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MIR-150 REGULA A CARGA PARASITÁRIA DE *LEISHMANIA INFANTUM* E OS NÍVEIS DE GZMB NAS PBMCS DE CÃES COM LEISHMANIOSE VISCERAL CANINA

Araçatuba

2022

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Dissertação 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 Mestre em Ciência Animal (Área de Medicina Veterinária Preventiva e Produção Animal).

Orientadora: Prof.ª Associada Valéria Marçal Felix de Lima

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CERTIFICADO DE APROVAÇÃO

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Aprovado como parte das exigências para obtenção do Título de Mestre em Ciência Animal, área: Medicina Veterinária Preventiva e Produção Animal pela Comissão Examinadora:

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Araçatuba, 27 de outubro de 2022.

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Jó 42:2

SOARES, M.F. et al. **miR-150 regula a carga parasitária de *Leishmania infantum* e os níveis de GZMB nas PBMCs de cães com Leishmaniose Visceral Canina.** 2022. 100 f. Dissertação (Mestrado) - Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2022.

RESUMO

A *Leishmania infantum* causa a leishmaniose visceral, uma doença tropical negligenciada que pode modular a resposta imune do hospedeiro por meio de pequenos RNAs não codificantes chamados microRNAs (miRNAs). Alguns miRNAs são expressos diferencialmente em células mononucleares do sangue periférico (PBMCs) de cães com leishmaniose visceral canina (LCan), incluindo o miR-150, cuja expressão está diminuída. Embora o miR-150 esteja negativamente correlacionado com a carga parasitária de *L. infantum*, não está claro se o miR-150 afeta diretamente a carga parasitária de *L. infantum* e (em caso afirmativo) como esse miRNA contribuiria para a infecção. Aqui, isolamos as PBMCs de 14 cães naturalmente infectados (grupo LCan) e seis cães saudáveis (grupo controle) e as tratamos *in vitro* com o mimetizador ou o inibidor do miR-150. Medimos a carga parasitária de *L. infantum* usando o qPCR e comparamos os tratamentos. Também medimos os níveis de proteína-alvo do miR-150 preditas *in silico* (STAT1, TNF-α, HDAC8 e GZMB) usando citometria de fluxo ou ensaios imunoenzimáticos. O mimetizador do miR-150 diminuiu a carga parasitária de *L. infantum* nas PBMCs de cães do grupo LCan. Também descobrimos que a inibição do miR-150 reduziu os níveis de GZMB. Esses achados demosntram que o miR-150 exerce uma função importante na infecção por *L. infantum* nas PBMCs caninas, o que poderia ser usado para mais estudos visando o desenvolvimento de drogas.

Palavras-chave: LCan. *Leishmania infantum*. miRNA. miR-150. GZMB

SOARES, M.F. et al. **miR-150 regulates the *Leishmania infantum* parasitic load and GZMB levels in peripheral blood mononuclear cells of dogs with canine visceral leishmaniasis.** 2022. 100 f. Dissertação (Mestrado) - Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2022.

ABSTRACT

Leishmania infantum causes visceral leishmaniasis, a neglected tropical disease that can modulate the host immune response by altering the expression of small non-coding RNAs called microRNAs (miRNAs). Some miRNAs are differentially expressed in peripheral blood mononuclear cells (PBMCs) of dogs with visceral canine leishmaniasis (CanL), like the down-regulated miR-150. Even though miR-150 is negatively correlated with *L. infantum* parasitic load, it is unclear if miR-150 directly affects *L. infantum* parasitic load and (if so) how this miRNA would contribute to infection. Here, we isolated PBMCs from 14 naturally infected dogs (CanL group) and six healthy dogs (Control group) and treated them *in vitro* with miR-150 mimic or inhibitor. We measured *L. infantum* parasitic load using qPCR and compared treatments. We also measured miR-150 *in silico* predicted target protein levels (STAT1, TNF-α, HDAC8, and GZMB) using flow cytometry or enzyme-linked immunosorbent assays. Increasing miR-150 activity diminished *L. infantum* parasitic load in CanL PBMCs. We also found that inhibition of miR-150 reduced GZMB levels. These findings demonstrate that miR-150 plays an important role in *L. infantum* infection in canine PBMCs, and these findings merit further studies aiming at drug development.

Key-words: CanL. *Leishmania infantum*. miRNA. miR-150. GZMB.

SUMÁRIO

1. INTRODUÇÃO GERAL

1.1 Aspectos epidemiológicos e clínicos das Leishmanioses

De maneira geral, a história da Leishmaniose está intrinsecamente ligada à história da atividade humana [1]. Essa doença se espalhou pelo mundo durante às primeiras migrações dos seres humanos na história e continua sendo um problema à nível mundial, com cerca de 0,9 a 1,7 milhões de pessoas sendo infectadas todos os anos e causando cerca de 20.000 a 40.000 mortes [2], sendo que 90% dos novos casos acontecem em 13 países (Afeganistão, Argélia, Bangladesh, Bolívia, Brasil, Colômbia, Etiópia, Índia, Irã, Peru, Sudão, Sudão do Sul e Síria) [2].

A Leishmaniose é um espectro de doenças causada por cerca de 20 espécies de protozoários do gênero *Leishmania* [3]. Esses parasitos apresentam duas formas principais: promastigota (flagelada) e amastigota (aflagelada), e sua principal forma de transmissão é através das picadas das fêmeas de mosquitos infectados dos gêneros *Phlebotomus* e *Lutzomyia* [4,5] . Esses insetos, também chamados de “mosquito-palha”, têm uma gama de diferentes hospedeiros, no qual se inclui o ser humano, roedores, marsupiais e o cão doméstico [1,6], o que faz com que a doença possa ser transmitida entre pessoas (potencial antroponótico) ou dos animais para as pessoas (potencial zoonótico) [7].

As fêmeas do mosquito-palha adquirem os parasitos do gênero *Leishmania* ao fazerem o repasto sanguíneo no hospedeiro, na sua forma amastigota [4,8]. O parasito então chega ao sistema digestivo do inseto, onde passa por diversas transformações até se tornar na forma promastigota metacíclica infectante [5,8]. Inclusive, o microbioma intestinal do mosquito é essencial para o desenvolvimento do parasito [9]. A fêmea infectada, ao novamente fazer o repasto sanguíneo, regurgita as formas infectantes, juntamente com proteínas salivares que auxiliam na infecção, transmitindo assim o parasito para o hospedeiro [8]. Com isso, o ciclo da *Leishmania* se mantém (Figura 1).

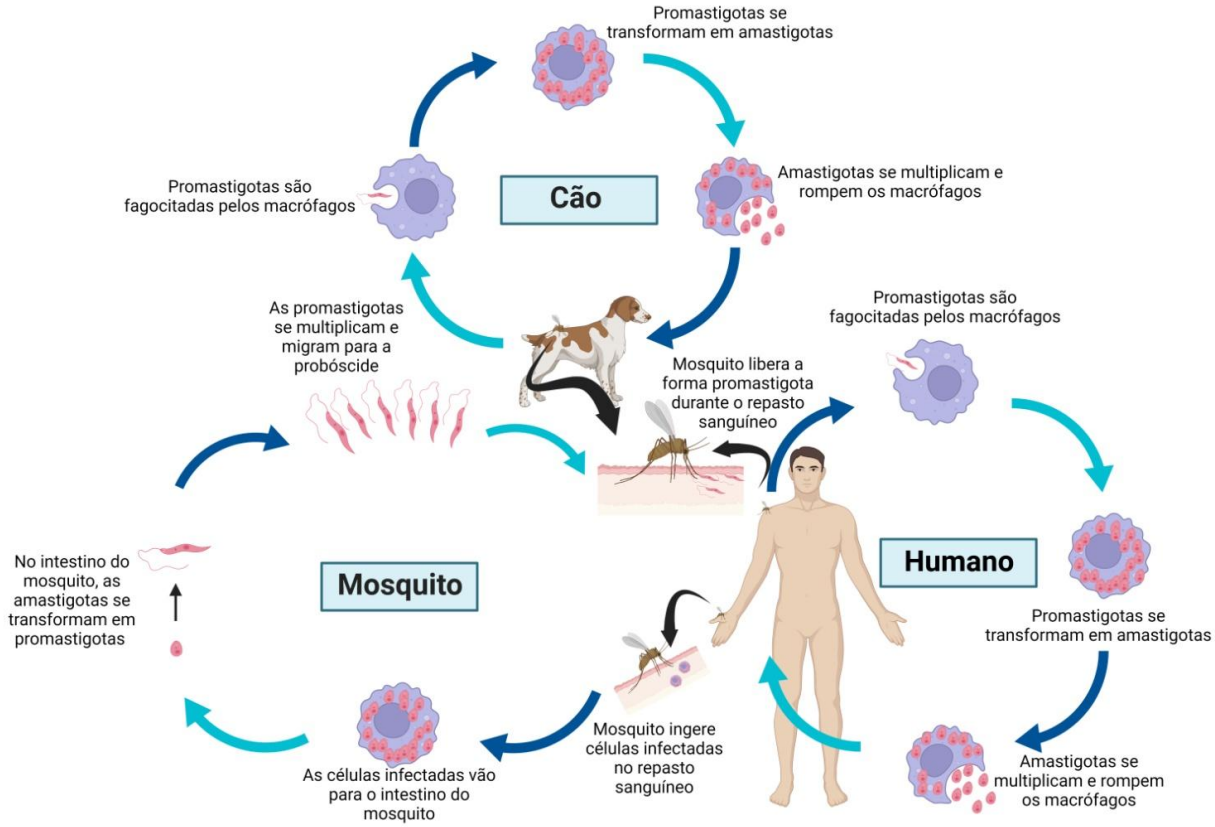

Fig. 1 Ciclo da *Leishmania* spp. As fêmeas do mosquito-palha liberam as formas promastigotas da *Leishmania* em humanos e em cães susceptíveis durante o repasto sanguíneo. Essas formas são então fagocitadas por fagócitos residentes, no qual se destacam os macrófagos. Nessas células, se diferenciam na forma amastigota e começam o processo de multiplicação intracelular, até o rompimento da célula. O mosquito-palha então se infecta ao ingerir células infectadas durante o repasto sanguíneo. No mosquito, a forma amastigota se converte na forma promastigota, principalmente nas porções médias do trato digestivo desses insetos. As formas promastigotas infectantes movem-se então para a probóscide dos insetos, onde infectam um novo hospedeiro susceptível (figura baseada em [7], criado com BioRender.com).

Apesar da morfologia muito similar, diferentes espécies de *Leishmania* causam dois tipos diferentes de forma clínica da doença: Leishmaniose cutânea (LC) e Leishmaniose Visceral (VL), também chamada de kalazar. Ainda, espécies diferentes de vetores e parasitos atuam em diferentes regiões do globo [1,5]. Em relação às espécies de *Leishmania*, no Velho Mundo as principais espécies que causam LC são *L. tropica* e *L. major*; e VL são *L. donovani* e *L. infantum*. Já no Novo Mundo, tem-se a LC causada principalmente por *L. amazonensis*, *L. mexicana* e *L. braziliensis*; e VL causada por *L. infantum* (syn. *chagasi*).

A forma clínica mais comum da doença é a LC, com cerca de 0,7 a 1,3 milhões de casos ocorrendo anualmente pelo mundo [2]. A LC apresente até três diferentes formas: Leishmaniose Cutânea Localizada (LCL), Leishmaniose Cutânea Difusa (LCD) e Leishmaniose Mucocutânea (LMC) [1,10]. Sua diferença está relacionada com o padrão de lesão cutânea. Enquanto LCL é caracterizada pela presença de lesões e úlceras de pele e, a LMC evolui para úlceras nas membranas do nariz, boca e garganta, podendo levar à uma desfiguração facial extensa.

Já a VL atinge cerca de 200.000 a 400.000 pessoas anualmente e é fatal em 95% dos casos sem tratamento [2]. Ainda, estimativas globais recentes, colocam a VL como a doença tropical negligenciada mais mortal do mundo [11,12]. Cerca de 90% dos casos acontecem em apenas seis países: Bangladesh, Brasil, Etiópia, Índia, Sudão e Sudão do Sul [2]. Os sintomas da VL incluem febre, perda de peso, hepatomegalia, esplenomegalia e anemia [1]. Na Índia, é causada principalmente pela *L. donovani* e os pacientes apresentam uma manifestação cutânea chamada Leishmaniose dermal pós-kalazar (LDPK), e possui transmissão antroponótica [3]. Já no Brasil, a VL é causada pela *L. infantum*, e possui potencial zoonótico, pois tem como um dos principais fatores de risco a proximidade com os cães domésticos infectados [13].

1.2 Leishmaniose Visceral Canina (LCan)

Desde que Nicolle e Comte descobriram a Leishmaniose Canina (LCan) [14], os cães domésticos têm sido considerados os principais reservatórios de VL, sendo importantes na transmissão da doença [13,15]. A transmissão ocorre principalmente por meio da picada das fêmeas do mosquito-palha, sendo no Brasil o *Lutzomyia longipalpis* a principal espécie [16,17]. Essa espécie possui atividade noturna e crepuscular, se alimentando principalmente em áreas com menos pelos no animal, como a cabeça, a ponte nasal, pavilhões auriculares e áreas inguinal e perianal [18].

Apesar da picada por *L. longipalpis* ser a principal forma de transmissão de *L. infantum* para os cães, formas alternativas também foram descritas na literatura, como a transmissão venérea [19], transplacentária [20] e transfusão sanguínea [21], além de outros vetores sugeridos, como pulgas [22,23] e

carrapatos [24,25]. Entretanto, todas essas alternativas aparentam ser de menor importância ao ser comparada com a transmissão pelo mosquito-palha.

Após a transmissão, os parasitos são fagocitados por neutrófilos e por células do sistema mononuclear fagocítico, principalmente macrófagos circulantes, onde se diferenciam na forma amastigota aflagelada, se reproduzindo de maneira assexuada até a ruptura da célula acontecer [3,7]. Os parasitos então invadem fagócitos mononucleares de diversos órgãos, como fígado, baço, linfonodos e medula óssea, levando ao desenvolvimento da doença [26].

No caso da LCan, o número e intensidade dos sinais clínicos estão relacionados com fatores como cepa do parasito, genética e imunidade do hospedeiro [18,26]. Por isso, até 50% dos cães infectados podem não exibir nenhum sinal clínico, sendo classificados como assintomáticos [27,28]. Esses cães apresentam uma forte resposta imune celular comparado aos cães sintomáticos [29,30]. Apesar de não apresentarem sinais clínicos, eles também são responsáveis por manter o ciclo de transmissão do parasito [31].

A outra metade dos cães infectados apresentam diferentes conjuntos de sinais clínicos inespecíficos, que começam a aparecer a partir de três meses e podem aparecer muitos anos após a infecção [26]. Dentre eles destacam-se lesões na pele, linfadenopatia, onicogrifose, perda de peso, hepatomegalia, esplenomegalia, e lesão renal [18]. A lesão de pele é o sinal clínico mais comum, com cerca de 90% dos cães apresentando esse sinal clínico [32,33]. Não existe uma ordem para o aparecimento dos sinais, porém a presença de insuficiência renal pode indicar a fase terminal da doença, levando o animal à óbito [18,26].

Como a LCan é uma doença sistêmica, pode-se observar alterações nos exames de hemograma, leucograma e perfil bioquímico dos cães doentes. A principal alteração vista no hemograma é a anemia, devido principalmente à doença renal crônica (diminuição da produção de eritropoetina) e às disfunções medulares [34–36].

A principal alteração vista no exame bioquímico de cães com LCan é o desbalanço das proteínas [6,36,37]. Esse desbalanço é representado por hiperproteinemia, com hipoalbuminemia e hipergamaglobulinemia, ocorrendo a inversão da razão albumina/globulina [6,37]. A hipoalbuminemia pode ser decorrente da anorexia, bem como insuficiência hepática ou como consequência

da doença renal [6]. Já a hipergamaglobulinemia está relacionada com a reação imunológica humoral exacerbada nos animais doentes [6].

Como os cães são os principais reservatórios urbanos da *L. infantum*, torna-se necessária a rápida detecção dos animais infectados na população ao observar-se os primeiros sinais clínicos da doença, principalmente em áreas endêmicas [36]. Como cães assintomáticos também podem transmitir a doença, os testes diagnósticos precisam ser corretamente utilizados e interpretados para evitar-se resultados falsos negativos e a propagação da doença na população [7]. Para isso, utiliza-se um conjunto de testes parasitológicos, sorológicos e moleculares [6,18].

Os métodos parasitológicos são o padrão-ouro na detecção da *L. infantum* em cães [6]. Nesse método, observa-se no microscópio óptico as formas amastigotas do parasito nos macrófagos de tecidos infectados, como medula óssea, linfonodos, baço e pele [26,38]. Apesar de alta especificidade, o método apresenta baixa sensibilidade, com 60-85% nas lâminas de medula óssea e 30-40% nos linfonodos [39].

Os métodos sorológicos são utilizados para a detecção de anticorpos específicos anti-*Leishmania* em cães [18]. Por sua facilidade e custo, são os métodos de eleição na rotina. Preferencialmente, utilizam-se técnicas quantitativas, como a Reação de Imunofluorescência Indireta (RIFI) e o Ensaio Imunoenzimático (ELISA) indireto [18]. A sensibilidade e especificidade desses testes depende do antígeno utilizado [26,40].

Para a triagem de diversas doenças, inclusive a LCan, métodos de imunocromatografia têm sido utilizados nas clínicas veterinárias [6,26]. São métodos sorológicos de baixo custo e com rápido resultado. No Brasil, utiliza-se o Dual Path Platform (DPP) para a triagem e o ELISA para a confirmação da doença [6,41].

Os métodos moleculares apresentam grande sensibilidade e especificidade, porém alto custo [6]. Na Medicina Veterinária, utiliza-se normalmente a Reação em Cadeia da Polimerase (PCR) ou a PCR em tempo real (qPCR) [6,18]. A qPCR pode ser utilizada em diversos tecidos, como sangue, medula óssea, linfonodos, fragmento de pele e swab conjutival [6,18,42]. Parte da sensibilidade e especificidade conferida à essa técnica decorre do primer utilizado durante a reação. Para a detecção do parasito, o

primer do DNA do kinetoplasto (kDNA) do parasito parece ser bastante sensível para a detecção direta do parasito nos tecidos [18,43]. É importante ressaltar que os resultados obtidos pelos métodos moleculares devem ser interpretados à luz de outras informações do cão, como seu histórico, sinais clínicos e o resultado de outros métodos diagnósticos [18].

No Brasil, os cães infectados por *L. infantum* podem seguir dois destinos: ou são eutanasiados ou iniciado o tratamento [17]. Até o momento, não existem evidências científicas que a eutanásia de cães infectados diminua a incidência de LV [44,45]. Já para a segunda alternativa, apesar da cura parasitológica ser rara, alguns cães desenvolvem a cura clínica da doença, aumentando assim sua expectativa de vida [6]. Apesar da cura clínica, a maioria dos cães continuam atuando como reservatórios do parasito [43]. Por isso, animais em tratamento devem utilizar-se de repelentes contra o mosquito, seja por meio de produtos “spot-on” ou por meio de coleiras [43].

Por meio da observação dos sinais clínicos da doença e o resultado dos testes diagnósticos, os cães podem ser classificados conforme a gravidade da doença [18], conforme demonstrado na tabela 1. O prognóstico e tratamento do animal está baseado no estágio clínico da LCan que o animal se encontra. De maneira geral, o alopurinol é considerado a droga de primeira-linha para o tratamento da LCan, seja associado com outra drogas ou usado como agente único [6,18,43]. Entretanto, no Brasil, o único medicamento autorizado para o uso no tratamento da LCan é a miltefosina [6].

Tabela 1. Estadiamento da Leishmaniose Visceral Canina baseado no status sorológico, sinais clínicos, achados laboratoriais e tipo de terapia e prognóstico (baseado em [18])

Estágio Clínico	Sorologia	Sinais Clínicos	Achados laboratoriais	Terapia	Prognóstico
Estágio I: Doença branda	Negativa à níveis baixos de anticorpos anti-*Leishmania*	Sinais clínicos brandos, como linfadenopatia periférica ou dermatite papular	Normalmente, sem anormalidades clinicopatológicas. Perfil renal normal (creatinina < 1,4 mg/dL; UPC < 0,5)	Apenas alopurinol/alopurinol + antimoniato de meglumina ou miltefosina	Bom
Estágio II: Doença moderada	Níveis baixos a altos de anticorpos anti-*Leishmania*	Além dos sinais do estágio I, podem apresentar lesões cutâneas difusas ou simétricas como dermatite esfoliativa,/onicogrifose, ulcerações (plano nasal, coxins plantares, proeminências ósseas, junções mucocutâneas), anorexia, perda de peso, febre e epistaxe	Anormalidades clinicopatológicas como anemia não regenerativa leve, hipergamaglobulinemia, hipoalbuminemia, síndrome de hiperviscosidade sérica. Subestágio—(a) perfil renal normal: creatinina < 1,4 mg/dl; não proteinúrico: UPC < 0,5. (b) Creatinina < 1,4 mg/dl; UPC = 0,5–1	Alopurinol + antimoniato de meglumina ou miltefosina	Bom a reservado
Estágio III: Doença severa	Níveis médios a altos de anticorpos anti-*Leishmania*	Além dos sinais listados nos estágios I e II, podem apresentar sinais originados de lesões por imunocomplexos: vasculite, artrite, uveíte e glomerulonefrite	Anormalidades clinicopatológicas listadas no estágio II Doença renal crônica (DRC) IRIS estágio I com UPC > 1 ou estágio II (creatinina 1,4–2 mg/dl)	Alopurinol + antimoniato de meglumina ou miltefosina Siga as diretrizes da IRIS para a DRC	Reservado à ruim
Estágio IV: Doença muito severa	Níveis médios a altos de anticorpos anti-*Leishmania*	Cães com sinais clínicos listados no estágio III. Tromboembolismo pulmonar ou síndrome nefrótica e doença renal terminal	Anormalidades clinicopatológicas listadas no estágio II DRC IRIS estágio III (creatinina 2-5 mg/dl) e estágio IV (creatinina > 5 mg/dl) Síndrome nefrótica: proteinúria marcada UPC > 5	Apenas alopurinol Siga as diretrizes da IRIS para a DRC	Ruim

UPC = relação proteína urinária por creatinina

Como os tratamentos atuais não garantem cura parasitológica e possuem diversos efeitos colaterais, entender os mecanismos imunológicos por trás da interação parasito-hospedeiro nos cães pode auxiliar no desenvolvimento de medicamentos cada vez mais eficazes contra a *L. infantum.*

1.3 Resposta Imune na LCan

A resposta imunológica contra *L. infantum* em cães é complexa e é objeto de muita investigação. Ademais, existem diversas abordagens que podem ser

feitas em relação à resposta imune do animal contra o parasito - como a abordagem por imunidade inata/adaptativa, imunidade órgão-específica e imunidade protetora/não-protetora. Para a nossa explicação, destacamos o desenvolvimento da imunidade/doença por meio do balanço pró/anti-inflamatório da imunidade inata/adaptativa.

Os macrófagos são as principais células parasitadas por *L. infantum*, porém não são as primeiras células a fagocitar o parasito. As primeiras células que realizam essa função são os neutrófilos [46–48]. Essas células da imunidade inata são utilizadas pelos parasitos do gênero *Leishmania* de maneira temporária, até serem finalmente fagocitadas pelos macrófagos [49]. Apesar de neutrófilos caninos conseguirem destruir a *L. infantum*, alguns parasitos conseguem evadir os mecanismos microbicidas do neutrófilos e sobreviverem no hospedeiro [46]. De fato, cada espécie de *Leishmania* possui uma forma diferente de evasão dos mecanismos microbicidas dos neutrófilos [49]. No caso da *L. infantum*, o principal mecanismo de evasão está relacionado com a prevenção de formação e destruição das NETs [49]. Por não conseguirem destruir completamente os parasitos, os neutrófilos podem estar atuando apenas como “Cavalos de troia” até a entrada da *L. infantum* nos macrófagos [48,49].

Nos macrófagos, a *L. infantum* permanece em uma estrutura chamada fagolisossomo, resultante da fusão dos fagossomos com as enzimas lisossomais [48]. Os macrófagos reconhecem a infecção por *L. infantum* através de “sensores” da imunidade inata, chamados receptores Toll-like (TLRs) [48,50]. Alguns TLRs, como o TLR-2, ativam os macrófagos e permitem a liberação de citocinas importantes para a resposta imune anti-*Leishmania*, como a interleucina 12 (IL-12) [51]. Além disso, os macrófagos ativados liberam espécies reativas de oxigênio (ROS) e óxido nítrico (NO) para destruir o parasito [52,53]; sendo esse último induzível pela enzima óxido nítrico sintase (iNOS), transcrita através da via de sinalização intracelular JAK/STAT1, dependente de IFN-γ [54].

Outro mecanismo da imunidade inata envolvido com a infecção de parasitos do gênero *Leishmania* é a ativação dos inflamassomas [55,56]. Esse mecanismo é estudado principalmente a partir da ativação do recepetor NOD-like (NLR) NLRP3 [55]. A ativação de NLRP3 leva a ativação da caspase-1 e produção de IL-1β, citocina importante na resposta imune inata contra o parasito [56]. Além disso, parasitos do gênero *Leishmania* podem ativar a via não-

canônica do inflamassoma por meio da caspase-11 [57]. A ativação do inflamassoma é prejudicial para diversas espécies de *Leishmania*, inclusive a *L. infantum* [55,58].

Apesar da resposta imune inata ser importante para o controle do parasito, ela não é suficiente para destruir todos os parasitos, já que eles desenvolveram mecanismos de evasão que garantem sua permanência em macrófagos [3,54,59]. Por exemplo, amastigotas de *L. infantum* resistem ao ambiente hostil dos macrófagos atrasando a formação do fagolisossomo [60,61] e produzindo antioxidantes para neutralizar os radicais livres [62–64]. Além disso, parasitos do gênero *Leishmania* podem bloquear a formação do inflamassoma [65,66]. Portanto, para evitar a multiplicação dos parasitos, os macrófagos contam com o auxílio da resposta imune adaptativa protetora [67].

A imunidade adaptativa protetora contra *L. infantum* em cães é predominantemente celular em comparação à uma resposta predominantemente humoral [53,68]. A resposta predominantemente celular é mediada por linfócitos T CD4+ do perfil Th1, por linfócitos T citotóxicos (T CD8+) e por citocinas secretadas por essas células, como o fator de necrose tumoral alfa (TNF-α) e o interferon gama (IFN-γ) [53,68]. As células T CD8+, além de contribuir com a secreção de citocinas, podem destruir as células infectadas por meio do conteúdo de seus grânulos (Perforina, granzima A, granzima B), impedindo a infecção de novas células [68,69].

O desenvolvimento de uma imunidade predominantemente Th1 pelos cães é multifatorial, dependendo de fatores como o estado nutricional, supressão do sistema imune e a genética individual dos animais [18]. Sobre a genética, é demonstrado que cães suscetíveis à LCan apresentam mutações em genes importantes para a resposta imune celular, como o gene *Slc11a1* (antigo *Nramp1*) e alelos do MHC classe-II [70–72]. Além disso, cães da raça *Ibizan Hound* apresentam maior resistência ao parasito por apresentarem maior resposta imune celular [73], reforçando o papel da genética para a resposta imune celular protetora.

A diferenciação de células T CD4+ imaturas em células Th1 é mediada por IL-12, secretada principalmente pelos macrófagos e células dendríticas [74]. As células Th1 então secretam citocinas como IFN-γ e TNF-α, que são cruciais para a ativação dos macrófagos e controle da multiplicação de *L. infantum*

nessas células [67,68]. Com isso, o sistema imune em geral adquire um perfil pró-inflamatório, que, em excesso, pode ser prejudicial ao hospedeiro [48,67].

Por isso, o sistema imune compensa o estado pró-inflamatório excessivo com a produção da citocina IL-10 [48,67]. Essa citocina é produzida normalmente em baixas quantidades na resposta pró-inflamatória e serve para antagonizar eventuais efeitos pró-inflamatórios exacerbados da resposta por IFN-γ [48,67]. Entretanto, por antagonizar os efeitos de IFN-γ, a IL-10 em maiores quantidades pode também diminuir os efeitos de destruição da *L. infantum* [48,67] . Portanto, o balanço entre a resposta inflamatória e a resposta reguladora é importante tanto para manter a destruição do parasito quanto para não causar lesões no hospedeiro [48].

Apesar de todo o mecanismo para a destruição do parasito, raramente o sistema imune consegue destruí-lo completamente. Consequentemente, com a cronicidade da doença e sem haver a destruição completa do parasito, ocorre a diferenciação de linfócitos T CD4+ imaturos em células T reguladoras 1 (Tr1) [48,75]. Essas células secretam tanto IFN-γ quanto IL-10, porém IL-10 em uma quantidade superior à IFN-γ [76]. Por consequência, há a diminuição da diferenciação de células T CD4+ para o perfil Th1 e diminuição da resposta microbicida nos macrófagos mediada por IFN-γ, contribuindo para a sobrevivência do parasito [48]. Essa mudança de perfil predominante de linfócitos T CD4+ imaturos de Th1 para Tr1 é essencial para que ocorra a mudança de um perfil inflamatório para um perfil mais regulador, no qual o parasito sobrevive e se multiplica [48]. Essa mudança é crucial para levar os linfócitos T à “exaustão” [48].

A “exaustão” de linfócitos T é marcada pela expressão de muitos receptores de superfície nessas células, como o programmed death ligand 1 (PD-1) [77]. A ligação dessas moléculas com seus ligantes (PD-L1), encontrado em macrófagos, faz com que haja aumento da apoptose desses linfócitos [78]. De fato, há aumento de apoptose de linfócitos T do sangue periférico e do baço de cães com LCan [79]. O aumento da apoptose dos linfócitos T culmina na maior sobrevivência e multiplicação da *L. infantum* [78].

Concomitantemente, as células B, que na doença subclínica desenvolvem baixos níveis de anticorpos específicos contra *L. infantum* [48], começam a desenvolver maiores níveis de anticorpos não específicos para o parasito,

levando ao quadro de hipergamaglobulinemia [48]. Esses anticorpos não são protetores, ao contrário, são prejudiciais ao organismo [48,80–82]. Por exemplo, esses anticorpos podem formar imunocomplexos que ativam a produção de IL-10 por macrófagos [48,80] e se depositam em órgãos, como o rim, podendo levar à uma insuficiência renal crônica, sinal clínico importante da LCan [18,83].

Além disso, recentemente têm-se estudado o desenvolvimento e o papel das células B reguladoras na progressão da doença. Essa população de células é capaz de produzir IL-10 em doenças de caráter autoimune e doenças crônicas, inclusive em cães com LCan [84–87]. A proporção de células B que produz IL-10 foi significativamente maior em cães com LCan que apresentaram sinais clínicos, mostrando sua importância para a progressão da doença [87]. A dinâmica está sintetizada na figura 2.

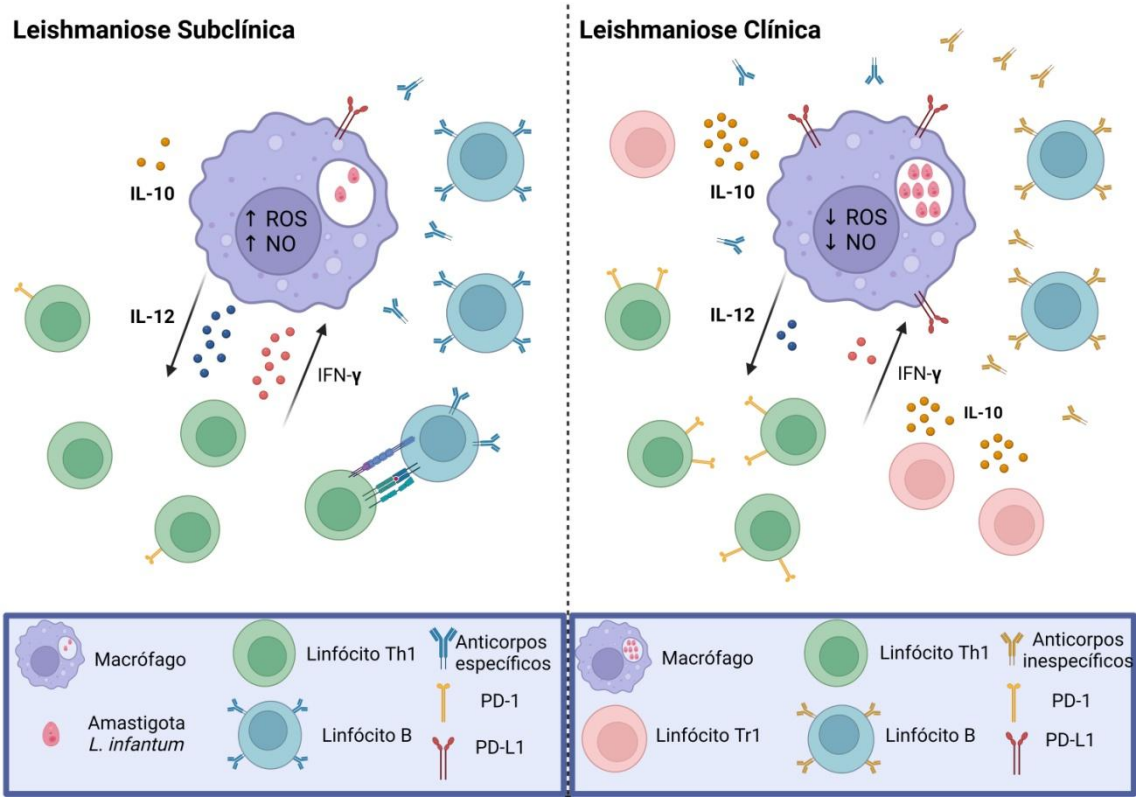

Fig. 2 Resposta imune na LCan subclínica e na LCan Clínica. Na LCan subclínica (esq.), observa-se um perfil pró-inflamatório, marcado pela ativação e diferenciação de linfócitos T CD4+ para Th1 e citocinas como IFN-γ e IL-12, com baixa produção de IL-10. Além disso, há ativação de macrófagos com menor multiplicação do parasito, e células B produzindo anticorpos específicos anti-*Leishmania.* Por não conseguir destruir os parasitos por completo, os linfócitos T CD4+ começam a se diferenciar em linfócitos T reguladores 1 (Tr1), que secretam grandes quantidades de IL-10. Ao mesmo tempo, tem-se menor secreção de IFN-γ e IL-12, levando o sistema imune a um perfil mais regulador. Linfócitos B começam a se multiplicar e secretar

anticorpos inespecíficos para *Leishmania*, que são prejudiciais ao hospedeiro. Por fim, o sistema imune entra em um estado de "exaustão", com aumento da expressão de PD-1 e PD-L1. Tudo isso leva a um aumento da sobrevivência do parasito, e favorece o aparecimento dos sinais clínicos (dir.) (figura baseada em [48], criado com BioRender.com).

Portanto, os sinais clínicos da LCan decorrem da diminuição de secreção de IFN-γ por linfócitos T CD4+ específicos para a *L. infantum*, aumento da secreção de IL-10 e da carga parasitária nos tecidos, e maiores níveis de anticorpos detectados nos testes sorológicos.

A pesquisa para tratamentos mais recentes contra *L. infantum* levam em consideração o entendimento de toda essa dinâmica do sistema imunológico apresentada. Por exemplo, a domperidona é utilizada por aumentar os níveis de resposta imune celular mediada por Th1[88,89]. Estudos também mostram que o bloqueio de PD-1 e PD-L1 aumenta a sobrevivência dos linfócitos T, com o aumento de TNF-α, da resposta imune celular e diminuição da carga parasitária de *L. infantum* em cães [78,90]. Logo, entender a imunologia da LCan é essencial para o desenvolvimento de tratamento mais eficazes contra a doença.

Recentemente, têm-se observado que mecanismos epigenéticos também estão envolvidos na resposta imunológica em infecções por *Leishmania* spp. [91], inclusive *L. infantum* em cães. Além de estudos envolvendo metilação de DNA, acetilação de histonas e histona desacetilases (HDACs), estudos apontam o controle pós-transcricional de genes relacionados com a imunidade através dos microRNAs (miRNAs) [91]. Portanto, entender como a infecção por *L. infantum* afeta a expressão e função de miRNAs em células de cães é vital para o auxílio no tratamento e controle desse parasito.

1.4 MicroRNAs (miRNAs)

O entendimento do controle pós-transcricional de genes foi revolucionado após a descoberta recente de uma classe diferente de RNA não-codantes, pequenos (20-24 nucleotídeos) chamados de microRNAs (miRNAs) [92]. A revolução foi tão grande, que a revista *Science* chamou-a de "*Breakthrough of the Year*" em 2002, além de render um Prêmio Nobel de Medicina, em 2006,

para Andrew Fire e Craig Mello, pela descoberta da interferência de RNAs na expressão de genes no modelo biológico de *Caenorhabditis elegans* [93] .

Apesar dos primeiros estudos terem sido realizados em *C. elegans*, descobriu-se que tanto a sequência quanto a função dos miRNAs são conservados em diferentes espécies, atestando sua relevância biológica [94]. Por exemplo, os primeiros estudos mostraram que a sequência de *let-7*, um dos primeiros miRNAs descobertos em *C. elegans*, eram conservados tanto em *Drosophila melanogaster* quanto no homem [95].

Por isso, cresceu-se o interesse em entender cada vez mais essas moléculas. Atualmente, muito se foi descoberto em relação à sua biogênese e mecanismo de ação, além de sua desregulação em condições patológicas e seu uso como diagnóstico, prognóstico e tratamento [96–99]. Alguns desses tópicos serão abordados aqui, além de uma abordagem em relação à infecção por *L. infantum* em cães.

Os miRNAs podem possuir diversas localizações no genoma (intergênicos, intrônicos e exônicos) [100], e são transcritos principalmente pela RNA polimerase II em um RNA de fita dupla, formando uma estrutura de *stem-loop*, ou seja, um tronco (*stem*) com terminal em forma de alça (*loop*), denominado de *primary microRNA* (pri-miRNA) [101]. O pri-miRNA é então clivado por um complexo formado principalmente por Drosha e DGCR8, em *precursor microRNA* (pre-miRNA), que possui o formato de "grampo de cabelo" [102].

Após o processo de maturação de pri-miRNA para pre-miRNA por Drosha/DGCR8, o pre-miRNA sai do núcleo em direção ao citoplasma através da formação de um complexo com a proteína Exportin-5 [103]. Uma vez 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* [104].

Em seguida, uma dessas fitas formadas, denominada "fita-guia", desempenhará suas funções juntamente com o complexo RISC [105]. Um dos principais componentes do complexo RISC são as proteínas Argonautas (AGO), principalmente AGO2 [105,106]. Todo o esquema, denominado de "via canônica" da biogênese dos miRNAs está representado na figura 3.

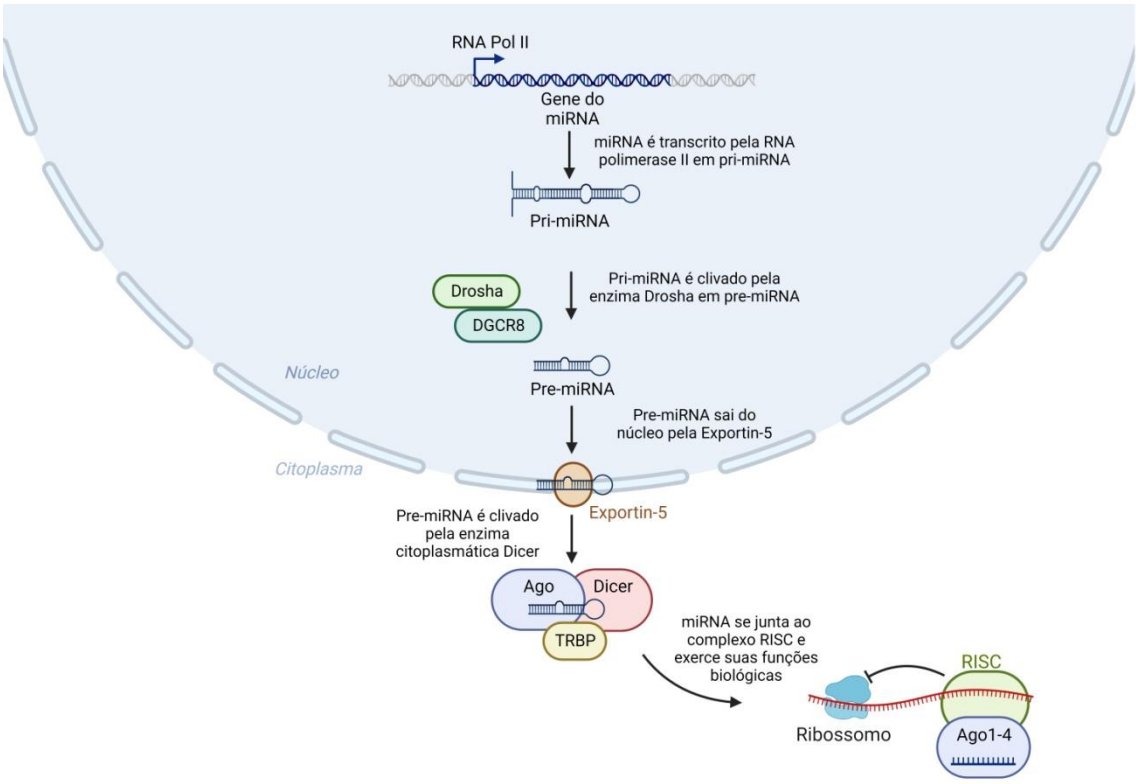

Fig. 3 Via canônica da biogênese dos miRNAs. A RNA polimerase II (RNA Pol II) sintetiza o *primary miRNA* (pri-miRNA) a partir do seu gene. O pri-miRNA é então clivado pelo complexo Drosha/DGCR8, formando o *precursor-miRNA* (pre-miRNA). O pre-miRNA é então exportando para fora do núcleo pela proteína 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 baseada em [107], criado com BioRender.com).

Classicamente, os miRNAs estão relacionados negativamente com seus mRNA-alvo [96]. Em outras palavras, 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. Inclusive, essa foi a dinâmica que possibilitou o descobrimento dos primeiros miRNAs em *C. elegans* [108–110].

Os miRNAs possuem pequenas regiões (6 - 8 nucleotídeos) que são cruciais para o reconhecimento do mRNA-alvo. Essas regiões são chamadas de "*seed*" [111]. A importância dessas regiões para o reconhecimento do mRNA-alvo é evidenciada no fato de que qualquer alteração nessa região pode causar o não reconhecimento do alvo, ou até mesmo o miRNA passa a ter um alvo

alternativo [112]. A ligação da região "*seed*" do miRNA do complexo RISC pode levar à desestabilização do mRNA-alvo por meio de sua clivagem [113].

Outras maneiras que o miRNA desempenha sua função é por meio da deadenilação seguida da remoção do 5' CAP de seu mRNA-alvo [114], e também via silenciamento transcricional mediado principalmente pro modificações na cromatina dose genes-alvo [115,116].

Recentemente, tem-se descoberto uma função oposta dos miRNAs, relacionada à promoção da transcrição e ao aumento da eficiência de tradução. A primeira está relacionada com o fato de miRNAs, como o *let-7i*, interagirem com *motifs* TATA-box no promotor proximal, que são importantes para a promoção da transcrição do gene [117]. Já o segundo pode ser visto com o miR-396-3 aumentando a tradução de TNF-α por interagir com elementos ricos em AU [118].

No organismo, os miRNAs podem ser secretados em vesículas extracelulares, como os exossomas [119,120]. Esse miRNAs circulantes permitem então a comunicação de células distantes entre si [120]. Além disso, as vesículas extracelulares e seu conteúdo podem ser utilizados como biomarcadores em diversas doenças, incluindo doenças infecciosas [119,121].

Atualmente, sabe-se que a expressão de miRNAs está desregulada em diversas doenças, tanto em humanos como em cães [122–124]. Essa desregulação está envolvida muitas vezes com a progressão da doença [125]. Por isso, os miRNAs têm sido estudados para serem alvos de diagnóstico, prognóstico e tratamento [96–99].

A desregulação de miRNAs também tem sido vista em células infectadas por parasitos do gênero *Leishmania* em diferentes organismos, inclusive *L. infantum* em cães [126–129]. Entender quais são esses miRNAs, os genes que estão sendo controlados por eles e o resultado na infecção por *L. infantum* é crucial para sua utilização como diagnóstico, prognóstico e tratamento.

1.5 miRNAs e a infecção por *Leishmania* spp.

Como visto anteriormente, parasitos do gênero *Leishmania* podem alterar a resposta imune para permanecerem vivos no hospedeiro. Uma das formas que

fazem isso é por meio da desregulação de miRNAs, os pequenos RNAs não-codantes responsáveis pelo controle da expressão gênica. Aqui, teremos uma breve revisão de como a *Leishmania* spp. utiliza esse mecanismo para modular a resposta imune e sobreviver em seus respectivos hospedeiros, com foco na *L. infantum* em cães.

Diferentes parasitos do gênero *Leishmania* modulam diferentes miRNAs em diferentes organismos, facilitando sua sobrevivência nos hospedeiros. Por exemplo, *L. major* pode alterar a expressão de 64 miRNAs em macrófagos humanos em diferentes períodos da infecção dentro de 24h [126]. Além disso, *L. major* e *L. donovani* podem alterar diferentes miRNAs em macrófagos e células dendríticas em humanos [127]. *L. donovani* também causa a alteração na expressão de miRNAs em macrófagos de camundongos [128]; e *L. infantum* em células mononucleares de sangue periférico (PBMCs) de cães [129].

É importante ressaltar que a alteração nos miRNAs não se dá apenas em fagócitos, como macrófagos e células dendríticas. Linfócitos T CD4+ cultivados com macrófagos infectados por *L. donovani* também possuem alteração na expressão dos seus miRNAs [130]. Interessantemente, os miRNAs que foram regulados positivamente estão relacionados com o perfil Th1 - imunidade adaptativa celular e perfil pró-inflamatório, enquanto os miRNAs regulados negativamente estavam relacionados com o perfil Th2. Ou seja, os genes que favorecem a resposta Th1 estavam regulados negativamente, enquanto os genes que favorecem a resposta Th2 estavam regulados positivamente. Dessa forma, os parasitos parecem poder direcionar uma mudança no perfil de linfócitos T CD4+ para o perfil Th2 através dos miRNAs, o que favorece sua sobrevivência no hospedeiro [130].

Alguns miRNAs parecem estar envolvidos com a diferenciação em células Th1 e Th2 em infecções por *Leishmania*. Um estudo *in silico* evidenciou que alguns miRNAs, como os miR-29b, miR-29a, miR-1272 e o miR-155 podem estar envolvidos na polarização Th1 e Th2 na infecção por *L. donovani* em humanos [131]. O próprio miR-155 já foi demonstrado como um miRNA importante para a polarização de linfócitos T para o perfil Th2 na patogênese de *L. major* [132]. Já a diminuição de miR-21 promove uma resposta Th1 e resistência a *L. donovani* em camundongos [133] e *L. infantum* em leucócitos

esplênicos de cães [134]. Esse miRNA está envolvido diretamente com a citocina IL-12 [133,134].

Esses parasitos podem ainda alterar a resposta imunológica do hospedeiro através dos miRNAs. Por exemplo, a *L. amazonensis* induz os miRNAs miR-294-3p e miR-721 em macrófagos de camundongos, que reduzem os níveis da óxido nítrico sintase 2 (NOS2) e de NO, aumentando assim a carga parasitária [135]. Além disso, a ativação de vias sinalização de TLR2, TLR4 e MyD88 regula positivamente o miRNA *let-7e*, e sua inibição aumenta a produção de NOS2 e NO, diminuindo a carga parasitária [136]. Ainda, a *L. donovani* pode ativar o fator induzível por hipóxia-1α (HIF-1α) e o miR-210, bloqueando vias de sinalização para a produção de citocinas pró-inflamatórias, como o TNF-α e a IL-12 [137], garantindo a sobrevivência do parasito.

Alguns mecanismos são tuilizados pela *Leishmania* spp. para a regulação de miRNAs. Por exemplo, a regulação de alguns miRNAs é devido ao aumento da proteína c-Myc em macrófagos humanos infectados por *L. donovani* [138]. Ainda, a glicoproteína 63 (gp63) desse parasito pode clivar a enzima Dicer1 e diminuir o miR-122 no fígado de camundongos [139].

Outro ponto importante a ressaltar é que o mesmo miRNA pode ter funções distintas dependendo da espécie de *Leishmania* ou manifestação clínica da doença (cutânea e visceral). Por exemplo, o miR-548d-3p está aumentado em células THP-1 humanas infectadas *in vitro* tanto por *L. braziliensis* como por *L. infantum* [140,141]. Porém, ao se inibir o miR-548d-3p nessas células, observa-se uma diminuição e aumento da carga parasitária, respectivamente [140,141].

Na LCan, nosso grupo observou que PBMCs de cães infectadas *in vitro* por *L. infantum* apresentaram a expressão diferencial de pelo menos 14 miRNAs, que podem estar envolvidos com vias de sinalização importantes para a sobrevivência do parasito ou a sua internalização, como a via de sinalização de MAPK [129]. Além disso, cães naturalmente infectados apresentaram uma diminuição do miR-122, encontrado em exossomas séricos [142], e expressão diferencial de diversos miRNAs em leucócitos esplênicos (LEs) e em PBMCs [134,143].

Dentre os miRNAs diferencialmente expressos nos LEs, observou-se aumento dos miR-21, miR-148a, miR-7 e miR-615; e diminuição dos miR-125a,

miR-125b e miR-150 [134]. Ao inibir-se a atividade miR-21 em LEs, houve aumento da citocina IL-12, diminuição de IL-10 e diminuição da carga parasitária de *L. infantum*, evidenciando a importância desse miRNA na patogênese da LCan nesse órgão nesses animais [134].

Já nas PBMCs, observou-se aumento dos miR-21, miR-424, miR-194, miR-451, miR-192, miR-503 e miR-371 em cães com LCan; e diminuição do miR-574 e do miR-150 [143]. Ademais, observou-se a correlação positiva entre os miR-194 e miR-371 e correlação negativa entre o miR-150 com a carga parasitária de *L. infantum* [143].

Assim como a LCan, outras doenças demonstraram diminuição do miR-150, como PBMCs de pacientes com HIV [144], células humanas infectadas com *Trypanosoma cruzi* [145] e soro de pacientes com COVID-19 [146]. Ademais, células HEK-293 transfectadas com o mimic do miR-150-5p tiveram a carga viral de SARS-CoV-2 diminuídas [146]. Ainda não se sabe se de fato há apenas correlação negativa entre o miR-150 e a carga parasitária de *L. infantum* ou uma relação de causa-efeito.

Predições *in silico* demonstraram que o miR-150 pode ter como alvo genes importantes para a resposta imunológica contra *L. infantum*, como o STAT1, TNF-α, HDAC8 e GZMB [143].

Como dito anteriormente, o STAT1 é um fator de transcrição encontrado em macrófagos, envolvido na sinalização de IFN-γ, aumentando a expressão de NO [54,147]. Além disso, STAT1 foi inversamente relacionado com o miR-150 em algumas doenças inflamatórias, como doença da artéria coronária em humanos e aterosclerose em um modelo de camundongos [148,149].

Já o TNF-α é uma citocina pró-inflamatória relacionada a uma resposta protetora de linfócitos Th1 contra *L. infantum* em cães, sendo considerado um biomarcador de resistência de *L. infantum* nesses animais [68,150]. O TNF-α já foi visto como um alvo direto do miR-150 em algumas doenças inflamatórias, como a encefalomielite autoimune experimental em camundongos e na resposta inflamatória induzida por LPS em culturas de células humanas [151,152], porém não se sabe se está diretamente relacionado com TNF-α na LCan.

HDAC8, outro possível alvo, é uma histona desacetilase responsável pelo controle epigenético da expressão gênica, incluindo genes relacionados a vias imunológicas [153,154]. Sua desregulação está ligada a muitas doenças

inflamatórias e infecciosas [153,155]. Embora as predições *in silico* apontem o *HDAC8* como um alvo direto do miR-150 [143], ainda não há estudos mostrando a relação direta entre deles.

GZMB (granzima B) é uma protease expressa em células NK e células T CD8+ [156]. Sua função é a ativação de vias apoptóticas através da ativação de caspases em células alvo [156]. Em camundongos knockout para miR-150, as células T CD8+ tiveram funções citolíticas prejudicadas, bem como menor expressão de *GZMB* [157]. Entretanto, nada se sabe da sua relação na infecção por *L. infantum*.

Visto que o miR-150 está diminuído nas PBMCs de cães com LCan e pode estar relacionado com a carga parasitária de *L. infantum* e a expressão de importantes genes-alvo para imunidade contra o parasito, nosso objetivo foi investigar a relação do miR-150 com a carga parasitária de *L. infantum*, bem como relacionar o miR-150 com o nível das proteínas STAT1, TNF-α, HDAC8 e GZMB.

2. CAPÍTULO 2 - MIR-150 REGULATES THE *LEISHMANIA INFANTUM* PARASITIC LOAD AND GZMB LEVELS IN PERIPHERAL BLOOD MONONUCLEAR CELLS OF DOGS WITH CANINE VISCERAL LEISHMANIASIS

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2.1 Resumo

A *Leishmania infantum* causa a leishmaniose visceral, uma doença tropical negligenciada que pode modular a resposta imune do hospedeiro por meio de pequenos RNAs não codificantes chamados microRNAs (miRNAs). Alguns miRNAs são expressos diferencialmente em células mononucleares do sangue periférico (PBMCs) de cães com leishmaniose visceral canina (LCan), incluindo o miR-150, cuja expressão está diminuída. Embora o miR-150 esteja negativamente correlacionado com a carga parasitária de *L. infantum*, não está claro se o miR-150 afeta diretamente a carga parasitária de *L. infantum* e (em caso afirmativo) como esse miRNA contribuiria para a infecção. Aqui, isolamos as PBMCs de 14 cães naturalmente infectados (grupo LCan) e seis cães saudáveis (grupo controle) e as tratamos *in vitro* com o mimetizador ou o inibidor do miR-150. Medimos a carga parasitária de *L. infantum* usando o qPCR e comparamos os tratamentos. Também medimos os níveis de proteína-alvo do miR-150 preditas *in silico* (STAT1, TNF-α, HDAC8 e GZMB) usando citometria de fluxo ou ensaios imunoenzimáticos. O mimetizador do miR-150 diminuiu a carga parasitária de *L. infantum* nas PBMCs de cães do grupo LCan. Também descobrimos que a inibição do miR-150 reduziu os níveis de GZMB. Esses achados demosntram que o miR-150 exerce uma função importante na infecção por *L. infantum* nas PBMCs caninas, o que poderia ser usado para mais estudos visando o desenvolvimento de drogas.

2.2 Abstract

Leishmania infantum causes visceral leishmaniasis, a neglected tropical disease that can modulate the host immune response by altering the expression of small non-coding RNAs called microRNAs (miRNAs). Some miRNAs are differentially expressed in peripheral blood mononuclear cells (PBMCs) of dogs with visceral canine leishmaniasis (CanL), like the down-regulated miR-150. Even though miR-150 is negatively correlated with *L. infantum* parasitic load, it is unclear if miR-150 directly affects *L. infantum* parasitic load and (if so) how this miRNA would contribute to infection. Here, we isolated PBMCs from 14 naturally infected

dogs (CanL group) and six healthy dogs (Control group) and treated them *in vitro* with miR-150 mimic or inhibitor. We measured *L. infantum* parasitic load using qPCR and compared treatments. We also measured miR-150 *in silico* predicted target protein levels (STAT1, TNF-α, HDAC8, and GZMB) using flow cytometry or enzyme-linked immunosorbent assays. Increasing miR-150 activity diminished *L. infantum* parasitic load in CanL PBMCs. We also found that inhibition of miR-150 reduced GZMB levels. These findings demonstrate that miR-150 plays an important role in *L. infantum* infection in canine PBMCs, and these findings merit further studies aiming at drug development.

2.3 Author summary

Visceral leishmaniasis is one of the most important neglected tropical diseases. In the New World, it is caused by the intracellular parasite *Leishmania infantum*. In urban areas, domestic dogs are the primary parasite reservoir. It is critical to study how this parasite survives in canine cells. One way *L. infantum* survives is through the regulation of host miRNAs. These miRNAs control the expression of critical genes related to *L. infantum* immunity. *L. infantum* can diminish miR-150 expression in canine cells; however, we do not know how vital this expression is to *L. infantum* parasitic load or if it is related to genes essential for the immune responses to the parasite. In the present study, we found that enhancing miR-150 action decreased *L. infantum* load, and inhibition of miR-150 was related to lower levels of an essential target gene. These findings shed light on the role that miR-150 exerts in *L. infantum* infection in dogs and may contribute to drug development.

2.4 Introduction

[1]Visceral leishmaniasis (VL) is a neglected tropical zoonotic disease caused by the protozoan *Leishmania infantum* in the New World. It can be fatal if left untreated [1]. Between 200,000 and 400,000 new cases and 20,000 to

[1] Revista PLOS Neglected Tropical Diseases (Anexo A).

40,000 deaths occur annually [1]. Dogs are the primary urban hosts, and human cases are associated with canine infections [2–4].

Leishmania alters host cell microenvironments by altering the expression of microRNAs (miRNAs), which are small non-coding RNAs that control translation [5–14]. This phenomenon is observed in all leishmaniasis forms, including VL, where immunity-related genes against the parasite can be altered [13–16]. The phenomenon occurs in various cell types following *Leishmania* spp. infections in macrophages [5, 6] dendritic cells [5,9], and T-cells [17], in humans [5, 6], mice [8, 10], and dogs [13–15].

Dogs develop canine visceral leishmaniasis (CanL) when infected with *L. infantum*. Parasites can alter peripheral blood mononuclear cells (PBMCs) miRNA expression in the early stages of infection [13]. Naturally infected dogs with moderate disease display altered expression of many miRNAs in their PBMCs, including miRNA-150 (miR-150) [14].

The miR-150 is a miRNA first described in hematopoietic cells [18]. It is related to NK, T, and B cells proliferation, apoptosis, and differentiation [18–20]. Because miR-150 regulates these immune cells, it can be called an "immuno-miR" [21].

Also, as miRNA are conserved among species, miR-150 is conserved among dogs and humans. The dog miR-150 shares the same sequence than human miR-150-5p [22]. In the dog genome, miR-150 is located in chromosome 1 [22].

Expression of miR-150 is diminished in naturally infected CanL PBMCs and negatively correlated with *L. infantum* parasitic load [14]. Other infectious diseases also showed downregulation of miR-150 [23–26], including SARS-CoV-2 in human serum [25] and AC16 cells infected with *Trypanosoma cruzi* [26].

Furthermore, miR-150 can target many genes, including genes related to the canine immune responses against *L. infantum* (e.g., STAT1, TNF-α, HDAC8, and GZMB), which in turn alter their respective protein levels [14] because miRNAs regulate translation of their target gene transcripts.

STAT1 is a transcription factor in macrophages involved in IFN-γ intracellular signaling [23, 24]. STAT1 promotes the expression of genes vital for the elimination of *L. infantum*, such as nitric oxide [27]. Furthermore, STAT1 was

inversely related to miR-150 in inflammatory diseases like coronary artery disease in humans [29] and atherosclerosis in a mouse model [30].

The second predicted target of miR-150 (TNF-α) is a pro-inflammatory cytokine related to a protective Th1 type T-cell response against *L. infantum* in dogs [31]. TNF-α is also a *L. infantum* resistance biomarker in dogs [32]. TNF-α was predicted to be a direct miR-150 target in inflammatory diseases such as experimental autoimmune encephalomyelitis in mice [33] and lipopolysaccharide-induced inflammatory responses in human cell cultures [34]; however, in CanL, it is unknown whether miR 150 is directly associated with TNF-α.

HDAC8 (another possible target) is a histone deacetylase responsible for the epigenetic control of gene expression [35], including genes related to immunological pathways [36]. Its dysregulation is associated with many inflammatory and infectious diseases [35,37]. Although *in silico* predictions point to HDAC8 as a miR-150 direct target [14], no studies showed a direct relationship between them.

GZMB is a serine-protease expressed in natural killer (NK) and T cytotoxic cells (CD8+ T-cells). Its function is the activation of apoptotic pathways through caspase activation in target cells [38]. In miR-150 KO mice, CD8+ T-cells had impaired cytolytic functions and lower GZMB expression [19].

In the present study, we show for the first time that increased miR-150 levels can decrease *L. infantum* parasitic load in PBMCs of CanL dogs. We also found that lowering miR-150 expression in CanL PBMCs leads to lower GZMB levels. These findings support a role for miR-150 in *L. infantum* infection on PBMCs and point to future CanL treatments [39].

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 00259-2020).

The CanL group consisted of 14 dogs naturally infected by *L. infantum*. All animals came from the Zoonosis Control Center of Araçatuba and were positive

for CanL according to immunochromatographic, serological, and molecular diagnoses. They exhibited at least two classical CanL clinical signs: onychogryphosis, lymphadenopathy, weight loss, skin lesions (ear-tip injuries, periocular lesions, and exfoliative dermatitis), or enlarged liver and spleen. The CanL dogs were classified according to their disease status [40].

The Control group consisted of six dogs with no significant hematologic and biochemical alterations, and all dogs were negative on all diagnostics tests.

All blood samples were obtained by jugular vein puncture and placed into two tubes: one without anticoagulant (for immunochromatographic and serological tests) and the other with EDTA (for red blood cell count, white blood cell count, and PBMCs isolation). Samples were processed immediately after collection.

2.5.2 Immunochromatographic and serologic diagnostics

We used immunochromatographic and serologic tests to confirm IgG anti-*Leishmania* antibodies' presence (CanL group) or absence (Control group). We used the CanL Dual Path Platform (DPP, Bio-Manguinhos, BR) for immunochromatography, following the manufacturer's instructions. As described elsewhere, we performed an indirect enzyme-linked immunosorbent assay (ELISA) for IgG anti-*Leishmania* detection for serological diagnostics [41]. A Spectra Count™ reader (Packard BioScience Company, CT, USA) with a 490 nm filter was used. Values above optical density 0.270 were considered positive [42].

2.5.3 PBMCs isolation

To isolate PBMCs from whole blood, we used Histopaque® 1077 (Sigma-Aldrich®, MO, USA) according to the manufacturer's instructions. Red blood cells (RBCs) were lysed using an in-house RBC lysis buffer (7.46 g/L NH_4Cl, 1.6 g/L EDTA, and 0.84 g/L sodium carbonate [Na_2CO_3]) at 4˚ C for 1 min. Cells were centrifuged at 2000 rpm for 5 min, followed by a phosphate buffer saline (PBS) pH 7.2 wash.

After these procedures, PBMCs were resuspended in 1 mL RPMI-1640 (Sigma-Aldrich®, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum, 0.03% L-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin. PBMCs were then counted in a Neubauer chamber. In dogs, the percentages of

lymphocytes and monocytes in the PBMCs were 59.5% and 10.2%, respectively [43].

2.5.4 PBMCs miRNA extraction and miR-150 expression

Total RNA from PBMCs miRNAs was extracted from both groups 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-150 expression, we used a methodology described elsewhere [14]. 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-150 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.

As SNORD96A and miR-150 presented similar reaction efficiency, miR-150 relative expression values were obtained using the $2^{-\Delta\Delta Ct}$ method [44]. All samples were tested in duplicate.

2.5.5 Transfection with miR-150 mimic and inhibitor in PBMCs

PBMCs were transfected with miR-150 mimic (5 nM), miR-150 inhibitor (50 nM), and All-Stars Negative control siRNA (scrambled) (Qiagen, MD, USA), using HiPerFect Transfection Reagent (Qiagen, MD, USA), following the manufacturer's guidelines.

Subsequently, transfected PBMCs (1.6×10^5/well) were cultured with RPMI-1640 medium in duplicate in 24-well cell culture plates for 48 h at 37 ºC in a 5% CO_2 incubator.

The transfection rate was evaluated using All-Stars HS Cell Death Control siRNA (Qiagen, MD, USA) at 50 nM. Cell Death Control siRNA is a blend of highly potent siRNAs targeting ubiquitously expressed genes essential for cell survival. Knockdown of these genes induces a high degree of cell death.

Therefore, the cell death rate indicates the transfection rate. The cell death rate was evaluated using Trypan Blue stain 0.4 % (Life Technologies, CA, USA) in a Neubauer chamber for optical microscopy. We obtained a mean transfection rate of 18% in the Control group and 20.2% in the CanL group.

2.5.6 DNA extraction and *L. infantum* parasitic load quantification

PBMCs DNA was extracted using the phenol-chloroform protocol [45]. Extracted DNA was analyzed on the NanoDrop™ ND-1000 spectrophotometer for purity evaluation (260/280) and quantification.

For *L. infantum* parasitic load quantification, qPCR was used, employing primers targeting the parasite's kinetoplast circular DNA (kDNA) (forward: 5′-GTGGGGGAGGGGCGTTCT-3′ and reverse: 5′-ATTTTACACCAACCCCCAGTT-3′). The 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 *L. 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 95 ºC at 15 s/ºC). Parasitic DNA load was determined in each sample by comparing each sample to the standard curve.

2.5.7 Flow cytometry for STAT1 analysis

PBMCs (1 x 10^4 cells) were incubated with fixation buffer 1% (paraformaldehyde in PBS pH 7.2) for 45 min at room temperature, protected from the light. Next, cells were centrifugated at 1800 rpm for 5 min, discarding the supernatants, and washed once with Permeabilization Buffer (Invitrogen, CA, USA). PBMCs were then resuspended in 50 µL of Permeabilization Buffer (Invitrogen, CA, USA) and incubated with anti-STAT1 primary antibody (MyBioSource, CA, USA) for 1 h at 4 ºC. Next, cells were washed, as mentioned

before. PBMCs were then incubated with anti-Rabbit IgG-FITC (Sigma-Aldrich®, St. Louis, MO, USA) for 30 min at 4 ºC, protected from the light.

Following a wash, PBMCs were resuspended in 100 µL of filtrated PBS before flow cytometry. Acquisition of 10,000 events was counted by experimental replicate on channel FL1, and analysis was performed using an Accuri C5 Flow Cytometer (BD Biosciences, NJ, USA) with BD Accuri C6 software, version 1.0.264.21.

2.5.8 TNF- α, HDAC8, and GZMB dosage

To quantify TNF-α, HDAC8, and GZMB, we performed capture ELISA of the cell culture supernatants using the DuoSet ELISA Development System for Canine TNF-α (R&D Systems, MN, USA), Human Histone Deacetylase 8/HDAC8 ELISA (RayBioTech Life, GA, USA), or Canine Granzyme B ELISA Kit (MyBioSource, CA, USA), according to the manufacturer instructions. Canine HDAC8 has a homology of 97.61% to human HDAC8. Plates were read using a SpectraCount reader (Packard BioScience Company, CT, USA) with a 450-nm filter.

2.5.9 Statistical analysis

First, we determined if the data were normally distributed using the D'Agostino-Pearson test. Next, we used the Mann-Whitney test to compare non-parametric unpaired variables (STAT1 and TNF-α levels to compare CanL and Control groups). The Wilcoxon test was used to compare non-parametric paired variables (STAT1 and TNF-α levels with mimic and inhibitor transfection). The Friedman test with Dunn's post hoc test was used for multiple comparisons between non-parametric paired variables (*L. infantum* parasitic load); the unpaired t-test for comparison between parametric and unpaired variables (miR-150 expression and GZMB level comparison between CanL and Control groups). The paired t-test was used to compare parametric and paired variables (GZMB levels with mimic and inhibitor transfection).

We used GraphPad Prism 8.0.2 software (GraphPad Software, CA, USA) for data analysis. Results were considered statistically significant when $p < 0.05$.

2.6 Results

2.6.1 Clinical and laboratory findings

All dogs from the CanL group showed at least two clinical signs compatible with CanL. The most common signs were skin lesions (100%) and onychogryphosis (64.3%). All dogs presented antibodies anti-*Leishmania*, as determined by DPP and indirect ELISA, and all of them contained the parasite DNA, measured by qPCR (Table 1). Dogs from the Control group presented no clinical signs and were negative on the diagnostic tests (Table 1).

Most dogs in the CanL group presented a significant reduction in RBC counts, globular volume, and hemoglobin (71.4%), whereas dogs from the Control group showed no significant alterations (S1 Table). Although dogs in the CanL group had lower leukocytes and lymphocytes counts, these differences were not statistically significant (Table S2).

For the biochemical profiles, infected dogs presented lower albumin values and higher globulin values and protein levels than the Control group (Table S3). Most CanL group dogs (78.6%) had a lower albumin/globulin ratio (Table S3). Based on clinical signs and laboratory findings, CanL dogs showed moderate CanL manifestations and were classified as CanL stage II [40].

2.6.2 miR-150 expression is diminished in PBMCs from CanL dogs

As previously shown [14], miR-150 could play a role in CanL immunity and is reduced in canine PBMCs. We compared the expression of miR-150 between the two groups and found that PBMCs from the CanL group had 2.6-fold less miR-150 than the Control group (Fig. 1).

Table 1. Clinical signs, serological and molecular diagnostics

Control				CanL			
Dog	Diagnostic		Clinical Signs	Dog	Diagnostic		Clinical Signs
	DPP and qPCR	ELISA (OD)			DPP and qPCR	ELISA (OD)	
1	-	0.022	No clinical signs	1	+	0.649	Onychogryphosis, weight loss, skin lesions (exfoliative dermatitis)

2	-	0.016	No clinical signs	2	+	1.192	Weight loss, skin lesions (periocular lesions)
3	-	0.043	No clinical signs	3	+	0.666	Onychogryphosis, skin lesions (exfoliative dermatitis, ear-tip injuries, periocular lesions)
4	-	0.057	No clinical signs	4	+	1.072	Lymphadenopathy, skin lesions (exfoliative dermatitis), enlarged liver and spleen
5	-	0.07	No clinical signs	5	+	1.007	Onychogryphosis, skin lesions (exfoliative dermatitis, ear-tip injuries, periocular lesions)
6	-	0.032	No clinical signs	6	+	0.759	Onychogryphosis and skin lesions (exfoliative dermatitis)
-	-	-	-	7	+	0.821	Lymphadenopathy, skin lesions (exfoliative dermatitis), enlarged liver and spleen
-	-	-	-	8	+	0.88	Lymphadenopathy, weight loss, skin lesions (periocular lesions)
-	-	-	-	9	+	1.007	Onychogryphosis, skin lesions (exfoliative dermatitis, ear-tip injuries)
-	-	-	-	10	+	0.575	Lymphadenopathy, onychogryphosis, skin lesions (exfoliative dermatitis, ear-tip injuries)
-	-	-	-	11	+	0.684	Lymphadenopathy and skin lesions (exfoliative dermatitis)
-	-	-	-	12	+	0.419	Lymphadenopathy, onychogryphosis, skin lesions (exfoliative dermatitis, skin lesions)
-	-	-	-	13	+	0.893	Lymphadenopathy, onychogryphosis, weight loss, skin lesions (exfoliative dermatitis, ear-tip injuries, periocular lesions), enlarged liver and spleen
-	-	-	-	14	+	0.768	Lymphadenopathy, onychogryphosis, skin lesions (exfoliative dermatitis, periocular lesions)
Mean		0.042				0.814	

CanL = Canine Visceral Leishmaniasis; DPP = Dual Path Platform test; OD = Optical Density. ELISA cut-off OD = 0.270

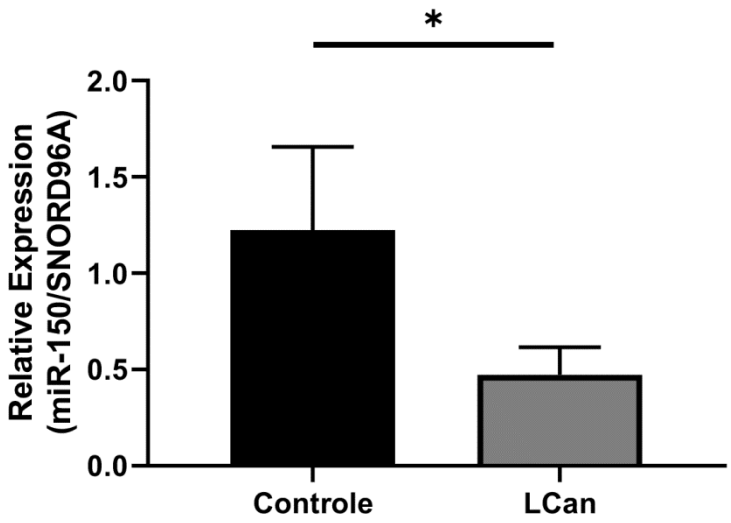

Fig 1. miR-150 expression in Control and CanL groups. PBMCs from the Control group (n = 5) and the CanL group (n = 8) were used to compare miR-150 expression by qPCR. We found that miR-150 expression was reduced in CanL PBMCs. Results are shown as mean ± standard error of the mean (SEM). The asterisk indicates significant differences (One-tailed unpaired t-test; * $p < 0.05$).

2.6.3 Increasing miR-150 activity in the PBMCs reduces *L. infantum* parasitic load

Next, we investigated whether miR-150 is a critical miRNA for *L. infantum* infection in PBMCs of CanL dogs. We transfected PBMCs from the CanL group with mimic and inhibitor of miR-150 and compared them with scrambled oligos (i.e., negative control) to determine possible differences in parasitic load. When we transfected with mimic, *L. infantum* parasitic load dropped more than 1.5 times compared to the scrambled group (Fig. 2). We saw no difference using the inhibitor (Fig. 2). These findings suggest that mimicking miR-150 in the PBMCs of CanL dogs can reduce *L. infantum* parasitic load.

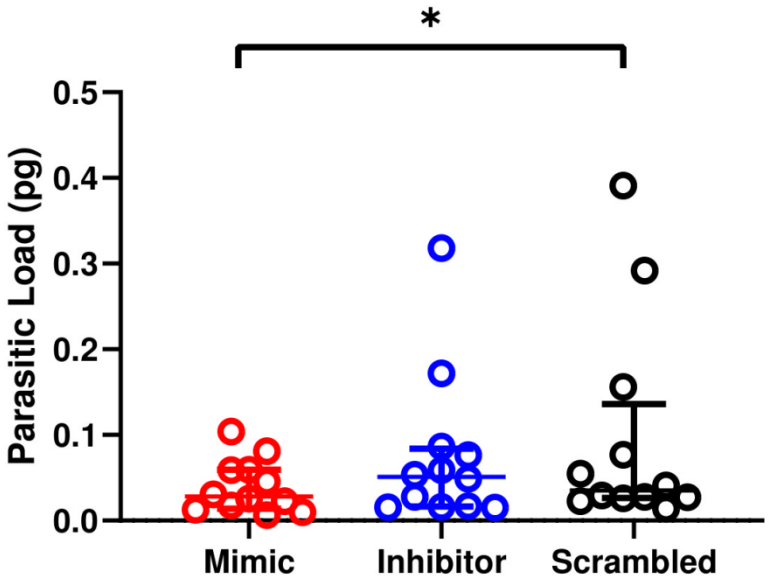

Fig 2. *L. infantum* parasitic load in canine PBMCs after miR-150 mimic or inhibitor transfection. PBMCs from the CanL group (n = 12) were transfected with miR-150 mimic, miR-150 inhibitor, or scrambled (negative control), and *L. infantum* parasitic load was compared. Results are shown as median ± interquartile range. The asterisk indicates significant differences (Friedman test with Dunn's post hoc test; * $p < 0.05$).

2.6.4 Protein levels of miR-150 predicted target genes vary between Control and CanL groups

Next, we asked by which targets miR-150 could be modulating host immunity. miR-150 has some previously predicted target genes, which are known to be essential for immunity against *L. infantum* in dogs. Hence, we investigated if STAT1, TNF-α, HDAC8, and GZMB could be involved in the CanL immunity. For this purpose, we compared their respective protein levels between CanL and Control groups. We found that STAT1 and TNF-α levels were higher in the CanL group than in the Control group (Figs. 3A and 3B, respectively). On the other hand, GZMB levels were lower in the CanL group (Fig. 3C). We saw no significant difference in the HDAC8 levels (S1 Fig). These findings suggest that STAT1, TNF-α, and GZMB levels differed between the groups and should be investigated as miR-150 target genes.

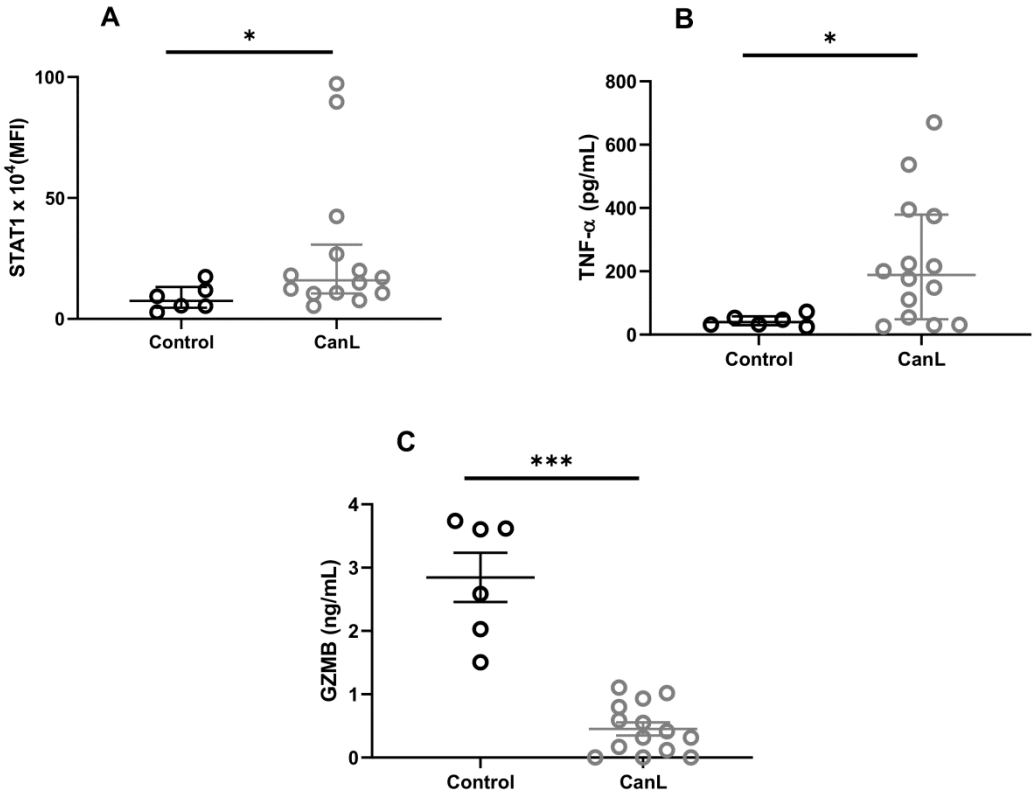

Fig 3. Comparison of STAT1, TNF-α, and GZMB levels between groups. We found that STAT1 (3A) and TNF-α (3B) levels were augmented, while GZMB levels were lower (3C) in the CanL group (n = 14) than in the Control group (n = 6). Results are expressed as median ± interquartile range (3A and 3B) or mean ± SEM (3C). Asterisks indicate significant differences (in 3A and 3B, Mann-Whitney test; * $p < 0.05$. In 3C, unpaired t-test; *** $p < 0.0001$).

2.6.5 Mimicking miR-150 in dog PBMCs does not affect STAT1, TNF-α, and GZMB levels in the CanL group

We demonstrated that mimicking miR-150 diminished *L. infantum* parasitic load. We also determined that some of the miR-150 predicted target genes had their protein expression altered in CanL PBMCs. Then, to determine if miR-150 is related to at least one of them, we transfected CanL PBMCs with miR-150 mimic and measured STAT1, TNF-α, and GZMB levels. We found no differences in any of the proteins analyzed (Fig. 4A, 4B, and 4C, respectively) compared to scrambled. We also found no differences in the Control group (S2 Fig). These findings suggest that diminished *L. infantum* parasitic load is not associated with these protein levels at this mimic concentration.

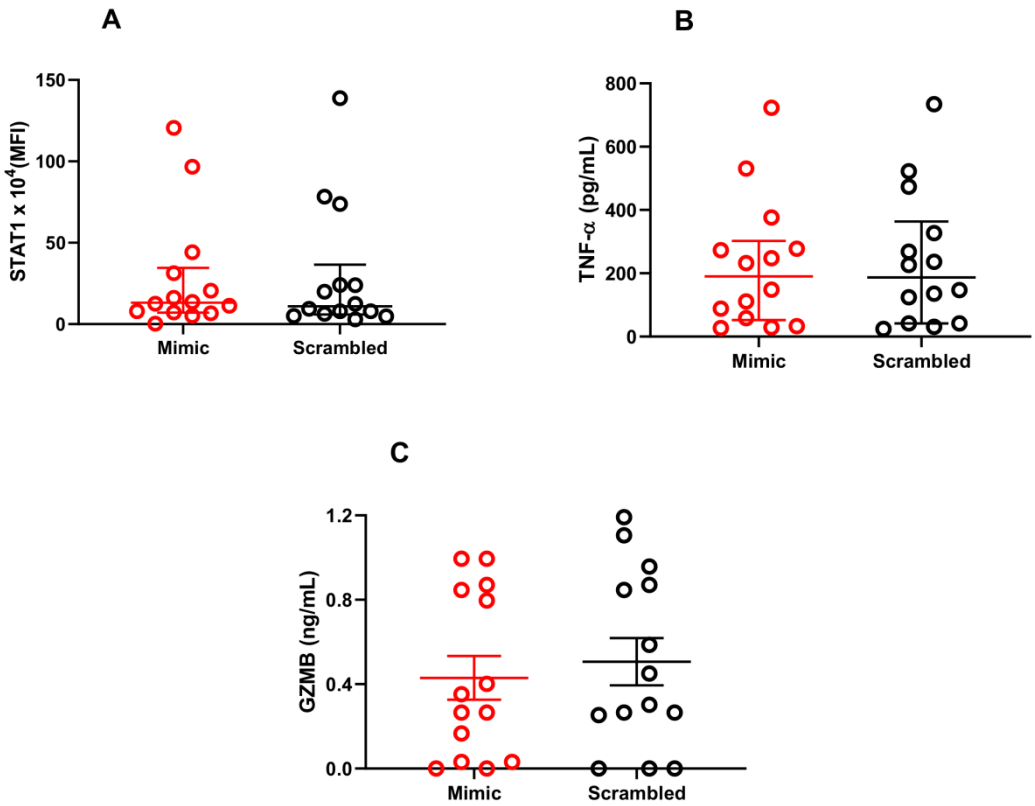

Fig 4. Comparison of STAT1, TNF-α, and GZMB levels after miR-150 mimic transfection in the CanL group PBMCs. PBMCs from the CanL group (n = 14) were transfected with the miR-150 mimic or scrambled (negative control), and STAT1, TNF-α, and GZMB levels were measured after 48 h. We found no significant differences in any protein level investigated (in 4A and 4B, Wilcoxon test; $p > 0.05$. In 4C, paired t-test; $p > 0.05$). Results are expressed as median ± interquartile range (in 4A and 4B) or mean ± SEM (in 4C).

2.6.6 Reducing miR-150 levels in the PBMCs decreased GZMB levels in the CanL group

miR-150 expression was lower in the CanL group than in the Control group. Therefore, we questioned whether reducing the miR-150 levels would generate differences in STAT1, TNF-α, and GZMB levels we observed between the two groups. To this end, we transfected CanL PBMCs with miR-150 inhibitor and measured STAT1, TNF-α, and GZMB protein levels. We observed that miR-150 inhibition did not decrease STAT1 and TNF-α levels (Fig. 5A and 5B, respectively), but it decreased GZMB levels compared to scrambled levels. We found no difference in the Control group (S3 Fig). This finding suggests that the lower levels of GZMB observed in the CanL group could be related to reduced miR-150 in this group.

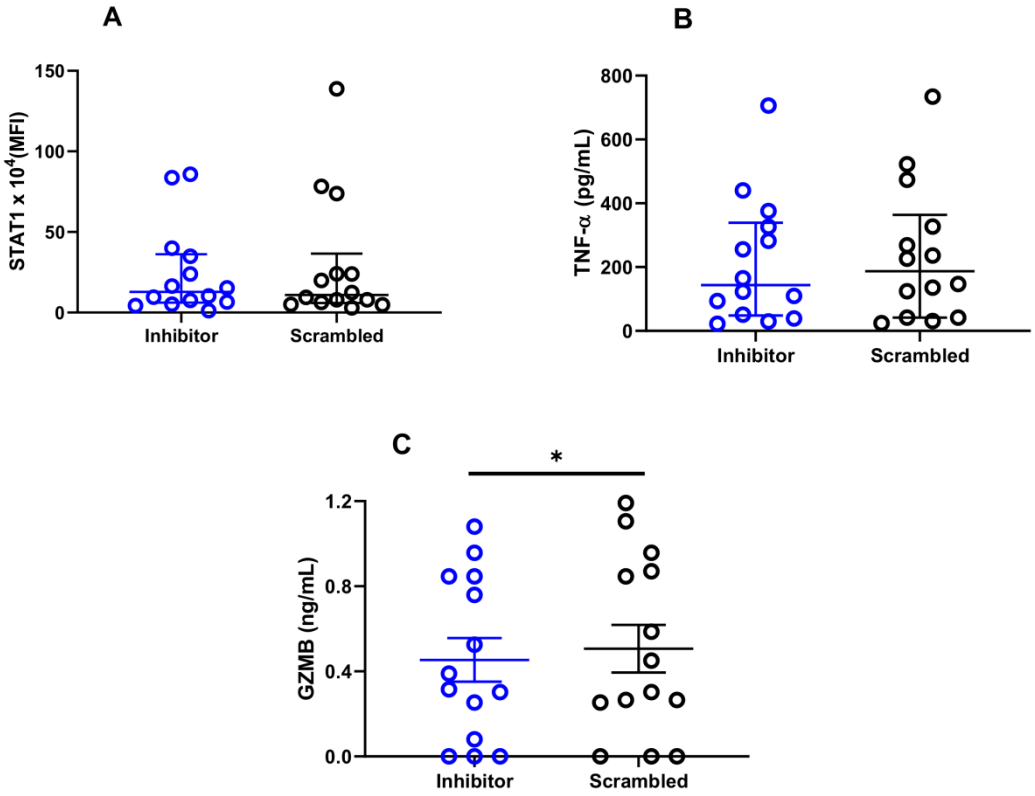

Fig 5. Comparison of STAT1, TNF-α, and GZMB levels after miR-150 inhibitor transfection in the CanL group PBMCs. PBMCs from the CanL group (n = 14) were transfected with the miR-150 inhibitor or scrambled (negative control), and the STAT1, TNF-α, and GZMB levels were measured after 48 h. Results are expressed as median ± interquartile range (5A and 5B) or mean ± SEM (5C). Asterisks indicate significant differences (in 5A and 5B, Wilcoxon test; $p > 0.05$. In 5C, paired t-test; * $p < 0.05$).

2.7 Discussion

Our group previously showed that miR-150 is reduced in the PBMCs of dogs naturally infected by *L. infantum* [14]. Here, we found that using miR-150 mimic reduced *L. infantum* parasitic load and that inhibition of miR-150 reduced GZMB protein levels.

Expression of miR-150 was reduced in CanL PBMCs, similar to inflammatory diseases like myasthenia gravis [46], in other infections like HIV [47] and SARS-CoV-2 [25], and in cells infected with *T. cruzi in vitro* [26]. The mechanism by which miR-150 is reduced in the CanL PBMCs remains unknown. Other *Leishmania* species can directly downregulate miRNAs in the host; for

example, *L. donovani* can downregulate miR-122 in the murine liver by targeting Dicer1, an essential enzyme for miRNA processing [10]. The reduction of miR-150 could also be related to a subset of T helper cells (CD4+ T- cells) called regulatory T-cells [48]. These cells can export miRNAs through the secretion of extracellular vesicles (EVs) [48], and miR-150 was released by regulatory T cell EVs [45, 46]. Further studies should focus on the mechanism involved in the reduction of miR-150 and the determination of which T-cell subsets that are associated with miR-150 downregulation.

Increasing miR-150 activity in canine PBMCs diminished *L. infantum* parasitic load. This finding confirmed that miR-150 is essential in the context of this parasitic infection. MiR-150 has been studied in viral infection [25]; when HEK T-cells were transfected with miR-150 at various concentrations, the SARS-CoV-2 viral load decreased in a dose-dependent manner. The authors also found that miR-150 targeted a critical viral gene, which reduced its corresponding protein levels [25]. Other miRNAs have been associated with *Leishmania* spp. parasitic load. For example, miR-21 was upregulated in canine splenic leukocytes, and inhibition of this miRNA diminished *L. infantum* parasitic load by increasing the Th1 response [15]. We hypothesized that miR-150 would decrease *L. infantum* parasitic load in the PBMCs through one of these mechanisms: (i) targeting a macrophage transcript vital for *L. infantum* infection and perpetuation; (ii) targeting an *L. infantum* gene directly; (iii) mediating an indirect effect, which could be related to T-cell action.

While seeking the mechanisms underlying the observed reduction in *L. infantum* parasitic load after miR-150 mimic transfection, we measured the protein levels of some *in silico* predicted miR-150 targets that could be related to immunity against *L. infantum*: STAT1, TNF-α, HDAC8, and GZMB [14].

STAT1 protein levels were higher in the CanL group than in the Control group. STAT1 is a transcription factor related to *Leishmania* spp. death [23, 47, 48]. Other *Leishmania* species have been shown to decrease STAT1 phosphorylation [52], phosphorylate a different STAT1 subset [53], and degrade it via proteasome [54]. *L. infantum* infection increased STAT1 expression in the early and chronic infection in mice spleen and liver [55], although the parasitic burden did not diminish in the chronic phase, suggesting that the immune system could not clear the infection [55]. Therefore, we believe that STAT1 is augmented

in the CanL group because the hosts used in our study are in a CanL chronic phase characterized by the absence of parasite clearance. This is the first study to compare STAT1 levels in PBMCs of CanL dogs.

TNF-α levels were higher in the CanL group. TNF-α is a pro-inflammatory cytokine related to *L. infantum* resistance in dogs [31,32]. Because our CanL group was composed of dogs in stage II of the disease, high TNF-α levels are expected as the immune system is fighting the parasite.

GZMB levels were lower in the CanL group. GZMB was diminished in the PBMCs of *L. major* naturally infected patients compared with endemic controls [56]. In a study with patients infected with *L. braziliensis*, peripheral CD8+ T-cells exhibited lower GZMB expression than healthy individuals [57]. These findings suggest that *Leishmania* spp. infection diminishes GZMB levels in PBMCs, specifically in CD8+ T-cells.

We didn't see any difference in HDAC8 levels between the groups. As HDAC8 is located mainly intracellularly, maybe our kit was unable to capture differences when comparing HDAC8 levels from cell culture supernatants.

Because STAT1, TNF-α, and GZMB protein levels differed between CanL and Control groups, we believe they could be a target for miR-150 and might be responsible for diminishing the *L. infantum* parasitic load in canine PBMCs. However, when we transfected these cells with miR-150 mimic, we found no differences in their protein levels. Despite decreasing *L. infantum* parasitic load, we believe that the miR-150 mimic transfection levels that we were able to achieve in these primary cells were not sufficient to elicit noticeable changes in STAT1, TNF-α, and GZMB levels. Therefore, we cannot discard the hypothesis that miR-150 could directly modulate them.

Although no changes in the protein levels were found with the miR-150 mimic, GZMB levels decreased when PBMCs were transfected with its inhibitor. GZMB is produced and secreted by NK and CD8+ T-cells. We hypothesized that this decrease might be due to a direct or indirect relationship between miR-150 and GZMB levels. Previous studies *in silico* showed that *GZMB* might be a direct target for miR-150 [14]; therefore, inhibition of miR-150 would increase GZMB levels and vice-versa. However, this inverse relationship was not observed in our conditions. In cutaneous lesions caused by *L. braziliensis*, *GZMB* was highly expressed, whereas miR-361-3p, a miRNA predicted to control *GZMB*

expression, was also highly expressed [58]. Such a positive correlation could result from miRNA action through mechanisms other than its classical binding to coding transcripts through sequence similarities [56, 57]. This possibility needs to be further investigated.

Furthermore, miR-150 could indirectly affect GZMB. Because GZMB is produced and secreted by NK and CD8+ T-cells, miR-150 could act directly in these cells, as miR-150 is essential for NK and CD8+ T-cell development [35, 58] and CD8+ T-cells cytolytic functions in mice [19]. Depleting miR-150 from CD8+ T-cells decreased *GZMB* expression following a challenge with *Listeria monocytogenes*, an intracellular bacterium, compared to control CD8+ T-cells in mice [19]. CD8+ T-cell responses are essential for parasite control in VL [31]. Therefore, studies clarifying the role of miR-150 in CanL regarding CD8+ T-cells are warranted.

We showed that miR-150 expression is downregulated in PBMCs of naturally infected CanL dogs. Increasing miR-150 activity (even in low concentrations) reduces *L. infantum* parasitic load. We also found that the miR-150 inhibition in these cells could be related to lower GZMB levels, which could be mediated by a direct or indirect effect of this miRNA. These results suggest an essential role for miR-150 in CanL and point to future studies on the expression of miR-150 in different T-cell subsets. We also expect that miR-150 could be used as a disease biomarker and a treatment for CanL.

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2.10 Supporting information

Table S1. Red blood cell counts in Control and CanL dogs.

Dog	RBC	Hemoglobin	GV	MCV	MCHC
Reference	**5.5-8.5 x10^{12}/L**	**12.0-18.0 g/dl**	**37-55 %**	**60-77 fL**	**32-36 %**
Control 1	8.23	18.5	54	65.61	34.26
Control 2	8.11	18.4	56	69.05	32.86
Control 3	6.78	17.1	52	76.7	32.88
Control 4	6.77	17	50	73.86	34
Control 5	6.33	14	42	66.35	33.33
Control 6	7.47	18.4	57	76.31	32.28
Mean	7.28[a]	17.23[a]	51.83[a]	71.31[a]	33.27[a]
CanL 1	5.32	11.4	34	63.91	33.53
CanL 2	4.91	11.6	34	69.25	34.12
CanL 3	5.5	14	39	70.91	35.9
CanL 4	4.55	10.4	32	70.33	32.5
CanL 5	5.77	12.3	37	64.12	33.24
CanL 6	5.33	11.7	35	65.67	33.43
CanL 7	4.55	10.4	33	72.53	31.52
CanL 8	5.4	12	37	68.52	32.43
CanL 9	5.66	13	38	67.14	34.21
CanL 10	4.04	9.1	29	71.78	31.38
CanL 11	4.12	10.7	31	75.24	34.52
CanL 12	1.22	3.2	9	73.77	35.56
CanL 13	3.44	8.4	25	72.67	33.6
CanL 14	4.77	11.5	33	69.18	34.85
Mean	4.61[b]	10.69[b]	31.86[b]	69.64[a]	33.63[a]

CanL = Canine visceral leishmaniasis group; RBC = red blood cells; GV = globular volume; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration. We used the Mann-Whitney test to compare RBC, Hemoglobin, and

GV; and an unpaired t-test to compare MCV and MCHC. Different letters (a,b) in the same column indicates significant difference ($p < 0.05$).

Table S2. White blood cell and platelet count in Control and CanL dogs.

Dog	Leukocytes	Neutrophils	Eosinophils	Basophils	Monocytes	Lymphocytes	Platelets
Reference	**6.0 - 17.0 x10^9/L**	**3000-11500 x10^6/L**	**150-1250 x10^6/L**	**Raros**	**150-1350 x10^6/L**	**1000-4800 x10^6/L**	**160-400 X10^3**
Control 1	9.9	5351	693	0	297	3567	331
Control 2	15.1	9513	453	0	151	4983	180
Control 3	8.6	6622	258	0	430	1290	120
Control 4	9.2	5704	368	0	276	2852	200
Control 5	10	6100	200	0	100	3600	240
Control 6	9.5	8740	0	0	285	475	260
Mean	10.38[a]	7005[a]	328.67[a]	0	256.5[a]	2794.5[a]	221.83[a]
CanL 1	8	6080	160	0	160	1600	400
CanL 2	10.8	8748	0	0	324	1728	380
CanL 3	7.4	5772	222	0	444	962	400
CanL 4	7.1	4047	0	0	142	2911	180
CanL 5	17	12240	1870	0	850	2040	280
CanL 6	6	4020	120	0	60	1800	160
CanL 7	2.2	0	0	0	0	0	160
CanL 8	8.2	4510	164	0	410	3116	200
CanL 9	6.6	4290	66	0	264	1980	160
CanL 10	6	4320	180	0	60	1440	250
CanL 11	11.9	9044	238	0	833	1785	220
CanL 12	6.9	5313	276	0	276	1035	300
CanL 13	4.8	2976	144	0	96	1584	180
CanL 14	13.7	8494	2055	0	548	2603	300
Mean	8.33[a]	5703.86[a]	392.5[a]	0	319.07[a]	1756[a]	255[a]

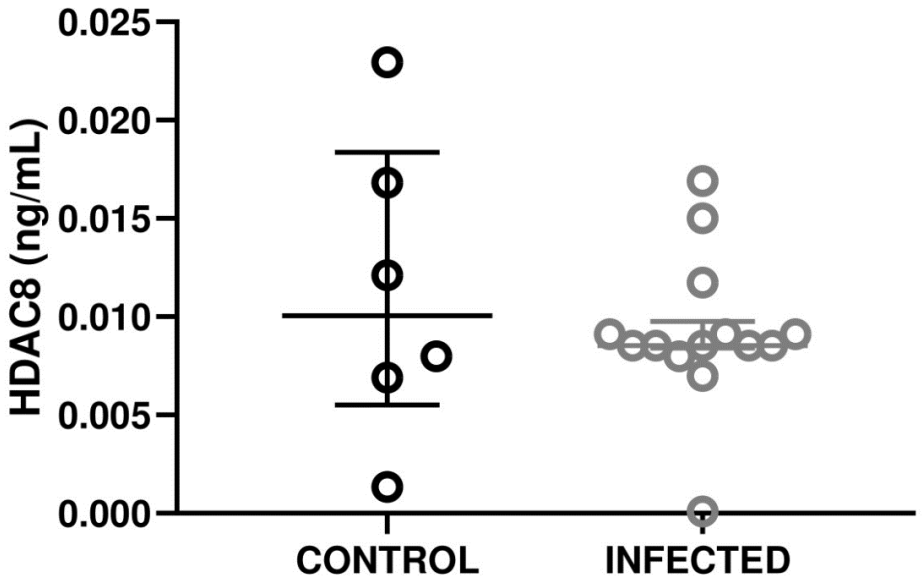
CanL = Canine Visceral Leishmaniasis group. We used the Mann-Whitney test to compare RBC, leukocytes, eosinophils, and platelets; and an unpaired t-test to compare neutrophils,

monocytes, and lymphocytes. Different letters (a,b) in the same column indicates significant difference ($p < 0.05$).

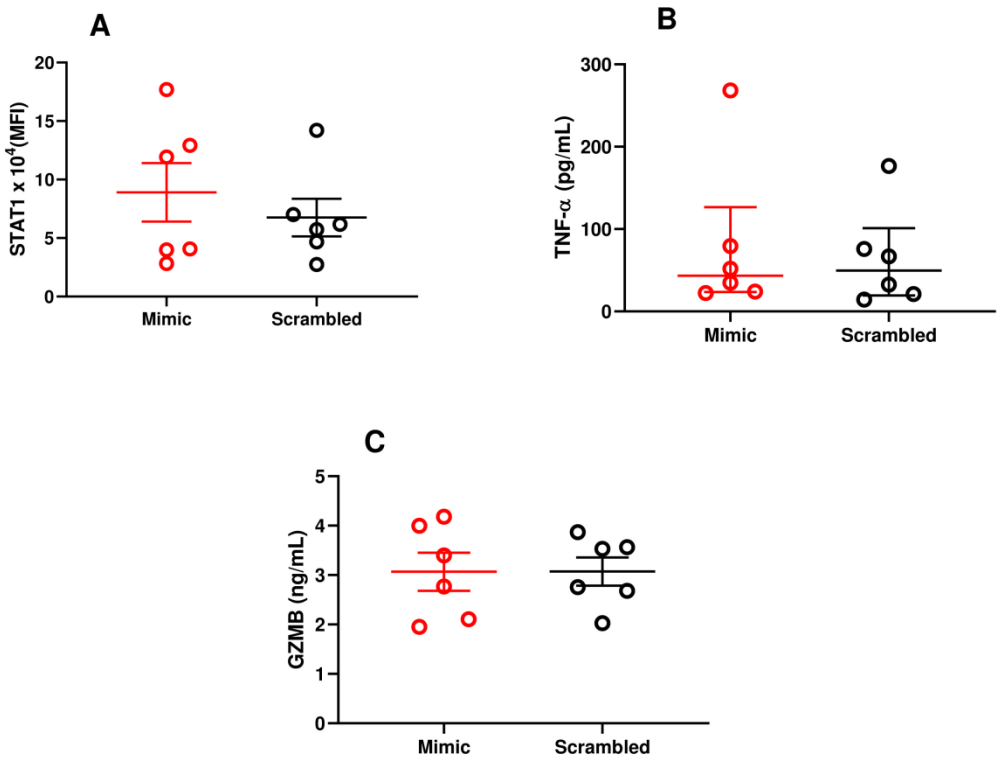
Table S3. Serum biochemical profile of Control and CanL dogs.

Dog	Albumin	Globulin	Total Protein	Creatinine	Urea	ALT	Alkaline Phosphatase	GGT
Reference	**2.6-3.3**	**2.7-4.4**	**5.4-7.1**	**0.5-1.5**	**10.03-50.03**	**21-102**	**20-156**	**1.2-6.4**
	g/L	**g/L**	**g/L**	**mg/dL**	**mg/dL**	**UI/L**	**UI/L**	**UI/L**
Control 1	3.7	3.1	6.8	1.2	-	41	-	-
Control 2	3.4	3.9	7.3	0.8	38	36	26	2.3
Control 3	3.2	3.1	6.3	1.2	38	42	73	3
Control 4	3.4	3.6	7	0.7	35	45	27	2.3
Control 5	3.3	4.7	8	0.9	31	30	21	2.3
Control 6	3.3	3	6.3	1.1	50	104	39	2.8
Mean	3.38[a]	3.57[a]	6.95[a]	0.98[a]	38.4[a]	49.67[a]	37.2[a]	2.54[a]
CanL 1	2	8.4	10.4	0.8	42	25	23	2.8
CanL 2	1.8	7.6	9.4	0.9	35.4	15	57	6
CanL 3	3	4.2	7.2	0.7	32	160	42	4
CanL 4	1.2	6.1	7.3	0.8	61	94	268	9
CanL 5	2.1	6.7	8.8	0.9	23	18	97	4
CanL 6	2.4	6.3	8.7	0.7	45	35	38	3.1
CanL 7	2.4	4.8	7.2	0.7	40	88	352	3.4
CanL 8	2.9	3.9	6.8	0.9	26	57	31	3
CanL 9	1.8	5.9	7.7	0.8	41	48	136	2.4
CanL 10	2	9.2	11.2	1	52	21	29	3
CanL 11	1.9	5.9	7.8	0.8	39.4	21	49	4.3
CanL 12	2.4	6.4	8.8	0.7	52	19	43	2
CanL 13	1.1	4.1	5.2	0.6	57	57	343	3
CanL 14	2.2	7.3	9.5	0.9	26	18	21	1.8
Mean	2.09[b]	6.2[b]	8.29[a]	0.8[b]	40.84[a]	48.29[a]	109.21[a]	3.7[a]

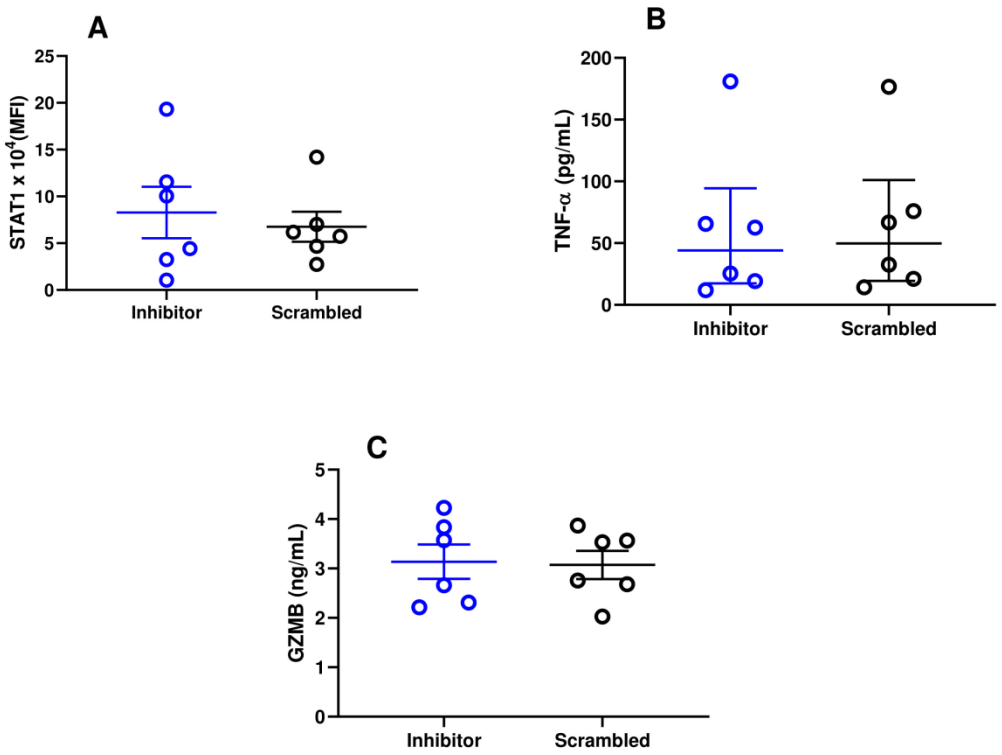
CanL = Canine visceral leishmaniasis group; ALT= alanine aminotransferase; GGT= gamma-glutamyl transferase. We used the Mann-Whitney test to compare ALT, alkaline phosphatase, and GGT; and an unpaired t-test to compare albumin, globulin, total protein, creatinine, and urea. Different letters (a,b) in the same column indicates significant difference ($p < 0.05$).



S1 Fig. Comparison of HDAC8 levels between the groups. miR-150 was predicted to target the HDAC8 protein gene. We showed that miR-150 is diminished in the CanL group, so any differences in HDAC8 levels between Control (n = 6) and CanL group (n = 14) could reveal a possible relationship with the miR-150. We found no statistically significant difference between the groups. Results are expressed as median ± interquartile range (Mann-Whitney test; $p > 0.05$).



S2 Fig. Comparison of STAT1, TNF-α, and GZMB levels after miR-150 mimic transfection in the Control group PBMCs. PBMCs from the Control group (n = 6) were transfected with the miR-150 mimic or scrambled (negative control) and the STAT1, TNF-α, and GZMB levels were measured after 48 h. We found no significant difference in any protein level investigated (in A and C, paired t-test; $p > 0.05$. In B, Wilcoxon test; $p > 0.05$). Results are shown as mean ± SEM (in A and C) and median ± interquartile range (in B).



S3 Fig. Comparison of STAT1, TNF-α, and GZMB levels after miR-150 inhibitor transfection in the Control group PBMCs. PBMCs from the Control group (n = 6) were transfected with the miR-150 inhibitor or scrambled (negative control) and the STAT1, TNF-α, and GZMB levels were measured after 48 h. We found no significant difference in any protein level investigated (in A and C, paired t-test; $p > 0.05$. In B, Wilcoxon test; $p > 0.05$). Results are shown as mean ± SEM (in A and C) and median ± interquartile range (in B).

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ANEXO A - NORMAS PARA PUBLICAÇÃO NA REVISTA PLOS NEGLECTED TROPICAL DISEASE

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Author Summary

We ask that all authors of research articles include a 150-200 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.

Authors should avoid the use of acronyms and complex scientific terms and write the author summary using the first-person voice. Authors may benefit from consulting with a science writer or press officer to ensure that they effectively communicate their findings to a general audience.

Introduction

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.

This section should also include a section with descriptions of any statistical methods employed. These should conform to the criteria outlined by the Uniform Requirements, as follows:

Submit detailed protocols for newer or less established methods. Well-established protocols may simply be referenced. Protocol documents for clinical trials, observational studies, and other **non-laboratory** investigations may be uploaded as supporting information.

We recommend and encourage you to deposit **laboratory protocols** in protocols.io, where protocols can be assigned their own persistent digital object identifiers (DOIs).

To include a link to a protocol in your article:

1. Describe your step-by-step protocol on protocols.io
2. Select **Get DOI** to issue your protocol a persistent digital object identifier (DOI)
3. Include the DOI link in the Methods section of your manuscript using the following format provided by protocols.io: http://dx.doi.org/10.17504/protocols.io.[PROTOCOL DOI]

At this stage, your protocol is only visible to those with the link. This allows editors and reviewers to consult your protocol when evaluating the manuscript. You can make your protocols public at any time by selecting **Publish** on the protocols.io site. Any referenced protocol(s) will automatically be made public when your article is published.

PLOS ONE offers an option for publishing peer-reviewed Lab Protocol articles, which describe protocols hosted on protocols.io articles. Read more information on Lab Protocol articles.

Results

The Results section should include all relevant positive and negative findings. The section may be divided into subsections, each with a concise subheading. The Results section should be written in past tense.

PLOS journals require authors to make all data underlying the findings described in their manuscript fully available without restriction, with rare exception.

Large data sets, including raw data, may be deposited in an appropriate public repository. See our list of recommended repositories.

For smaller data sets and certain data types, authors may provide their data within supporting information files accompanying the manuscript. Authors should take care to maximize the accessibility and reusability of the data by selecting a file format from which data can be efficiently extracted (for example, spreadsheets or flat files should be provided rather than PDFs when providing tabulated data).

For more information on how best to provide data, read our policy on data availability. PLOS does not accept references to “data not shown.”

As outlined in the Uniform Requirements, authors that present statistical data in the Results section should do the following:

Give numeric results not only as derivatives (for example, percentages) but also as the absolute numbers from which the derivatives were calculated, and specify the statistical significance attached to them, if any. Restrict tables and figures to those needed to explain the argument of the paper and to assess supporting data. Use graphs as an alternative to tables with many entries; do not duplicate data in graphs and tables. Avoid nontechnical uses of technical terms in statistics, such as "random" (which implies a randomizing device), "normal," "significant," "correlations," and "sample."

Discussion

The Discussion should be concise and tightly argued. It should start with a brief summary of the main findings. It should include paragraphs on the generalizability, clinical relevance, strengths, and limitations of your study.

You may wish to discuss the following points also:

- How do the conclusions affect the existing knowledge in the field?
- 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.

Authors are responsible for ensuring that anyone named in the Acknowledgments agrees to be named.

PLOS journals publicly acknowledge the indispensable efforts of our editors and reviewers on an annual basis. To ensure equitable recognition and avoid any appearance of partiality, do not include editors or peer reviewers—named or unnamed—in the Acknowledgments.

Do not include funding sources in the Acknowledgments or anywhere else in the manuscript file. Funding information should only be entered in the financial disclosure section of the submission system.

References

Any and all available works can be cited in the reference list. Acceptable sources include:

- Published or accepted manuscripts
- Manuscripts on preprint servers, providing the manuscript has a citable DOI or arXiv URL.

Do not cite the following sources in the reference list:

- Unavailable and unpublished work, including manuscripts that have been submitted but not yet accepted (e.g., "unpublished work," "data not shown"). Instead, include those data as supplementary material or deposit the data in a publicly available database.

- Personal communications (these should be supported by a letter from the relevant authors but not included in the reference list)
- Submitted research should not rely upon retracted research. You should avoid citing retracted articles unless you need to discuss retracted work to provide historical context for your submitted research. If it is necessary to discuss retracted work, state the article's retracted status in your article's text and reference list.

Ensure that your reference list includes full and current bibliography details for every cited work at the time of your article's submission (and publication, if accepted). If cited work is corrected, retracted, or marked with an expression of concern before your article is published, and if you feel it is appropriate to cite the work even in light of the post-publication notice, include in your manuscript citations and full references for both the affected article and the post-publication notice. Email the journal office if you have questions.

References are listed at the end of the manuscript and numbered in the order that they appear in the text. In the text, cite the reference number in square brackets (e.g., "We used the techniques developed by our colleagues [19] to analyze the data"). PLOS uses the numbered citation (citation-sequence) method and first six authors, et al.

Do not include citations in abstracts.

Make sure the parts of the manuscript are in the correct order *before* ordering the citations.

Formatting references

Because all references will be linked electronically as much as possible to the papers they cite, proper formatting of references is crucial.

PLOS uses the reference style outlined by the International Committee of Medical Journal Editors (ICMJE), also referred to as the "Vancouver" style. Example formats are listed below. Additional examples are in the ICMJE sample references.

A reference management tool, EndNote, offers a current style file that can assist you with the formatting of your references. If you have problems with any reference management program, please contact the source company's technical support.

Journal name abbreviations should be those found in the National Center for Biotechnology Information (NCBI) databases.

Supporting information

Authors can submit essential supporting files and multimedia files along with their manuscripts. All supporting information will be subject to peer review. All file types can be submitted, but files must be smaller than 20 MB in size.

Authors may use almost any description as the item name for a supporting information file as long as it contains an "S" and number. For example, "S1 Appendix" and "S2 Appendix," "S1 Table" and "S2 Table," and so forth.

Supporting information files are published exactly as provided, and are not copyedited.

Supporting information captions

List supporting information captions at the end of the manuscript file. Do not submit captions in a separate file.

The file number and name are required in a caption, and we highly recommend including a one-line title as well. You may also include a legend in your caption, but it is not required.

Example caption

S1 Text. Title is strongly recommended. Legend is optional.

In-text citations

We recommend that you cite supporting information in the manuscript text, but this is not a requirement. If you cite supporting information in the text, citations do not need to be in numerical order.

Figures and Tables

Figures

You can include figures in the main manuscript file at initial submission. If the manuscript reaches the revise stage, prepare and submit each figure as an individual file.

Cite figures in ascending numeric order at first appearance in the manuscript file.

Figure captions

Insert figure captions in manuscript text, immediately following the paragraph where the figure is first cited (read order). Don't include captions as part of the figure files themselves or submit them in a separate document.

At a minimum, include the following in your figure captions:

- A figure label with Arabic numerals, and "Figure" abbreviated to "Fig" (e.g. Fig 1, Fig 2, Fig 3, etc). Match the label of your figure with the name of the file uploaded at submission (e.g. a figure citation of "Fig 1" must refer to a figure file named "Fig1.tif").
- A concise, descriptive title

The caption may also include a legend as needed.

Tables

Cite tables in ascending numeric order upon first appearance in the manuscript file.

Place each table in your manuscript file directly after the paragraph in which it is first cited (read order). Do not submit your tables in separate files.

Tables require a label (e.g., "Table 1") and brief descriptive title to be placed above the table. Place legends, footnotes, and other text below the table.

Data reporting

All data and related metadata underlying the findings reported in a submitted manuscript should be deposited in an appropriate public repository, unless already provided as part of the submitted article.

Repositories may be either subject-specific (where these exist) and accept specific types of structured data, or generalist repositories that accept multiple data types. We recommend that authors select repositories appropriate to their field. Repositories may be subject-specific (e.g., GenBank for sequences and PDB for structures), general, or institutional, as long as DOIs or accession numbers are provided and the data are at least as open as CC BY. Authors are encouraged to select repositories that meet accepted criteria as trustworthy digital repositories, such as criteria of the Centre for Research Libraries or Data Seal of Approval. Large, international databases are more likely to persist than small, local ones.

To support data sharing and author compliance of the PLOS data policy, we have integrated our submission process with a select set of data repositories. The list is neither representative nor exhaustive of the suitable repositories available to authors. Current repository integration partners include Dryad and FlowRepository. Please contact data@plos.org to make recommendations for further partnerships.

Instructions for PLOS submissions with data deposited in an integration partner repository:

- Deposit data in the integrated repository of choice.
- Once deposition is final and complete, the repository will provide you with a dataset DOI (provisional) and private URL for reviewers to gain access to the data.
- Enter the given data DOI into the full Data Availability Statement, which is requested in the Additional Information section of the PLOS submission form. Then provide the URL passcode in the Attach Files section.

If you have any questions, please email us.

Accession numbers

All appropriate data sets, images, and information should be deposited in an appropriate public repository. See our list of recommended repositories.

Accession numbers (and version numbers, if appropriate) should be provided in the Data Availability Statement. Accession numbers or a citation to the DOI should also be provided when the data set is mentioned within the manuscript.

In some cases authors may not be able to obtain accession numbers of DOIs until the manuscript is accepted; in these cases, the authors must provide these numbers at acceptance. In all other cases, these numbers must be provided at full submission.

Identifiers

As much as possible, please provide accession numbers or identifiers for all entities such as genes, proteins, mutants, diseases, etc., for which there is an entry in a public database, for example:

- Ensembl
- Entrez Gene
- FlyBase
- InterPro
- Mouse Genome Database (MGD)
- Online Mendelian Inheritance in Man (OMIM)
- PubChem

Identifiers should be provided in parentheses after the entity on first use.

Small and macromolecule crystal data

Manuscripts reporting new and unpublished three-dimensional structures must include sufficient supporting data and detailed descriptions of the methodologies used to allow the reproduction and validation of the structures. All novel structures must have been deposited in a community endorsed database prior to submission (please see our list of recommended repositories).

Small molecule single crystal data

Authors reporting X-Ray crystallographic structures of small organic, metal-organic, and inorganic molecules must deposit their data with the Cambridge Crystallographic Data Centre (CCDC), the Inorganic Crystal Structure Database (ICSD), or similar community databases providing a recognized validation functionality. Authors are also required to include the relevant structure reference numbers within the main text (e.g. the CCDC ID number), as well as the crystallographic information files (.cif format) as Supplementary Information, along with the checkCIF validation reports that can be obtained via the International Union of Crystallography (IUCr).

Macromolecular structures

Authors reporting novel macromolecular structures must have deposited their data prior to submission with the Worldwide Protein Data Bank (wwPDB), the Biological Magnetic Resonance Data Bank (BMRB), the Electron Microscopy Data Bank (EMDB), or other community databases providing a recognized validation functionality. Authors must include the structure reference numbers within the main text and submit as Supplementary Information the official validation reports from these databases.

Striking image

You can upload a visually striking image alongside your submission, which we may use to showcase your article through PLOS' online channels. We choose the monthly issue image from the striking images submitted with articles scheduled for publication.

Submission Criteria

- Choose an image that represents the article in a striking and eye-catching way.
- It can be derived from a figure or supporting information file from the paper, and it may be a cropped portion of an image or the entire image.

- Alternatively, you can create or source an image, as long as it adheres to our CC BY license.
- High resolution: between 300-600 dpi
- Single panel
- Ideally avoid added details like text, scale bars, and arrows.

How to Submit

1. Submit your striking image to the submission system using the file type “Striking Image”.
2. Upload a separate file with the corresponding caption.

If no striking image is uploaded, a member of the journal team will choose an appropriate image, which may be a figure from the submission or a separately sourced CC BY image.

Additional Information Requested at Submission

Financial Disclosure Statement

This information should describe sources of funding that have supported the work. If your manuscript is published, your statement will appear in the Funding section of the article.

Include your statement in the Financial Disclosure section of the initial submission form.

The statement should include:

- Specific grant numbers
- Initials of authors who received each award
- URLs to sponsors’ websites

Also state whether any sponsors or funders (other than the named authors) played any role in:

- Study design
- Data collection and analysis
- Decision to publish
- Preparation of the manuscript

If they had no role in the research, include this sentence: “The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.”

If the study was unfunded, include this sentence as the Financial Disclosure statement: “The author(s) received no specific funding for this work."

Competing interests

The corresponding author is asked at submission to declare, on behalf of all authors, whether there are any financial, personal, or professional interests that could be construed to have influenced the work.

Any relevant competing interests of authors must be available to editors and reviewers during the review process and will be stated in published articles.

Related manuscripts

When submitting a manuscript, all authors are asked to indicate that they do not have a related or duplicate manuscript under consideration (or accepted) for publication elsewhere. If related work has been or will be submitted elsewhere or is in press elsewhere, then a copy must be uploaded with the article submitted to PLOS. Reviewers will be asked to comment on the overlap between related submissions.

Preprints

PLOS encourages authors to post preprints to accelerate the dissemination of research. Posting a manuscript on a preprint server does not impact consideration of the manuscript at any PLOS journal.

Authors posting preprints on bioRxiv or medRxiv can choose to concurrently submit their manuscripts to relevant PLOS journals through the direct transfer service.

Authors submitting manuscripts in the life and health sciences to *PLOS Neglected Tropical Diseases* may choose to have PLOS forward their submission to bioRxiv or medRxiv, depending on the scope of the paper, for consideration for posting as a preprint.

Reviewer and editor suggestions

We ask authors to suggest suitable editors and at least four potential reviewers when submitting their manuscript. Bear in mind any potential competing interests when making these suggestions. It is not appropriate to suggest recent collaborators or other researchers at your institution. See our policy on competing interests for more information.

Guidelines for Specific Study Types

Study design, reporting, and analyses are assessed against all relevant research and methodological technique standards held by the community. Guidelines for specific study types are outlined below.

Human and animal research

All research involving humans and animals must have been approved by the authors' institutional review board or equivalent committee(s), and that board must be named by the authors in the manuscript. For research involving human participants, informed consent must have been obtained (or the reason for lack of consent explained, e.g. the data were analyzed anonymously) and all clinical investigation must have been conducted according to the principles expressed in the Declaration of Helsinki. It must be stated in the Methods section of the paper whether informed consent was written or oral. If informed consent was oral, it must be stated in the paper: (a) why written consent could not be obtained, (b) that the IRB approved the use of oral consent, and (c) how oral consent was documented.

Authors should be able to submit, upon request, a statement from the research ethics committee or institutional review board indicating approval of the research. We also encourage authors to submit a sample of a patient consent form, and may require submission on particular occasions.

All animal work must have been conducted according to relevant national and international guidelines. In accordance with the recommendations of the Weatherall report, *The use of non-human primates in research*, we specifically require authors to include details of animal welfare and steps taken to ameliorate suffering in all work involving non-human primates. The institution that approved the study must be named, and it must be stated in the paper that the study was conducted adhering to the institution's guidelines for animal husbandry.

Clinical trials

PLOS follows the World Health Organization's (WHO) definition of a clinical trial:

A clinical trial is any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes [...] Interventions include but are not restricted to drugs, cells and other biological products, surgical procedures, radiologic procedures, devices, behavioural treatments, process-of-care changes, preventive care, etc.

Registering Clinical Trials

All clinical trials submitted to PLOS journals must be entered in a publicly accessible registry approved by the WHO or ICMJE. See the list of approved registries.

PLOS journals consider prospective trial registration (that is, registration before participant enrollment has begun) to be best publication practice, as recommended by the ICMJE. Clinical trials that began to enroll participants before ICMJE recommendations took effect on July 1, 2005 may be retrospectively registered.

More information about trial registration, including the WHO definition of a clinical trial, is in the ICMJE FAQ.

PLOS Neglected Tropical Diseases is unlikely to publish clinical trials that are not prospectively registered. We recognize, however, that in rare cases late registration may occur for exceptional reasons that merit consideration. Authors seeking evaluation by *PLOS Neglected Tropical Diseases* of a non–prospectively registered clinical trial must provide a compelling reason for lack of prospective registration.

In addition, as for all PLOS journals, authors wishing to submit a clinical trial that was not publicly registered before participant enrollment began must register the trial retrospectively in a publicly accessible registry. They must also:

- Register all related clinical trials and confirm they have done so in the Methods section
- Explain in the Methods the specific reasons for failing to register before participant enrollment
- Confirm that future trials will be registered prospectively

PLOS journal editors may decline to further consider any clinical trial for which, in the editor's judgment, absence of prospective registration raises concerns of selective publication or selective reporting of research outcomes.

PLOS supports the public disclosure of all clinical trial results, as mandated, for example, by the 2007 FDA Amendments Act. Prior disclosure of results on a clinical trial registry site will not affect consideration.

Required Documentation

Clinical trial reports must adhere to the relevant reporting guidelines for their study design, such as CONSORT for randomized controlled trials, TREND for non-randomized trials, and other specialized guidelines as appropriate.

For all clinical trial submissions, authors must include the following:

- Registration details (reported in the Methods section and in the submission form)
- CONSORT checklist or relevant reporting guideline (uploaded as supporting information)
- CONSORT flow diagram (uploaded as Fig 1)
- Trial protocol (uploaded as supporting information)
- Details of prior approval for human subjects research by an institutional review board (IRB) or equivalent ethics committee(s)

The submission will not be considered if documentation is not provided. The checklist, flow diagram, and protocol will be published with the article if the manuscript is accepted.

The manuscript file must include the following information:

- An explanation of any deviation from the trial protocol
- Description of informed consent obtained from participants
- Any information on statistical methods or participants not indicated in the CONSORT documentation

Systematic reviews and meta-analyses

Submissions with systematic reviews and meta-analyses are considered research articles. Submit these manuscripts with the "Research Article" type in the submission system.

Reports of systematic reviews and meta-analyses must adhere to the PRISMA Statement or alternative guidelines appropriate to the study design, and include the completed checklist and flow diagram to accompany the main text. Authors must complete the appropriate reporting checklist not only with page references, but also with sufficient text excerpted from the manuscript to explain how they accomplished all applicable items.

Abstracts should follow PRISMA for Abstracts, using the PLOS abstract format. Authors must also state within the Methods section of their paper whether a protocol exists for their systematic review, and if so, provide a copy of the protocol as supporting information.

The journal supports the prospective registration of systematic reviews. Authors whose systematic review was prospectively registered (e.g., in a registry such as PROSPERO) should provide the registry number in their abstract. Registry details and protocols will be made available to editors and reviewers, and included with the paper if the report is ultimately published.

Diagnostic studies

Reports of studies of diagnostic accuracy must adhere to the STARD requirements or alternative guidelines appropriate to the study design (see the EQUATOR web site) and include a completed checklist as supporting information. Authors must complete the appropriate reporting checklist not only with page references, but also with sufficient text excerpted from the manuscript to explain how they addressed all applicable items.

Observational studies

For observational studies, including case control, cohort, and cross-sectional studies, authors must adhere to the STROBE Statement or alternative guidelines appropriate to the study design (see the EQUATOR web site) and include a completed checklist as supporting information. Authors must complete the appropriate reporting checklist not only with page references, but also with sufficient text excerpted from the manuscript to explain how they addressed all applicable items.

For observational studies, authors are required to clearly specify (a) What specific hypotheses the researchers intended to test, and the analytical methods by which they planned to test them; (b) What analyses they actually performed; and (c) When reported analyses differ from those that were planned, authors must provide transparent explanations for differences that affect the reliability of the study's results.

If a prospective analysis plan (from the study's funding proposal, IRB or other ethics committee submission, study protocol, or other planning document written before analyzing the data) was used in designing an observational study, authors must include the relevant prospectively written document with the manuscript submission for access by editors and reviewers and eventual publication alongside the accepted paper. If no prospectively written document exists, authors should explain how and when they determined the analyses being reported.

Microarray experiments

Reports of microarray experiments must conform to the MIAME guidelines, and the data from the experiments must be deposited in a publicly accessible database.

Other Article Types

If you are submitting content other than a research article, read the guidelines for other article types.

You may be eligible for APC support

Many institutional partners globally have publishing agreements with PLOS to allow their corresponding authors to publish with reduced or no APCs. To determine if your corresponding author is eligible, please visit our institutional partners page to determine what kind of agreement your institution has with PLOS.

If your corresponding author is affiliated with a participating institution, they must follow the instructions below to demonstrate eligibility.

If your corresponding author is not from a participating institution and requires assistance paying publishing fees, please consider applying for a fee waiver at submission.

ANEXO B - CERTIFICADO DO COMITÊ DE ÉTICA

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"

CAMPUS ARAÇATUBA
FACULDADE DE ODONTOLOGIA
FACULDADE DE MEDICINA VETERINÁRIA

CEUA - Comissão de Ética no Uso de Animais
CEUA - Ethics Committee on the Use of Animals

CERTIFICADO

Certificamos que o Projeto de Pesquisa intitulado **"Papel do miR-150 na indução de resposta imune contra Leishmania infantum em cães"**, Processo FOA nº 00259-2020, sob responsabilidade de Valéria Marçal Félix de Lima apresenta um protocolo experimental de acordo com os Princípios Éticos da Experimentação Animal e sua execução foi aprovada pela CEUA em 01 de Dezembro de 2020.

VALIDADE DESTE CERTIFICADO: 31 de Dezembro de 2022.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 31 de Janeiro de 2023.

CERTIFICATE

We certify that the study entitled **"The role of miR-150 in the immune response against Leishmania infantum in dogs"**, Protocol FOA nº 00259-2020, under the supervision of Valéria Marçal Félix de Lima presents an experimental protocol in accordance with the Ethical Principles of Animal Experimentation and its implementation was approved by CEUA on December 01, 2020.

VALIDITY OF THIS CERTIFICATE: December 31, 2022.
DATE OF SUBMISSION OF THE FINAL REPORT: January 31, 2023.

Prof. Associado Guilherme de Paula Nogueira
Coordenador da CEUA
CEUA Coordinator