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CÂMPUS DE ARAÇATUBA

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MicroRNA-194 regula a carga parasitária e a produção de óxido nítrico dependente de IL-1β em células mononucleares do sangue periférico de cães com leishmaniose

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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 Doutor em Ciência Animal (Medicina Veterinária Preventiva e Produção Animal).

Orientador: Prof. Dra. Valéria Marçal Felix de Lima

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“Que maravilha é ninguém precisar esperar um único momento para melhorar o mundo”

Anne Frank

COSTA, S.D. **MicroRNA194 regula a carga parasitária e a produção de óxido nítrico dependente de IL-1β em células mononucleares do sangue periférico de cães com leishmaniose**. 2023. 96 f. Tese (Doutorado) Ciência Animal, Faculdade de Medicina Veterinária - Universidade Estadual Paulista, Araçatuba, 2023.

RESUMO

Os cães domésticos são os principais reservatórios urbanos de *Leishmania infantum*, o agente causador da leishmaniose visceral (LV). Na leishmaniose canina (CanL), a modulação da resposta imune do hospedeiro pode estar associada à expressão de pequenos RNAs não codificantes denominados microRNA (miR). A expressão do miR-194 aumenta em células mononucleares do sangue periférico (CMSP) de cães com leishmaniose com correlação positiva com a carga parasitária. Estudamos CMSP de 5 cães saudáveis e 28 cães com leishmaniose. A expressão basal do miR-194 foi analisada utilzando qPCR. As CMSP foram transfectadas com Mimic e Inibidor sintéticos do miR-194 e cultivadas a 37º C, 5% de CO_2 por 48 horas. A expressão de possíveis alvos do miR-194 foi analisada: iNOS, NO, T-Bet, GATA3 e FoxP3 foram analisadas por citometria de fluxo; a expressão gênica de TRAF6 foi analisada por qPCR, e o nível das citocinas IL-1β, IL-4, IL-6, IL-10, TNF-α, IFN-γ e TGF-β no sobrenadantes de cultura celular foi mensurada por Ensaio Imunoenzimático de Captura (ELISA). A carga parasitária foi analisada por citometria e qPCR. Ensaio funcional com Inibitor do miR-194 e bloqueio de IL-1β e avaliação da produção de NO também foram realizados. Transfecção com o mimic do miR-194 promoveu um aumento da carga parasitária. Não houve alterações significativas na expressão de T-bet, GATA3 ou FoxP3 com aumento ou inibição do miR-194. A inibição de miR-194 aumentou IL-1β e NO em CMSP de cães doentes, e o bloqueio de IL-1β após a inibição de miR-194 diminuiu os níveis de NO. Esses achados sugerem que o miR-194 é regulado positivamente em CMSP de cães com leishmaniose e regula positivamente a carga parasitária, possivelmente diminuindo a produção de NO dependente da IL-1β. Esses resultados aumentam nossa compreensão dos mecanismos moleculares de evasão da resposta imune pelo parasita e a identificação de possíveis alvos terapêuticos.

Palavras-chave: Leishmaniose visceral. Cães. MicroRNAs. Genes. Imunologia

COSTA, S.F. **MicroRNA-194 regulates parasitic load and IL-1β-dependent nitric oxide production in the peripheral blood mononuclear cells of dogs with leishmaniasis.** 2023. 96 f. Tese (Doutorado) Ciência Animal, Faculdade de Medicina Veterinária - Universidade Estadual Paulista, Araçatuba, 2023.

ABSTRACT

Domestic dogs are the primary urban reservoirs of *Leishmania infantum*, the causative agent of visceral leishmaniasis. In canine leishmaniasis (CanL), modulation of the host's immune response may be associated with the expression of small non-coding RNAs called microRNA (miR). MiR-194 expression increases in peripheral blood mononuclear cells (PBMCs) of dogs with leishmaniasis with a positive correlation with parasite load. We studied PBMCs from 5 healthy dogs and 28 dogs with leishmaniasis. Basal expression of miR-194 was measured using qPCR. PBMCs were transfected with synthetic miR-194 mimic and inhibitor, and cultured at 37 ºC, 5% CO_2 for 48 hours. Expression of possible targets iNOS, NO, T-Bet, GATA3, and FoxP3 were measured using flow cytometry; gene expression of TRAF6 was measured using qPCR, and level of cytokines IL-1β, IL-4, IL-6, IL-10, TNF-α, lFN-γ, and TGF-β in cell culture supernatants was measured using capture enzyme-linked immunosorbent assays. Parasite load was measured using cytometry and qPCR. Functional assays followed by IL-1β blockade and assessment of NO production were also performed. Transfection with miR-194 mimic promoted an increase in the parasite load. There were no significant changes in T-bet, GATA3, or FoxP3 expression with miR-194 enhancement or inhibition. Inhibition of miR-194 increased IL-1β and NO in PBMCs from diseased dogs, and blockade of IL-1β following miR-194 inhibition decreased NO levels. These findings suggest that miR-194 is upregulated in PBMCs from dogs with leishmaniasis and increases parasite load, possibly decreasing NO production via IL-1β. These results contribute to our understanding of the mechanisms of immune evasion by the parasite and the identification of possible therapeutic targets.

Keywords: Visceral leishmaniasis. Dogs. MicroRNA. Genes. Immunology

SUMÁRIO

1 INTRODUÇÃO GERAL

1.1 Epidemiologia da Leishmaniose Visceral

Leishmanioses são doenças zoonóticas causadas por diferentes espécies de protozoários do gênero *Leishmania* e são transmitidos para animais e humanos por meio de insetos da família *Psychodidae* (1). As diferentes espécies do protozoário causam um conjunto de síndromes clínicas em humanos que podem comprometer a pele (Leishmaniose Cutânea - LC), membranas mucosas (Leishmaniose Mucocutânea - LM) e vísceras (Leishmaniose Visceral - LV) (2). A LV é a forma mais severa das leishmanioses e causada pelas espécies *Leishmania (L.) donovani* e *L. infantum* no velho mundo e *L. infantum* (sinônimo de *L. chagasi*) no novo mundo (2). A LV é caracterizada por episódios irregulares de febre, anemia, aumento do baço e fígado (1,2), com uma letalidade de mais de 90% dos casos em que os pacientes não são submetidos a nenhum tipo de tratamento (1–3).

A ocorrência da LV é amplamente distribuída no mundo, principalmente em regiões tropicais e subtropicais. Estima-se que de 50.000 a 90.000 novos casos da infecção ocorram anualmente em todo mundo, sendo que apenas de 25 a 45% dos casos são reportados a Organização Mundial de Saúde (OMS) (2). Nas américas, uma média de 3.500 casos de LV são reportados anualmente com uma taxa de mortalidade de 7% (1). Ainda nas américas, a LV é reportada em 13 países (Argentina, Bolívia, Brasil, Colômbia, Costa Rica, El Salvador, Guatemala, Honduras, Mexico, Nicaragua, Paraguai, Uruguai e Venezuela) (1).

O Brasil é o principal país endêmico para LV nas américas (2) e concentra 90% dos casos registrados na região (4). Dados do Ministério da Saúde do Brasil demonstram que em 2021 foram registrados 1683 casos de LV no país com 165 óbitos, sendo a maioria dos casos registrados na região Nordeste (46%), seguido das regiões Norte (16%), Sudeste (14%), Centro Oeste (5%) e Sul (1%) (4). Dos casos de LV registrados no Brasil no ano de 2021, 61 foram no estado de São Paulo, com 10 mortes (4). No estado de São Paulo, a doença foi notificada pela primeira vez no município de Araçatuba no ano de 1999, quando foi confirmado o primeiro caso canino autóctone no município (5) e desde de então, entre 1999 a 2019 foram 8.553 casos registrado no estado com 266 óbitos (6).

A transmissão de *L. infantum* no Brasil ocorre pela picada da fêmea do flebotomíneo hematófago *Lutzomyia longipalpis* (7), embora outras espécies de flebotomíneo já tenham sido identificadas como vetores do protozoário (8). Mesmo com a dispersão dos flebotomíneos para quase todas as regiões do país, a ausência do vetor em áreas onde existem casos de LV sugere a existência de outros modos de transmissão do parasita (9). Outros artrópodes que se alimentam de sangue, como carrapatos (10,11) ou pulgas (10) também já foram descritos como possíveis transmissores de *L. Infantum*, entretanto, não há uma conclusão sobre o papel desses ectoparasitas no ciclo transmissão da doença (12). Já foi descrito também a ocorrência de transmissão venérea (13), vertical (14–16) e por transfusão sanguínea (17).

Os cães domésticos (*Canis familiaris*) são os principais reservatórios de *L. infantum* em áreas urbanas (18) e em áreas endêmicas há uma correlação entre a prevalência de cães soropositivos e o número de casos da doença em humanos (19,20). Essa relação entre cães soropositivos e LV humana acontece devido à proximidade dos cães com os humanos, pelo alto parasitismo na pele de cães infectados, principalmente em regiões ulceradas (21), pela alta prevalência de cães infectados em áreas endêmicas e poucos apresentarem a forma sintomática da doença (22) e pela dificuldade de diagnóstico no grande número de cães doentes assintomáticos (23) . Portanto, devido à importância dos cães na manutenção do ciclo epidemiológico da LV, sugere-se que o controle da infecção e/ou doença no cão pode contribuir para o controle efetivo da doença no homem (24).

1.2 Leishmaniose Canina

A infecção canina por *L. infantum*, tem início após a inoculação de formas promastigotas metacíclicas na pele durante o repasto sanguíneo da fêmea do flebotomíneo. Embora não existam evidências diretas de todas as etapas do processo de instalação da doença no cão, no homem ou mesmo no modelo murino, o cenário geral parece ser o descrito a seguir. As promastigotas de *L. infantum* são fagocitadas por células fagocíticas residentes ou recrutadas como macrófagos e neutrófilos (25). No interior das células fagocíticas, as promastigotas (que exibem flagelo aparente) se aderem aos fagossomos e transformam-se em formas amastigotas (que possuem flagelo restrito à bolsa flagelar) (26). Neutrófilos contendo leishmania podem sofrer

apoptose e serem fagocitados por macrófagos, transferindo assim a sua carga parasitária. Os macrófagos produzem óxido nítrico (NO) para eliminação do parasita (27), no entanto, nesse primeiro momento a ativação de mecanismos leishmanicidas dos macrófagos não é suficiente para evitar a propagação do parasita (28).

Sem uma contenção apropriada, as amastigotas se multiplicam por divisão binária dentro dos macrófagos e são transportadas para o linfonodo drenante e a partir daí, no interior dos macrófagos, disseminam-se para diversos órgãos ricos em células do sistema fagocítico mononuclear (29) como o baço, fígado e a medula óssea (25). Nesses órgãos, as leishmanias continuam a proliferar no interior dos macrófagos até ocasionar a ruptura da célula e dessa forma infectar outros macrófagos (30), processo que induz a uma inflamação crônica, frequentemente granulomatosa, que promove alterações funcionais e estruturais nos órgãos afetados (31,32) e dessa maneira, causar a doença.

A Leishmaniose Canina (CanL em inglês), termo utilizado devido a infecção por *L. infantum* nos cães poder afetar tanto a pele quanto as vísceras (22) é manifestada por lesões e sintomas característicos. Os sinais clínicos mais frequentes da CanL são lesões na pele, linfoadenopatia, onicogrifose, perda de peso, caquexia, febre e doença renal (33). Alterações laboratoriais como anemia não regenerativa, hipoalbuminemia, hiperglobulinemia, trombocitopenia, proteinúria, azotemia renal e aumento de enzimas hepáticas também são encontradas nos cães doentes (34). O número e a intensidade dos sinais clínicos são determinados por um conjunto de fatores que envolvem a cepa do protozoário, a genética e principalmente o estado imunológico do hospedeiro (23). Desta maneira, alguns cães controlam a infecção por um período de tempo sem o aparecimento de sinais (estado subclínico ou assintomático) (22,35), enquanto outros apresentam evolução aguda ou progressiva da doença (estado clinico ou sintomático) (23).

1.3 Resposta imunológica na Leishmaniose Canina

A resposta imunológica desenvolvida pelo cão frente à infecção por *L. infantum* é complexa e determinante para a resistência ou susceptibilidade a doença (27,36). A imunidade inata é a primeira linha de defesa contra o protozoário. Após a inoculação na derme pela picada do flebotomíneo, a maioria das promastigotas de leishmania

são mortas pela ação do sistema complemento (27), embora a saliva do flebotomíneo possa inibir a ação do complemento e consequentemente induzir a falha da resposta imune já no momento da inoculação (37). Moléculas das leishmanias sobreviventes à ação do complemento são detectadas por receptores da imunidade inata presentes em células residentes como células de Langerhan, dendríticas, natural killer e células recrutadas como monócito/macrófagos e neutrófilos (26,38). Entre os receptores imunes inatos estão os receptores Toll-like (TLRs), que utilizam proteínas adaptadoras como o MyD88 para desencadear a produção de citocinas pró-inflamatórias, tais como TNF-α, IFN-γ e IL-12, bem como moléculas coestimuladoras B7-1/B7-2 e CD40 em macrófagos que se ligam respectivamente com o CD28 e CD40L de células T e ativam momentaneamente mecanismos leshmanicidas dos macrófagos infectados e induzem a resposta imune adaptativa (39,40).

Outro mecanismo da imunidade inata envolvido nas infecções por *Leishmania* spp. é a ativação dos inflamossomas (38,41). Os inflamossomas podem ser ativados por duas vias diferentes: a partir da ativação do receptor NODlike (NLR) NLRP3, um receptor intracelular que reconhece PAMPS (Padrões Moleculares Associados aos Patógenos) do parasita e também pela ativação de receptores da família de TNF e IL-1β (38). A ativação de NLRP3 leva a ativação da caspase-1 e clivagem da pré-IL-1β em IL-1β ativa, citocina importante na resposta imune inata contra o parasito (41). Além disso, parasitos do gênero Leishmania podem ativar também a via não-canônica do inflamassoma por meio da caspase-11 (42) . A ativação do inflamassoma aumenta a ativação de mecanismos leishmanicidas contra espécies de Leishmania, entre elas a *L. infantum* (41,43).

Apesar de todos os mecanismos da resposta inata para o controle do parasita, a ativação da resposta imune adaptativa é essencial para o controle da infecção. Como demonstrado na figura 1, tanto a resposta imune celular (Th1) quanto a resposta imune humoral (Th2) são ativadas na CanL, e a progressão da doença depende do balanço entre as respostas Th1 e Th2 (24,35,36,44). O recrutamento eficiente de células apresentadoras de antígenos (APCs) na resposta inata pode resultar em uma ativação bem sucedida da resposta imune celular (Th1), essencial para resistência à *L. infantum* nos cães infectados e predominante em cães em estados subclínicos e/ou assintomático da infecção (36,44) (Figura 1). Durante resposta Th1, células apresentadoras de antígeno, incluindo células dendríticas, capturam leishmanias, ou

fragmentos delas na derme e migram para região T do linfonodo de drenagem (25,45). Durante essa migração, as células dendríticas amadurecem e desenvolvem a capacidade de expressar moléculas coestimulatórias e produzir IL-12 (46). Já no linfonodo, após o processamento do antígeno, as células dendríticas que expressam peptídeos de leishmania acoplados ao complexo principal de histocompatibilidade (MHC classe I e classe II), expressam moléculas coestimulatórias membros da família B7 (CD80 e CD86) (47) e produzem IL-12 (48), são capazes de estimular a proliferação e a diferenciação de linfócitos T naïve em linfócitos T efetores Th1 (T CD4+ e T CD8+). As células T CD4+ Th1 migram para os tecidos afetados pela infecção e produzem IFN-γ e TNF-α e essas citocinas ativam mecanismos leishmanicidas, incluindo a produção de óxido nítrico, e espécies reativas de oxigênio (H2O2 e íon superóxido) nos macrófagos infectados pelo protozoário (48–50). Além disso, linfócitos T CD8+ também podem produzir IFN-γ ou exercer atividade citotóxica sobre células infectadas pela leishmania (51).

No entanto, a susceptibilidade à infecção por *L. infantum* nos cães e progressão da CanL é associada a uma acentuada resposta imune humoral (Th2) (52) com produção das citocinas IL-4 e IL-10 (53–55) que inibem a resposta imune celular Th1 específica ao parasita promovendo assim o aumento da carga parasitária (24) (Figura 1). As células B, que na doença subclínica produzem baixos níveis de anticorpos específicos contra *L. infantum* começam a produzir maiores níveis de anticorpos não específicos ao parasita, levando ao quadro de hipergamaglobulinemia (56). A produção exacerbada de anticorpos não específicos ao parasito leva a formação de imunocomplexos (56,57) que se depositam em órgãos como os rins, podendo levar à uma insuficiência renal crônica em estágios mais avançados da doença no cão (58). Além disso, os imunocomplexos induzem a produção de IL-10 por macrófagos diminuindo assim a ativação de mecanismos leishmanicidas (30,56,57). Recentemente têm-se estudado o desenvolvimento e o papel das células B reguladoras na progressão da doença. As células B regulatórias são capazes de produzir IL-10 em doenças autoimune e doenças crônicas, inclusive na CanL (59,60) onde a proporção de células B que produz IL-10 é maior em cães com leishmaniose e sintomáticos, sugerindo um papel importante dessas células na progressão da doença (60).

Portanto, conhecer os mecanismos moleculares pelos quais o parasita regula as respostas imunológicas, de maneira favorável à sua sobrevivência, é fundamental para entender a progressão da doença e também identificar possíveis alvos terapêuticos para a CanL.

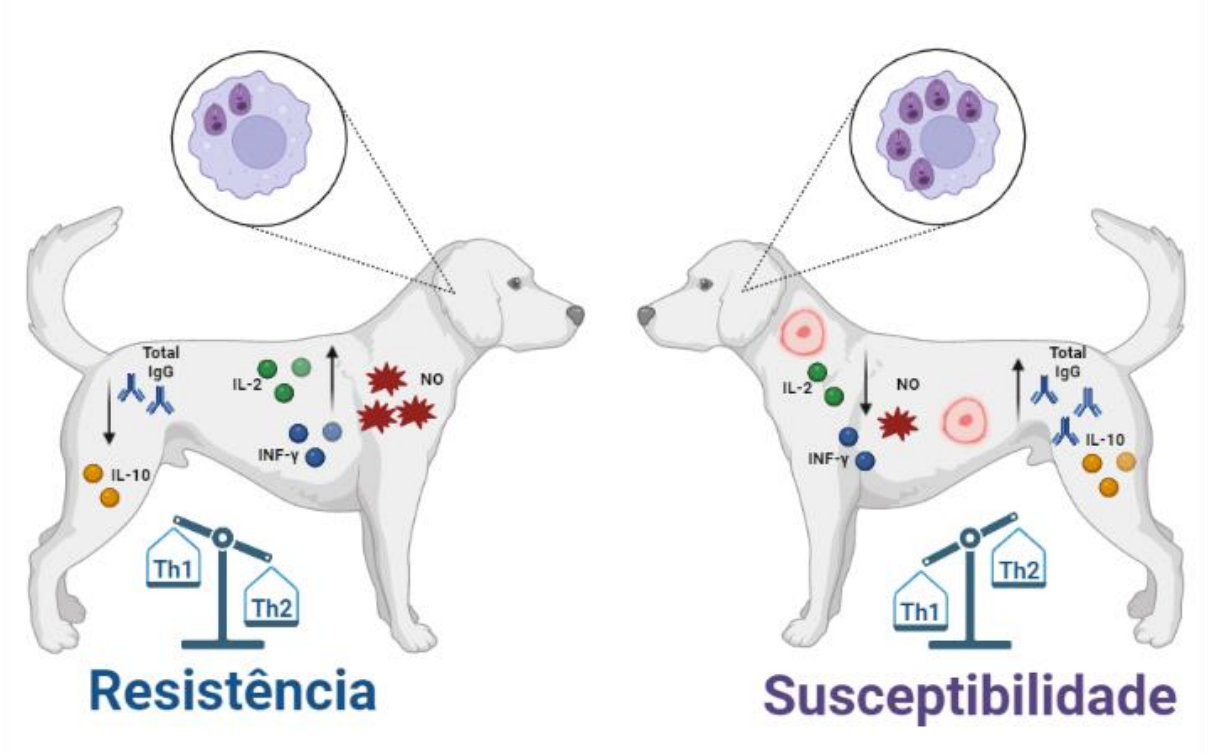

Figura 1. Na leishmaniose canina tanto a resposta imune celular Th1 quando a resposta imune humoral Th2 são ativadas e o balanço entre as duas respostas determina a resistência ou progressão da doença. Cães resistentes à infecção e assintomáticos desenvolvem uma eficiente resposta imune celular (Th1) em detrimento a resposta imune humoral (Th2) com produção das citocinas IL-2, IFN-γ e ativação de NO nas células infectadas para o controle da proliferação do parasita. Em contrapartida, cães susceptíveis e sintomáticos desenvolvem uma exacerbada resposta Th2 em detrimento a resposta Th1, com alta produção de anticorpos e produção da citocina IL-10 que inibe a ativação de mecanismos leishmanicidas e promove a replicação do parasita. (Figura criada pelo aplicativo BioRender, baseadas em 24,35,36,44).

1.4 MicroRNA (miRs)

Existem muitas evidências da função dos microRNAs (miRNAs) na regulação da expressão gênica e da tradução de proteínas que são fundamentais para o

desenvolvimento, função e diferenciação de vários tipos celulares do sistema imunológico (61,62).

MicroRNAs são RNAs pequenos, não codificadores, envolvidos na regulação do RNA mensageiro (mRNA) em nível pós-transcricional, muitas vezes atuando como inibidores da tradução e consequentemente, regulando a atividade celular. Os miRNAs se ligam ao 3'UTR do RNA mensageiro (mRNA) alvo, resultando na maioria dos casos, na inibição da tradução ou degradação direcionada do próprio mRNA (63).

Os genes que codificam os miRNAs estão localizados em várias partes do genoma como regiões intergênicas, intrôns e exôns (64). A biogênese dos miRNAs tem início com a transcrição dos miRNAs, principalmente pela RNA polimerase II em um RNA de fita dupla, formando uma estrutura de stem-loop, um tronco (stem) com terminal em forma de alça (loop), denominado de primary microRNA (pri-miRNA) (65). Após a transcrição do pri-miRNA, ele é clivado por um complexo formado principalmente por pelas enzimas Drosha e DGCR8, em precursor microRNA (pre-miRNA), que possui o formato de "grampo de cabelo" (66).

Após a maturação do pre-miRNA, ele é transportado para o citoplasma das células pela formação de um complexo com a proteína Exportin-5 (66). No citoplasma, o "grampo de cabelo" do pre-miRNA é incorporado ao complexo proteico de silenciamento induzido por RNA (RISC), onde é processado pela ribonuclease tipo III Dicer em um duplex miRNA/miRNA* (67). Em seguida, uma dessas fitas formadas, denominada "fita-guia", desempenhará suas funções juntamente com o complexo RISC (68). Um dos principais componentes do complexo RISC são as proteínas Argonautas (AGO), principalmente AGO2 (68,69). Para melhor entendimento, toda a biogênese de miRNAS é demonstrada na figura 2.

Desde a descoberta dos miRNAs, eles sempre foram relacionados negativamente com seus mRNA-alvo onde o aumento da expressão de um miRNA específico está relacionado com a diminuição na quantidade de proteína traduzida a partir do mRNA-alvo desse miRNA (70). No entanto, funções opostas dos miRNAs como promoção da transcrição e aumento da eficiência da tradução também já foram demonstradas em situações específicas (71,72). miRNAs como let-7i que se liga especificamente com o motifs TATA-box do gene da IL-2 e eleva a produção de mRNA e proteína de IL-2 em linfócitos T CD4(+) (72). Já o miR-396-3 aumenta a tradução de TNF-α por interagir com elementos ricos em AU (71).

A expressão desregulada de miRNAs ocorre em vários tipos de doenças e muitas vezes é associada à progressão e agravamento dessas doenças como na doença de Alzheimer (73), artrite reumatoide (74), doenças renais (75), doenças cardiovasculares (76), infecções virais (77–79) e em vários tipos de canceres (80). Sendo assim, vários estudos são conduzidos na tentativa de identificar a expressão diferencial de miRNAS em várias doenças e dessa maneira utiliza-los como meios de diagnóstico, prognósticos e possíveis alvos terapêuticos.

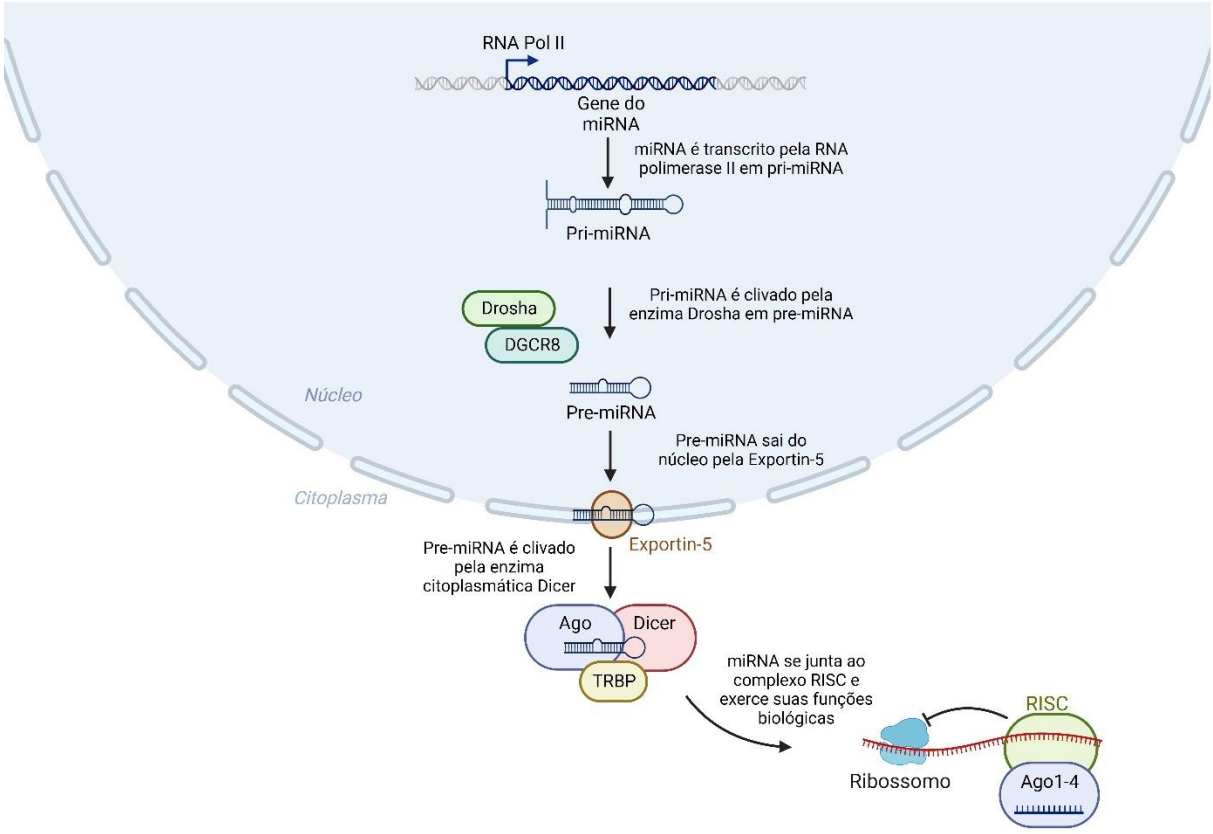

Figura 2. Via canônica da biogênese dos miRNAs. A RNA polimerase II (RNA Pol II) sintetiza o primary miRNA (pri-miRNA) a partir do seu gene. O pri-miRNA é então clivado pelo complexo Drosha/DGCR8, formando o precursor-miRNA (pre-miRNA). O pre-miRNA é então exportando para fora do núcleo pela proteína Exportin-5. No citoplasma, o pre-miRNA é clivado pela ribonuclease Dicer, juntamente com proteínas acessórias, em duas fita-simples de miRNA (duplex miRNA/miRNA*). Uma dessas fitas simples, chamada de "fita-guia" se une ao complexo RISC, onde exerce suas funções biológicas (figura retirada da dissertação de mestrado “MIR-150 regula a carga parasitária de *Leishmania infantum* e os níveis de GZMB nas PBMCS de cães com leishmaniose visceral canina”. Soares et al., 2023).

1.5 MicroRNA (miRs) na Leishmaniose Canina

Nas infecções por *Leishmania spp.*, vários estudos demonstram os miRNAs como reguladores chave das respostas celulares em células murinas, humanas e caninas (81–87), com papel importante na regulação da resposte imune celular (Th1) (85,88), sugerindo portanto, os miRNAs como alvo terapêutico na leishmaniose (84,88,89).

A expressão de um repertório de diferentes miRNAs já foi descrita pelo nosso grupo de pesquisa no baço e sangue de cães naturalmente infectados por *L. infantum* quando comparado com cães saudáveis (90,91). Análises do microarranjo e validação por PCR demonstraram aumento da expressão dos miR-21, miR-148a, miR-7, miR-615 e diminuição dos miR-125a, miR-125b e miR-150 em leucócitos esplênicos (91). Já em células mononucleares do sangue periférico (CMSP), aumento na expressão dos miRNAs miR-21, miR-192, miR-194, miR-424, miR-371, miR-451 e miR-503 e menor expressão dos miRNAs miR-150 e miR-574 foi observado (90). Os dados do microarranjo foram submetidos à análise *in sílico* e assim foi possível identificar possíveis vias e alvos dos miRNAs diferencialmente expressos em CMSP e validados pelo programa Ingenuity Pathway Analysis (QIAGEN®). Os miRNAs diferencialmente expressos nas CMSP apresentaram alvos em diversas vias canônicas relacionados a resposta imune, como por exemplo crosstalk entre células dendríticas e células NK, via STAT3, papel antiproliferativo do TOB na sinalização de células T, sinalização de PTEN e receptor de morte celular (90).

Dentre os miRNAs que tem a sua expressão alterada em CMSP de cães com leishmaniose, está o miR-194. Na análise da expressão do miR-194 por PCR em CMSP dos cães naturalmente infectados com *L. infantum*, o miR-194 mostrou-se 3,08 vezes mais expresso em CMSP de cães doentes quando comparado aos dos cães não infectados e apresentou correlação positiva com a carga parasitária nas CMSP (90). O miR-194 é conservado entre as espécies, inclusive entre cães e humanos, sendo denominado na espécie humana de miR-194-5P (92). Em cães, o miR-194 está localizado no cromossomo 38 (92).

O miR-194 está envolvido na regulação dos transcritos de genes em várias vias canônicas e dentro dessas vias, regula transcritos de genes relacionados com a resposta imunológica. Dentre eles, destacamos os transcritos de MAPK1, que aparece

regulado pelo miR-194 em 9 vias diferentes, seguidos de SOCS2, 2 vias e TRAF6 em 2 vias (90).

O gene MAPK1 codifica a proteína quinase 2 regulada por sinais extracelulares (ERK2), um membro da família das proteínas quinases ativadas por mitógeno (MAP Quinases). A ERK2, às vezes referida como p42-MAPK, juntamente com a outra isoforma ERK1 ou p44-MAPK participam da via ERK MAPKS (93). A via ERK MAPKS é ativada em resposta a fatores de crescimento, hormônios e estímulos pró-inflamatórios (94,95). O sinal mitógeno é transmitido do citoplasma para o núcleo por meio da fosforilação de várias quinases que participam da via, o que resulta na translocação nuclear da ERK1 e ERK2 e a ativação de uma série de fatores de transcrição (93,96). As funções e regulação da via ERK MAPKs estão envolvidas em diferentes fases e componentes do sistema imunológico (97).

Em camundongos deficientes de ERK2, foi observado aumento na expressão de GATA3 e FoxP3 e diminuição na expressão de T-bet em células T do baço (98). O bloqueio de ERK1/2 em macrófagos peritoneais e da medula óssea de camundongos estimulados com LPS, diminuiu mRNA e proteína TNF-α (99). A regulação negativa de MAPK1 do parasita tem sido associada a resistência ao tratamento com Antimoniais na LV humana causada *por L. donovani* (100,101). Já em macrófagos de camundongos deficientes de ERK1/2 e infectados *in vitro* por *L. amazonensis*, o parasita diminui a fagocitose e a produção de TNF-α e aumenta a produção de IL-10 (102). Esses achados demonstram que a via ERK MAPK1 pode regular vários mecanismos importantes da resposta imune do cão frente a infecção por *L. infantum*, entretanto, o papel de MAPK1 e sua regulação pelo miR-194 na CanL ainda não foram demostrados.

O gene SOCS2 codifica a proteína supressora da sinalização de citocinas 2 (SOCS2). Essa proteína é um membro da família SOCS e sua principal atividade é atenuar a transdução de sinal desencadeada por citocinas (103). A SOCS2 regula a sinalização de citocinas dependentes das vias de sinalização JAK/ STAT em células da imunidade inata e adaptativa, regulando a produção de vários mediadores como TNF-α e IL-12 (104,105) e a diferenciação de células T (106). O SOCS2 é alvo direto do miR-194, e regula negativamente a expressão de SOCS2 (107).

Nenhuma alteração na carga parasitária e produção de citocinas foi observada em células do linfonodo de camundongos Knockout para SOCS2 e infectado por *L.*

major (108). No entanto, em células T CD4+ de camundongos knockout para SOCS2, o aumento de mRNA e proteína IL-4 e IL-10 e de mRNA de GATA3 foi observado, favorecendo a diferenciação e a produção de citocinas Th2 (106). Em contraste, a redução da expressão de mRNA de IFN-y e TNF-α foi observada no baço e coração de camundongos knockout para SOCS2 infectados por *Trypanossoma cruzi* (109). Juntos esses dados sugerem que a inibição de SOCS2 pode estimular a resposta imune Th2, tipo de resposta que é associada a progressão da leishmaniose em cães, no entanto o papel do SOCS2 e sua regulação por miR-194 ainda não foram estudados na CanL.

O gene TRAF6 codifica a proteína fator 6 associada ao receptor de TNF (TRAF6). Essa proteína é um membro de família TRAF e está envolvida na transdução de sinal de membros da super família dos receptores de TNF e na via de sinalização TLR, além das vias de sinalização via receptor de osteoprotegerina OPGL e CD40 (110,111). Após a estimulação dessas vias, o TRAF6 interage com várias proteínas quinases, resultando na ativação do NF-κB e na produção de citocinas pró-inflamatórias (112,113). O TRAF6 é alvo do miR-194 e a super expressão do miR-194 diretamente diminui a expressão de TRAF6 e, consequentemente, diminuiu o mRNA de TNF-α, IL-1, IL-6 em células de núcleo pulposo de discos intervertebrais de camundongos (114) e os níveis de TNF-α e TGF-β em monócitos humanos de linhagem (115).

Além da regulação de citocinas pró-inflamatórias por TRAF6 e baixa expressão de iNOS também foi observada em macrófagos de camundongos knockout para TRAF6 em resposta a IL-1 (110) . Em modelos murinos de infecção por *L. donovani,* o parasita inibe a ligação IRAK-1/TRAF6 prejudicando a sinalização da via TLR em macrófagos (116), e redução da morte de leishmania foi observado após o bloqueio de TRAF6 (117). Esses estudos sugerem um papel importante de TRAF6 na regulação da resposta pró-inflamatória e ativação de mecanismos leishmanicidas em células infectadas, entretanto, se os mesmos mecanismos são regulados por TRAF6 na CanL, assim como a regulação de TRAF6 pelo miR-194 ainda precisam ser elucidados na doença em cães.

Dado o envolvimento do miR-194 em diferentes componentes da resposta imunológica, como diferenciação de células T, produção de citocinas pró-inflamatórias, anti-inflamatórias e imunorreguladoras, e a ativação de mecanismos

leishmanicidas, seria possível o miR-194 regular positivamente a carga parasitária em CMSP de cães com leishmaniose. Dessa maneira, esse estudo teve como objetivo elucidar por quais mecanismos o miR-194 pode aumentar a carga parasitária no sangue de cães doentes. Portanto, esse estudo avaliou o papel regulatório do miR-194 na expressão de fatores de transcrição T-bet, GATA3 e FoxP3, na produção das citocinas pró-inflamatórias IL-1β, IL-6, TNF-α e IFN-γ, anti-inflamatória IL-4 e imunorreguladoras IL-10 e TGF-β, a ativação de mecanismos leishmanicidas iNOS e NO e a expressão de TRAF6 em CMSP, e posteriormente a correlação com a carga parasitária, visando identificar mecanismos moleculares de evasão da resposta imune do parasita, assim como possíveis alvos terapêuticos para o tratamento da CanL.

2 CAPÍTULO 1 - MicroRNA-194 regulates parasitic load and IL-1β-dependent nitric oxide production in the peripheral blood mononuclear cells of dogs with leishmaniasis

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2.1 Resumo

Os cães domésticos são os principais reservatórios urbanos de Leishmania infantum, o agente causador da leishmaniose visceral (LV). Na leishmaniose canina (CanL), a modulação da resposta imune do hospedeiro pode estar associada à expressão de pequenos RNAs não codificantes denominados microRNA (miR). A expressão do miR-194 aumenta em células mononucleares do sangue periférico (CMSP) de cães com leishmaniose com correlação positiva com a carga parasitária. Estudamos CMSP de 5 cães saudáveis e 28 cães com leishmaniose. A expressão basal do miR-194 foi analisada utilzando qPCR. As CMSP foram transfectadas com Mimic e Inibidor sintéticos do miR-194 e cultivadas a 37º C, 5% de CO2 por 48 horas. A expressão de possíveis alvos do miR-194 foi analisada: iNOS, NO, T-Bet, GATA3 e FoxP3 foram analisadas por citometria de fluxo; a expressão gênica de TRAF6 foi analisada por qPCR, e o nível das citocinas IL-1β, IL-4, IL-6, IL-10, TNF-α, IFN-γ e TGF-β no sobrenadantes de cultura celular foi mensurada por Ensaio Imunoenzimático de Captura (ELISA). A carga parasitária foi analisada por citometria e qPCR. Ensaio funcional com Inibitor do miR-194 e bloqueio de IL-1β e avaliação da produção de NO também foram realizados. Transfecção com o mimic do miR-194 promoveu um aumento da carga parasitária. Não houve alterações significativas na expressão de T-bet, GATA3 ou FoxP3 com aumento ou inibição do miR-194. A inibição de miR-194 aumentou IL-1β e NO em CMSP de cães doentes, e o bloqueio de IL-1β após a inibição de miR-194 diminuiu os níveis de NO. Esses achados sugerem que o miR-194 é regulado positivamente em CMSP de cães com leishmaniose e regula positivamente a carga parasitária, possivelmente diminuindo a produção de NO dependente da IL-1β. Esses resultados aumentam nossa compreensão dos mecanismos moleculares de evasão da resposta imune pelo parasita e a identificação de possíveis alvos terapêuticos.

2.2 Abstract

Domestic dogs are the primary urban reservoirs of *Leishmania infantum*, the causative agent of visceral leishmaniasis. In Canine Leishmaniasis (CanL), modulation of the host's immune response may be associated with the expression of small non-coding RNAs called microRNA (miR). MiR-194 expression increases in peripheral blood mononuclear cells (PBMCs) of dogs with leishmaniasis with a positive correlation with

the parasite load. We studied PBMCs from 5 healthy dogs and 28 dogs with leishmaniasis. The basal expression of miR-194 was measured using qPCR. PBMCs were transfected with miR-194 scramble, mimic, and inhibitor and cultured at 37º C, 5% CO_2 for 48 hours. The expression of possible targets was measured: iNOS, NO, T-Bet, GATA3, and FoxP3 were measured using flow cytometry; gene expression of TRAF6 was measured using qPCR, and the production of cytokines IL-1β, IL-4, IL-6, IL-10, TNF-α, IFN-γ, and TGF-β in cell culture supernatants was measured using capture enzyme-linked immunosorbent assays (ELISA). Parasite load was measured using cytometry and qPCR. Functional assays followed by miR-194 inhibitor and IL-1β blockade and assessment of NO production were also performed. The mimic of miR-194 promoted an increase in parasite load. There were no significant changes in T-bet, GATA3, or FoxP3 expression with miR-194 enhancement or inhibition. Inhibition of miR-194 increased IL-1β and NO in PBMCs from diseased dogs, and blockade of IL-1β following miR-194 inhibition decreased NO levels. These findings suggest that miR-194 is upregulated in PBMCs from dogs with leishmaniasis and increases parasite load, possibly decreasing NO production via IL-1β. These results increase our understanding of the mechanisms of evasion of the immune response by the parasite and the identification of possible therapeutic targets.

2.3 Author summary

Visceral leishmaniasis (VL) is a zoonosis in tropical and subtropical regions. In the new world, it is caused by the obligate intracellular protozoan *Leishmania infantum*. In urban areas, domestic dogs are the primary reservoir. Increase in canine leishmaniasis (CanL) contributes to the increase in VL in humans. One mechanism of parasite survival is through regulation of microRNA (miRNA), responsible for translational control of transcripts related to anti-parasite immunity. One example is miR-194, which is increased in the peripheral blood mononuclear cells (PBMCs) of dogs with leishmaniasis. In the present study, we found that increasing miR-194 increases parasite load, and decreasing miR-194 increases IL1β and NO. Subsequently, we found that the increase in NO depends on IL1β. Since NO its directly involved in parasite load control, these findings contribute to understanding how the parasite evades immune responses and paves the way for the discovery of new therapeutic targets.

2.4 Introduction[1]

Visceral Leishmaniasis (VL) is a zoonosis caused in the new world by the obligate intracellular protozoan *Leishmania infantum* (syn. *Leishmania chagasi*) [1]. VL is one of the primary parasitic diseases with the potential for outbreaks and death worldwide; it occurs primarily in tropical and subtropical regions, with an estimated 50.000 to 90.000 new cases annually [2]. Brazil is an endemic region for VL, with 97% of the cases registered in the Americas [2].

The domestic dog is the primary urban reservoir [3]; in endemic regions, there is a correlation between seropositive dogs and the number of cases in humans [4,5]. In canine leishmaniasis (CanL), infection-resistant dogs develop a cellular immune response (Th1) with the production of cytokines IL-2, IL-12, and IFN-γ [6,7] stimulating production of nitric oxide (NO) in infected macrophages and parasite death [8]. By contrast, dogs susceptible to the disease develop a humoral immune response (Th2) with high antibody titers [6] and production of IL-4 and IL-10 cytokines, that inhibit activation of leishmanicidal mechanisms, resulting in increased parasite loads [9].

Modulation of immune response in favor of the parasite in CanL may be associated with the differential expression of microRNAs (miRNAs), which are small non-coding RNAs that bind to their target messenger RNA via base complementarity, resulting in the regulation of translation by messenger RNA degradation [10,11]. In CanL, differential expression of several miRNAs, miR-21, miR-148a, and miR-615 in splenic leukocytes and of miR-21, miR-159, miR-451, miR-192, miR-194, and miR371 in peripheral blood mononuclear cells (PBMCs) can impact immunity-related targets [12,13].

In PBMCs from dogs with leishmaniasis, miR-194 is increased and positively correlates with parasite load [13], possibly regulating critical immune response targets against the parasite. MiR-194 is conserved across species, including humans and dogs [14]. Dogs share the same sequence as human miR-194-5p, located in dogs on chromosome 38 [14].

In silico analyses demonstrate that the MAPK1 gene transcript is target of miR-194 in PBMCs from dogs with leishmaniasis [13]. MAPK1 can act on various elements of the immune response [15]. The MAPK1 gene encodes extracellular signal-

[1] O artigo está formatado de acordo com as normas da Revista PLOS Neglected Tropical Diseases. Vide Anexo A.

regulated protein kinase 2 (ERK2), a member of the mitogen-activated protein (MAP) kinase family. ERK2 acts in the differentiation of helper T cells and regulatory T cells, inducing a decrease in T-bet and increasing the expression of GATA3 and Foxp3 [16]. In VL caused by *Leishmania donovani*, parasite downregulation of MAPK1 is associated with resistance to treatment with Antimonials [17,18]. In ERK1/2-deficient murine macrophages infected *in vitro* with *Leishmania amazonensis*, a decrease in phagocytosis and TNF-α production and an increase in IL-10 production were observed [19]. These findings demonstrate that ERK MAPK1 pathway can regulate T-bet, GATA3, FoxP3 expression and TNF-α and IL-10 production which participate immune response against *Leishmania infantum* infection, however, the regulation of these mechanisms by miR-194 in CanL has not yet been demonstrated.

Another transcript predicted as a target of miR-194 in CanL is cytokine signaling suppressor protein 2 (SOCS2) [13]. This protein is a member of the SOCS family whose primary activity is the attenuation of cytokine-triggered signal transduction [20]. No changes in parasite load and cytokine production were observed in lymph node cells from SOCS2 knockout mice infected with *L. major* [21]. There are no studies on the role of SOCS2 in VL; however, the absence of SOCS2 in mice stimulated the differentiation of TCD4 cells to the Th2 profile in the spleen, with increased production of IL-4, IL-10, and GATA3 [22]. Furthermore, the absence of SOCS2 also decreases the expression of the Th1 cytokine IFN-γ in the spleen and heart of mice infected with *Trypanosoma cruzi.* Together, these data suggest that SOCS2 inhibition promotes an increase in IL-4 and IL-10 cytokines and the differentiation of T helper cells to Th2, a response profile associated with the progression of CanL, however, whether miR -194 regulate Th2 in sick dogs remains to be verified.

TRAF6 transcript is also a predicted target of miR-194 in CanL [13]. The TNF receptor-associated factor 6 protein (TRAF6), participates in signal transduction after stimulation of TNF family receptors and in the TLR, OPGL, and CD40 signaling pathway [24,25], resulting in NF-κB activation and pro-inflammatory cytokine production [26,27]. TRAF6 participates in NF-κB activation in murine macrophages infected by *Leishmania donovani* [28,29], and NF-κB activation promotes the expression of TNF-α and iNOs in infected cells [28]. TRAF6 is a target of miR-194 [30,31], and its inhibition positively regulated the expression of TRAF6 and the

production of cytokines IL-1, IL-6, and TNF-α in disk pulposus cells of rats [31] and TNF-α and TGF-β in a human monocytic lineage [30]. These studies suggest an important role for TRAF6 in regulating the pro-inflammatory response and activation of leishmanicidal mechanisms in infected cells, however, whether the same mechanisms are regulated by TRAF6 in CanL, as well as the regulation of TRAF6 by miR-194, still needs to be determined elucidated in the disease in dogs.

In this study, we determined whether miR-194 regulates parasite load. We measured the expression of transcription factors T-bet, GATA3, and FoxP3 and studied the leishmanicidal elements iNOS and NO in PBMCs from dogs with lesihamiasis. We measured production of cytokines IL-1β, lL-4, IL-6, IL-10, TNFα, IFN-γ, and TGFβ in culture supernatants. We also analyzed the expression of TRAF6 in these cells. We demonstrated that an increase in miR-194 activity increases parasite load, and its inhibition increases IL-1β and NO in PBMCs from dogs with leishmaniasis. We also demonstrated that increase in NO levels following reduction of miR-194 activity depended on IL-1β in PBMCs from diseased dogs.

2.5 Methods

2.5.1 Canine screening and sample collection

The Animal Experimental Research Ethics Committee approved the study, with the approval of the Animal Use Ethics Committee (CEUA) from São Paulo State University (process number 00624-2018).

To compose the infected group, we selected 28 adult dogs, of various breeds, male and female with natural leishmaniasis infections. The infected dogs came from the Zoonosis Control Center of Araçatuba/SP Brazil and presented with positive serological tests for anti-leishmania antibodies, according to indirect enzyme-linked immunosorbent assay (ELISA) [32] and immunochromatographic (rapid test DPP /Bio-Manguinhos, BR), and for *Leishmania* spp. DNA according to qPCR [33]. All dogs were symptomatic and exhibited at least three typical clinical signs of the disease in dogs, including onychogryphosis, lymphadenopathy, hepatomegaly and splenomegaly, cachexia, alopecia, periocular lesion, and skin lesions.

We selected 5 healthy dogs of various breeds, male and female for the control group. The dogs came from private owners after providing a consent form.

Healthy dogs did not show clinical, hematologic, or biochemical alterations, and the serological and molecular tests described above were negative for leishmaniasis.

Blood samples were collected by jugular vein puncture, and 8 mL were placed in plastic tubes containing K_2EDTA (Becton-Dickson, USA), intended for obtaining mononuclear cells and carrying out the hemogram. Another 4 mL was placed in plastic tubes without anticoagulant for serology and biochemical measurements.

2.5.2 PBMC isolation

PBMCs from the dogs were isolated using a gradient of Histopaque® 1077 (Sigma®, USA) following the manufacturer's instructions. Red cells were lysed using a lysis buffer containing 7.46 g/L of ammonium chloride and washed three times in phosphate-buffered saline (PBS) pH 7.2. Cells were resuspended in 1 mL of RPMI 1640 supplemented with 10% fetal bovine serum, penicillin-G 100 IU/mL, streptomycin 100 µg/mL, and L-glutamine 2 mmol/L (Sigma®, USA).

2.5.3 DNA extraction and determination of Leishmania species in dogs with leishmaniasis

The extraction of $1x10^6$ total DNA from PBMCs from dogs with leishmaniasis was performed using a commercial DNAeasy® kit (Qiagen, Valencia, California, 91355, USA) following the manufacturer's instructions. The final DNA elution was 30 µL. The Leishmania spp. was determined using PCR-RFLP [34], comparing the sample restriction profile with the profile obtained from *Leishmania infantum* (IOC/L0575-MHOM/BR/ 2002/LPC-RPV). We used a positive control and water as a negative control.

2.5.4 PBMC miRNA extraction and miR-194 expression

Total RNA from PBMC miRNAs was extracted using the mirVana™ miRNA Isolation kit (Invitrogen, CA, USA), following the manufacturer's instructions. Isolated RNAs were then analyzed on a NanoDrop™ ND-1000 spectrophotometer (NanoDrop™, Thermo Fisher, MA, USA) for purity evaluation (260/280) and quantification.

For miR-194 expression, we used a methodology described elsewhere [13]. Briefly, cDNA was synthesized using a miScript RT II kit (Qiagen, MD, USA), as recommended by the manufacturer. Next, RT-qPCR was performed using a commercially available primer specific for *Canis familiaris* miR-194 and the

endogenous reference RNA SNORD96A (Qiagen, MD, USA). Amplification conditions consisted of an activation step of 95 ˚C for 15 min, followed by 40 cycles of 94 ˚C for 15 s, 55 ˚C for 30 s, and 70 ˚C for 30 s for denaturation, annealing, and extension, respectively. We also generated a ten-fold standard curve using a cDNA pool serial dilution to evaluate reaction efficiency.

Because SNORD96A and miR-194 presented similar reaction efficiencies, miR-194 relative expression values were obtained using the $2^{-\Delta\Delta Ct}$ method [35]. All samples were tested in duplicate.

2.5.5 Transfection with miR-194 mimic and inhibitor in PBMCs

MiR-194 activity was altered in PBMCs of dogs using transfection of chemically-modified double-stranded RNA that mimics or inhibts microRNA with HiPerfect Transfection Reagent (QIAGEN, USA). Following the manufacturer's recommendations, PBMCs at 1.6 $x10^5$ were transfected with All Stars Negative control siRNA (5 nM), miR-194 control transfection (Scrambled), miR-194 mimic (5 nM) (Mimic), or miR-194 inhibitor (50 nM) (Inhibitor) (miScript miRNA Mimic and Inhibitor Qiagen, USA). For the evaluation of the transfection rate, siRNA AllStars HS Cell Death Control (50nM) (Qiagen, USA) was used, and cell death was analyzed using light microscopy after staining with trypan blue according to the manufacturer's recommendations. Transfected cells were placed in culture in 24-well plates with complete RPMI medium at 37 ºC in a 5% CO_2 incubator. After 48 hours, the cells and supernatants were collected to evaluate transfection rate, cell viability, and evaluation of targets of miR-194. Mean transfection rates were 20% in the control group and 22% in the infected group.

2.5.6 DNA extraction and Leishmania infantum parasitic load quantification

PBMC DNA was extracted using a phenol-chloroform protocol [36]. Extracted DNA was analyzed on the NanoDrop™ ND-1000 spectrophotometer for purity evaluation (260/280) and quantification.

For *Leishmania infantum* parasitic load quantification, qPCR was used, employing primers that amplify the intergenic spacer internal transcript (ITS1) segment of the parasite rRNA gene at a concentration of 10mM (5'TCCAGCACATTTTGCGA GTA3' and 5'CCACACAGGTTTCTTCTTTATTTGG3') [37]. qPCR reaction was standardized with 30 ng purified genomic DNA, 12.5 µL SYBR® Green JumpStart™

Taq ReadyMix™ (Sigma-Aldrich®, St. Louis, MO, USA), 5 pmol of each primer, and 9.5 µL of ultrapure H_2O in a final reaction volume of 25 µL. We also generated a ten-fold standard curve using extracted DNA from *Leishmania infantum* promastigotes (MHOM/BR00/MER02) for parasite quantification. Reactions were performed using a Mastercycler® RealPlex2 system (Eppendorf, CT, USA) under the following conditions: Initial heating of 94 ºC for 2 min, followed by 40 cycles of denaturation (94 ºC for 15 s), annealing, and extension (58 ºC for 1 min). After these steps, a dissociation curve of the amplified fragment was determined (95 ºC for 15 s, then 60 ºC to 95º C at 15 s/ºC). Parasitic DNA load was determined in each sample by comparing each sample to the standard curve.

2.5.7 Quantification of parasite load, NO, iNOS, T-bet, GATA3, and FoxP3 using flow cytometry

To quantify the parasite load, the method described by Di Giorgio et al. [38] was used with modifications. After 48 hours of culture at 37º C and 5% CO_2, PBMCs were harvested and incubated for 60 minutes at room temperature in 1x PBS containing 1% paraformaldehyde (Sigma). Cells were centrifuged at 400 *g* for 5 minutes. Permeabilization of cell membranes and parasitophorous vacuoles was performed by resuspending the cells in ethanol (Sigma) for 60 minutes at –20° C. Cells were washed with 2% bovine serum albumin (BSA, Sigma) in 1x PBS and incubated for 60 minutes at 4 °C with anti-LPG *Leishmania* monoclonal antibody (ABD, Serotec) diluted 1/250. After three successive washes with PBS/BSA, amastigotes were stained with 1 µM of PE-conjugated anti-IgG2a (Sigma) and FITC-conjugated anti-human CD14 monoclonal antibody (Bio-Rad) for 60 minutes at 4° C. Cells were labeled for monocytes (CD14+ cells) and positivity for gp63.

To determine intracellular NO levels, PBMCs were centrifuged at 400 *g* for 5 minutes, resuspended in a minimum volume of PBS, and stained with DAF-2DA (2M) (Invitrogen-Leiden Molecular Probes, Netherlands) for 60 minutes at 37° C in the presence of 5% CO_2. Samples without markings were used as a negative control to delimit the negative populations of the analyzed samples.

For analysis of iNOS production, PBMCs were fixed with 500 µl of fixation buffer (Invitrogen) and incubated for 10 min at room temperature. Cells were centrifuged at 400 *g* for 5 minutes and washed twice with permeabilization buffer (Invitrogen). Then, PBMCs were resuspended with 50 µl of permeabilization buffer

(Invitrogen) and incubated with anti-human iNOS antibodies conjugated to PE and its respective isotype control (BIORBYT) for 60 minutes at 4º C. Cells were washed with PBS/BSA and stored at 4°C in the dark until analysis.

To determine the expression of T-bet, GATA3, and FoxP3 transcription factors, T CD3+ lymphocytes were incubated. PBMCs were suspended in PBS containing 1% BSA, 0.1% azide, and 20% FBS to block Fc receptors and incubated for 30 minutes at room temperature. Cells were centrifuged at 400 *g* for 5 minutes and incubated with FITC-conjugated Anti-Dog CD3 monoclonal antibodies and their respective isotype controls (Bio-Rad, USA). PBMCs were then fixed with 500 µl of fixation buffer (Invitrogen) and incubated for 45 minutes at room temperature. Cells were centrifuged at 400 *g* for 5 minutes and washed twice with permeabilization buffer (Invitrogen). Then, cells were resuspended with 50 µl of permeabilization buffer (Invitrogen) and incubated with anti-human T-bet (BioLegend, USA), anti-human GATA3 (BioLegend, USA), and anti-human Foxp3 (eBioscience) antibodies conjugated to PE and with their respective isotype control during 90 minutes at 4° C. Cells were washed with PBS/BSA and stored at 4° C in the dark until analysis.

For all targets, the acquisition of 10.000 events was performed on an Accuri C5 flow cytometer (BD Biosciences, USA) and analyzed using BD Accuri C6 software, version 1.0.264.21 (BD Biosciences, CA, USA).

2.5.8 Quantification of cytokines IL-1β, IL-4, IL-6, IL-10, TNF-α, IFN-y, and TGF-β by capture ELISA

Detection of cytokines in the PBMC culture supernatants was performed using the DuoSet ELISA Development Systems kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

2.5.9 Relative gene expression of TRAF6

After transfection with miR-194 mimic and inhibitor and culture for 48 hours at 37 °C and 5% CO_2, total RNA was extracted from PBMCs using a commercial RNeasy Mini Kit® (Qiagen, Valencia, California, 74104, USA) according to the manufacturer's recommendations. Then, RNA was eluted in nuclease-free water, and the concentration of the extracted RNA (ng/µL) and the degree of purity (A260 nm/A280 nm coefficient) were evaluated using a spectrophotometer (NanoDrop Technologies ND 1000 UV/VIS, USA). Cells were frozen at –80 °C to perform reverse

transcription subsequently. cDNA production was performed using a commercial QuantiTect Reverse Transcription kit (Qiagen, Valencia, California, 205311, USA) with 100 ng of RNA and oligo (dT) primer in a final volume of 20 µL. cDNA was frozen at –20 °C until analysis.

TRAF6 gene expression was determined by RT-qPCR using a Mastercycler-Ep Realplex 4-S thermal cycler (Eppendorf North America, Westbury, NY, USA). The RT-qPCR reactions were standardized with 1ul of cDNA, 4 µl of HOT FIREPoL® EvaGreen® qPCR MIX Plus (no ROX) 5X (SOLIS BIODYNE), 100 nM of each oligonucleotide primer, and 13.2µl of ultrapure water in a volume of final reaction of 20 µl. Specific primers for TRAF6 were designed using the Primer3Plus1 software (UNTERGASSER et al., 2012), selecting the fragment 5'TCTGCAAAGCTTGCATCA TC3' and 5'AAGGTACGTTGGCACTG GAG3'. Amplification conditions were an initial incubation of 2 min at 50 °C and 2 min at 95 °C, followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min, and 60 °C for 1 min. At the end of amplification, a dissociation curve of the amplified fragment was determined under the following conditions: 95 °C for 15 s, 60 °C for 15 s, followed by 20 minutes until reaching 95 °C for 15 s. Nuclease-free water (Sigma-Aldrich Co, St. Louis, USA) was used as a negative control, and samples were evaluated in duplicate. Reaction efficiency values, coefficients of determination, and angular coefficients (slopes) were obtained from the amplification of seven serial dilutions of a cDNA pool. To analyze the results, the relative gene expression was performed with efficiency correction as previously described [39], using the geometric mean of reference genes beta-actin (5'CCAGCAAGGATGAAGATCAAG3' and 5'TCTGCTGGAAGGTGGACA G3') and HPRT-1 (5'CACTGGGAAAACAATGCAGA3' and 5'ACAAAGTCAGGTT TATAGCCAACA3') [40].

2.5.10 Functional assay with IL-1β blockade after transfection with miR-194 inhibitor in PBMCs from dogs with leishmaniasis

For the functional assay with IL-1β neutralization, PBMCs from dogs with leishmaniasis were transfected with miR-194 Inhibitor as described in section 2.4.5, and treated with canine IL-1β/IL-1F2 beta (R&D System) at 400 ng/mL with its respective isotype control (Sheep IgG Isotype Control-Invitrogen/USA) according to the manufacturer's recommendations. Cells were cultured in 24-well plates with complete RPMI medium at 37 ºC and 5% CO_2. After 48 hours, cells were stained with DAF-2DA (2M) (Invitrogen-Leiden Molecular Probes, The Netherlands) for 60 minutes

at 37 °C in 5% CO_2 for NO detection. IL-1β was evaluated in PBMCs culture supernatants by capturing ELISA to assess blocking efficiency (S1 Fig).

2.5.11 Statistical analysis

Statistical analysis was performed using GraphPad Prism v6 software (GraphPad Software, Inc., La Jolla, CA, USA). All statistical variables were tested for normality using the Shapiro-Wilk test. To compare the values corresponding to the parasite load and production of iNOS, NO, T-bet, GATA3, FoxP3, IL-1β, IL-4, IL-6, IL-10, TNF-α, IFN-y, TGF-β, and TRAF6 expression between treatments within groups, Friedman's test with Dunn's post-test was used. Mann-Whitney test was used to compare the results between the control and infected groups. Values were considered significant when $p < 0.05$.

2.6 Results

2.6.1 All dogs with leishmaniasis were infected with *Leishmania infantum*

To determine the species of *Leishmania* present in the infected dogs, Restriction fragment polymorphism (RFLP)-PCR was performed. All sick dogs (Infected group) were infected with *Leishmania infantum* (S2 Fig).

2.6.2 Clinical and laboratory findings

Dogs with leishmaniasis (Infected group) showed at least three characteristic clinical signs of the disease, the most frequent clinical sign being skin lesions (86% [24/28]), followed by lymphadenopathy (75% [21/28]), onychogryphosis (68% [19/28]), cachexia (50% [14/28]), seborrhea (36% [10/28]), alopecia and periocular lesions (32% [9/28]), and hepatosplenomegaly (28% [8/28]). Healthy dogs (Control group) showed no clinical signs (S1 Table). Dogs in the infected group tested positive for the anti-*Leishmania* antibodies by indirect ELISA [32] and the rapid DPP test. They tested positive for *Leishmania* spp. DNA by qPCR (Supplementary Table 1). Healthy dogs (Control group) were negative for all serological and molecular diagnostic tests for leishmaniasis (S1 Table).

Dogs with leishmaniasis showed a significantly lower red blood cell count, hemoglobin, hematocrit, and serum albumin concentration and significantly higher serum globulin concentrations than healthy dogs (control group) (S2-S4 Tables). Based on clinical and laboratory findings, sick dogs showed moderate manifestations

of leishmaniasis and were classified in stage II of the disease, as proposed by Solano-Gallego et al. [41]. Healthy dogs (control group) showed no alterations in the blood count (S1 and S2 Tables) or biochemical parameters (S4 Table).

2.6.3 mirR-194 is increased in PBMCs from dogs with leishmaniasis

MiR-194 expression was elevated in the PBMCs of dogs with leishmaniasis [13]. To validate the increase in miR-194, we measured the expression of miR194. Expression of miR-194 was significantly higher in the PBMCs of dogs in the infected group than in the Control group (Fig 1).

Fig 1. MiR-194 expression in PBMCs from healthy dogs and those with leishmaniasis. Expression of miR-194 in PBMCs from healthy dogs (Control Group N = 5) and dogs with leishmaniasis (Infected Group N = 10) was quantified using qPCR. Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data for each animal. Asterisks indicate significant differences (Mann-Whitney test, $p < 0.05$).

2.6.4 Mimic miR-194 increased parasite load in PBMCs from dogs with leishmaniasis

Progression and worsening of CanL are associated with parasite proliferation [42]. MiR-194 was positively correlated with increased parasite load in the PBMCs of dogs with leishmaniasis [13]. To determine the role of miR-194 in regulating parasite burden, PBMC from diseased dogs were transfected with miR-194 mimic and miR-194 inhibitor, and after 48 hours of culture at 37°C and 5% CO_2, parasite burden was determined. Parasite load increased after transfection with miR-194 mimic according

to flow cytometry (Fig 2A) and quantification of *Leishmania* spp. DNA using qPCR (Fig 2B).

Fig 2. Quantifying parasite load in PBMCs from dogs with leishmaniasis after transfection with Mimic and miR-194 Inhibitor. PBMCs were transfected with Mimic and Inhibitor of miR-194. After 48 hours of culture at 37 ºC and 5% CO_2, parasite load was quantified using flow cytometry (N 18) (A) and qPCR (N = 9) (B). Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data for each animal. Asterisks indicate significant differences (Friedman's test followed by Dunn's test, $p < 0.05$)

2.6.5 Inhibitor miR-194 increased NO levels in PBMCs from dogs with leishmaniasis

In CanL, parasite control depends on the activation of leishmanicidal mechanisms such as iNOS activation [43,44] and NO production in infected cells [45–47]. To determine whether miR-194 regulates iNOS and NO production, PBMCs from healthy dogs and those with leishmaniasis were transfected with Mimic and Inhibitor of miR-194. After 48 hours of culture at 37º C and 5% CO_2, spontaneous production of iNOS and NO was measured using flow cytometry. There were no significant differences in basal iNOS and NO production between PBMCs from healthy dogs and those with leishmaniasis (S3 Fig). The same result was observed for iNOS and NO production in PBMCs from healthy dogs after increasing and decreasing miR-194 activity (Figs 3A and 3C). Interestingly, there was a tendency to increase iNOS production, followed by a significant increase in NO production after transfection with a miR-194 inhibitor in PBMCs from dogs with leishmaniasis (Figs 3B and 3D).

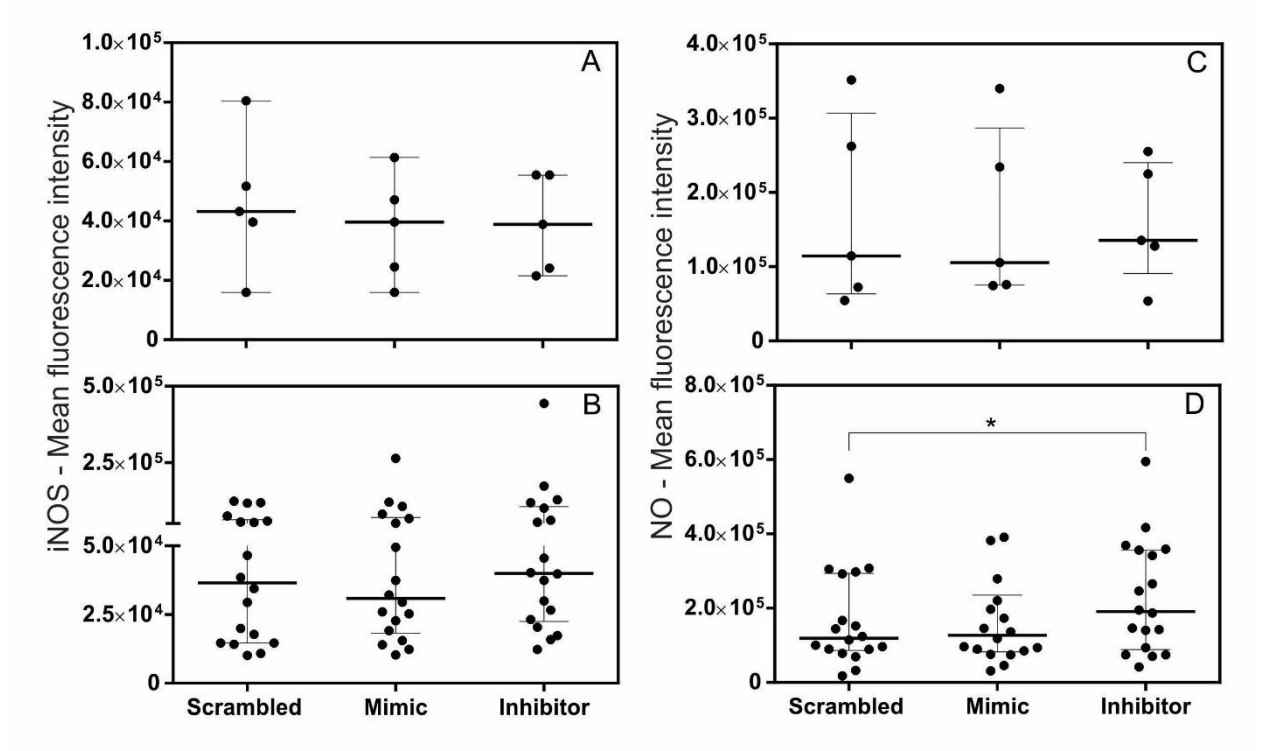

Fig 3. iNOS and NO production in PBMCs from healthy dogs and those with leishmaniasis after transfection with Mimic and Inhibitor of miR-194. PBMCs were transfected with miR-194 Mimic and Inhibitor. After 48 hours of culture at 37º C and 5% CO_2, PBMCs were incubated with anti-human iNOS antibodies conjugated to PE and the respective isotype controls to evaluate iNOS expression. To evaluate NO production, cells were incubated with the DAF-2DA (2M) probe (Invitrogen-Leiden Molecular Probes, Holland), and the mean fluorescence intensity was measured using flow cytometry. The evaluation was performed on PBMCs from healthy dogs (Control group, N = 5) (A and C) and dogs with leishmaniasis (Infected group, N = 18) (B and D). Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data for each animal. Asterisks indicate significant differences (Friedman's test followed by Dunn's test, $p < 0.05$).

2.6.6 miR-194 did not regulate the expression of transcription factors T-bet, GATA3, or FoxP3 in PBMCs from dogs

Progression of leishmaniasis in dogs is associated with an exacerbated humoral immune response (Th2) or the development of an immunosuppressive state at the expense of an effective cellular immune response (Th1) [1,48,49]. Treg cells have been associated with a protective role during infection in dogs [50–52].

Generation of subsets of Th1, Th2, and Treg cells involves the expression of transcription factors T-bet [53], GATA3 [54], and FoxP3 [55], respectively. To determine whether there is a regulatory role for miR-194 in the differentiation of TCD4+ cells subsets, PBMCs from healthy dogs and those with leishmaniasis were transfected with miR-194 Mimic and Inhibitor and cultured for 48 hours at 37º C and 5% CO_2. There was no significant difference in the basal expression of transcription factors between healthy dogs and those with leishmaniasis (S4 Fig). The same result was observed with the increase or decrease of miR-194 activity in T-bet expression (Figs 4A and 4B), GATA3 (Figs 4C and 4D), and FoxP3 (Figs 4E and 4F).

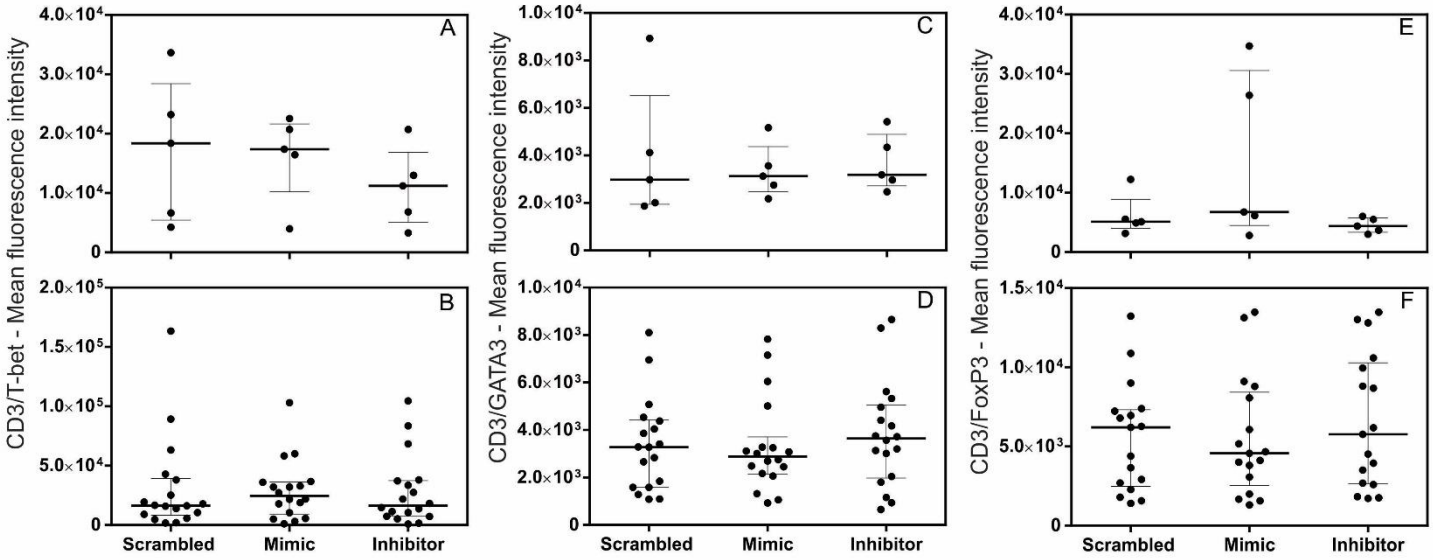

Fig 4. Quantification of T-bet, GATA3, and FoxP3 transcription factors in lymphocytes from healthy dogs and those with leishmaniasis after transfection with miR-194 Mimic and Inhibitor. PBMCs were transfected, and after 48 hours of culture at 37º C and 5% CO_2, they were incubated with FITC-conjugated canine anti-CD3 followed by anti-human T-bet, anti-human GATA3, and anti-human FoxP3 antibodies (conjugated to PE). Their respective isotype controls and the mean fluorescence intensity were evaluated using flow cytometry. The evaluation was performed on PBMCs from healthy dogs (Control group, N = 5) (A, C, and E) and dogs with leishmaniasis (Infected group, N = 18) (B, D, and F). Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data for each

animal. Asterisks indicate significant differences (Friedman's test followed by Dunn's test, $p < 0.05$

2.6.7 miR-194 Inhibitor increased production of IL-1β in the supernatants of PBMC culture from dogs with leishmaniasis but did not alter the production of IL-6, IL-4, TNF-α, IFN-y, TGF-β, or IL -10

Pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and IFN-y have been associated with resistance in leishmaniasis [1,56]. In contrast, immunoregulatory cytokines such as TGF-β and IL-10 and anti-inflammatory such as IL-4 are associated with disease susceptibility [48,57].

Test for a possible regulatory role of miR-194 in the production of cytokines IL-1β, IL-6, IL-4, TNF-α, IFN-y, TGF-β, and IL-10, PBMCs from healthy dogs and those with leishmaniasis were transfected with miR-194 Mimic and Inhibitor. After 48 hours of culture at 37º C and 5% CO_2, supernatants were collected, and the cytokines were measured using capture ELISA.

IL-4 and TGF-β were increased in the PBMCs supernatants from dogs with leishmaniasis, and the other cytokines did not show significant differences between the groups (S5 Fig).

Regarding the pro-inflammatory cytokines IL-1β, IL-6, TNF-α, and IFN-y, only IL-1β increased in PBMC culture supernatants from dogs with leishmaniasis after transfection with the miR-194 inhibitor (Figure 5B). In contrast, of healthy dogs post transfection, there was no alteration in IL-1β in the PBMC supernatants (Fig 5A). No changes were observed in the production of IL-6 (Figs 5C and 5D), TNF-α (Figs 5E and 5F), or IFN-y (Figs 5G and 5H) in the PBMC culture supernatants of healthy dogs and those with leishmaniasis after transfection with miR-194 Mimic and Inhibitor. This same result was found for the production of the anti-inflammatory cytokine IL-4 (Figs 6A and 6B) and the immunoregulatory cytokines IL-10 (Figs 6C and 6D) and TGF-β (Figs 6E and 6F).

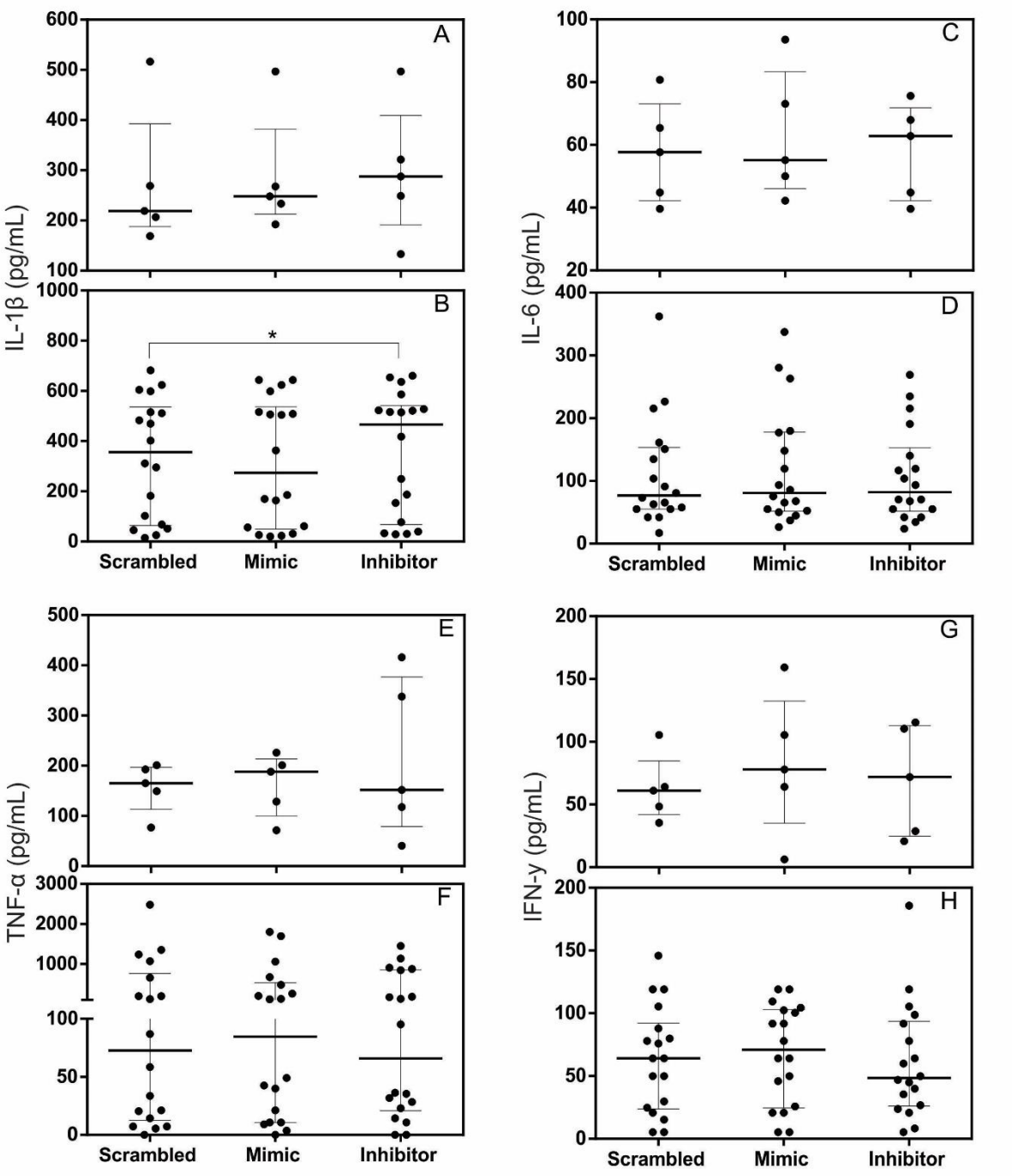

Fig 5. Quantification of pro-inflammatory cytokines in the supernatants of cultured PBMCs from healthy dogs and those with leishmaniasis after transfection with miR-194 Mimic and Inhibitor. PBMCs were transfected, and after 48 hours at 37º C and 5% CO_2, the culture supernatants were collected, and the production of pro-inflammatory cytokines IL-1-β, IL-6, TNFα and IFN-y were evaluated using capture ELISA. The evaluation was performed on the PBMC culture supernatants of healthy dogs (Control group, N = 5) (A, C, E and G) and dogs with

leishmaniasis (Infected group, N = 18) (B, D, F and H). Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data from each animal. Asterisks indicate significant differences (Friedman's test followed by Dunn's test, $p < 0.05$).

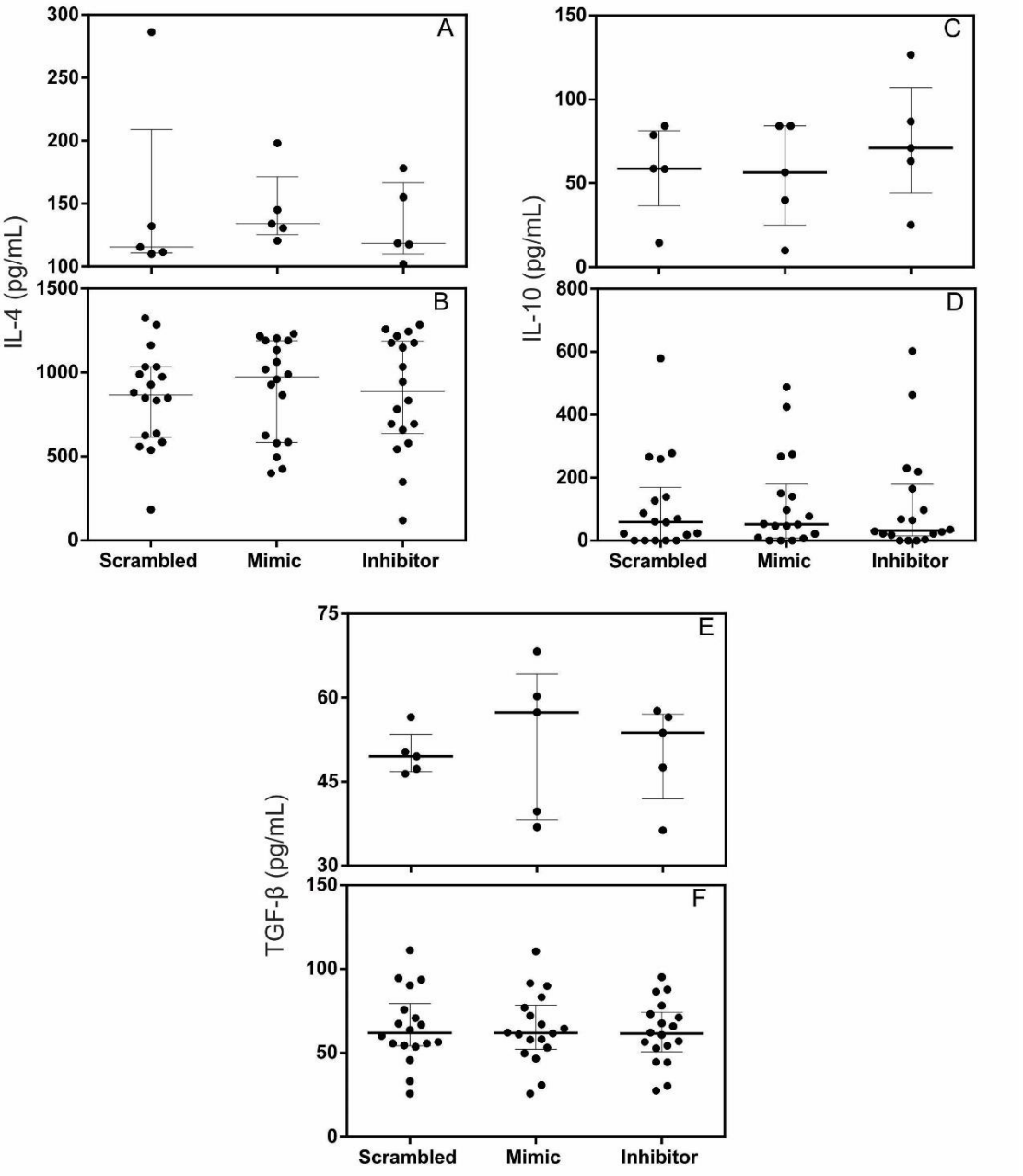

Fig 6. Quantification of anti-inflammatory and immunoregulatory cytokines in the supernatants of cultured PBMCs from healthy dogs and dogs with leishmaniasis after transfection with miR-194 Mimic and Inhibitor. PBMCs were transfected, and after 48 hours at 37º C and 5% CO_2, the culture supernatants were collected, and the production of anti-inflammatory cytokine IL-4 and immunoregulatory

cytokines IL-10 and TGF-β, were evaluated using capture ELISA. The evaluation was performed on the PBMC culture supernatants of healthy dogs (Control group, N = 5) (A, C, and E) and dogs with leishmaniasis (Infected group, N = 18) (B, D, and F). Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data from each animal. Asterisks indicate significant differences (Friedman's test followed by Dunn's test, $p < 0.05$).

2.6.8 miR-194 did not regulate expression of the TRAF6 in dog PBMC

In silico analyzes demonstrated that TRAF6 is a target of miR-194 in the PBMCs of dogs with leishmaniasis [13]. In rat spinal disk pulposus cells, miR-194 regulates TRAF6 translation and inhibits its stimulation on the expression of several genes, including IL-1 [31]. To assess whether miR-194 regulates TRAF6 expression in PBMCs from healthy dogs and those with leishmaniasis, cells were transfected with miR-194 Mimic and Inhibitor after 48 hours of culture at 37º C and 5% CO_2, TRAF6 relative expression was analyzed using qPCR. Relative expression of TRAF6 was lower in PBMCs from dogs with leishmaniasis than those from healthy dogs (S6 Fig). There was no change in relative expression of TRAF6 with increasing and decreasing miR-194 activity in PBMCs from healthy dogs (Fig 7A) and dogs with leishmaniasis (Fig 7B).

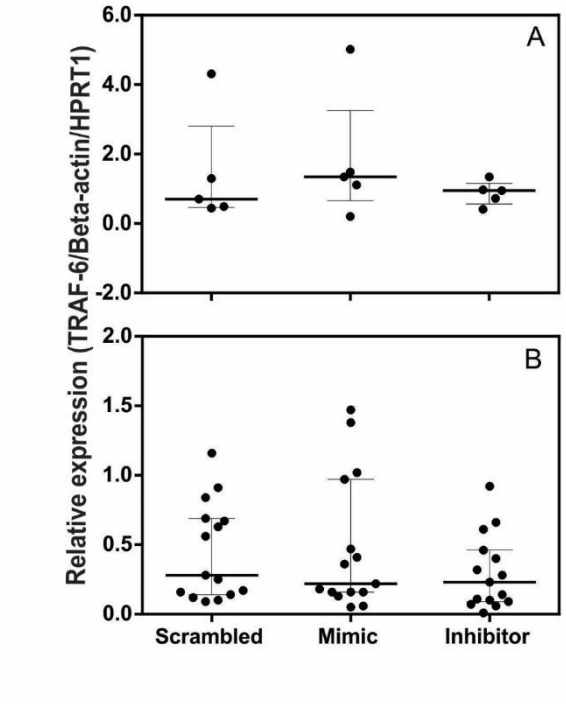

Fig 7. Relative gene expression of TRAF6 in PBMC from healthy dogs and those with leishmaniasis after transfection with miR-194 Mimic and Inhibitor. PBMCs were transfected, and after 48 hours of culture at 37º C and 5% CO_2, relative gene expression of TRAF6 was analyzed using qPCR. Gene expression was performed in PBMCs from healthy dogs (Control group, N = 5) (A) and dogs with leishmaniasis (Infected group, N = 15) (B). Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data from each animal. Asterisks indicate significant differences (Friedman's test followed by Dunn's test, $p < 0.05$).

2.6.9 IL-1β blockade decreases NO after miR-194 inhibition in PBMCs from dogs with leishmaniasis

MiR-194 inhibition increased IL-1β and NO production in PBMCs from dogs with leishmaniasis. Adding IL-1β to cultured macrophages from mice infected with *Leishmania amazonensis* increased nitrite production in culture supernatants [56]. To determine whether NO production depends on IL-1β in PBMCs from dogs with leishmaniasis after miR-194 inhibition, a functional assay with IL-1β blockade was performed. PBMCs from dogs with leishmaniasis were transfected with miR-194 inhibitor with IL-1β blocking antibody. After 48 hours of culture at 37º C and 5% CO_2, NO production was evaluated using flow cytometry. IL-1β blockade reversed the positive effect of miR-194 inhibition, with decreased NO production (Fig 8).

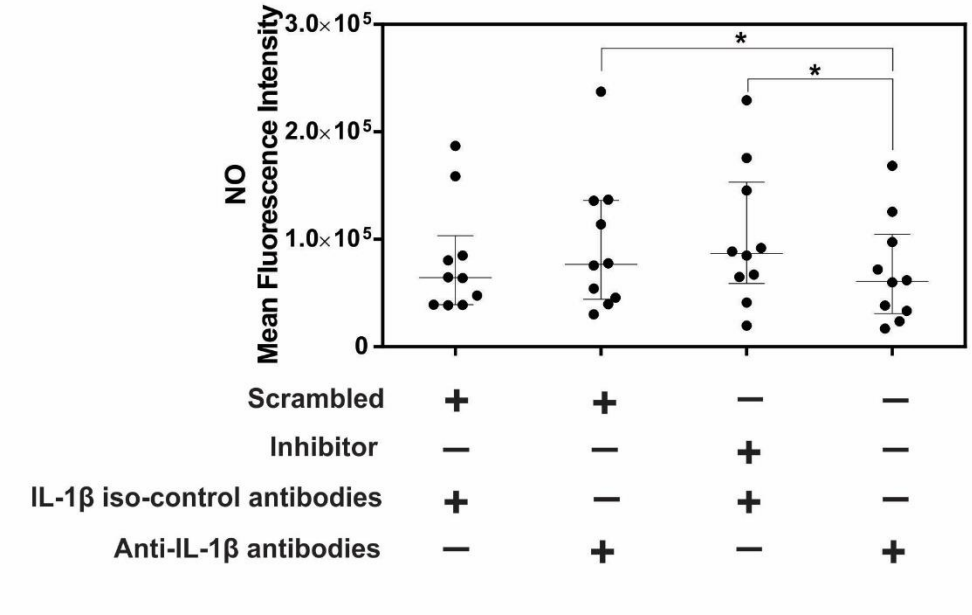

Fig 8. NO levels in PBMCs from dogs with leishmaniasis after transfection with miR-194 inhibitor and IL-1β blockade. PBMCs were transfected with miR-194 inhibitor and canine anti-IL1β antibody. After 48 hours of culture at 37º C and 5% CO_2, the cells were incubated with the DAF-2DA (2M) probe (Invitrogen-Leiden Molecular Probes, The Netherlands), and the mean fluorescence intensity of NO was evaluated using flow cytometry. Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data for each animal. Asterisks indicate significant differences (Friedman's test followed by Dunn's test, $p < 0.05$).

2.7 Discussion

Increased parasite load in dogs naturally infected with *Leishmania infantum* is associated with the inability to establish an effective cellular immune response (Th1) and an exacerbated stimulation of the humoral immune response (Th2). There is evidence that miR-194 positively regulates parasite load in CanL [13], possibly affecting gene expression and translation of proteins fundamental for immune system development, function, and cell differentiation. In the present study, molecular tools were used to increase and decrease miR-194 activity in PBMCs from dogs, with and without leishmaniasis, to evaluate its functional role in activating leishmanicidal mechanisms. We also measured the expression of transcription factors related to cell differentiation T helper profile Th1, Th2, and regulatory T cells, pro-inflammatory, anti-inflammatory, and immunoregulatory cytokines, and their correlation with parasitic load.

MiR-194 was increased in the PBMCs of dogs with leishmaniasis. Increased activity of miR-194 using mimic increased parasite load in PBMCs of diseased dogs. In a previous study, there was an increase in miR-194 in the PBMCs of dogs with leishmaniasis, which positively correlated with parasite load [13]. There are no studies on the role of miR-194 in parasite load in CanL. In H1N1 influenza virus infection, increased miR-194 activity induced an increase in viral load *in vitro* in human lung cells, and *in vivo* in mice [58]. In both models, miR-194 downregulated the production of IFN-α and IFN-β, facilitating viral replication. These findings suggest that miR-194 participates in upregulating the replication of intracellular parasites, including CanL; however, the mechanisms by which miR-194 regulates parasite load in diseased dogs are unknown.

In CanL, high levels of iNOS and NO are associated with decreased parasitic load [59]. We observed that after miR-194 activity was decreased with an inhibitor in the PBMCs of dogs with leishmaniasis, there was an increase in iNOS followed by NO, suggesting a possible regulation of this factor by miR-194. In addition to its microbicidal effect on monocytes and macrophages, NO is produced from iNOS in other immune system cells, such as T lymphocytes [60] and dendritic cells [61]. In human and mouse T lymphocytes and dendritic cells, NO exerts several immunoregulatory effects, including a decrease in apoptosis [62] an increase in MHC II expression [63,64], and increased chemotaxis directed by CCL19 [65]. Although these mechanisms have not yet been described in CanL, it is possible that NO, in addition to its leishmanicidal effects, has immunoregulatory functions.

MAPK1 is a predicted target of mir-194 in CanL [13]. It encodes the ERK2 protein involved in various stages and components of the immune system [15]. In mice with ERK2 protein deficiency, there is increased expression of GATA3 and FoxP3 and decreased expression of T-bet in T lymphocytes of lymph nodes and the spleen [16]. In our study, miR-194 did not regulate the expression of transcription factors T-bet, GATA3, or FoxP3 in CanL, suggesting that miR-194 has no role in the MAPK1/ERK2 pathway in the expression of these transcription factors in sick dogs.

We observed higher levels of IL-4 and TGF-β in the supernatants of PBMCs from dogs with leishmaniasis than from healthy dogs, confirming the regulatory role of these cytokines in CanL. IL-4 increases in the blood of dogs infected with *Leishmania infantum* with the onset of clinical signs [66]. High levels of TGF-β have been observed in the supernatant of spleen cell cultures [67] and the lymph nodes associated with disease progression in dogs [57]. No significant change was observed in the expression of IL-4 and TGF-β or IL-10 and IL-6, TNFα, and IFN-y after increasing and decreasing miR-194 activity. These findings suggest that in CanL, miR-194 has no regulatory role in the expression of these cytokines.

By contrast, there was an increase in IL-1β following a decrease in miR-194 activity in the supernatant of PBMCs from dogs with leishmaniasis. Analysis of the sequences of the IL-1β and miR-194 in dogs in the “TargetScan” program, demonstrate that there is no complementarity between the sequences [68], suggesting an indirect regulation of IL-1β by miR-194.

Several studies showed that miR-194 decreases inflammatory response, negatively regulating the production of pro-inflammatory cytokines (e.g., IL-1β) and inhibiting the activation of the NF-κB pathway by various targets, and in various cell types [31,69,70]. MiR-194 inhibits the NF-κB pathway by decreasing the expression of TRAF6 in cells of the disk pulposus of the intervertebral disk of rats [31], decreases the expression of neutrophilin 1 in human astrocytes in neurodegenerative diseases [69], and decreases the chemokine CXCR4 in murine alveolar macrophages in acute lung injury [70]. In these studies, decreasing miR-194 activity increased IL-1β production and its regulatory targets. *In silico* analyses demonstrated that TRAF6 is a target of miR-194 in PBMCs of dogs with leishmaniasis [13], and previous studies demonstrated that TRAF6 is a target of miR-194 [31,70,71]. However, miR-194 appears to regulate IL1-β production in CanL by another pathway, as we did not observe changes in TRAF6 expression after transfection of PBMCs with miR-194 mimic and inhibitor.

Expression of TRAF6 was lower in PBMCs from dogs with leishmaniasis, when compared to healthy dogs, suggesting that possible targets of TRAF6 may be downregulated. Low expression of iNOS in macrophages and TNF-α in peripheral blood leukocytes was observed in TRAF6-knockout mice [24]. In addition, bone marrow and spleen dendritic cells from TRAF6-deficient mice have lower expression of MHCII and surface costimulatory molecules B7.2, as well as lower production of pro-inflammatory cytokines IL-6 and IL-12 in response to stimulation with CD40L or various TLR ligands [72,73]. These anti-inflammatory effects observed in the absence or decrease of TRAF6 (primarily in the production of cytokines) may be the result of a deficient activation of the NF-κB pathway. In fact, in murine macrophages infected by *Leishmania donovani*, the parasite inhibits IRAK-1/TRAF6 binding and consequently decreases phosphorylation of IkB, an NF-kB inhibitor [29]. These results suggest that the decrease in TRAF6 expression in PBMCs from dogs with leishmaniasis downregulates inflammatory response to the parasite; nevertheless, further studies are needed to identify the role of TRAF6 in CanL.

We observed that the decrease in miR-194 activity in PBMCs from dogs with leishmaniasis increased NO and IL-1β in the culture supernatants. IL-1β regulates NO production in cells infected by intracellular protozoa [56,74]. To clarify the modulatory role of IL-1β in NO production, IL-1β blockade was performed after miR-

194 activity decreased in PBMCs from dogs with leishmaniasis. IL-1β blockade significantly decreased NO production, suggesting a regulatory role for IL-1β in NO production. *Leishmania* induces NLRP3 inflammasome activation in macrophages *in vitro* and in mice *in vivo*. The production of IL-1β and the IL1-β-IL-1R and MyD88 signaling pathways increase NO production [56]. Furthermore, miR-194 can downregulate inflammasome activation in inflammatory cells by significantly decreasing IL-1β production [71]. In the present study, we did not analyze inflammasome activation following increases and decreases in miR-194 activity. The mechanism by which miR-194 regulates IL-1β in CanL remains unknown.

We conclude that miR-194 is upregulated in PBMCs from dogs naturally infected with *Leishmania infantum* and increases parasite load, possibly decreasing IL-1β-dependent NO production. These findings contribute to the understanding of the molecular mechanisms by which the parasite evades the host's immune response, in addition to identifying targets for new therapies.

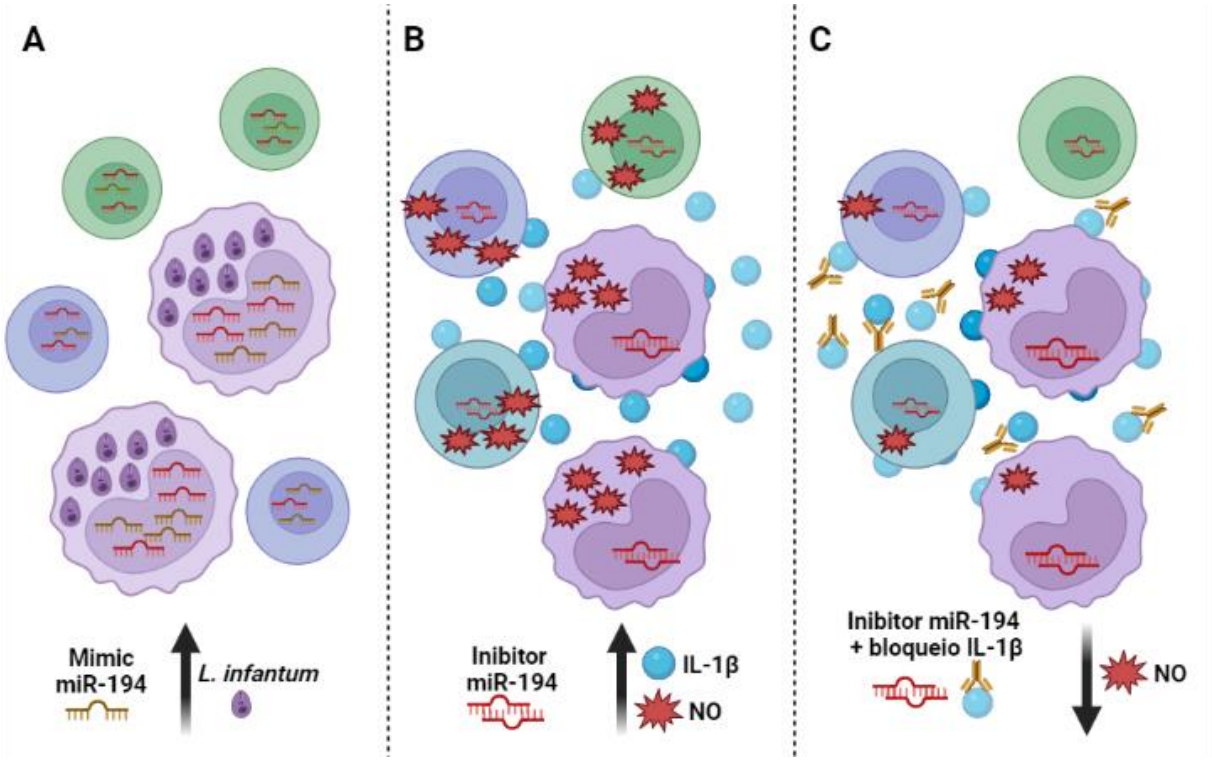

Fig 8. Graphical abstract: A - MiR-194 is increased in PBMC from dogs with leishmaniasis and miR-194 mimic increases parasite load. **B** miR-194 inhibitor

increases IL-1β and NO production. **C** miR-194 inhibitor+IL-1βB neutralization decreases NO production. Figure created by the BioRender software.

2.8 Acknowledgments

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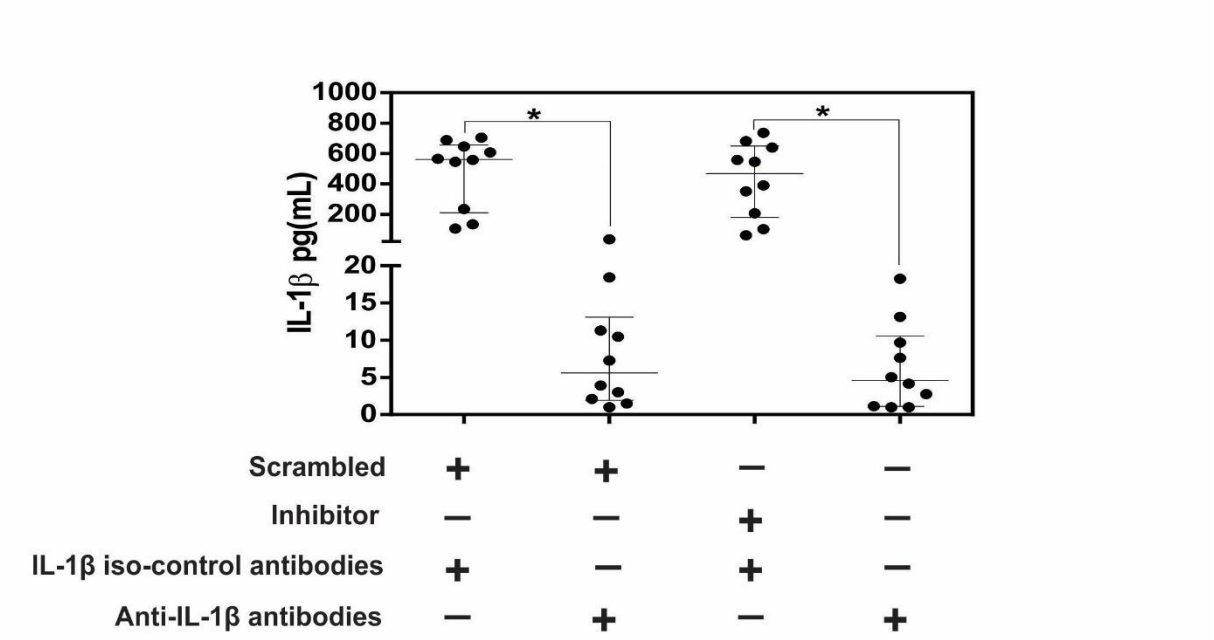
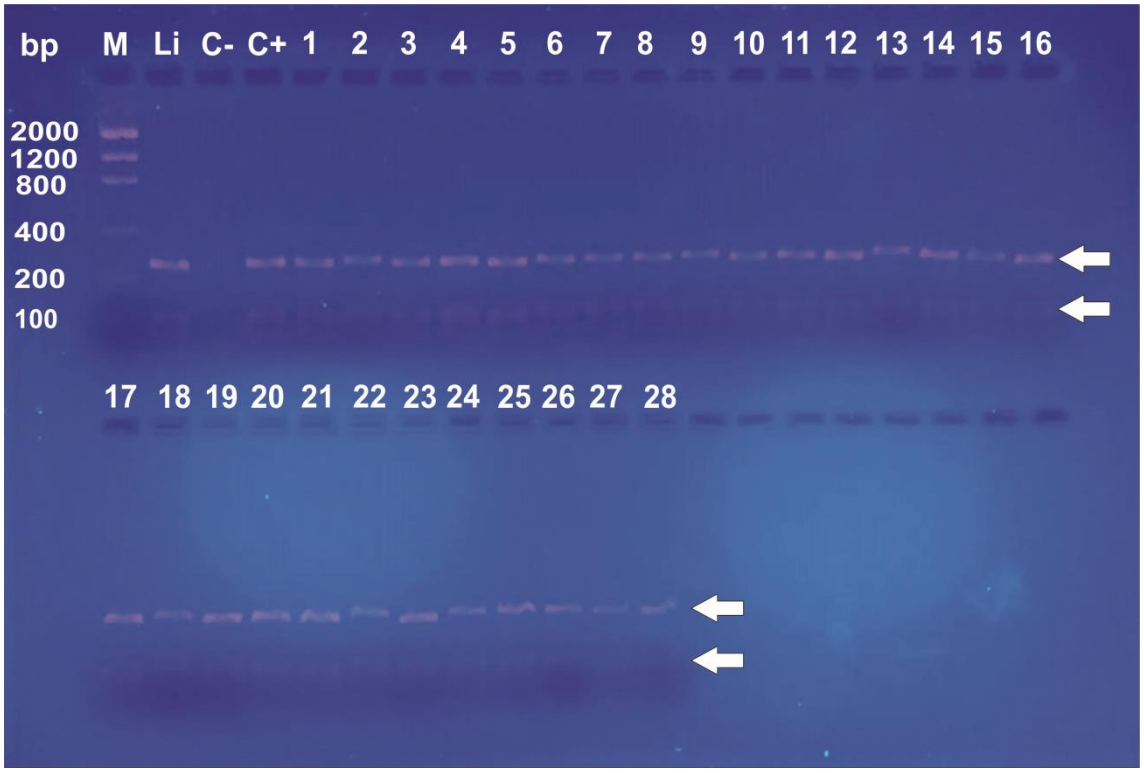
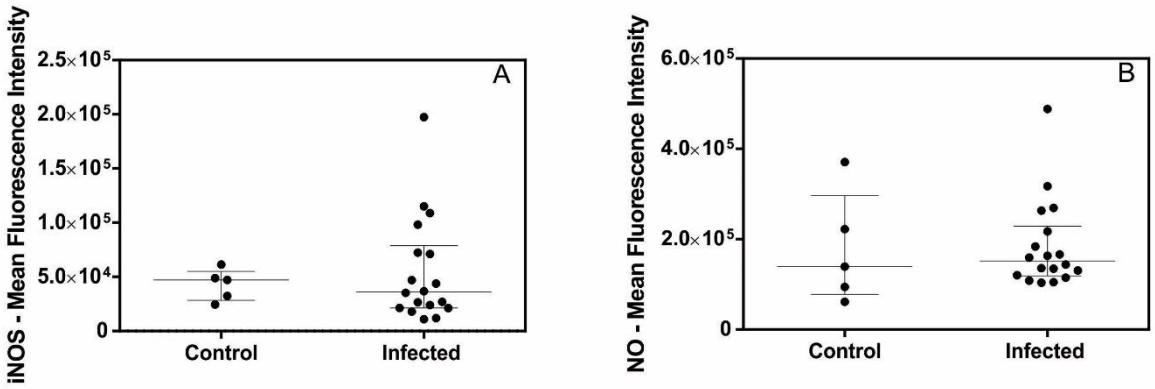
2.10 Supporting information



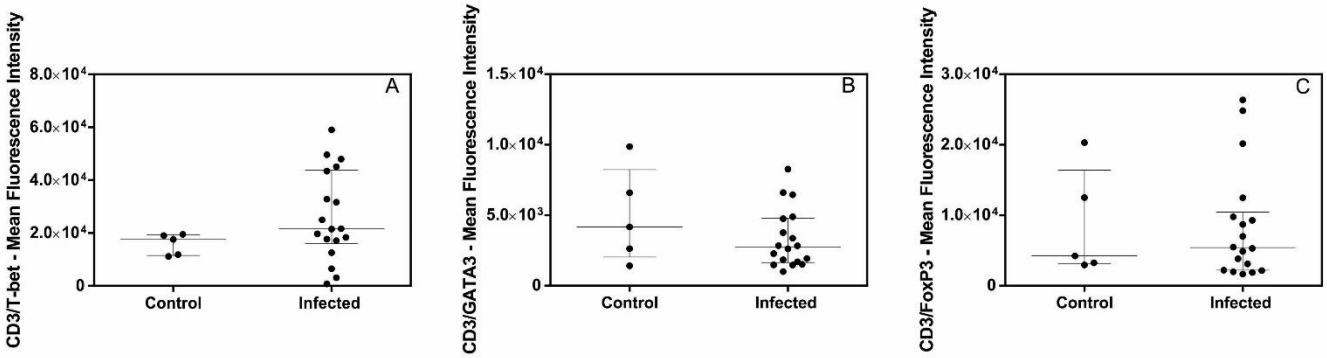
S1 Fig. Quantifying IL-1-β in the PBMC supernatant cultures of dogs with leishmaniasis after transfection with miR-194 Inhibitor and IL-1-β blockade. PBMC were transfected with Scramble and miR-194 Inhibitor or IL-1-β blocking antibody or respective Iso-controls. After 48 hours of culture at 37 ºC and 5% CO_2, the culture supernatants were removed, and capture ELISA evaluated IL-1-β cytokine production. Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data for each animal. Asterisks indicate significant differences (Friedman's test followed by Dunn's test, $p < 0.05$).



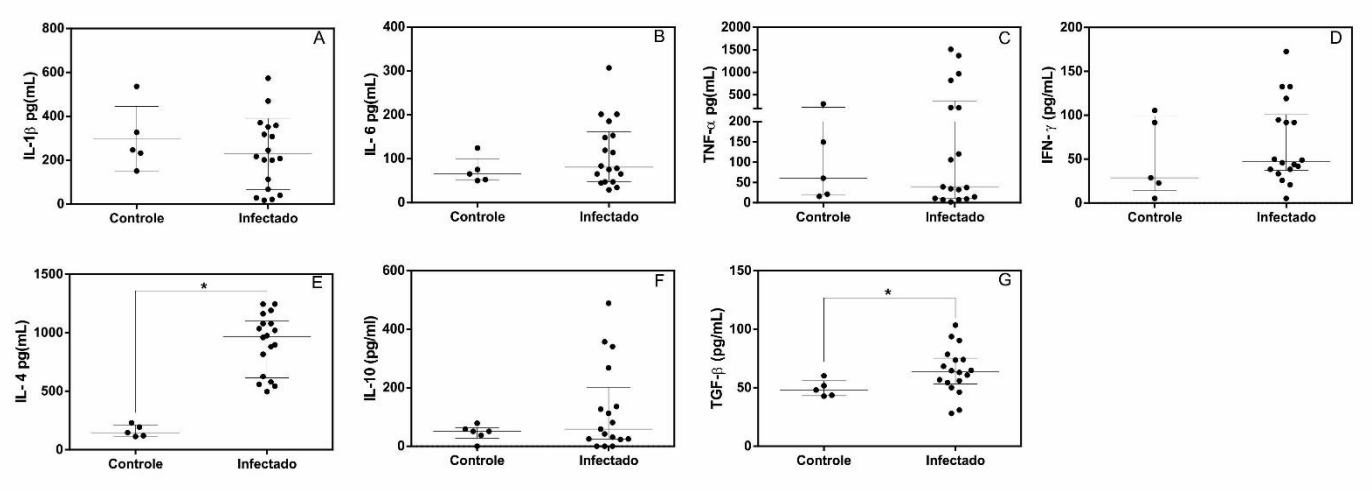
S2 Fig. RFLP analysis of ITS1-PCR fragments amplified from DNA samples of standard isolates using the Hae III enzyme. M: molecular marker (100 bp); Li: *Leishmania infantum*. Samples 1 to 28 showed an identical sampling profile to *L. infantum*. RFLPs were identified on 3% agarose gels stained with gel red and indicated by an arrow.



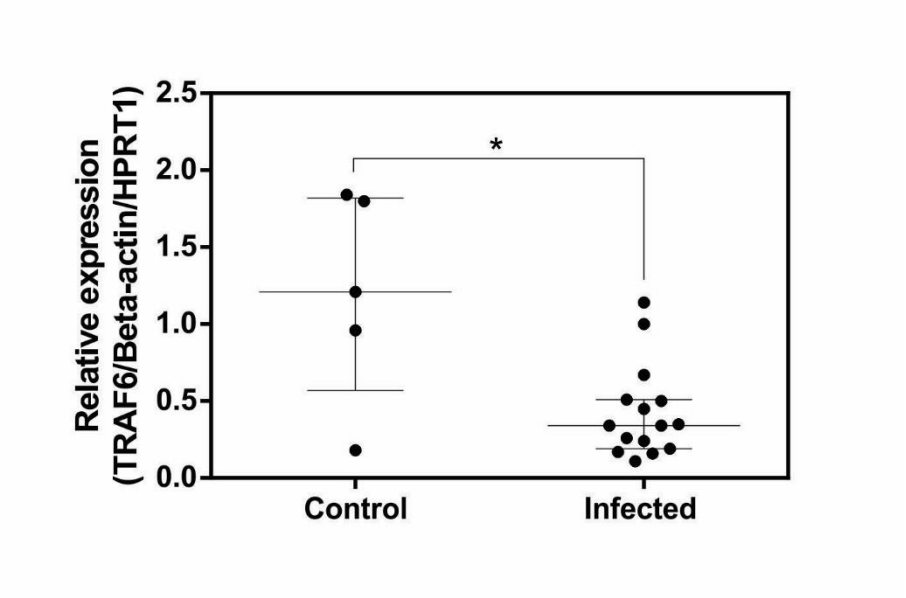
S3 Fig. Basal levels of iNOS and NO in PBMCs from healthy dogs and dogs with leishmaniasis. PBMCs were cultured at 37 ºC and 5% CO2 for 48 hours and then incubated with PE-conjugated anti-human iNOS antibodies and their respective control isotypes to assess iNOS expression. To evaluate NO production, cells were incubated with the DAF-2DA (2M) probe (Invitrogen-Leiden Molecular Probes, Netherlands), and the mean fluorescence intensity was evaluated using flow cytometry. The evaluation was performed on PBMCs from healthy dogs (Control group, N = 5) (A) and dogs with leishmaniasis (Infected group, N = 18) (B). Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data for each animal. Asterisks indicate significant differences (Mann-Whitney test, $p < 0.05$).



S4 Fig. Basal quantification of transcription factors T-bet, GATA3, and FoxP3 in lymphocytes from healthy dogs and dogs with leishmaniasis. PBMC were cultured for 48 hours at 37 ºC and 5% CO2 and then were incubated with FITC-conjugated anti-canine CD3, human anti-T-bet, human anti-GATA3, and human anti-FoxP3 antibodies, conjugated to PE. Their respective and mean fluorescence intensity was evaluated using flow cytometry. The evaluation was performed on PBMCs from healthy dogs (Control group N = 5) and dogs with leishmaniasis (Infected group N = 18). T-bet (A), GATA3 (B), and FoxP3 (C). Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data for each animal. Asterisks indicate significant differences (Mann-Whitney test $p < 0.05$).



S5 fig. Basal quantification of cytokines in PBMC culture supernatants from healthy dogs and dogs with leishmaniasis. PBMCs were cultured for 48 hours at 37 ºC and 5% CO2. The supernatants were removed, and the production of cytokines IL-1-β (A), IL-6 (B), TNFα (C), IFN-y (D), IL-4 (E), IL-10 (F), and TGF-β (G) were measured using capture ELISA. The evaluation was performed on the PBMC culture supernatants from healthy dogs (Control group, N = 5) and dogs with leishmaniasis (Infected group, N = 18). Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data for each animal. Asterisks indicate significant differences (Mann-Whitney test, $p < 0.05$)



S6 Fig. Basal relative gene expression of TRAF6 in PBMCs from healthy dogs and dogs with leishmaniasis. PBMCs were cultured at 37 ºC and 5% CO2 for 48 hours, and then the relative expression of TRAF6 was analyzed using qPCR. Data are expressed as the median and interquartile range (25 and 75). Symbols represent individual data for each animal. Asterisks indicate significant differences (Mann-Whitney test, $p < 0.05$).

S1 Table. Clinical signs, serological and molecular diagnosis of dogs with leishmaniasis (Infected group) and healthy dogs (Control group).

Dogs	Sex	Clinical signs	Diagnosis		
			DPP	PCR	ELISA O.D.
Infected 1	M	Cachexia, seborrhea, skin lesions	+	+	0.920
Infected 2	F	Lymphadenopathy, onychogryphosis, alopecia, skin lesions	+	+	0.978
Infected 3	M	Lymphadenopathy, onychogryphosis, cachexia, alopecia	+	+	1.207
Infected 4	F	Lymphadenopathy, hepatosplenomegaly, onychogryphosis, seborrhea, alopecia, skin lesions	+	+	1.267
Infected 5	F	Lymphadenopathy, onychogryphosis, cachexia	+	+	0.968
Infected 6	F	Lymphadenopathy, onychogryphosis, cachexia, seborrhea, alopecia, skin lesions	+	+	0.923
Infected 7	M	Lymphadenopathy, hepatosplenomegaly, onychogryphosis, skin lesions	+	+	0.552
Infected 8	M	Onychogryphosis, cachexia, seborrhea, periocular lesion, skin lesions	+	+	0.764
Infected 9	F	Lymphadenopathy, onychogryphosis, cachexia, seborrhea, alopecia, periocular lesion, skin lesions	+	+	0.649
Infected 10	M	Lymphadenopathy, hepatosplenomegaly, onychogryphosis, seborrhea, periocular lesion, skin lesions	+	+	0.912
Infected 11	M	Lymphadenopathy, hepatosplenomegaly, seborrhea, skin lesions	+	+	0.295
Infected 12	M	Lymphadenopathy, onychogryphosis, seborrhea, periocular lesion, skin lesions	+	+	0.511
Infected 13	F	Lymphadenopathy, alopecia, skin lesions	+	+	0.781
Infected 14	M	Lymphadenopathy, periocular lesion, skin lesions	+	+	0.986
Infected 15	M	Cachexia, seborrhea, skin lesions	+	+	0.687
Infected 16	F	Cachexia, onychogryphosis, skin lesions	+	+	0.649
Infected 17	M	Lymphadenopathy, hepatosplenomegaly, cachexia, skin lesions	+	+	1.219
Infected 18	M	Lymphadenopathy, cachexia, skin lesions	+	+	1.192
Infected 19	M	Cachexia, periocular injury, skin lesions	+	+	0.666
Infected 20	F	Lymphadenopathy, onychogryphosis, cachexia, skin lesions	+	+	0.570
Infected 21	M	Lymphadenopathy, onychogryphosis, alopecia, skin lesions	+	+	0.547
Infected 22	F	Lymphadenopathy, onychogryphosis, periocular lesion, skin lesions	+	+	0.814
Infected 23	F	Lymphadenopathy, periocular lesion, skin lesions	+	+	0.964
Infected 24	M	Lymphadenopathy, onychogryphosis, cachexia, skin lesions	+	+	0.792
Infected 25	M	Lymphadenopathy, hepatosplenomegaly, onychogryphosis, cachexia, alopecia	+	+	0.523
Infected 26	F	Lymphadenopathy, hepatosplenomegaly, onychogryphosis, seborrhea, periocular lesions, skin lesions	+	+	0.678
Infected 27	M	Lymphadenopathy, onychogryphosis, alopecia, skin lesions	+	+	1.328
Infected 28	F	Hepatosplenomegaly, onychogryphosis, periocular lesion	+	+	1.209
Control 1	F	No clinical signs	-	-	0.112
Control 2	M	No clinical signs	-	-	0.070
Control 3	F	No clinical signs	-	-	0.094
Control 4	F	No clinical signs	-	-	0.045
Control 5	M	No clinical signs	-	-	0.016

Abbreviations: (DPP) Immunochromatographic test; (PCR) Polymerase Chain Reaction; (O.D.) Optical Density.

S2 Table. Red blood cell parameters

Dogs	Values Reference	Red Cells 5.5-8.5 x10^{12}/L	Hemoglobin 12.0-18.0 g/dl	Hematocrit 37-55 %	MCV 60-77 fL	MCHC 32-36 %
Infected 1		4.05	7.8	25	61.73	31.20
Infected 2		3.84	9.1	25	65.10	36.40
Infected 3		4.30	9.5	27	62.79	35.19
Infected 4		4.06	10.0	28	68.97	35.71
Infected 5		2.82	6.6	20	70.92	33.00
Infected 6		4.05	10.2	30	74.07	34.00
Infected 7		3.07	7.6	23	74.92	33.04
Infected 8		4.77	10.1	29	60.80	34.83
Infected 9		5.44	11.2	34	64.34	32.00
Infected 10		4.33	9.0	28	64.67	32.14
Infected 11		5.40	11.0	35	64.34	34.29
Infected 12		5.01	11.8	36	66.18	32.78
Infected 13		5.22	11.7	35	67.05	33.43
Infected 14		5.77	14.5	44	76.26	32.95
Infected 15		5.32	11.4	34	63.91	33.53
Infected 16		4.55	10.5	32	70.33	32.81
Infected 17		4.91	11.6	34	69.25	34.12
Infected 18		5.50	14.0	39	70.91	35.90
Infected 19		5.01	11.4	33	65.87	34.55
Infected 20		5.05	11.9	34	67.33	35.00
Infected 21		5.8	11.2	36	60.24	31.85
Infected 22		4.07	11.8	37	69.42	30.98
Infected 23		3.04	7.2	20	65.79	36.00
Infected 24		4.55	10.3	30	65.93	34.33
Infected 25		2.64	6.4	18	68.18	35.56
Infected 26		5.44	10.4	34	62.50	30.59
Infected 27		4.73	10.3	30	63.42	34.33
Infected 28		3.55	7.2	22	61.97	32.73
	Mean±SD	**4.51±0.89[a]**	**10.2±2.3[a]**	**30±6.2[a]**	**66.69±4.21[a]**	**33.69±1.59[a]**
Control 1		6.77	17.0	52	76.81	32.69
Control 2		6.33	14.0	42	66.35	33.33
Control 3		7.07	17.0	49	69.31	34.69
Control 4		7.93	18.3	53	66.83	34.53
Control 5		8.11	18.4	56	69.05	32.86
	Mean±SD	**7.24±0.76[b]**	**16.9±1.7[b]**	**50±5.3[b]**	**67.67±4.20[a]**	**33.62±0.93[a]**

Infected: dogs with leishmaniasis. Control: healthy dogs. RBC: red blood cells, MCV: mean corpuscular, MCHC: mean corpuscular hemoglobin concentration volume. a,b The same letters in the same column indicate no statistical difference using unpaired t-test.

S3 Table. White blood cells and platelet counts.

Dogs	Values Reference	Neutrophils 3.000-11.500 x10^6/L	Eosinophils 150-1.250 x10^6/L	Basophils Raros x10^6/L	Monocytes 150-1.350 x10^6/L	Lymphocytes 1.000-4.800 x10^6/L	Platelets 160-400 X10^1
Infected 1		6.831	198	0	396	2.475	220
Infected 2		13.783	358	0	1.253	2.506	400
Infected 3		4.320	72	0	288	1.800	140
Infected 4		6.035	0	0	425	2.040	200
Infected 5		9.176	0	0	248	2.976	160
Infected 6		3.780	240	0	180	1.800	280
Infected 7		4.464	372	0	279	4.185	200
Infected 8		9.724	429	0	1.144	3.003	180
Infected 9		4.029	0	0	316	3.555	160
Infected 10		8.400	0	0	120	3.480	160
Infected 11		5.775	0	0	154	1.771	200
Infected 12		5.829	0	0	174	2.697	300
Infected 13		2.920	40	0	400	1.000	160
Infected 14		5.610	425	0	185	2.380	200
Infected 15		6.080	160	0	160	1.600	400
Infected 16		5.226	234	0	178	2.262	280
Infected 17		8.748	0	0	324	1.728	380
Infected 18		5.772	222	0	444	1.962	400
Infected 19		12.060	268	0	154	1.038	300
Infected 20		6.424	264	0	188	2.024	280
Infected 21		3.660	300	0	180	1.860	180
Infected 22		6.141	489	0	178	2.492	220
Infected 23		11.760	147	0	735	2.058	180
Infected 24		3.431	147	0	194	1.128	160
Infected 25		2.680	0	0	179	1.280	200
Infected 26		5.694	546	0	156	1.404	160
Infected 27		6.696	390	0	620	4.460	180
Infected 28		5.412	0	0	198	1.990	160
	Mean±SD	**6.445±2.79^a**	**189±174^a**	**0±0^a**	**266±148^a**	**2.248±0.88^a**	**230±82^a**
Control 1		5.192	616	0	352	2.640	180
Control 2		6.100	200	0	150	3.600	240
Control 3		3.630	110	0	155	1.705	160
Control 4		6.930	945	0	155	2.520	180
Control 5		9.513	453	0	151	4.983	180
	Mean±SD	**6.273±2.18^a**	**464±335^a**	**0±0^a**	**192±89^a**	**3.090±1.254^a**	**188±30^a**

Infected: dogs with leishmaniasis. Control: healthy dogs. a,b The same letters in the same column indicate no statistical difference using unpaired t-test.

S4 Table. Sera biochemical profile.

Dogs	Reference values	Albumin 2.6-3.3 g/L	Globulin 2.7-4.4 g/L	T. Protein 5.4-7.1 g/L	Creatinine 0.5-1.5 mg/dL	Urea 10.03-50.03 mg/dL	ALT 21-102 UI/L	AP 20-156 UI/L	GGT 1.2-6.4 UI/L
Infected 1		1.8	9.0	10.8	0.7	30	37	75	6.0
Infected 2		1.6	6.4	8.0	0.8	32	26	120	2.0
Infected 3		1.1	5.2	6.3	1.0	60	21	109	1.9
Infected 4		1.9	6.8	8.7	0.7	49	92	92	1.0
Infected 5		1.0	7.4	8.4	1.5	87	25	135	1.0
Infected 6		2.7	8.8	11.5	0.5	28	159	51	1.7
Infected 7		1.6	9.0	10.6	0.8	63	20	17	1.9
Infected 8		1.9	8.9	10.8	0.5	25	25	107	1.1
Infected 9		1.9	7.3	9.2	0.9	44	37	76	1.3
Infected 10		1.9	5.1	7.0	0.5	23	27	135	1.4
Infected 11		1.7	7.1	8.8	0.7	25	37	35	1.3
Infected 12		1.5	9.1	10.6	1.6	70	34	38	2.8
Infected 13		2.2	8.0	10.2	1.0	44	22	32	1.7
Infected 14		2.3	6.2	8.5	0.7	30	32	76	3.2
Infected 15		2.0	8.4	10.4	0.8	42	25	23	2.8
Infected 16		1.6	7.9	9.5	1.0	51	37	67	2.1
Infected 17		1.8	7.6	9.4	0.9	35	15	57	6.0
Infected 18		2.0	4.8	7.2	0.7	32	160	42	4.0
Infected 19		1.5	6.3	7.8	0.6	21	45	22	2.2
Infected 20		1.7	4.5	6.2	0.5	23	57	141	2.1
Infected 21		1.1	5.9	7.0	0.8	33	24	96	1.8
Infected 22		2.0	7.3	10.0	0.7	22	40	73	2.0
Infected 23		1.0	6.0	7.0	1.1	73	78	151	2.0
Infected 24		1.2	5.7	6.9	1.4	56	69	30	3.0
Infected 25		1.3	5.7	8.0	1.3	42	57	27	1.5
Infected 26		2.3	10.0	12.3	0.7	20	50	51	3.0
Infected 27		1.6	10.0	11.6	1.1	36	23	96	2.5
Infected 28		1.8	5.9	7.7	0.6	33	66	171	2.3
	Mean±SD	**1.7±0.4a**	**7.1±1.5[a]**	**7.6±0.8[a]**	**0.8±0.3[a]**	**40±17[a]**	**47±36[a]**	**76±44[a]**	**2.3±1.2[a]**
Control 1		3.3	4.3	7.1	0.9	44	34	35	4.1
Control 2		3.3	4.7	8.0	0.9	31	30	21	2,3
Control 3		3.2	4.8	9.0	0.6	28	22	71	4.0
Control 4		3.2	3.8	7.0	0.9	44	33	69	2.7
Control 5		3.4	3.9	7.3	0.8	38	36	26	2.3
	Mean±SD	**3.2±0.8[b]**	**4.3±0.4[b]**	**8.9±1.7[a]**	**0.8±0.1[a]**	**37±7.3[a]**	**31±5.[a]**	**44±23[a]**	**3.0±0.9[a]**

Infected: dog with leishmaniasis. Control: healthy dogs. ALT: alanine aminotransferase, AST: aspartate aminotransferase, AP: Alkaline phosphatase, GGT: gamma glutamyl transferase. a,b The same letters in the same column indicate no statistical difference using unpaired t-test.

APÊNDICE A – REFERÊNCIAS DA INTRODUÇÃO GERAL

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ANEXO A NORMAS DE PUBLICAÇÃO DA REVISTA PLOS NEGLECTED TROPICAL DISEASE

Submission Guidelines

PLOS Neglected Tropical Diseases publishes original research articles of importance to the NTDs community and the wider health community. We will consider manuscripts of any length; we encourage the submission of both substantial full-length bodies of work and shorter manuscripts that report novel findings that might be based on a more limited range of experiments.

The writing style should be concise and accessible, avoiding jargon so that the paper is understandable for readers outside a specialty or those whose first language is not English. Editors will make suggestions for how to achieve this, as well as suggestions for cuts or additions that could be made to the article to strengthen the argument. Our aim is to make the editorial process rigorous and consistent, but not intrusive or overbearing. Authors are encouraged to use their own voice and to decide how best to present their ideas, results, and conclusions.

PLOS Neglected Tropical Diseases is committed to the highest ethical standards in medical research. Accordingly, we ask authors to provide specific information regarding ethical treatment of research participants, patient consent, patient privacy, protocols, authorship, and competing interests. We also ask that reports of certain specific types of studies adhere to generally accepted standards. Our requirements are based on the Uniform Requirements for Manuscripts Submitted to Biomedical Journals, issued by the International Committee for Medical Journal Editors.

Style and Format

Manuscript Organization

Most manuscripts should be organized as follows. Instructions for each element appear below.

- Title
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- Introduction
- Methods
- Results
- Discussion
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- Supporting information Captions

Uniformity in format facilitates the experience of readers and users of the journal. To provide flexibility, however, the Results and Discussion can be combined into one Results/Discussion section.

Other elements

- Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately.
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The compiled submission PDF includes low-resolution preview images of the figures after the reference list. The function of these previews is to allow you to download the entire submission as quickly as possible. Click the link at the top of each preview page to download a high-resolution version of each figure. Links to download Supporting Information files are also available after the reference list.

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Title

Include a full title and a short title for the manuscript.

Titles should be written in sentence case (only the first word of the text, proper nouns, and genus names are capitalized). Avoid specialist abbreviations if possible. For clinical trials, systematic reviews, or meta-analyses, the subtitle should include the study design.

Author names and affiliations

Enter author names on the title page of the manuscript and in the online submission system.

On the title page, write author names in the following order:

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Each author on the list must have an affiliation. The affiliation includes department, university, or organizational affiliation and its location, including city, state/province (if applicable), and country. Authors have the option to include a current address in addition to the address of their affiliation at the time of the study. The current address should be listed in the byline and clearly labeled “current address.” At a minimum, the address must include the author’s current institution, city, and country.

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Do not include any citations in the Abstract. Avoid specialist abbreviations.

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We ask that all authors of research articles include a 150- to 200-word non-technical summary of the work, immediately following the Abstract. Subject to editorial review and author revision, this short text is published with all research articles as a highlighted text box.

Distinct from the scientific abstract, the Author Summary should highlight where the work fits in a broader context of life science knowledge and why these findings are important to an audience that includes both scientists and non-scientists. Ideally aimed to a level of understanding of an undergraduate student, the significance of the work should be presented simply, objectively, and without exaggeration.

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The Introduction should put the focus of the manuscript into a broader context. As you compose the Introduction, think of readers who are not experts in this field.

Include a brief review of the key literature. If there are relevant controversies or disagreements in the field, they should be mentioned so that a non-expert reader can delve into these issues further. The Introduction should conclude with a brief statement of the overall aim of the experiments and a comment about whether that aim was achieved.

Methods

This section should provide enough detail for reproduction of the findings. Protocols for new methods should be included, but well-established protocols may simply be referenced. Detailed methodology or supporting information relevant to the methodology can be published on our web site.

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- How can future research build on these observations and what are the key experiments that must be done?

Acknowledgments

Those who contributed to the work but do not meet our authorship criteria should be listed in the Acknowledgments with a description of the contribution.

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References

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