiu na cultura de leucócitos esplênicos de cães com CanL estimulados com PGE_2. Concluímos que a infecção por *Leishmania* modula os receptores de PGE_2 em cães, e que a ligação da PGE_2 aos receptores pode ativar a capacidade microbicida das células.

Palavras – Chave: Prostaglandina. Receptores de prostaglandina. *Leishmania.* Cães.

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ABSTRACT

Canine leishmaniasis (CanL) is caused by the intracellular parasite *Leishmania infantum.* Prostaglandin E2 (PGE_2) exerts potent regulatory effects on the immune system in *Leishmania* infection, but in CanL has not yet been studied. In this study, PGE_2 and PGE_2 receptor levels and the regulatory effect of PGE_2 on arginase activity; NO_2; the IL-10, IL-17 and TNF-α and parasite load in the presence of agonists, antagonists and inhibitors were evaluated in cultures of splenic leukocytes obtained from dogs with CanL. Our results showed that splenic leukocytes from dogs with CanL had lower EP2 receptor levels than splenic leukocytes from healthy animals. We observed that NO_2 levels decreased when the cells were treated with PGE_2 receptor agonist (EP1/EP2/EP3), a PGE_2 receptor antagonist (AH-6809) or a COX-2 inhibitor (NS-398) and that TNF-α and IL-17 cytokine levels decreased when the cells were treated with a PGE_2 receptor agonist (EP2) or stimulated with PGE_2. The parasite load in splenic leukocyte cell cultures from dogs with CanL decreased after stimulation of the cells with PGE_2. We conclude that the infection of dogs by *Leishmania* modulates PGE_2 receptors and speculate that the binding of PGE_2 to its receptors may activate the microbicidal capacity of cells.

Keywords: Prostaglandin. Receptors Prostaglandin E. *Leishmania*. Dogs.

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1 INTRODUÇÃO GERAL

A Leishmaniose canina (CanL) é uma doença sistêmica em cães causada por protozoários do gênero *Leishmania*, da ordem Kinetoplastida e família Trypanosomatidae (1).

Em relação ao ciclo biológico, o protozoário se apresenta sob duas formas: promastigota e amastigota. A forma promastigota flagelada, encontrada no intestino delgado do inseto vetor (hospedeiro intermediário), é transmitida ao hospedeiro durante o repasto sanguíneo, pela fêmea do flebótomo *Lutzomyia longipalpis*; já a forma amastigota, parasita intracelular aflagelado, é encontrado nas células do sistema fagocíticos mononuclear do hospedeiro vertebrado (2).

A Leishmaniose Visceral (LV) tem sido relatada em pelo menos 98 países, e 94% dos casos de LV está centralizado em apenas sete países: Brasil, Etiópia, Índia, Quênia, Somália, Sudão do Sul e Sudão (3). Estima-se que 500.000 novos casos de LV ocorrem anualmente, com cerca de 12 milhões de pessoas infectadas (3). No Brasil, a LV é altamente endêmica em estados da região Nordeste, como Ceará, Bahia, Maranhão, Piauí, Pernambuco, Rio Grande do Norte e Paraíba (4). Atualmente, está em expansão no estado de São Paulo. A migração das zonas rurais para as zonas urbanas, o rápido crescimento e urbanização não planejada conduziu a uma rápida expansão da LV no Brasil e em outros países da América do Sul (5).

O primeiro relato da ocorrência de CanL no estado de São Paulo ocorreu em 1998 na cidade de Araçatuba. Os dados do Ministério da Saúde mostram que na região Sudeste, o número de casos de 2000 e 2017 (subiram de 314 para 908, respectivamente), e o mais alarmante é que o número de mortes foi quase treze vezes maior, saltando de 9 para 113 (6).

Os cães são de extrema importância na manutenção do ciclo epidemiológico da doença, devido ao parasitismo intenso na pele, podendo infectar os seres humanos (7). A leishmaniose em cães é prevalente em áreas endêmicas podendo atingir 20 a 40% da população (8).

O parasito é transmitido para os cães principalmente pela picada de flebotomíneos, mas já foi visto que há possibilidade de outras formas de transmissão por artrópodes vetores, como carrapatos (9), pulgas (10) e também por transfusão sanguínea (11).

No hospedeiro vertebrado canino, o parasita pode causar lesões e sintomas que são característicos de CanL, embora alguns cães infectados possam ser assintomáticos, sintomáticos e oligossintomáticos (12) e alguns passam evoluir para a cura espontânea (13). A forma assintomática representa 20 a 40% da população de soro positivo, dos quais 80% realmente desenvolvem a doença (14).

Os principais sinais clínicos da CanL são: apatia, anorexia, perda de peso, febre, palidez das mucosas, perda de massa muscular, linfoadenopatia periférica generalizada, hepatomegalia, esplenomegalia, epistaxe e lesões cutâneas (15).

O estadiamento clínico da CanL é realizado com base no status sorológico, sinais clínicos, achados laboratoriais, onde são classificados em estágio de I a IV. O cão no estágio I é classificado como: doença leve, negativo para titulação de anticorpos anti-*leishmania*, sinais clínicos leves, como linfadenopatia periférica ou dermatite papular, normalmente sem anormalidade nos exames laboratoriais. No estágio II, o cão pode apresentar: doença moderada, com titulação de anticorpos anti-*leishmania* variando de baixo a alto, sinais clínicos do estágio I como também lesões cutâneas, ulceras nasais, anorexia, perda de peso e febre, com anormalidades nos exames clínicos patológicos como hipergamaglobulinemia, hipoalbuminemia e funções renais dentro dos parâmetros de normalidade. No estágio III, apresenta doença grave, com titulação de anticorpos anti-*leishmania* variando de médio a alto, e além dos sinais clínicos listados nos estágios I e II apresenta sinais oriundos de lesões do complexo imune como vasculite, artrite e uveite, com anormalidades clínico patológicas listadas no estágio II ainda doença renal crônica. O estágio IV é classificado como doença grave, e os cães apresentam níveis elevados de anticorpos, com sinais clínicos listados no estádio III e tromboembolismo pulmonar e doença renal terminal, com anormalidades clínico patológicas, doença renal crônica e síndrome nefrótica (15).

A supressão da imunidade celular é o aspecto mais importante na patogênese e progressão da doença canina. A ausência da resposta das células T aos antígenos de *Leishmania* spp. é observada *in vivo*, com teste de Montenegro negativo (16). Em cães infectados com *Leishmania infantum*, ocorre uma redução do número de linfócitos T (17) e a desorganização da polpa branca do baço (18).

A imunossupressão associada com a infecção crônica ocorre devido a altas taxas de apoptose das células T e esse mecanismo pode contribuir para a

desorganização da polpa branca do baço e diminuição dos níveis de células T no sangue periférico (19).

A imunidade protetora em cães tem geralmente sido associada com resposta imunológica celular, manifestada por resposta linfoproliferativa positiva a antígenos de *Leishmania* spp. (20) e produção de citocinas, tais como IFN-gama e TNF-α, as quais são necessárias para ativação de macrófagos e morte de parasitas intracelulares (21,22).

Os macrófagos têm papel importante na resposta imunológica contra *Leishmania* spp, pois são células parasitadas e, além disso, desencadeiam a resposta adaptativa (23). Os macrófagos M1 ou M2 dominantes direcionam os linfócitos T para produzir respostas de Th1 ou Th2, respectivamente, amplificando ainda mais as respostas do tipo M1 ou M2 nas respostas imunológicas à infecção, tumor ou inflamação (24). Nos macrófagos M1 a L-arginina é metabolizada pela óxido nítrico sintase induzível (iNOS) para gerar NO e citrulina (24), matando o parasita, enquanto que em macrófagos M2, a L-arginina é degradada pela arginase em ornitina e ureia (24).

A arginase leva à produção de poliaminas que promovem o crescimento intracelular da *Leishmania* spp. (23). Alta atividade da arginase já foi observada no sangue de pacientes com LV (25) sendo considerado um marcador do agravamento da doença humana (26). A inibição da arginase reduz o número de parasitas e retarda a evolução da doença em camundongos infectados por *Leishmania* (27). Em macrófagos de camundongos infectados com *L. donovani* também foi observado um aumento da atividade arginase (23).

Nos cães infectados com *L. infantum* foi observado aumento no perfil de macrófago M2 em amostras de pele, linfonodo e baço (28), porém em cães naturalmente infectados por *Leishmania* spp. a atividade arginase em macrófagos do linfonodo foi reduzida e um aumento de NO_2 foi observado no sobrenadante da cultura, sugerindo a polarização de macrófagos para o perfil M1 (29).

O NO_2 é uma importante molécula efetora diretamente envolvida na atividade microbicida e citotóxica dos macrófagos humanos (30) e de camundongos (31). Sua produção já foi observada em sobrenadante de macrófagos caninos infectados *in vitro* com promastigotas de *L infantum* (32) e pode estar envolvido na proteção contra a infecção natural por *Leishmania* spp. (33).

A liberação do NO_2 por macrófagos humanos infectados por *Leishmania* spp. e a sua atividade microbicida pode ser regulada pela PGE_2 (30), porém em cães com CanL a função reguladora de PGE_2 na produção de NO_2 não foi caracterizada no baço, principal sítio de replicação do parasita.

A PGE_2 regula múltiplos aspectos da inflamação em diferentes células imunes (34), além disso, tem potentes propriedades imunossupressoras incluindo a inibição da ativação dos macrófagos, além disso, pode induzir quimiotaxia de leucócitos e modulação de citocinas (35,36). É conhecida por exercer sua ação por ligações distintas à membrana celular associada à proteína G ligada a guanosina trifosfato heterotrimétrica acoplada a diferentes receptores prostanóides denominados receptores EP1, EP2, EP3 e EP4 (37). A proteína G acoplada a estes receptores pode desencadear diferentes vias de transdução, que podem gerar a ativação celular através dos receptores EP1, EP2 e EP4 ou inibição das respostas celulares através do receptor EP3 (38).

O aumento da PGE_2 já foi observado em sobrenadante de cultura de macrófagos de linfonodos de cães com CanL, podendo regular a produção do TNF-α nesses cães (29). Em macrófagos humanos infectados com *L. infantum* a PGE_2 diminui a carga parasitária, mediada por NO_2 (30). A PGE_2 aumenta expressão de iNOS em macrófagos murinos infectados por *Leishmania* atuando na ativação de macrófagos (31). Em camundongos infectados *in vitro* com *Leishmania major,* a carga parasitária diminui na presença dos agonistas de PGE_2 atuando nos receptores EP2 e EP4 (39). A PGE_2 também desempenha um papel regulador na produção das citocinas IL-17 e TNF-α em camundongos infectados com *Leishmania donovani* (40).

A citocina 17 (IL-17) conhecida como uma citocina inflamatória, desempenha um papel de proteção contra parasitas intracelulares, tais como a *Leishmania.* A IL-17 atua sinergicamente com o IFN-γ para potenciar a produção de NO_2 e atividade leishmanicida em macrófagos de camundongos infectados com *L. infantum* (41). Estudo com cães infectados com *L. infantum*, mostrou que a IL-17 foi positivamente correlacionada com a expressão de RNAm pela iNOS e IFN-γ. Portanto a IL-17 parece desempenhar um papel na redução do crescimento do parasita através da ativação da iNOS, sugerindo um papel protetor na infecção por *L. infantum* em cães (41). Em camundongos nocautes infectados para a subunidade Ebi3 da citocina IL-27 (Ebi3-/-) ocorre aumento da produção de IL-17 nos órgãos alvos, aumentando o fluxo de neutrófilos que auxiliam no controle da carga parasitária (42). O aumento da

IL-17 também já foi observado no soro de pacientes com LV (43). Em células esplênicas de camundongos infectados com *L. donovani,* a PGE_2 regula negativamente a produção da IL-17, ajudando na sobrevivência do parasita (40). Porém em cães infectados com *L. infantum* ainda não foi avaliado se a PGE_2 regula a produção da IL-17.

O TNF-α está associado à proteção na CanL através do mecanismo de resposta Th1 (44), portanto o controle da sobrevivência do parasita em macrófago canino foi associado ao aumento desta citocina (21). O TNF-α já foi observado em maiores concentrações no linfonodo (29,45), baço, fígado (46) e sangue (47) de cães com CanL. O papel protetor dessa citocina foi observado no baço de cães com CanL, em que fraca correlação negativa foi observada entre a expressão de TNF-α e a carga parasitária no baço (22). Em camundongos, o TNF-α é essencial para o controle da LV (48). Já foi observado também que o TNF-α aumenta a produção de NO_2 em macrófagos caninos (49) e, que no sobrenadante de cultura de leucócitos totais do linfonodo de cães naturalmente infectado a PGE_2, aumenta a produção de TNF-α (29). Em macrófagos murinos infectados por *L. donovani* o agonista do receptor de PGE_2 (EP2) diminuiu a produção de TNF-α (40). Porém no baço de cães infectados com *L. infantum* ainda não foi investigado se a PGE_2 regula a produção do TNF-α.

Por outro lado, a IL-10 tem sido indicada como a principal citocina supressora da resposta imune protetora em humanos e em modelos de murinos infectados com LV (50). A IL-10 é produzida por diferentes tipos de células, tais como macrófagos, células dendríticas, células B e diferentes subtipos de linfócitos T (51,52). Além disso, a IL-10 pode atuar diretamente sobre as células T $CD4^+$ através da inibição da proliferação e produção de IL-12, IFN-γ, IL-4, IL-5 e TNF-α (52–54). O aumento da IL-10 já foi observado no baço (55), linfonodo (29,45) e sangue periférico (56) de cães com CanL quando comparado aos cães saudáveis. Em macrófagos peritoneais e células esplênicas de camundongos infectados com *L. donovani* a produção de IL-10 é regulada pela PGE_2 (40). Na CanL a regulação de IL-10 por PGE_2 não foi caracterizada.

Estudo com agonista de receptores de PGE_2 em macrófagos peritoneais de camundongos infectados *in vitro* com *Leishmania major*, mostrou que na presença dos agonistas dos receptores de PGE_2 (EP2 e EP4) diminuiu a carga parasitária, já na presença dos agonistas dos receptores de PGE_2 (EP1 e EP3) aumentou a carga parasitária, sugerindo que os agonistas dos receptores de PGE_2 (EP2 e EP4) tem um

papel crucial na redução da carga parasitária (39). O aumento da expressão do receptor EP2, em macrófagos peritoneais de camundongos infectados, está relacionado com o aumento da sobrevivência da *L. donovani* (40), suportando seu papel na diminuição da atividade microbicida. Em cães, nenhum estudo avaliou o papel dos receptores de PGE_2 na patogênese da CanL e poucos estudos caracterizaram o papel dos receptores de PGE_2 em cães (57).

Objetivo do presente estudo foi avaliar em cultura de leucócitos esplênicos de cães com CanL e cães saudáveis o efeito regulador da PGE_2 com o estímulo de agonistas, antagonista, de inibidor e da PGE_2 na atividade da arginase, carga parasitária, NO_2, e citocinas IL-10, IL-17 e TNF-α.

2 CAPÍTULO 1 - REGULATORY EFFECT OF PGE_2 ON MICROBICIDAL ACTIVITY AND INFLAMMATORY CYTOKINES IN CANINE LEISHMANIASIS

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Parasite Immunology – Anexo A

2.1 Abstract

Canine leishmaniasis (CanL) is caused by the intracellular parasite *Leishmania infantum.* Prostaglandin E2 (PGE_2) exerts potent regulatory effects on the immune system in *Leishmania* infection, but in CanL has not yet been studied. In this study, PGE_2 and PGE_2 receptor levels and the regulatory effect of PGE_2 on arginase activity; NO_2; the IL-10, IL-17 and TNF-α and parasite load in the presence of agonists, antagonists and inhibitors were evaluated in cultures of splenic leukocytes obtained from dogs with CanL. Our results showed that splenic leukocytes from dogs with CanL had lower EP2 receptor levels than splenic leukocytes from healthy animals. We observed that NO_2 levels decreased when the cells were treated with PGE_2 receptor agonist (EP1/EP2/EP3), a PGE_2 receptor antagonist (AH-6809) or a COX-2 inhibitor (NS-398) and that TNF-α and IL-17 cytokine levels decreased when the cells were treated with a PGE_2 receptor agonist (EP2) or stimulated with PGE_2. The parasite load in splenic leukocyte cell cultures from dogs with CanL decreased after stimulation of the cells with PGE_2. We conclude that the infection of dogs by *Leishmania* modulates PGE_2 receptors and speculate that the binding of PGE_2 to its receptors may activate the microbicidal capacity of cells.

Keywords: Prostaglandin E2, Receptors, *Leishmania* spp, Dogs

2.2 Introduction

In Brazil, visceral leishmaniasis is caused by the intracellular parasite *Leishmania infantum*, which is transmitted by the bite of the female sandfly mosquito (Phlebotominae) (1). Ninety-four percent of cases of visceral leishmaniasis (VL) worldwide occur in only seven countries: Brazil, Ethiopia, India, Kenya, Somalia, South Sudan and Sudan (2). According to one estimate, 500,000 new cases of VL occur annually, and approximately 12 million people are infected with this parasite (3). Dogs are considered the main urban source of *L. infantum* (4) due to the intense parasitism of canine skin by this organism, and contact with infected dogs can lead to infection in humans (5). The main clinical signs of canine leishmaniasis (CanL) are apathy, anorexia, weight loss, fever, mucosal pallor, loss of muscle mass, generalized peripheral lymphadenopathy, hepatomegaly, splenomegaly, epistaxis and cutaneous lesions (6).

Increased levels of PGE_2 have been observed in the culture supernatants of macrophages obtained from the lymph nodes of dogs with CanL with a regulatory role in the production of TNF-α in dogs with CanL (7). In human macrophages infected with *L. infantum,* PGE_2 regulates the parasite load in a manner that is mediated by NO_2 (8). Activation of macrophages by PGE_2 increase the expression of iNOS in murine macrophages infected with *Leishmania* (9). PGE_2 is known to act through four distinct prostanoid receptors (EP1, EP2, EP3 and EP4) that are coupled to G proteins (10). EP1, EP2 and EP4 receptors activate the cellular response, whereas the EP3 receptor inhibits the cellular response (11). In mice infected *in vitro* with *Leishmania major,* the parasite load decreased in the presence of PGE_2 agonists that act on EP2 and EP4 receptors (12). PGE_2 plays a regulatory role in the production of cytokines IL-17 and TNF-α in mice infected with *Leishmania donovani* (13).

In light of the regulatory action of PGE_2 on the cellular immune response in dogs (7), the present study was designed to evaluate the regulatory effects of PGE_2 on cultured splenic leukocytes obtained from dogs with CanL and from healthy dogs; the effects of stimulation by agonists, antagonist, inhibitor and PGE_2 on arginase activity, parasite load, NO_2 and the levels of the cytokines IL-10, IL-17 and TNF were assessed.

2.3 Material and methods

2.3.1 Approval by the Ethics Committee

This study was approved by the Comitê de Ética em Pesquisa Experimental Animal (Ethics Committee on Experimental Animal Research, COBEA) and the Comitê de Ética no Uso Animal (Ethics Committee on Animal Use, CEUA) of the São Paulo State University "Júlio de Mesquita Filho" (UNESP), Campus of Araçatuba, School of Veterinary Medicine (FMVA) on 04/15/2011 according to process 00977-2016.

2.3.2 Triage of animals

Twenty adult dogs with CanL were used in this study. The dogs were of both sexes, two to five years of age, and of various breeds and weights. The dogs were obtained from the Centro de Controle de Zoonoses de Araçatuba (Center for Zoonosis Control of Araçatuba, CCZA). All of the animals were seropositive for the *L. infantum* antigen by indirect ELISA (14), were symptomatic, and presented at least three clinical signs of CanL: onychogryphosis, lymphadenopathy, periocular lesions, skin lesions and cachexia (STable 1). The infected dogs had stage two disease (6) and were classified based on physical examination, anti-*Leishmania* antibody levels as determined by indirect ELISA (14), complete blood count (STable 2A and B) and evaluation of their serum biochemical profiles (STable 3). Molecular diagnosis was also performed for the detection of parasite DNA in spleen samples (STable 1). The spleen fragments were obtained after euthanasia of the animals at the Center for Zoonosis Control of Araçatuba (CCZA). The healthy group consisted of 10 dogs of various breeds and weights; these animals were negative by PCR and had negative serology for *Leishmania* spp. (14) (STable 4). The owners of these animals provided written informed consent to the procedure that was performed. All animals showed complete blood counts (STable 5 A and B) and biochemical profiles (STable 6) within the reference values for the species.

2.3.3 Collection of samples

Dogs with CanL were euthanized by intravenous injection of barbiturates (Tiopental, Cristália Itapira, SP) followed by 19.1% potassium chloride solution according to state law (São Paulo, 2006). After euthanasia, spleen fragments were collected and maintained in RPMI 1640 medium (Sigma, St. Louis, MO, USA)

supplemented with 10% heat-inactivated foetal bovine serum (FBS) (Gibco, Waltham, MA, USA), 0.03% L-glutamine (Sigma, St. Louis, MO, USA), 100 IU/mL penicillin (Sigma, St. Louis, MO, USA) and 100 mg/mL streptomycin (Sigma, St. Louis, MO, USA). Spleen fragments were removed from healthy dogs by surgical excision and were maintained at 4°C in RPMI 1640 cell culture medium supplemented as described by (15). The surgical procedure used to remove spleen fragments from healthy dogs was performed at the Luiz Quintiliano de Oliveira Veterinary Hospital, FMVA-UNESP.

2.3.4 Culture of splenic leukocytes

Splenic leukocytes were obtained from $2cm^3$ spleen fragments that were macerated and added to 10 mL of complete RPMI 1640 medium. After removal of the cell debris by passage of the macerated suspension through a cell strainer (Cell Strainer, BD Biosciences, USA), the suspension was processed with 5 mL of lysis buffer containing 7.46 g/L ammonium chloride (NH4Cl) at 4°C for 10 min, centrifuged at 2000 rpm for 5 minutes, and washed three times with phosphate-buffered saline (PBS) at pH 7.2.

The splenic leukocytes ($5x10^6$ cells per well) were incubated for 18 hours at 37 °C in an incubator containing 5% CO_2 in the presence of agonists of the PGE_2 EP1 and EP3 receptors (17-phenyl-trinor-prostaglandin E2, Santa Cruz Biotechnology, Dallas, USA 1 nM), the EP2 receptor (R-Butaprost, Santa Cruz Biotechnology, Dallas, USA, 1 nM) or the EP4 receptor (TCS 2510, Santa Cruz Biotechnology, Dallas, USA, 1 nM) (13), an antagonist of PGE_2 EP1 and EP2 receptors (AH-6809, 10 μM) (13), or a COX-2 inhibitor (NS-398, 1 mM) (13) (Santa Cruz Biotechnology, Dallas, USA). PGE_2 (1 mM) (Cayman Chemical, Michigan, USA) was also used (13).

The culture supernatants were harvested, stored at -80°C and used to determine the production of PGE_2, NO_2 and the cytokines TNF-α, IL-10 and IL-17. The cells were used to evaluate PGE_2 receptor expression (EP1, EP2, EP3 and EP4) and to determine parasite load. Another fraction of cells was stored in the presence of a serine and cysteine protease inhibitor (Complete EDTA-free, Roche, Germany) at -20°C for the determination of arginase activity.

2.3.5 Indirect ELISA

Sera obtained from the dogs were analysed by indirect ELISA using total promastigote lysate *Leishmania (L.) infantum* (MHOM/BR00/MER02) as the antigen. ELISA was performed according to the technique described by (14).

2.3.6 DNA extraction and amplification by real-time PCR

DNA was extracted from the samples using a commercial kit (DNAEasy, Qiagen, Valencia, CA, USA) according to the manufacturer's recommendations. The extracted DNA was quantified in a spectrophotometer 260/280 (NanoDrop Thermo Fisher Scientific, Massachusetts, USA) for evaluation of its purity and stored at -20°C.

Parasite load quantification was performed using real-time PCR with primers that amplify the ITS1 intergenic region of the rRNA gene (5'-CTGGATCATTTTCCGATG-3' and 5'-TGATACCACTTATCGCACTT-3'). PCR was performed in a total volume of 12 µL containing 10 pmol of each primer, 2 µL of DNA (50 ng) and 6.25 µL of Quantifast SYBER Green Mix (Qiagen, Valencia, CA, USA) in a CFX96 Real-Time apparatus (Bio-Rad Laboratories, CA, USA) as follows: initial denaturation at 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 30 seconds, and a step for dissociation of the amplified fragment. For each reaction, a standard curve was obtained using DNA from *Leishmania infantum* promastigotes (MHOM/BR00/MER02) at dilutions corresponding to 10^8 to 10^1 parasites.

2.3.7 Evaluation of the expression of EP1, EP2, EP3 and EP4 receptors in $CD14^+$ cells

The expression of EP1, EP2, EP3 and EP4 receptors was evaluated by flow cytometry. Splenic leukocytes were suspended in PBS containing 5% bovine serum albumin and 10% autologous serum for 30 minutes at 4°C for Fc receptor blockade (FcR). After centrifugation at 2000 rpm at 4°C, the cells were resuspended and incubated with primary polyclonal antibodies against EP1, EP2, EP3 and EP4 receptors (Santa Cruz Biotechnology, Dallas, USA) for 1 h at 4°C. These antibodies cross-react with canine EP1, EP2, EP3 and EP4 receptors when used at the dilution recommended by the manufacturer. After two washes with saline solution containing 2% fetal bovine serum, the cells were incubated with a secondary antibody conjugated to phycoerythrin (PE) at a dilution of 1:100 (Santa Cruz Biotechnology, Dallas, USA) or with the respective control isotypes (R&D Systems, Minneapolis, MN, USA) for 30 minutes at 4°C in the dark. The cells in each experimental group were evaluated for

fluorescence using a flow cytometer (Accuri C5, BD Biosciences CA, USA). The fluorescence of PE was detected in channel FL2. The log of the fluorescence intensity was measured using BD Accuri C6 software (BD Biosciences, CA, USA). The acquisition was performed with 20,000 closed events. Data analysis was performed using BD Accuri C6 software, Version 1.0.264.21 (BD Biosciences CA, USA).

2.3.8 Measurement of arginase activity

The arginase activity of spleen cell lysates was determined. For this, $5x10^6$ cells were lysed in 50 µL of 0.1% Triton X-100 followed by agitation for 30 minutes at 37°C. The lysate was then incubated with 50 µL of $MnCl_2$ (10 mM) and 50 mM Tris-HCl, and the enzyme was activated by heating the sample at 56°C for 10 min. Hydrolysis of L-arginine was performed by adding 25 µL of 0.5 M L-arginine (pH 9.7) and stirring the sample at 37°C for 60 minutes. The reaction was terminated by the addition of 400 µL of H_2SO_4 (96%):H_3PO_4 (85%):H_2O (1:3:7 v/v/v). The concentration of urea in each sample was measured based on the light absorption of the sample at 540 nm after the addition of 25 µL of α-isonitrosopropiophenone (ISPF) (dissolved in 100% ethanol) and heating at 100 ºC for 45 minutes (16).

2.3.9 Quantification of NO_2

NO_2 levels in the supernatants of splenic leukocyte cultures were determined using standard Griess reagent (17). Briefly, 100 µL of supernatant from splenic leukocyte cultures was mixed with an equal volume of Griess reagent containing 1% sulfanilamide (Sigma-Aldrich, St. Louis, MO, USA) diluted in 5% H_3PO_4 and 0.1% N-(1-naphthyl)-ethylenediamine (Sigma-Aldrich, St. Louis, MO, USA). After a 10 minute incubation at room temperature, the plates were read in a spectrophotometer (Packard Bio Science Company, Meriden, CT, EUA) with a 540 nm filter. The NO_2 concentration was determined by comparison of the obtained values with those obtained using a standard curve of 0.78 to 200 µM nitrite (NO_2).

2.3.10 Determination of IL-10, TNF-α and IL-17 levels

The concentrations of the cytokines IL-10, TNF-α and IL-17 in the supernatants of the splenic leukocyte cultures were determined by capture ELISA using a commercial reagent (Duo SET® Canine IL-10, TNF-α and IL-17, R&D Systems, Minneapolis, MN, USA). The assay was performed according to the manufacturer's

instructions. The plates were read using a Spectra Count™ reader (Packard Bio Science Company, Meriden, CT, EUA) with a 450nm filter. All measurements were performed in duplicate.

2.3.11 Determination of PGE_2 levels

Dosage of PGE_2 was determined by competitive ELISA of supernatants obtained from splenic leukocyte cultures using a commercial prostaglandin E2 reagent (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendations.

2.3.12 Quantification of parasites in cell culture

For quantification of parasites in the cell cultures, the flow cytometry method of (18) was used with modifications. Briefly, at the end of the incubation period, the cells were harvested and incubated for 60 minutes at room temperature in PBS (pH 7.2) containing 1% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA). After centrifugation of the samples at 400 g for 5 minutes, permeabilization of the membrane and of the parasitophorous vacuole was performed by resuspending the cells in ethanol (Sigma-Aldrich, St. Louis, MO, USA) for 60 minutes at -20°C. The cells were then washed with 2% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) in PBS and incubated for 60 minutes at 4°C with monoclonal antibody GP63 (ABD, Serotec Kidlington, United Kingdom). After three successive washes with PBS, the amastigotes were stained with 1 µL of anti-IgG conjugated to phycoerythrin (PE) (Sigma-Aldrich, St. Louis, MO, USA) and with an anti-human CD14 monoclonal antibody conjugated to fluorescein isothiocyanate (FITC) (ABD, Serotec Kidlington, United Kingdom) for 60 minutes at 4°C. The cells were then washed with PBS and stored at 4°C in the dark until analysis. Cells from each experimental group were evaluated for intracellular fluorescence by flow cytometry (Accuri C5, BD Biosciences CA, USA). FITC fluorescence was collected in Channel FL1, and PE fluorescence was collected in channel FL2. Fluorescence analysis was performed using BD Accuri C6 software (BD Biosciences CA, USA). The acquisition was performed with 20,000 closed events.

2.4 Results

2.4.1 Evaluation of PGE_2 receptors

Although PGE_2 receptors play a role in various infections (11), their role in CanL has not yet been studied. We observed that expression of the EP2 receptor was lower in splenic leukocyte cultures from dogs with CanL than in splenic leukocyte cultures from healthy dogs. No difference in the expression of EP1, EP3 or EP4 receptors was observed (Figure 1A). The overlap histogram shown in Figure 1B is representative of the expression of EP2 in splenic leukocytes from dogs with CanL and in splenic leukocytes from healthy dogs.

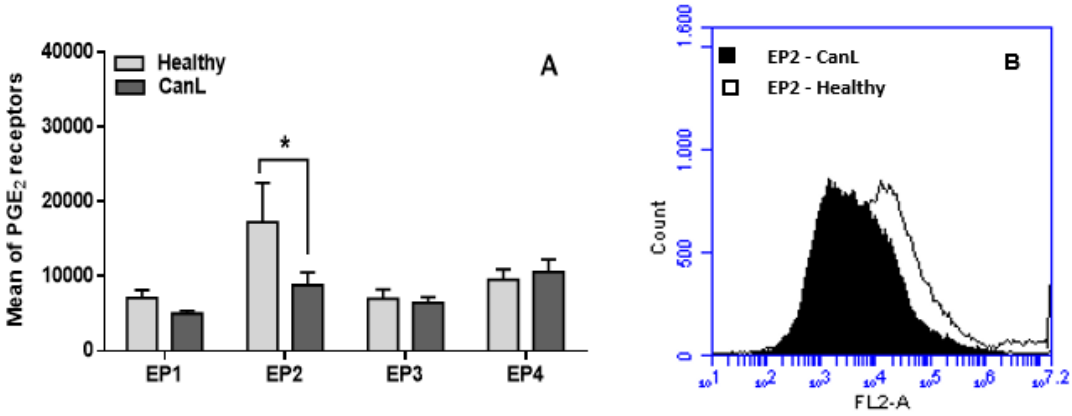


Figure 1: (**A**) Expression of PGE_2 receptors (EP1, EP2, EP3 and EP4) in splenic leukocytes from dogs with CanL (N = 20) and from healthy dogs (N = 13). (**B**) Representative flow cytometry overlap histogram showing EP2 expression in splenic leukocytes from dogs with CanL and healthy dogs. The statistical test used was the Mann-Whitney test ($p < 0.05$). The graphs in panel **A** show the mean ± the standard error of the mean.

2.4.2 Assessment of arginase activity

The arginase-1 pathway is very important in the regulation of infection by *Leishmania* spp. In dogs with CanL, splenic leukocytes show an increased M2 profile (19), but the relationship of this phenomenon to PGE_2 has not yet been clarified. Therefore, the arginase activity of splenic leukocytes from dogs with CanL was evaluated after exposure of the cells to PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) and PGE_2 itself.

The arginase activity of splenic leukocytes from dogs with CanL was higher than that of splenic leukocytes from healthy dogs (Figure 2). Treatment of splenic leukocytes obtained from healthy dogs with PGE_2 receptor agonists (EP2 and EP4), a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or PGE_2 itself increased arginase activity, whereas no difference in arginase activity was observed after treatment of the cells with PGE_2 receptor agonists (EP1 and EP3). No difference in the arginase activity of splenic leukocytes from dogs with CanL was observed after

treatment of the cells with PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or PGE_2 itself (Figure 2).

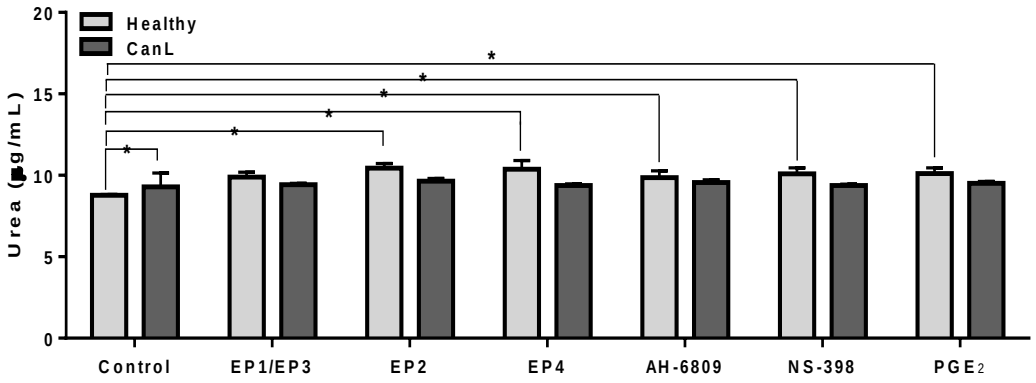


Figure 2: Arginase activity of splenic leukocytes obtained from dogs with CanL (N = 20) and from healthy dogs (N = 10) after treatment of the cells with PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or PGE_2. The Mann-Whitney test ($p < 0.05$) was used for comparisons between groups. Dunn's test was used for multiple comparisons ($p < 0.05$). The graph shows the mean ± the standard error of the mean.

2.4.3 Determination of NO_2

Because PGE_2 is known to play a regulatory role in the production of NO_2 (8), the NO_2 levels in the culture supernatants of splenic leukocytes obtained from dogs with CanL and from healthy dogs were evaluated in the presence of PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor and PGE_2 itself.

The culture supernatants of splenic leukocytes from dogs with CanL contained higher concentrations of NO_2 than the culture supernatants of splenic leukocytes from healthy dogs (Figure 3). The NO_2 concentration in the culture supernatants from splenic leukocytes of dogs with CanL decreased when the cells were treated with PGE_2 receptor agonists (EP1/EP3 and EP2), with a PGE_2 receptor antagonist (AH-6809) or with a COX-2 inhibitor (NS-398), but no difference in NO_2 concentration was observed in the presence of the PGE_2 receptor agonist (EP4) or PGE_2 itself (Figure 3). In splenic leukocytes from healthy dogs, no changes were observed after treatment of the cells with PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or PGE_2 itself (Figure 3).

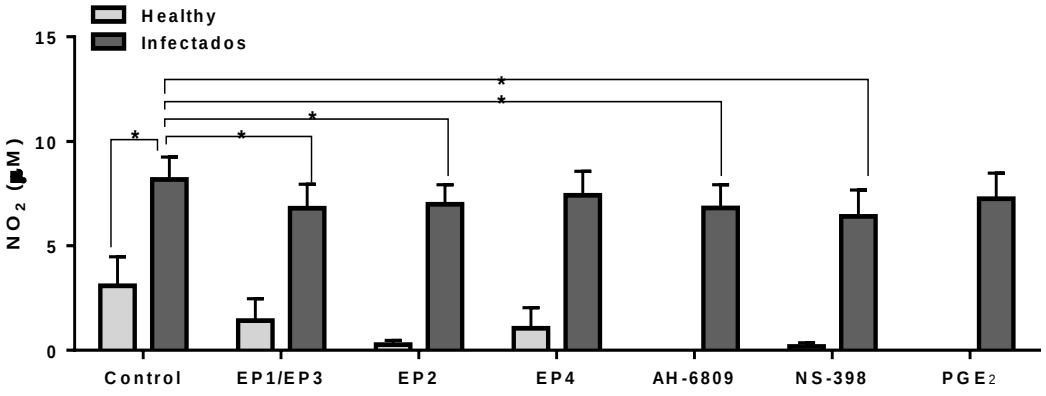


Figure 3: Concentrations of NO_2 in the culture supernatants of splenic leukocytes obtained from dogs with CanL (N = 19) and from healthy dogs (N = 10) after treatment of the cells with PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or stimulation with PGE_2. The Mann-Whitney test ($p < 0.05$) was used for comparisons between groups. Dunn's test was used for multiple comparisons ($p < 0.05$). The graph shows the mean ± the standard error of the mean.

2.4.4 Determination of IL-10, TNF-α and IL-17 levels

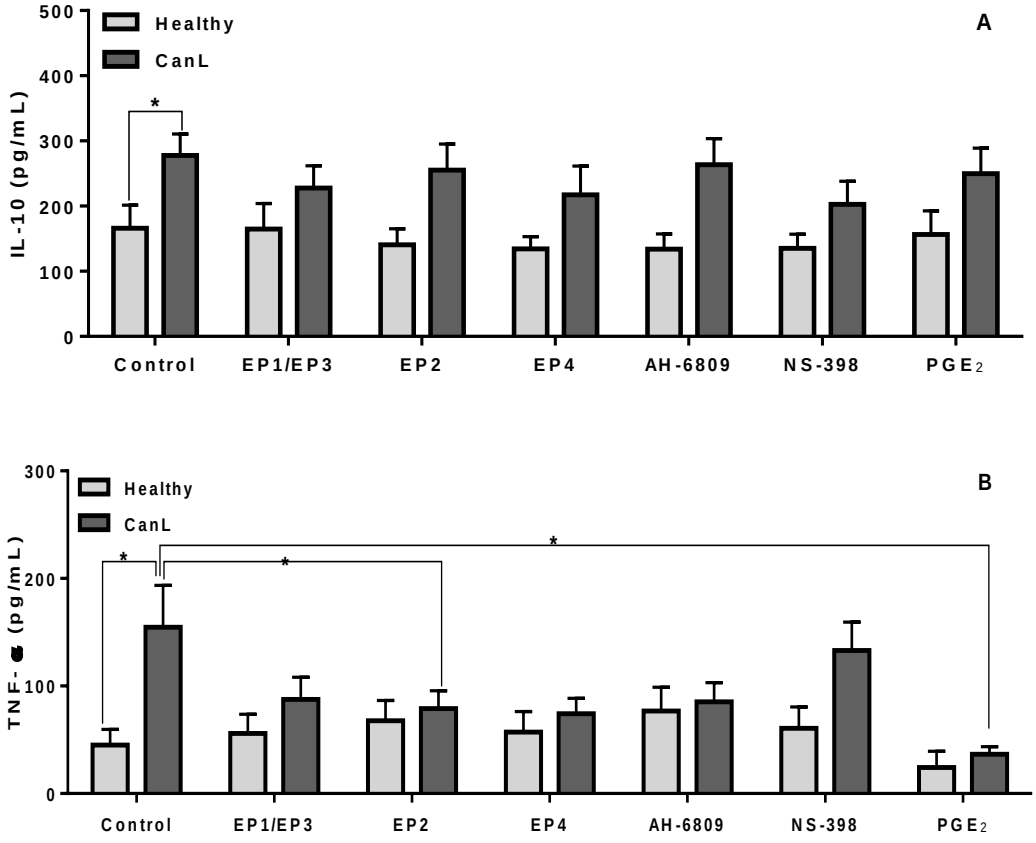
The cytokines IL-10, TNF-α and lL-17 regulate the immune response in dogs with CanL and are associated with the control (20,21) and multiplication of this parasite (22). We investigated whether PGE_2 regulates the production of these cytokines by cultured canine splenic leukocytes in the presence of PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or PGE_2 itself.

The concentration of IL-10 in the culture supernatants of splenic leukocytes obtained from dogs with CanL was higher than that in the culture supernatants of splenic leukocytes obtained from healthy dogs (Figure 4A). Treatment with PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or PGE_2 itself did not alter the concentration of IL-10 in the culture supernatants (Figure 4A).

The concentration of TNF-α in the culture supernatants of splenic leukocytes obtained from dogs with CanL was higher than that in the culture supernatants of splenic leukocytes obtained from healthy dogs (Figure 4B). Treatment with a PGE_2 receptor agonist (EP2) or the addition of PGE_2 resulted in a decrease in the TNF-α concentration in the supernatants of cell cultures from dogs with CanL, a difference that was not observed after the other treatments (Figure 4B). Treatment of splenic leukocytes from healthy dogs with PGE_2 receptor agonists, a PGE_2 receptor

antagonist (AH-6809), a COX-2 inhibitor (NS-398) or PGE_2 itself did not alter the concentration of TNF-α in the culture supernatant (Figure 4B).

The IL-17 concentration in the culture supernatants of splenic leukocytes obtained from dogs with CanL was higher than that in the culture supernatants of splenic leukocytes obtained from healthy dogs (Figure 4C). The concentration of IL-17 in the supernatants of cultured splenic leukocytes from dogs with CanL decreased when the cells were treated with a PGE_2 receptor agonist (EP2), but in the presence of other treatments, there was no difference (Figure 4C). Treatment of splenic leukocytes from healthy dogs with PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or PGE_2 itself did not alter the concentration of IL-17 in the culture supernatant (Figure 4C).



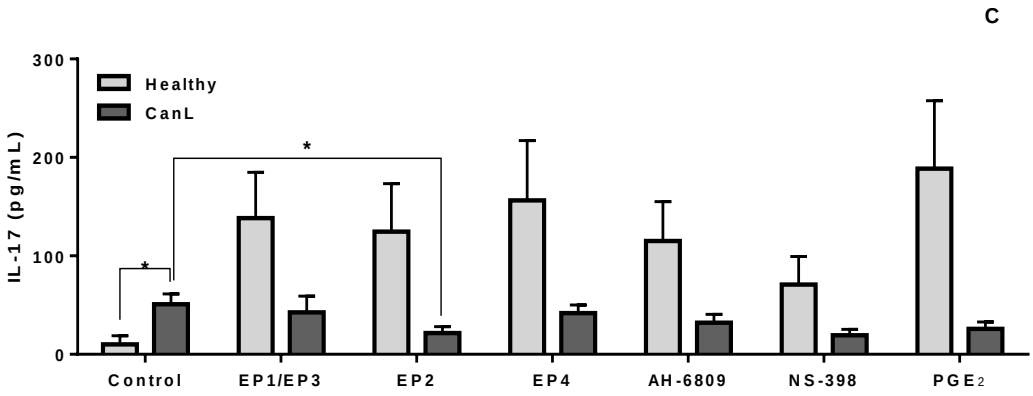


Figure 4: (**A**) Concentration of IL-10 in the culture supernatants of splenic leukocytes obtained from dogs with CanL (N = 18) and from healthy dogs (N = 6) after treatment of the cells with PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or stimulation with PGE_2. (**B**) Concentration of TNF-α in the culture supernatants of splenic leukocytes from dogs with CanL (N = 19) and healthy dogs (N = 6) after treatment of the cells with PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or stimulation with PGE_2. (**C**) Concentration of IL-17 in the culture supernatants of splenic leukocytes from dogs with CanL (N = 15) and healthy dogs (N = 4) after treatment of the cells with PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or stimulation with PGE_2. The Mann-Whitney test ($p < 0.05$) was used for comparisons between groups. Dunn's test was used for multiple comparisons ($p < 0.05$). The graphs show the mean ± the standard error of the mean.

2.4.5 Determination of PGE_2

Increased PGE_2 production by cultured cells obtained from the lymph nodes of dogs with CanL has been reported to be associated with increased NO_2 and TNF-α levels (7). Because the spleen is an important target organ of CanL, PGE_2 levels in the culture supernatants of canine splenic leukocytes were determined in the presence of PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809) and a COX-2 inhibitor (NS-398).

The concentration of PGE_2 in the culture supernatants of splenic leukocytes from healthy dogs was higher than that in the culture supernatants of splenic leukocytes from dogs with CanL (Figure 5). In leukocytes from dogs with CanL, we observed an increase in the PGE_2 concentration in the culture supernatant after treatment of the cells with PGE_2 receptor agonists (EP1 and EP3), but no such difference was observed after the other treatments (Figure 5). In leukocytes from healthy dogs, we observed a decrease in the concentration of PGE_2 in the culture supernatant after treatment of the cells with a COX-2 inhibitor (NS-398), but no difference was observed after the other treatments (Figure 5).

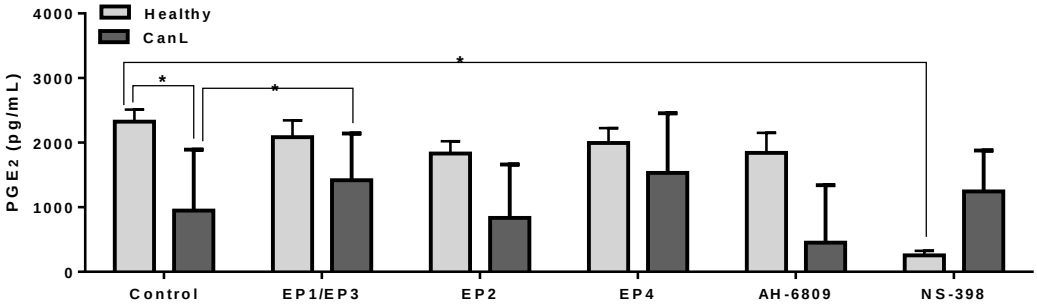


Figure 5: Concentrations of PGE_2 in the culture supernatants of splenic leukocytes obtained from dogs with CanL (N = 12) and from healthy dogs (N = 7) after treatment of the cells with PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or stimulation with PGE_2. The Mann-Whitney test ($p < 0.05$) was used for comparisons between groups. Dunn's test was used for multiple comparisons ($p < 0.05$). The graph s the mean ± the standard error of the mean.

2.4.6 Evaluation of parasite load

Specific signalling by various types of PGE_2 receptors may regulate anti-*Leishmania* activity (12). Therefore, the parasite load in splenic macrophages from dogs with CanL was evaluated in the presence of PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) and PGE_2 itself.

The parasite load in macrophage cultures from dogs with CanL decreased after treatment of the cells with a PGE_2 receptor antagonist (AH-6809) and after stimulation with PGE_2, but no differences in parasite load were observed after the other treatments (Figure 6A).

In the figure showing the cytometry results, spleen CD14+ cells from dogs with CanL were used (Figures 6B) to determine the parasite load. The overlap histograms for CD14+/GP63+ spleen cells show the parasite load in splenic macrophages from dogs with CanL after treatment of the cells with a PGE_2 receptor antagonist (AH-6809) (Figure 6C) or with PGE_2 itself (Figure 6D).

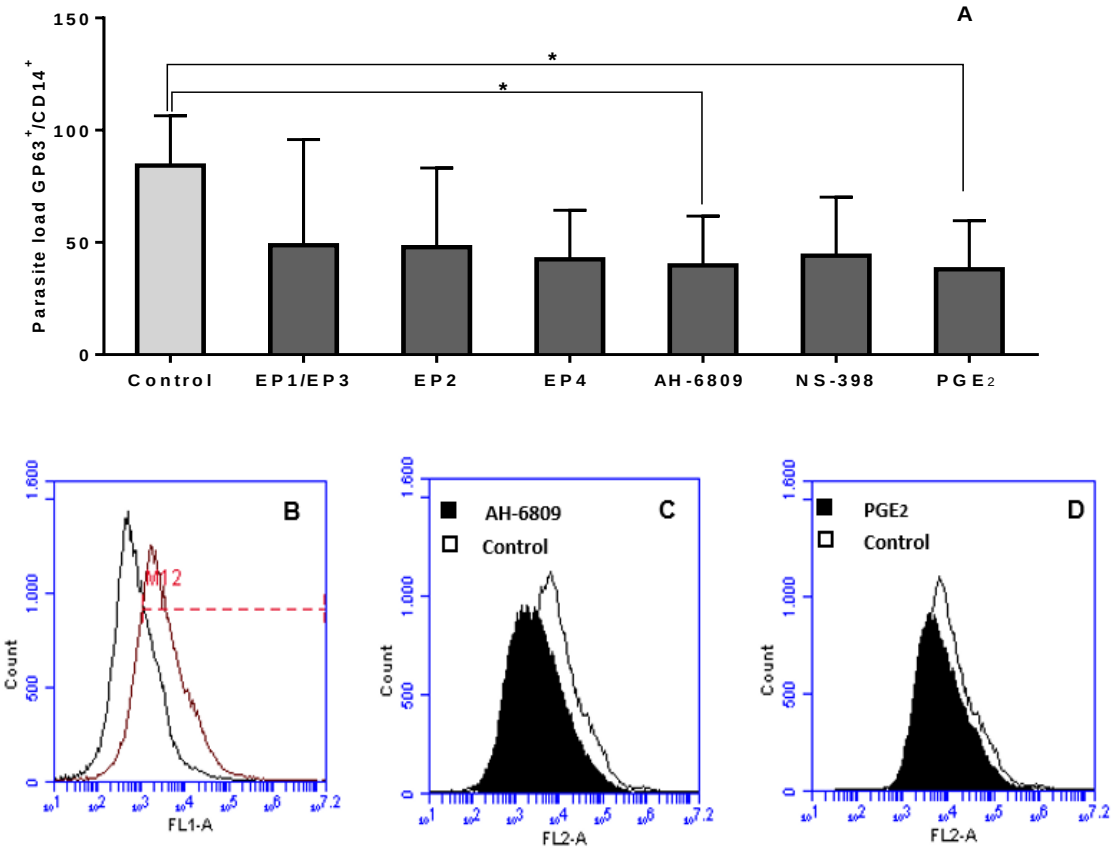


Figure 6: (**A**) Evaluation of the parasite load in cultures of splenic macrophages from dogs with CanL (N = 19) after treatment of the cells with PGE_2 receptor agonists, a PGE_2 receptor antagonist (HA-6809), a COX-2 inhibitor (NS-398) or stimulation with PGE_2. (**B-D**) Flow cytometry results showing representative overlap histograms with gate in FL1-A in splenic macrophages from dogs with CanL before (**B**) and after treatment with (**C**) a PGE_2 receptor antagonist (AH-6809) or (**D**) PGE_2. The statistical test used Dunn's multiple comparison ($p < 0.05$). The graphs show the mean ± the standard error of the mean.

2.5 Discussion

We investigated whether *Leishmania infantum* infection in dogs modulates the lipid mediator PGE_2, its receptors, arginase activity, NO_2, the levels of cytokines IL-10, TNF-α and IL-17 and parasite load in splenic leukocytes obtained from dogs with CanL and from healthy dogs. We observed that EP2 receptor expression was lower in splenic leukocytes from dogs with CanL than in splenic leukocytes from healthy dogs. Culture supernatants of splenic leukocytes from dogs with CanL showed elevated NO_2 levels, and the levels decreased when the cells were treated with PGE_2 receptor agonists (EP1/EP2/EP3), a PGE_2 receptor antagonist (AH-6809) or a COX-2 inhibitor (NS-398). The levels of the cytokines TNF-α and IL-17 in the culture supernatants of splenic leukocytes from dogs with CanL decreased when the cells were treated with a PGE_2 EP2 receptor agonist; TNF-α levels also decreased when the cells were

stimulated by PGE_2. The parasite load in splenic macrophage cultures from dogs with CanL decreased in the presence of PGE_2.

Our results show that the PGE_2 receptor EP2 is expressed at a lower level in leukocytes from dogs with CanL than in leukocytes from healthy dogs. Contrasting results were observed in peritoneal macrophages from mice infected with *L. donovani*; in those experiments, the EP2 receptor showed increased expression (13), suggesting that different parasites and hosts may regulate the expression of this receptor differently.

We observed that splenic leukocytes from dogs with CanL displayed increased arginase activity. High levels of arginase in peripheral blood mononuclear cells (PBMCs) of patients with VL have been shown to be associated with the severity of the disease (23). In addition, arginase expression in macrophages promotes parasite growth in *in vitro* experimental models of leishmaniasis (24,25), suggesting that in dogs with CanL arginase activity is associated with immune suppression, which aggravates the disease.

The arginase activity of leukocytes from dogs with CanL did not change in the presence of PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or PGE_2 itself. In contrast, in a biopsy of a patient with diffuse cutaneous leishmaniasis caused by infection with *L. amazonense*, PGE_2 increased the M2 profile (26). Our results suggest that PGE_2 is not directly related to the regulation of arginase 1 in dogs with CanL.

We found that the arginase activity of splenic leukocytes obtained from healthy dogs increased in the presence of PGE_2 receptor agonists (EP2 and EP4), a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) and PGE_2 itself. This suggests that PGE_2 is involved in the conversion of macrophages into M2. This conversion was not observed in infected dogs, possibly due to a predominance of the M2 profile (19).

The NO_2 levels in the culture supernatants of splenic leukocytes from dogs with CanL were higher than those in the supernatants of splenic leukocytes from healthy dogs. In other studies, elevated NO_2 levels were found in culture supernatants from mononuclear cells obtained from the peripheral blood of dogs with CanL (27), in culture supernatants of macrophages from dogs infected with *L. infantum* (28) and in culture supernatants of lymph node macrophages from dogs with CanL (7). These observations confirm that NO_2 is involved in the response to *Leishmania* in dogs.

The NO_2 levels in the culture supernatants of splenic leukocytes from dogs with CanL decreased in the presence of PGE_2 receptor agonists (EP1/EP3 and EP2), in the presence of a PGE_2 receptor antagonist (AH-6809) and in the presence of a COX-2 inhibitor (NS-398), but no changes in NO_2 levels were observed after treatment of cultured cells from healthy animals with these agents. Similar to our findings in canine macrophages infected with *Leishmania*, indomethacin decreased NO_2 levels (8), suggesting a regulatory mechanism for PGE_2 in the production of NO_2.

Arginine is a common substrate for the synthesis of urea, NO_2, polyamines, creatinine, proline and glutamate. L-ornithine is a product of arginase activity and is necessary for the production of polyamines and proline (29). In splenic leukocytes from dogs with CanL, PGE_2 appears to regulate arginine metabolism to produce NO_2 rather than urea.

IL-10 has been indicated as the main cytokine suppressor of the protective immune response in humans and in murine models of VL infection (22,30). In culture supernatants of splenic leukocytes from dogs with CanL, we observed increased IL-10 levels compared to the levels in culture supernatants of splenic leukocytes from healthy dogs. The results confirm the regulatory role of this cytokine in the spleen (31), lymph nodes (7,32) and peripheral blood of dogs with CanL (33). However, treatment of splenic leukocytes from dogs with CanL with PGE_2 receptor agonists, a PGE_2 receptor antagonist (AH-6809), a COX-2 inhibitor (NS-398) or PGE_2 itself did not alter the production of IL-10. This contrasts with previous results obtained in mice, in which PGE_2 selectively regulates cAMP signalling to increase the production of IL-10; in mice, this modulatory effect can be triggered by EP1 or EP4 receptors (34). The results suggest that PGE_2 is not involved in the production of IL-10 in dogs.

TNF-α is associated with protection against CanL through the Th1 response mechanism (35). The control of parasite survival in canine macrophages was associated with increased levels of this cytokine (36). The concentration of TNF-α was higher in culture supernatants of splenic leukocytes from dogs with CanL than in culture supernatants of splenic leukocytes from healthy dogs. Similar to our findings, high expression of TNF-α was observed in the spleen (37), lymph nodes (7,32) and blood (38) of dogs with CanL; the results confirm that TNF-α is involved in the immune response of dogs at all infection sites. The decrease in the production of TNF-α in cultures of splenic leukocytes from dogs with CanL in the presence of PGE_2 and a PGE_2 receptor agonist (EP2) observed in our study has been observed previously in

macrophages from mice infected with *L. donovani* (13), suggesting that PGE_2 signalling via the EP2 receptor may be involved in the decrease in TNF-α levels in CanL.

Cytokine 17 (IL-17), which is known as an inflammatory cytokine, plays a protective role against intracellular parasites such as *Leishmania*. IL-17 acts synergistically with IFN-γ to potentiate NO_2 production and leishmanicidal activity in macrophages of mice infected with *L. infantum* (21). It has been reported that PGE_2 can also decrease the secretion of Th17 cytokines in *Leishmania* mice infection (13). We observed high concentrations of IL-17 in the culture supernatants of splenic leukocytes obtained from dogs with CanL. Increased IL-17 levels have also been observed in the serum of patients with VL (39) and in the culture supernatants of splenic leukocytes from mice infected with *L. infantum* (21). Our results confirm the role of IL-17 in the infection of dogs with CanL.

We have shown that treatment with a PGE_2 EP2 receptor agonist decreases the production of IL-17 by splenic leukocytes obtained from dogs with CanL. Similarly, the PGE_2 EP2 receptor agonist also decreases IL-17 levels in mice infected with *L. donovani* (13). We suggest that PGE_2 regulates the production of IL-17 through the PGE_2 EP2 receptor the spleen in CanL.

We observed low PGE_2 concentrations in the culture supernatants of splenic leukocytes obtained from dogs with CanL. Similarly, low concentrations of PGE_2 were observed in the serum of dogs with CanL, and the magnitude of the decrease in PGE_2 level was associated with the severity of the disease (40). This confirms that PGE_2 may be associated with the ability to control infection.

In turn, the concentration of PGE_2 was higher in culture supernatants from splenic leukocytes obtained from healthy dogs compared to those of leukocytes obtained from dogs with CanL. In another study, a high concentration of PGE_2 was observed in the culture supernatants of leukocytes obtained from the lymph nodes of dogs with CanL (7). This difference may be related to the differences in the immune response to different sites of infection, as greater parasite load was evidenced in the spleen than in the lymph nodes (41).

The parasite load in cultures of splenic macrophages from dogs with CanL decreased in the presence of the PGE_2 receptor antagonist (AH-6809) and in the presence of PGE_2 itself. In other studies, PGE_2 decreased the parasite load in canine macrophages (8), and the PGE_2 receptor antagonist (AH-6809) decreased the parasite

load in peritoneal macrophages of mice infected with *L. donovani* (13). Our results suggest that PGE_2 is involved in the control of leishmanicidal activity in dogs with CanL.

In the presence of the COX-2 inhibitor NS-398, we observed no difference in the parasite load. In contrast, NS-398 was reported to decrease the parasite load in peritoneal macrophages of mice infected with *L. donovani* (42). This difference may be related to the NS-398 inhibitor concentration, which was 25-fold lower in our study than in the study of (42).

We conclude that *Leishmania* infection regulates PGE_2 receptors in dogs and that the binding of PGE_2 to receptors can activate the microbicidal capacity of cells.

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2.7 Support information

STable 1: Main clinical signs associated with CanL; serological and molecular diagnosis of dogs with CanL.

Animals	Sex	ELISA/optical density > 0.270	PCR	Clinical signs
Infected 1	Male	1.472	Positive	Onychogryphosis, Cachexia, Skin lesions, Alopecia
Infected 2	Male	1.504	Positive	Onychogryphosis, Cachexia, Skin lesions, Alopecia
Infected 3	Female	1.321	Positive	Onychogryphosis, Cachexia, Skin lesions, Alopecia, Lesions in the ear
Infected 4	Male	0.838	Positive	Onychogryphosis, cachexia, Skin lesions, Alopecia, Lesions in the ear, Hepatosplenomegaly
Infected 5	Female	0.678	Positive	Onychogryphosis, Cachexia, Skin lesions, Alopecia
Infected 6	Female	1.016	Positive	Lymphadenopathy, Hepatosplenomegaly, Onychogryphosis
Infected 7	Male	1.483	Positive	Onychogryphosis, Skin lesions, Lesions in the ear
Infected 8	Female	1.123	Positive	Lymphadenopathy, Onychogryphosis, Cachexia
Infected 9	Female	0.755	Positive	Onychogryphosis, Cachexia, Skin lesions, Alopecia, Lesions in the ear, Periocular lesion
Infected 10	Male	0.698	Positive	Onychogryphosis, Skin lesions, Periocular lesion, Cachexia
Infected 11	Male	0.398	Positive	Onychogryphosis, cachexia, Skin lesions, Alopecia, Periocular lesion
Infected 12	Female	0.926	Positive	Hepatosplenomegaly, Onychogryphosis, Lesions in the ear, Skin lesions, Periocular lesion
Infected 13	Female	0.430	Positive	Onychogryphosis, Skin lesions, Lesions in the ear
Infected 14	Female	0.484	Positive	Lymphadenopathy, Onychogryphosis, Hepatosplenomegaly
Infected 15	Male	0.723	Positive	Onychogryphosis, Skin lesions, Periocular lesion, Cachexia
Infected 16	Female	1.055	Positive	Onychogryphosis, Cachexia, Skin lesions, Lesions in the ear, Periocular lesion
Infected 17	Male	0.523	Positive	Onychogryphosis, Cachexia, Skin lesions, Periocular lesion
Infected 18	Male	0.757	Positive	Lymphadenopathy, Onychogryphosis, Lesions in the ear, Skin lesions
Infected 19	Female	0.916	Positive	Lymphadenopathy, Onychogryphosis, Hepatosplenomegaly, Skin lesions
Infected 20	Female	0.659	Positive	Lymphadenopathy, Onychogryphosis, Hepatosplenomegaly, Periocular lesion

STable 2A. Complete blood cell counts of dogs in the infected group.

Animals	Red Blood Cells	GV	Haemoglobin	MCV	MCHC
Reference values	**5.5 - 8.5 x10^6/µL**	**37 - 55%**	**12 - 18 g/dL**	**60 - 77 fL**	**32 - 36%**
Infected 1	3.3	20.1	7.1	60.0	35.3
Infected 2	7.8	53.2	19.0	67.9	35.7
Infected 3	5.2	34	11	63	32
Infected 4	2.0	14.3	4.3	72.2	38.2
Infected 5	4.2	28	8.3	58	28
Infected 6	1.8	12.4	3.9	67.8	31.4
Infected 7	4.7	34.9	10.1	74.1	28.9
Infected 8	2.7	18.5	6.2	69.8	33.5
Infected 9	3.3	22.6	7.4	67.8	33.7
Infected 10	6.5	46.8	16.2	72.7	25.1
Infected 11	2.4	14.4	5.5	73.2	31.6
Infected 12	2.6	17.0	5.9	65.0	34.7
Infected 13	3.3	20.9	7.1	64.1	33.9
Infected 14	2.5	18.1	5.8	73.0	32.0
Infected 15	0.8	5.6	1.7	71.3	30.3
Infected 16	2.3	15.3	5.3	68.4	34.6
Infected 17	4.1	41.7	9.5	103.1	22.7
Infected 18	3.7	24.7	7.5	66.8	30.3
Infected 19	2.6	15.9	4.9	60.5	30.8
Infected 20	3.3	21.2	6.1	65.1	28.7

Abbreviations: GV (globular volume); MCHC (mean corpuscular haemoglobin concentration); MCV (mean corpuscular volume)

STable 2B: Complete blood cell counts of dogs in the infected group.

Animals	Leukocytes	Lymphocytes	Neutrophils	Monocytes	Platelets
Reference values	**6 - 17 x10³/µL**	**1.000 - 4.800/µL**	**3.000 - 11.000/µL**	**150 - 1.350/µL**	**160 - 430x10³/µL**
Infected 1	11.6	4.988	5.800	696.0	36.0
Infected 2	8.6	1.729	6.370	455.0	242.0
Infected 3	19.0	6.543	12.123	334	110.0
Infected 4	7.5	3.675	3.600	150.0	102.0
Infected 5	15.2	5.932	8.679	539	121.0
Infected 6	12.6	5.040	6.930	378.0	43.0
Infected 7	15.0	7.130	7.440	310.0	113.0
Infected 8	5.8	1.298	4.130	354.0	145.0
Infected 9	11.6	4.872	6.032	696.0	103.0
Infected 10	25.9	4.488	20.856	528.0	168.0
Infected 11	4.7	1.470	2.989	196.0	124.0
Infected 12	3.1	1.240	1.736	62.0	30.0
Infected 13	6.7	1.428	4.964	272.0	292.0
Infected 14	12.1	1.677	9.933	516.0	121.0
Infected 15	3.0	480	2.310	120.0	73.0
Infected 16	6.1	744	5.146	186.0	383.0
Infected 17	10.4	2.553	7.326	555.0	22.0
Infected 18	14.7	4.077	9.664	906.0	167.0
Infected 19	9.1	1.615	7.030	475.0	166.0
Infected 20	3.5	936	2.412	180.0	41.0

STable 3: Biochemical profiles of infected dogs.

Animals	Albumin	ALT	AST	Creatinine	Total Protein	Urea
Reference values	**26 - 33 g/L**	**21 - 102 UI/L**	**23 – 66 UI/L**	**0.5 - 1.5 mg/dL**	**54 - 71 g/L**	**1.67-8.33 mg/dL**
Infected 1	12.9	170.3	95.8	0.7	81.7	23.9
Infected 2	13.3	13.7	78.7	0.5	84.9	4.7
Infected 3	10.3	17.4	25.1	0.6	68.4	3.9
Infected 4	10.2	22.8	55.1	0.5	50.7	1.7
Infected 5	5.5	9.9	14.4	0.5	41.5	2.3
Infected 6	9.4	22.7	49.3	0.7	64.2	7.8
Infected 7	24.8	441.3	378.3	0.6	81.2	5.1
Infected 8	8.3	33.5	41.9	1.0	60.0	14.8
Infected 9	14.6	16.8	44.5	0.7	76.8	4.5
Infected 10	6.1	12.3	50.2	0.4	43.5	12.3
Infected 11	15.5	33.8	42.3	0.6	35.9	6.2
Infected 12	7.5	9.2	21.9	0.3	35.7	4.4
Infected 13	15.0	15.7	40.5	0.8	76.6	1.7
Infected 14	7.6	6.1	44.6	0.6	36.6	8.4
Infected 15	9.0	14.7	97.6	0.9	42.5	14.0
Infected 16	13.2	7.4	53.8	1.3	69.9	8.9
Infected 17	24.9	59.6	80.3	0.5	68.1	6.7
Infected 18	13.5	60.9	78.0	4.6	52.0	52.9
Infected 19	38.3	38.3	71.7	7.7	70.7	2.3
Infected 20	19.0	31.4	58.6	9.8	92.3	5.5

Abbreviations: ALT (alanine aminotransferase); AST (aspartate aminotransferase)

STable 4. Clinical data associated with the serological and molecular diagnosis of healthy dogs.

Animals	Sex	ELISA/optical density < 0.270	PCR	Clinical signs
Healthy 1	Female	0.037	Negative	No clinical signs
Healthy 2	Female	0.041	Negative	No clinical signs
Healthy 3	Male	0.086	Negative	No clinical signs
Healthy 4	Female	0.038	Negative	No clinical signs
Healthy 5	Male	0.040	Negative	No clinical signs
Healthy 6	Female	0.030	Negative	No clinical signs
Healthy 7	Male	0.010	Negative	No clinical signs
Healthy 8	Female	0.033	Negative	No clinical signs
Healthy 9	Female	0.048	Negative	No clinical signs
Healthy 10	Male	0.047	Negative	No clinical signs
Healthy 11	Female	0.050	Negative	No clinical signs
Healthy 12	Male	0.054	Negative	No clinical signs
Healthy 13	Male	0.045	Negative	No clinical signs

STable 5A. Complete blood cell counts of healthy dogs.

Animals	Red Blood Cells	GV	Haemoglobin	MCV	MCHC
Reference values	**5.5 - 8.5 x10^6/µL**	**37 - 55%**	**12 - 18 g/dL**	**60 - 77 fL**	**32 - 36%**
Healthy 1	5.5	41.6	13.7	75.9	32.6
Healthy 2	6.7	41.0	14.1	73.1	34.3
Healthy 3	7.2	52.4	17.9	72.3	34.1
Healthy 4	5.7	41.0	14.0	71.8	34.1
Healthy 5	7.4	52.8	18.7	71.7	35.4
Healthy 6	7.2	53.2	18.1	74.0	34.0
Healthy 7	8.1	58.3	19.7	71.9	33.7
Healthy 8	7.3	55.9	19.0	76.5	33.9
Healthy 9	8.6	62.3	21.3	72.2	34.2
Healthy 10	6.0	41.8	14.0	69.6	33.4
Healthy 11	6.5	47.0	15.8	72.5	33.6
Healthy 12	6.4	45.0	15.1	70.0	33.4
Healthy 13	7.8	53.2	19.0	67.9	35.7

Abbreviations: GV (globular volume); MCHC (mean corpuscular haemoglobin concentration); MCV (mean corpuscular volume)

STable 5B: Complete blood cell counts of healthy dogs.

Animals	Leukocytes	Lymphocytes	Neutrophils	Monocytes	Platelets
Reference values	**6 - 17 x10^3/µL**	**1.000 - 4.800/µL**	**3.000 - 11.000/µL**	**150 - 1.350/µL**	**160 - 430x10^3/µL**
Healthy 1	12.1	1.750	10.000	375.0	331.0
Healthy 2	7.8	1.394	6.068	328.0	241.0
Healthy 3	12.4	2.944	8.960	512.0	317.0
Healthy 4	8.0	2.214	5.740	82.0	240.0
Healthy 5	10.3	1.768	8.424	104.0	288.0
Healthy 6	13.4	4.590	8.640	135.0	535.0
Healthy 7	11.3	1.938	9.234	114.0	238.0
Healthy 8	7.7	1.404	5.694	624.0	211.0
Healthy 9	15.0	5.425	8.835	775.0	175.0
Healthy 10	5.8	1.062	4.543	177.0	258.0
Healthy 11	10.0	2.750	7.810	220.0	296.0
Healthy 12	8.8	2.156	6.270	392.0	145.0
Healthy 13	10.0	1.976	7.488	520	242

STable 6: Biochemical profiles of healthy dogs.

Animal	Albumin	ALT	AST	Creatinine	Total Protein	Urea
Reference values	**26 - 33 g/L**	**21 - 102 UI/L**	**23 – 66 UI/L**	**0.5 - 1.5 mg/dL**	**54 - 71 g/L**	**1.67 - 8.33 mg/dL**
Healthy 1	28.63	26.84	22.61	0.77	72.58	2.69
Healthy 2	31.44	32.71	55.87	0.91	61.23	5.12
Healthy 3	32.53	22.50	40.06	0.81	70.62	6.70
Healthy 4	35.01	45.07	29.65	1.07	67.32	8.41
Healthy 5	33.17	38.63	38.64	1.07	66.02	4.59
Healthy 6	35.29	39.30	25.70	0.87	68.42	4.49
Healthy 7	33.00	101.25	63.85	0.99	57.72	8.66
Healthy 8	26.82	28.85	24.75	0.73	50.73	4.25
Healthy 9	30.65	107.77	42.25	0.67	78.25	2.41
Healthy 10	35.32	40.25	24.72	0.78	59.84	4.42
Healthy 11	33.9	41.9	61.9	1.0	59.2	4.9
Healthy 12	26.1	33.6	34.1	1.1	62.8	6.4
Healthy 13	29.8	59.5	41.0	1.4	55.7	8.3

Abbreviations: ALT (alanine aminotransferase); AST (aspartate aminotransferase)

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APÊNDICE - REFERÊNCIAS DA INTRODUÇÃO GERAL

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ANEXO A - NORMAS PARA PUBLICAÇÃO NA REVISTA PARASITE IMMUNOLOGY

Author Guidelines

Contents

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• **Commissioned Review or Article** – by invitation only
• **Brief Definitive Reports**- preliminary findings of research in progress or a case report of particular interest (1500 word limit).
• **Letters to the Editor**–are welcomed (400 word limit).
• **Meeting Reports**– reports on meetings or parts of meetings concerned with parasite immunology. These reports will be commissioned, but the editors will be pleased to receive reports that might be used

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vi. Funding statement
vii. Data availability statement
viii. Author contributions
ix. Abstract and keywords
x. Main text
xi. Acknowledgements
xii. References
xiii. Tables (each table complete with title and footnotes)
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Sample references follow:

Journal article
1. King VM, Armstrong DM, Apps R, Trott JR. Numerical aspects of pontine, lateral reticular, and inferior olivary projections to two paravermal cortical zones of the cat cerebellum. J Comp Neurol 1998;390:537-551.

Book
2. Voet D, Voet JG. Biochemistry. New York: John Wiley & Sons; 1990. 1223 p. Please note that journal title abbreviations should conform to the practices of Chemical Abstracts.

Internet Document
9. American Cancer Society. Cancer Facts & Figures 2003. http://www.cancer.org/downloads/STT/CAFF2003PWSecured.pdf. Accessed March 3, 2003.

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If the corresponding author(s) can provide a satisfactory explanation, source data and reverse the image processing underlying the aberrations, the revised figure will be accepted for publication without reporting to the institution.

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Source: **When things go wrong: correcting the scientific record**

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