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

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microRNA-148a REGULA CITOCINAS INFLAMATÓRIAS E ATIVIDADE MICROBICIDA NA LEISHMANIOSE CANINA

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GABRIELA TORRES REBECH

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

Orientadora: Profª. Drª Valéria Marçal Felix de Lima

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“Aparta-te do mal, e pratica o que é bom; procura a paz, e empenha-te por alcançá-la”

Bíblia Sagrada: Salmos, 34:14.

“A gente Brilha. Forte ou fraco, mas brilha!
Um dia é sol e no outro é lua.
Brilho que ilumina a rua.
A casa, o beco ou a avenida.
Brilho de vela, de estrela e de lanterna.
Intensidade? Não importa!
Só não deixe de brilhar. ”
Gabriela T. Rebech

Rebech, GT. **microRNA-148a regula citocinas inflamatórias e atividade microbicida na leishmaniose canina**. 2022. 66 f. Tese (Doutorado) – Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2022.

RESUMO

A leishmaniose canina (LCan) é um grave problema de saúde pública, pois animais infectados facilitam a transmissão do protozoário *Leishmania* para humanos pela picada do vetor flebotomíneo durante o repasto sanguíneo. A progressão da LCan está relacionada à supressão efetiva da resposta imune, possivelmente associada a pequenos RNAs chamados microRNAs (miR), que podem afetar a tradução do mRNA em proteínas e, consequentemente, regular o funcionamento das células. O aumento de miR-148a em leucócitos esplênicos (LS) de cães com LCan foi observado em estudos anteriores e análises *in silico* identificaram possíveis vias envolvidas na regulação da resposta imune que são afetadas por este miR. Portanto, através do uso de cultura celular e transfecção, *in vitro*, avaliamos o envolvimento de miR-148a, na regulação de citocinas inflamatórias como TNF-α, IL-6, IL-12, IL-1β, iNOS, expressão de MHCII, CD80, CD3, dos fatores de transcrição T-bet (Th1) e GATA-3 (Th2) e sua relação com a carga parasitária em LS de cães com LCan. Primeiramente, LS coletados de cães saudáveis e doentes (LCan), foram transfectados com oligonucleotídeos inventariados de inibidor e mimetizador de miR-148a. Após 48 horas, a expressão das proteínas MHCII, CD80, iNOS, CD3, Tbet, GATA-3 foi avaliada por citometria de fluxo e a concentração de TNF-α, IL-12, IL-6 e IL-1β foi medida no sobrenadante de cultura por ELISA de captura. A transfecção de LS com mimetizador de miR-148a diminuiu a concetração de iNOS nas células e de TNF-α, IL-6 e IL-12 no sobrenadante do LS cultivado de cães LCan. Curiosamente, a transfecção com inibidor de miR-148a diminuiu a carga parasitária nas células. Esses resultados sugerem um papel regulador negativo deste miR na resposta imune à infecção por *L. infantum*. Concluímos que miR-148a pode afetar a resposta imune regulando citocinas inflamatórias durante LCan. Nossos resultados contribuem para o entendimento da complexa interação hospedeiro/parasito na leishmaniose canina e podem auxiliar no desenvolvimento de possíveis tratamentos.

Palavras-chave: LCan. microRNA-148a. Resposta imune. Fator de transcrição. Citocina.

Rebech, GT. **microRNA-148a regulates inflammatory cytokines and microbicidal activity in canine leishmaniasis**. 2022. 66 f. Tese (Doutorado) – Faculdade de Medicina Veterinária, Universidade Estadual Paulista, Araçatuba, 2022.

ABSTRACT

Canine leishmaniasis (CanL) is a serious public health concern, because infected animals facilitate transmission of the *Leishmania* protozoan to humans by the bite of sandfly vector during the blood meal. Progression of CanL is related to effective suppression of immune response, possibly associated with small RNAs called microRNAs (miR), which can affect mRNA translation into proteins and, consequently, regulate cells function. The increase of miR-148a in splenic leukocytes (SL) of dogs with CanL was observed in previous studies and *in silico* analysis identified possible pathways involved in immune response regulation that are affected by this miR. Therefore, through the use of, *in vitro*, cell culturing and transfection, we evaluated the involvement of miR-148a, in regulation of inflammatory cytokines such as TNF-α, IL-6, IL-12, IL-1β, iNOS, MHCII expression, CD80, CD3, of the T-bet (Th1) and GATA-3 (Th2) transcription factors and their relationship with parasite load in SL of dogs with CanL. Primary, SL obtained from healthy and diseased dogs (CanL), were transfectedwith miR-148a mimic and inhibitor inventoried oligonucleotides. After 48 hours, expression of MHCII, CD80, iNOS, CD3, Tbet, GATA-3 proteins was evaluated by flow cytometry and concentration of TNF-α, IL-12, IL-6 and IL-1β was measured in culture supernatant by capture ELISA. Transfection of SL with miR-148a mimic decreased iNOS concentration in the cells and TNF-α, IL-6 and IL-12 in the supernatant of the cultured SL from CanL dogs. Interestingly, transfection with miR-148a inhibitor decreased parasite load in cells. These results suggest a negative regulatory role of this miR in immune response to *L. infantum* infection. We conclude that miR-148a can affect immune response by regulating inflammatory cytokines during CanL. Our results contribute to the understanding of the complex host/parasite interaction in canine leishmaniasis and could assist the development of possible treatments.

Keywords: CanL, microRNA-148a. Immune response. Transcription factor. Cytokine.

SUMÁRIO

1 INTRODUÇÃO GERAL

1.1 Aspectos epidemiológicos e clínicos da Leishmaniose

A leishmaniose é uma doença negligenciada endêmica em mais de 90 países com estimativa de 12 milhões de pessoas infectadas no mundo, podendo chegar a 1,6 milhões de novos casos ao ano e ocasionar entre 20 e 30 mil mortes ao ano [1]. Afeta principalmente o leste da África com altos índices de mortalidade, porém, em 2019, mais de 90% dos novos casos registrados pela Organização Mundial de Saúde ocorreram em dez países, como o Brasil, Etiópia, Índia, Eritreia, Iraque, Quênia, Nepal, Somália, Sudão e Sudão do Sul [2].

A leishmaniose é causada por protozoários do gênero *Leishmania* (*L.*), transmitidos por mosquitos fêmeas da família *Phlebotominae* e gênero *Lutzomyia* spp durante o repasto sanguíneo [3]. A transmissão da leishmaniose varia entre antroponótica, ocorre entre humano – inseto vetor – humano e é endêmica no Afeganistão, República Islâmica do Irã, Paquistão e República Árabe Síria como também em países europeus; zoonótica, ocorre entre humano - vetor - outros mamíferos, endêmica em países da América Latina e ocorre principalmente em ambiente doméstico, onde os cães são considerados principais reservatório do parasito [4–6].

Apresenta-se de três principais formas nos humanos: visceral, também chamada de kala-azar (mais severa); cutânea (mais comum) e muco cutânea [2]. Nas américas, as formas cutânea e muco cutânea estão presentes em 20 países, sendo endêmica em 18 e apresentam média de 55 mil casos reportados por ano, enquanto a forma visceral está presente em 13 países possuindo uma média de 3.500 casos ao ano e, em 2020, o Brasil reportou 97% dos casos de leishmaniose visceral (LV) em humanos, registrado nas américas [2].

No Brasil, a leishmaniose era inicialmente considerada uma zoonose de animais silvestres e afetava, ocasionalmente, quem tivesse contato com florestas. Mas, devido ao desmatamento ela avançou para a zona rural e periurbana. Na década de 1980 a doença era registrada em 19 Unidades Federadas, já em 2003 todas as Unidades da Federação registraram autoctonia [7]. Entre o período de 1993 a 2012,

foram registrados 26.965 casos autóctones ao ano com aumento expressivo nos anos 1994 e 1995 com 22,83 e 22,94 casos/ 100.000 habitantes, respectivamente [7].

No estado de São Paulo entre os anos de 1999 e 2019 foram notificados 8.553 casos de leishmaniose visceral humana atingindo uma taxa de 8,7% de letalidade e neste mesmo período, 107 cidades relataram casos autóctones representando 16,5% do estado [8]. Nos anos de 2020, 2021 e até fevereiro de 2022, no estado de São Paulo, foram registrados, consecutivamente, 87, 57 e 10 casos com 8, 6 e 1 mortes, sendo Bauru, Araçatuba e Marília as cidades com maior número de casos registrados [9].

O município de Araçatuba foi o primeiro a registrar a presença de *Lutzomyia longipalpis*, o principal vetor da leishmaniose, no perímetro urbano em 1997 e logo depois, em 1998, foi detectado a presença de *L. infantum,* subgênero responsável por ocasionar a forma visceral da doença, em amostras de cães com suspeita clínica. No ano seguinte, em 1999, os primeiros casos de leishmaniose visceral autóctones em humanos do estado de São Paulo foram registados em Araçatuba e também na cidade de Birigui [10].

Em Araçatuba, o número de casos de leishmaniose visceral humana sofreu aumento importante no ano de 2021 com 5 casos registrados apenas no período de janeiro a julho, enquanto que em 2020, apenas 1 caso foi registrado [11]. Estes dados refletem a importância do monitoramento da doença na cidade afim de combater seu avanço e evitar maiores danos a sociedade. Dados epidemiológicos da leishmaniose em cães apontam aumento significativo no número de casos pois, de janeiro a julho de 2021, 529 animais foram recolhidos e eutanasiados na cidade Araçatuba, representando 60% do número total registrado no ano 2020 [11].

O diagnóstico da leishmaniose, é feito a partir de diversos métodos os quais são considerados de acordo com o aspecto clínico e laboratorial apresentado, podendo variar entre testes moleculares como a Reação em Cadeia da Polimerase “Polymerase Chain Reaction” (PCR): identificação e/ou quantificação do material genético do protozoário na amostra coletada [12]; teste parasitológico: identificação do protozoário por microscopia de luz no material coletado e fixado em lâmina; testes sorológicos: como Ensaio Imuno Enzimático “Enzyme Linked Immunosorbent Assay” (ELISA) baseado na identificação de anticorpos IgG e teste imunocromatografico, uma forma fácil e rápida para obtenção de resultado qualitativo [13].

A leishmaniose canina (LCan), é um sério problema de saúde pública pois, os cães são os principais reservatórios de *Leishmania* no ambiente doméstico e podem favorecer o contágio de novos vetores devido ao alto parasitismo na pele e, por estarem próximo aos humanos, facilitam a transmissão da doença. Entretanto, entre os cães domésticos, estudos investigam a transmissão da doença através de pulgas e carrapatos assim como sem o intermédio de vetores, por transfusão sanguínea, transplacentária ou venérea, por exemplo [13,14].

Após a transmissão do protozoário, as formas promastigotas metacíclicas atingem rapidamente os microvasos do hospedeiro e são fagocitadas, principalmente, por macrófagos [15] e outras células fagocíticas como neutrófilos e células dendríticas, recrutadas para o local afetado [16]. Dentro da célula fagocitária, o parasito passa para a forma amastigota, onde se multiplica por divisão binária no vacúolo parasitóforo, até ocasionar a ruptura da célula parasitada e se espalhar pelo tecido, infectando novas células e, completando seu ciclo de vida [16].

O equilíbrio da resposta imunológica torna-se necessário para promover a resistência do hospedeiro à doença [17]. Entretanto, esta resposta é prejudicada e resulta na falha do controle parasitário [18]. A grande quantidade de parasitas na pele, o aumento da inflamação e altos níveis de apoptose de células como macrófagos e linfócitos, estão diretamente relacionados aos sinais apresentados por cães sintomáticos e podem ser grandes aliados na progressão da doença [19].

Existem sinais e sintomas característicos da LCan como apatia, anorexia, febre, perda de massa muscular, linfoadenomegalia, hepatomegalia, esplenomegalia, epistaxe e lesões cutâneas [20]. Entretanto, alguns cães infectados podem ser assintomáticos ou oligossintomáticos e alguns podem até apresentar evolução para a cura espontânea [21].

Os diversos sinais e sintomas apresentados pelos cães infectados durante o desenvolvimento da LCan, dificultam a identificação da doença e também a conduta a ser tomada pelo médico veterinário. Sendo assim, um grupo de pesquisa (LeishVet) desenvolveu um sistema de quatro estágios utilizando parâmetros como sinais clínicos, anormalidades clínico-patológicas e status sorológico para identificar e dividir os animais infectados de acordo com o grau de severidade da doença, facilitando o prognóstico e tratamento dos cães doentes [13].

Os cães são classificados no estágio I, quando apresentam a forma branda da doença, com titulação de anticorpos anti-leishmania abaixo do ponto de corte ou

ausente, apresentando sinais clínicos discretos como linfadenopatia periférica ou dermatite papular, exames laboratoriais dentro dos valores normais ou com alterações discretas. Cães classificados no estágio II, apresentam a forma moderada da doença, podem apresentar titulação de anticorpos anti-leishmania variando de baixo a alto, sinais e sintomas característicos do estágio I como também, lesões cutâneas, anorexia, febre, anormalidades como hipergamaglobulinemia e hipoalbuminemia, mas apresentar funções renais dentro dos parâmetros de normalidade. No estágio III da doença, os cães apresentam a forma grave, com titulação de anticorpos anti-leishmania variando de médio a alto, sinais e sintomas apresentados nos estágios I e II, além de lesões causadas por imunocomplexos como vasculite, artrite e uveíte, com anormalidades clínico patológicas do estágio II e também apresentar doença renal crônica. Já o estágio IV é classificado como doença grave, no qual os cães apresentam níveis elevados de anticorpos, com sinais e sintomas apresentados no estágio III, além de tromboembolismo pulmonar e doença renal terminal, com anormalidades clínico patológicas [13,14,20].

1.2 Resposta imune na LCan

Após a infecção pela *L. infantum*, células dendríticas, liberam de IL-12 para o meio externo, e estimulam células Natural Killer (NK) que liberam IFN-γ [22] responsável por induzir a ativação de macrófagos e a produção de óxido nítrico sintase induzível (iNOS), uma enzima que catalisa a L-arginina resultando na produção de óxido nítrico (NO) [23]. O NO, é um radical livre, gasoso, inorgânico, incolor que exerce a função de mediar reações citotóxicas de células imunes, sendo capaz de destruir patógenos e células tumorais [24]. Os macrófagos também produzem o fator de necrose tumoral alfa (TNF-α) que tem ação autócrina, para aumentar ainda mais a produção de microbicidas e radicais livres [25].

Nos primeiros meses de infecção por *L. Infantum*, cães sintomáticos apresentam maior concentração de nitrito (NO_2), um indicador da produção de NO, no sobrenadante de cultura de macrófagos derivados de mononucleares do sangue periférico, em comparação aos cães assintomáticos [26]. Porém, após oito meses, o nível de NO é significativamente maior no grupo assintomático. Desta forma, pode-se dizer que no início da infecção, a produção de NO pode indicar uma atividade

microbicida nos animais infectados, mas não a resistência, já os níveis mais elevados de NO observado em cães assintomáticos, pode sugerir função protetora dessa molécula a longo prazo [27]. Cães com alta carga parasitária esplênica apresentam diminuição de citocinas pró-inflamatórias como IFN-γ, TNF-α, IL-12 e IL-6, e aumento de citocinas anti-inflamatórias como IL-10 e TGF-β em comparação aos cães com baixa carga parasitária [28].

O tipo de resposta observada nos cães sintomáticos, é caracterizada pelo predomínio da resposta humoral, com detecção de anticorpos anti-leishmania, entre 45 dias até 3 meses após a infecção por *L. infantum*, podendo estar correlacionados ao aparecimento dos sintomas durante o desenvolvimento da doença [29]. Estes cães apresentaram aumento de anticorpos IgG, IgA e IgM, quando comparados a cães assintomáticos, enquanto os cães assintomáticos apresentam aumento da linfoproliferação e baixos níveis de anticorpos específicos em comparação aos cães sintomáticos, refletindo a ineficiência da resposta humoral à doença [29].

As citocinas secretadas são responsáveis por determinar qual tipo de linfócito T será ativado [30]. Por exemplo, a secreção de IFN-γ ou lL-12, desencadeará a ativação de linfócitos tipo T *helper* (Th) 1, enquanto a IL-4, desencadeará uma resposta tipo Th2 [30]. A IL-12 secretada pelas células dendríticas inicia uma resposta Th1 relacionada a citotoxidade com a ativação de células NK, além da produção de citocinas pró-inflamatórias como o IFN-γ [25] que consequentemente, induz a produção de NO nas células fagocitárias, principalmente macrófagos [31].

A produção do IFN-γ também está relacionada às células TCD4+ e ativação das células TCD8+ citotóxicas, as quais desempenham função importante na morte do parasito [27]. A resposta de células tipo Th2, está relacionada à imunidade humoral e a produção de citocinas anti-inflamatórias participantes da regulação da resposta imune celular. A IL-10 é uma das citocinas presentes na resposta Th2 fortemente relacionada à progressão da leishmaniose, pois durante a resposta ao parasito, esta citocina é responsável pelo controle da inflamação através da não ativação de macrófagos e supressão de linfócitos Th1 e das citocinas IL-12 e IFN-γ [16], além de favorecer a sobrevivência das células B e a diferenciação de plasmócitos [32].

O recrutamento, ativação e a expansão dos linfócitos T para o local inicial e o desenvolvimento de uma resposta apropriada, estão relacionados ao tipo de antígeno apresentado pelo complexo principal de histocompatibilidade de classe II (“major histocompatibility complex-class II” MHC-II) de células apresentadoras de

antígeno “antigen presenting cell” (APC) e o envolvimento de moléculas co-estimulatórias [33] pertencentes a família B7, como a B7-1 (CD80) e a B7-2 (CD86) [34]. Esta ligação induz sinais positivos responsáveis por estimular a ativação e a diferenciação celular e o aumento da produção de citocinas, entretanto, a supressão da expressão de CD80 durante a LCan já foi observada [34,35].

A supressão da imunidade protetora durante a leishmaniose, pode ser uma das causas do aumento dos sinais clínicos nos cães sintomáticos e é caracterizada pelo baixo número de linfócitos TCD4+ no sangue periférico, aumento de interleucinas anti-inflamatórias como a IL-10 [36], o declínio de interleucinas pró-inflamatórias essenciais para a sobrevivência de células T [37] e de IFN-γ, essencial para a ativação de macrófagos participantes na morte de parasitas e células parasitada [38]. Já a proteção do hospedeiro, pode estar relacionada a uma resposta efetiva caracteriza pelo aumento da resposta celular e produção de citocinas pró-inflamatórias como IFN-γ e o TNF-α [16].

A IL-1β, uma citocina de perfil Th1, desempenha ação pró-inflamatória, vasodilatadora, é produzida por inflamassomos e secretada por várias células [39]. Durante a LCan, a IL-1β está aumentada no baço e no cérebro regulando negativamente a carga parasitária no tecido cerebral, mas não influenciando a carga parasitária no baço [40], divergência relacionada à compartimentalização da resposta imune observada na LCan [41].

Na LCan, o baço é um dos órgãos mais afetados durante a infecção, juntamente com a pele e a medula óssea. É o local onde a resposta ao parasita será montada e onde ocorrerá ou não, o processo de ativação celular [18,21], entretanto, o alto parasitismo ocorrente neste órgão pode resultar em desestruturações morfológicas como hipertrofia e hiperplasia da polpa branca e desestruturação folicular [28], além da substituição de macrófagos por linfócitos [20].

1.3 MicroRNAs e a Resposta Imune

A regulação da resposta imune tem sido relacionada a atividade de uma classe de pequenos RNAs não codificadores, chamados de microRNA (miR) [42,43]. Os miRs apresentam cerca de 19 a 25 nucleotídeos, transcristos no interior da célula onde poderão ser degradados ou reconhecer um mRNA específico, se ligar a ele por

complementariedade e, consequentemente, controlar a expressão gênica [44,45]. Essa atividade dos miRs resulta na alteração da expressão de proteínas e de inúmeras atividades celulares [43,46] também relacionadas a resposta imunológica [42,43].

Na LCan o aumento da expressão de miR-21, miR-192, miR-194, miR-424, miR-371, miR-451 e miR-503 e a diminuição da expressão de miR-150 e miR-574, foi observado através da técnica de microarranjo em células mononucleares do sangue periférico [47]. Já em leucócitos esplênicos, a expressão de miR-21, miR-148a, miR-7, miR-615 está aumentada enquanto a expressão de miR-125a, miR-125b e miR-150 está diminuída [48].

A análise in silico dos dados do microarranjo mostrou vias e alvos dos miRs diferencialmente expressos que foram processados através do programa Ingenuity Pathway Analysis (QIAGEN®) e os miRs diferencialmente expressos apresentaram diversas vias canônicas e alvos relacionadas com a resposta imunológica [48].

Entre os miRs alterados observados na análise do microarranjo de leucócitos esplênicos dos cães infectados, o miR-148a mostrou-se 2,29 vezes mais expresso quando comparado aos cães não infectados e apresentou forte correlação positiva com a carga parasitária esplênica [48].

O miR-148a, apresenta homologia completa entre humanos, camundongos e cães [miRBase]. Está envolvido na regulação de genes relacionados a resposta imunológica, como à ativação da STAT3 [48] importante regulador transcricional responsável por estimular a expressão de mediadores da imunidade inata, como também a granulopoiese importante para eliminação de fungos e bactérias [49], a sinalização de PTEN [48] que pode regular negativamente a via PI3K/AKT de diferenciação celular e melhorar o desenvolvimento da resposta imune eficiente na infecção por *Leishmania* [50], a apoptose mediada por linfócitos T citotóxicos, a sinalização de TGF-β, ativação de Th1 e Th2 e a via de apresentação de antígenos [48].

O papel regulatório do miR-148a na imunidade já foi relatado várias doenças [51–53]. A mimetização do miR-148a resultou na diminuição da capacidade de apresentação de antígeno, proliferação de células T antígeno específicas e a produção de citocinas como IL-6, IL-12, TNF-α e IFN-β em células dendríticas imaturas e maturas de camundongos experimentalmente sensibilizadas por lipopolisacarídeos (LPS) [54], diminuição de IL-1β e TNF-α na endometrite

experimentalmente induzida por LPS em camundongos [53] e redução de TNF-α, IL-1β e IL-6 em macrófagos experimentalmente infectados com *Mycobacterium marinum* [55]. Entretanto, a regulação da resposta imune pelo miR-148a na LCan ainda não foi estudada.

Em cães se evidencia uma alta produção de anticorpos, que tem efeitos deletérios. É possível que a hipergamaglobulinemia observada possa estar associada a alta produção de miR-148a, uma vez que este miR também participa na diferenciação de células plasmáticas sendo o miR mais abundante nestas células em estágios iniciais, e altamente expressos em linfócitos B ativos [56].

Desta forma, sabendo-se da participação do miR-148a na resposta imunológica e sua ação sobre fatores de transcrição que influenciam a diferenciação da resposta Th1/Th2, ativação de citocinas e regulação de moléculas envolvidas na apresentação de antígeno, torna-se necessário estudar o papel deste miR, na resposta do hospedeiro a essa zoonose, visando a geração de tratamento futuro para leishmaniose.

2 CAPÍTULO 1 – MIR-148a REGULATES INFLAMMATION AND MICROBICIDAL ACTIVITY IN CANINE LEISHMANIASIS

Short Title: miR-148a in canine leishmaniasis immunoregulation

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2.1 Resumo

A leishmaniose canina (LCan) é um grave problema de saúde pública, pois animais infectados facilitam a transmissão do protozoário *Leishmania* para humanos pela picada do vetor flebotomíneo durante o repasto sanguíneo. A progressão da LCan está relacionada à supressão efetiva da resposta imune, possivelmente associada a pequenos RNAs chamados microRNAs (miR), que podem afetar a tradução do mRNA em proteínas e, consequentemente, regular o funcionamento das células. O aumento de miR-148a em leucócitos esplênicos (LS) de cães com LCan foi observado em estudos anteriores e análises *in silico* identificaram possíveis vias envolvidas na regulação da resposta imune que são afetadas por este miR. Portanto, através do uso de cultura celular e transfecção, *in vitro*, avaliamos o envolvimento de miR-148a, na regulação de citocinas inflamatórias como TNF-α, IL-6, IL-12, IL-1β, iNOS, expressão de MHCII, CD80, CD3, dos fatores de transcrição T-bet (Th1) e GATA-3 (Th2) e sua relação com a carga parasitária em LS de cães com LCan. Primeiramente, LS coletados de cães saudáveis e doentes (LCan), foram transfectados com oligonucleotídeos inventariados de inibidor e mimetizador de miR-148a. Após 48 horas, a expressão das proteínas MHCII, CD80, iNOS, CD3, Tbet, GATA-3 foi avaliada por citometria de fluxo e a concentração de TNF-α, IL-12, IL-6 e IL-1β foi medida no sobrenadante de cultura por ELISA de captura. A transfecção de LS com mimetizador de miR-148a diminuiu a concetração de iNOS nas células e de TNF-α, IL-6 e IL-12 no sobrenadante do LS cultivado de cães LCan. Curiosamente, a transfecção com inibidor de miR-148a diminuiu a carga parasitária nas células. Esses resultados sugerem um papel regulador negativo deste miR na resposta imune à infecção por *L. infantum*. Concluímos que miR-148a pode afetar a resposta imune regulando citocinas inflamatórias durante LCan. Nossos resultados contribuem para o entendimento da complexa interação hospedeiro/parasito na leishmaniose canina e podem auxiliar no desenvolvimento de possíveis tratamentos.

Palavras-chave: LCan. microRNA-148a. Resposta imune. Fator de transcrição. Citocina.

2.2 Abstract

Canine leishmaniasis (CanL) is a serious public health concern, because infected animals facilitate transmission of the *Leishmania* protozoan to humans by the bite of sandfly vector during the blood meal. Progression of CanL is related to effective suppression of immune response, possibly associated with small RNAs called microRNAs (miR), which can affect mRNA translation into proteins and, consequently, regulate cells funtion. The increase of miR-148a in splenic leukocytes (SL) of dogs with CanL was observed in previous studies and in silico analysis identified possible pathways involved in immune response regulation that are affected by this miR. Therefore, through the use of, *in vitro*, cell culturing and transfection, we evaluated the involvement of miR-148a, in vitro, in regulation of inflammatory cytokines such as TNF-α, IL-6, IL-12, IL-1β, iNOS, MHCII expression, CD80, CD3, of the T-bet (Th1) and GATA-3 (Th2) transcription factors and their relationship with parasite load in SL of dogs with CanL. Primary, SL obtained from healthy and diseased dogs (CanL), were transfectedwith miR-148a mimic and inhibitor inventoried oligonucleotides. After 48 hours, expression of MHCII, CD80, iNOS, CD3, Tbet, GATA-3 proteins was evaluated by flow cytometry and concentration of TNF-α, IL-12, IL-6 and IL-1β was measured in culture supernatant by capture ELISA. Transfection of SL with miR-148a mimic decreased iNOS in the cells and TNF-α, IL-6 and IL-12 in the supernatant of the cultured SL from CanL dogs. Interestingly, transfection with miR-148a inhibitor decreased parasite load in SL cells. These results suggest a negative regulatory role of this miR in immune response to *L. infantum* infection. We conclude that miR-148a can affect immune response by regulating inflammatory cytokines during CanL. Our results contribute to the understanding of the complexhost/parasite interaction in canine leishmaniasis and could assist the development of possible treatments.

Keywords: CanL, microRNA-148a. Immune response. Transcription factor. Cytokine.

2.3 INTRODUCTION

Visceral leishmaniasis (VL) is a neglected disease caused by *Leishmania* (*L.*) species protozoan and transmitted by the bite of infected female phlebotomine sandflies [1]. Visceral leishmaniasis is the most serious form of disease [2] and, in 2019, Brazil reported 97% of all VL cases in the Americas [1]. Domestic dogs are natural reservoirs of *Leishmania* in urban areas [2,3] because they present high parasite load in the skin and they live in close proximity with humans facilitating transmission [4–6].

During CanL, the spleen is one of the most parasitized organs throughout the infection process [7–9]. In this organ, a mixed Th1/Th2 immune response is observed and symptomatic dogs present predominance of Th2 response, contributing to parasite development [10–13]. In contrast, resistance to CanL has been associated with IL-12-induced Th1 protective immune response [3], pro-inflamatory cytokine production [12] namely IFNγ, IL-12, IL-6, TNF [14] and inducible nitric oxide synthase (iNOS) leishmanicidal activity [11,15,16].

Leishmania infection can suppress host immune response [12,17]. It has been observed that high parasite load implies in a low cytokine expression in the canine spleen [14]. In fact, low splenic iNOS and IL-12 mRNA levels have been associated to parasite persistence during CanL [18]. Notwithstanding, TNF-α is increased in spleen during CanL [19,20] and this cytokine was negatively related to parasite load in spleen of dogs [14].

MicroRNAs (miR) are small non-coding RNAs that work as post-transcriptional gene regulators [21,22], affecting immune response in various diseases [22]. Recent studies have shown changes in miR expression in peripheral blood mononuclear cells (PBMC) [23] and in SL [24] from dogs naturally infected with *L. infantum*.

Microarray analysis revealed a 2.29 fold increase of miR-148a in SL during CanL, and *in silico* analysis showed canonical pathways directly regulated by miR-148a, including PTEN signaling, cytotoxic T lymphocyte-mediated apoptosis of target cells, Th1 and Th2 activation pathway, antigen presentation among other pathways related to immune responses that is indirectly regulated by this miR, as cytokine production [24].

Revista Plos One (ANEXO A)

MicroRNA-148a members of the miR-148/-152 family located in humans on chromosome 7, 12 and 17, and in mouse on chromosome 6, 15 and 11, respectively [25]. This miR can modulate genes involved in the innate and the adaptive immunity, in particular antigens of the major histocompatibility complex (MHC) [25,26], in addition to being involved to cancer initiation, tumor growth and metastasis formation [25].

According to miRBase Sequence Database, a database of published miRNA sequences [27], miR148-a present complete homology between canine, murine and human. Previously, was observed that miR-148a can reduces concentration of cytokines as IL-6, IL-12, IL-1β and TNF-α in experimentally induced inflammatory process in human microglial cell line [28] and murine models [26,29], but the role of miR148 in immunological aspects during CanL, has not been studied yet.

In the present study, we demonstrate that transfection with a miR-148a mimic resulted in downregulation of the pro-inflammatory cytokine expression iNOS, TNF-α, IL-6 and IL-12, whereas the use of a miR-148a inhibitor reduced parasitic load in cultured SL, obtained from dogs that were naturally infected with *L. infantum*.

2.4 Materials and method

2.4.1 Animal screening and samples collection

This study was approved by the Animal Experimental Research Ethics Committee (COBEA), with approval of Animal Use Ethics Committee (CEUA) of UNESP– Universidade Estadual Paulista "Júlio de Mesquita Filho (process 00624-2018).

For control group composition, five healthy mongrel animals were selected (3 females and 2 males), without hematological and biochemical alterations and negative dianognosis following (immunochromatographic (Dual-Path Platform (DPP®), serological [30] and molecular analysis [31] for CanL.

For infected group composition, we selected fourteen adult mongrel dogs that were naturally infected by *L. infantum*. These dogs came from the Araçatuba Zoonoses Control Center and presented positive diagnosis following immunochromatographic (Dual-Path Platfor (DPP®), serological [25] and molecular analysis [26] for CanL.

All dogs were symptomatic and had at least three of following clinical

signs: onychogryphosis, weight loss, ear-tip injuries, lymphadenomegaly, cachexia, alopecia, periocular and skin lesions (S1 Table).

Blood samples were obtained by jugular vein puncture from both groups (infected and healthy) and placed in tubes without anticoagulant to obtain serum for indirect ELISA [25] (S1 Table) and biochemical profile (S2 Table), also tubes containing EDTA (BD®, USA) to perform blood cell count (S3 Table), all samples processed immediately after collection.

Infected dogs were euthanized by barbiturate anesthesia (Tiopental, Cristália Itapira, SP), followed by intravenous injection of 19.1% potassium chloride by the same route, as recommended for the control of Visceral Leishmaniasis, in compliance with local legislation. After euthanasia, a $2cm^3$ fragment of spleen was collected for SL isolation. Spleen fragments in control dogs were removed by surgical excision [13]. All spleen fragments were maintained in complete RPMI-1640 medium (Sigma®, USA) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco®, USA), 0.03% L-glutamine (Sigma®, USA), 100 IU/mL of penicillin (Sigma®, USA) and 100 mg/mL of streptomycin (Sigma®, USA), for SL isolation, until processing.

2.4.2 Isolation of SL

Splenic leukocytes were obtained from a $2cm^3$ fragment that was macerated and added to 10ml RPMI-1640 medium (Sigma, USA) supplemented with 10% heat inactivated fetal bovine serum, 0.03% L-glutamine, and 100IU/mL penicillin and 100mg/mL streptomycin. After removal of cell debris with a 100µm cell strainer (BD Falcon Cell strainer, USA), suspension was processed with 5mL of red blood cell lysis buffer containing 8g/L ammonium chloride (NH_4Cl), 1,6g/L EDTA, 0,16g/L calcium carbonate (Na_2CO_3) at 4°C for 10 minutes, centrifuged at 2000rpm for 5 minutes, and washed with phosphate buffered saline (PBS) at pH 7.2, three times. Cells were then counted in a Neubauer chamber.

2.4.3 Serological diagnosis by ELISA

Samples were analyzed by ELISA assay using total antigen from lysed promastigotes [25]. The antigen was coated overnight with 20µg/ml protein pH 9.6, then washed three times in PBS containing 0.05% Tween 20 (washing buffer) and saturated for 1 hour with 150 µl/well of a mixture of PBS and 10% FCS at room

temperature. Next, the preparation was washed again three times with washing buffer. Blocking buffer/Tween (100µl of serum sample (1/400) diluted in PBS, pH 7.2, containing 0.05% Tween 20 and 10% FCS) was added to each well and incubated at room temperature for 3h, followed by three washes with washing buffer. Subsequently, 100µl/well of anti-dog IgG conjugated with horseradish peroxidase (Sigma, St. Louis, MO, USA) at appropriate dilution in blocking buffer/Tween was added, incubated at room temperature for 1 hour and washed. Substrate solution (0.4mg/ml o-phenylenediamine (Sigma) and 0.4µl/ml H2O2 in phosphate citrate buffer, pH 5.0) was added at 100 µl/well and developed for 5 Min at room temperature. Reaction was stopped with 50µl of 3M H2SO4. Absorbance was measured at 490 nm using a Tecan microplate reader (Sunrise model ref. 16039400). Negative and positive controls were included on each plate. Positive controls obtained from a hyperimmune animal were included. The cut-off was determined using the mean +3 SD of the readings obtained from post transfection, was performed using the commercial mirVana kit for isolation serum samples of healthy dogs from non-endemic leishmaniasis areas.

2.4.4 DNA extraction and determination of *Leishmania* species

Extraction of total DNA from $5x10^4$ SL with commertial kit (Kit Dneasy (Qiagen)) following the manufacturer's instructions. Determination of the *Leishmania* species was performed by PCR-RFLP (Restriction Fragment Length Polymorphism) [26], comparing the restriction profiles of the sample with a profile obtained from *L. infantum* (IOC / L0575-MHOM / BR / 2002 / LPC-RPV), *L. braziliensis* (IOC / L0566-MHOM / BR / 1975 / M2903) and *L. amazonensis* (IOC / L0575-MHOM / BR / 1967 / PH8) as positive controls, and water as a negative control (S1 Fig).

2.4.5 Extraction and quantification of total RNA

Extraction of total RNA, including miRNAs, from $5x10^4$ SL was performed using phenol (Life Technologies, USA), following manufacturer’s instructions. After RNA isolation, samples were stored at -80°C. RNA samples were analyzed by spectrophotometer (NanoDrop, Thermo Scientific, USA) for purity evaluation (260/280) and quantification.

2.4.6 Real-time PCR for miR-148a

To confirm upregulation of miR-148a in dogs with CanL, as seen in [24],

real-time PCR (qPCR) was performed. cDNA production was performed using the miScript RT II kit (Qiagen, USA), as recommended by the manufacturer. A total of 1µg of RNA was used for each sample with the 5x miScript Hiflex Buffer, in a final volume of 20µl. Mix was incubated for 60 min at 37ºC, followed by 5 min at 95ºC to inactivate the miScript Reverse Transcriptase. Next, qPCR was performed using commercially available specific primer for the *Canis familiaris* miR-21 and the endogenous reference SNORD96A (miScript, Qiagen). SYBR Green system (myScript SYBR Green PCR kit, Qiagen) was used in a real-time thermal cycler (RealPlex, Eppendorf). Amplification conditions consisted of an initial activation step of 95ºC for 15 min followed by 40 cycles of 94ºC for 15 seconds, 55ºC for 30 seconds and 70ºC for 30 seconds, for denaturation, annealing and extension, respectively. For miRNA analysis, a standard curve was performed with serial dilution of a pool of cDNAs. Quantification of miR148 was performed by converting the sample cycle threshold values to a concentration (ng/µl), based on the standard curves, which were generated using 10-fold serial dilutions of the cDNA pool. Values obtained for the target miRNA were then divided by SNORD96A values, in order to obtain normalized target values for each sample. All samples were run in duplicate.

2.4.7 Transfection with miR-148a mimic and inhibitor in SL

Splenic leukocytes were cultured (1,6x105 cells/replicate) in triplicate in a 24-well plate for 48h at 37°C in 5% CO2. All Stars Negative control siRNA (scrambled), miR-148a mimic (5nM) and miR-148a inhibitor (50nM) (miScript miRNA Mimic and Inhibitor Qiagen, USA) were used and SL were transfected using 3µL of Hiperfect (Qiagen, USA) in each well, following manufacturer's instructions. For the evaluation of transfection rates, AllStars HS Cell Death Control siRNA (Qiagen, USA) was used at a final concentration of 50nM. Transfection rate was measured by flow cytometry using 7-AAD Viability Staining Solution (BioLegend, USA) according to manufacturer's instructions, and cellular death was evaluated using trypan blue at a Neubauer chamber for optical microscopy. A medium transfection rate of 20% was obtained for both groups studied.

2.4.8 Flow cytometry analysis in SL

For flow cytometry analysis, $1x10^4$ cells were incubated with Fc blocking buffer (10% of bovine fetal serum) for 30 min at room temperature. Cells were

centrifuged at 1800rpm for 7 minutes and then incubated with anti-dog MHC class II conjugated with fluorescein isothiocyanate (FITC) (Bio-Rad, USA) and anti-dog Anti-CD80 monoclonal antibody (Biolegend) phycoerythrin (PE) conjugated or mouse anti-dog CD3/FITC (Bio-Rad). Next, anti MHCII/CD80, MHCII/iNOS, CD3/Tbet and CD3/GATA-3 double tagging SL were analyzed, posteriorly analyzed by flow cytometer. To avoid non-specific binding, cells were incubated with respective control isotypes. Parasite load quantification followed the methods described by [32], with modifications. After 48 hours in culture, cells were harvested and incubated for 60 minutes at room temperature in 1x PBS containing 1% paraformaldehyde (Sigma). Then, they were centrifuged at 400 g for 5 minutes, and permeabilization of the membrane and the parasitophorous vacuole was performed by resuspending the cells in ethanol (Sigma) for 60 minutes at -20°C. Cells were washed with 2% bovine serum albumin (BSA, Sigma) in PBS1x, and incubated for 60 minutes at 4°C with anti-LPG-*Leishmania* monoclonal antibody (ABD, Serotec) diluted 1/250.

For parasite load quantification, we used the method described by [33], with modifications. After 48 hours in culture, cells were harvested and incubated for 60 minutes at room temperature in 1x PBS containing 1% paraformaldehyde (Sigma). Then, they were centrifuged at 400 g for 5 minutes, and permeabilization of the membrane and the parasitophorous vacuole was performed by resuspending the cells in ethanol (Sigma) for 60 minutes at -20°C. Cells were washed with 2% bovine serum albumin (BSA, Sigma) in PBS1x, and incubated for 60 minutes at 4°C with anti-LPG-Leishmania monoclonal antibody (ABD, Serotec) diluted 1/250. After three consecutive washes with PBS/BSA, amastigotes were stained with 1 µM of anti-IgG2a conjugated to phycoerythrin (PE) (Sigma), and also with monoclonal antibody anti-human CD14 conjugated to fluorescein isothiocyanate (FITC) (Bio Rad) for 60 minutes at 4°C. Cells were washed in PBS/BSA buffer and stored at 4°C in the dark until the time of analysis. All acquisitions were counted in 10,000 events by experimental replicate on channel FL1 and FL2, and cytometric analysis was performed with an Accuri C5 Flow Cytometer (BD Biosciences, USA) using BD Accuri C6 software, version 1.0.264.21 (BD Biosciences, USA).

2.4.9 Capture ELISA for TNF-α, IL-12, IL-6 and IL-1β quantification

After 48h of transfection, supernatant from the cultured SL was collected, centrifuged at 2500rpm and stored at -80°C until further analysis.Detection of cytokines

was performed using DuoSet ELISA Development Systems kits (R&D Systems, Minneapolis, MN, USA), following the manufacturer's instructions.

2.4.10 Statistical analysis of data obtained

For statistical analysis, data were submitted to normality tests D'Agostino & Peanson omnibus, Shapiro-Wilk and KS and then, non-parametric tests were used. Mann-Whitney test was used to compare protein expression in SL between control and infected groups. Friedman with Dunn's multiple comparison test was used for treatment comparison after transfection with mimics and inhibitor. Friedman with Dunn's multiple comparison test was used for treatment comparison after transfection and IL-12 and TNF-α neutralization. Results were considered significant when $p<0.05$. Graphpad Prism 6 program (Graph Pad Software, Inc. CA. USA) was used to perform data analysis.

2.5 Results

2.5.1 miR-148a expression is increased in splenic leukocyte (SL) of dog with Canine Leishmaniasis (CanL)

To confirm the increase in miR-148a expression in CanL, real-time PCR was performed with SL from dogs of both groups. We observed higher expression of miR-148a, in infected compared to control group (Fig 1).

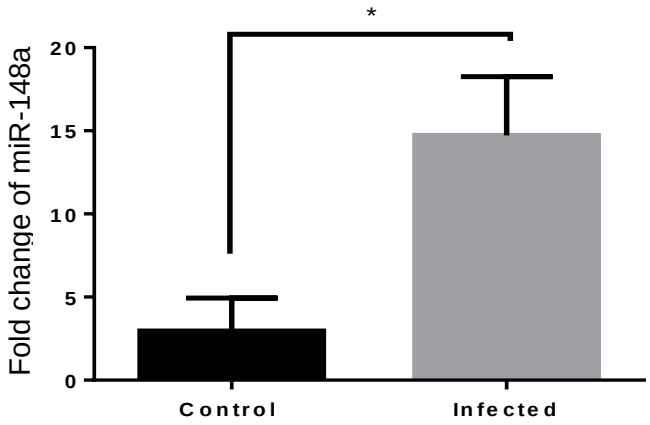
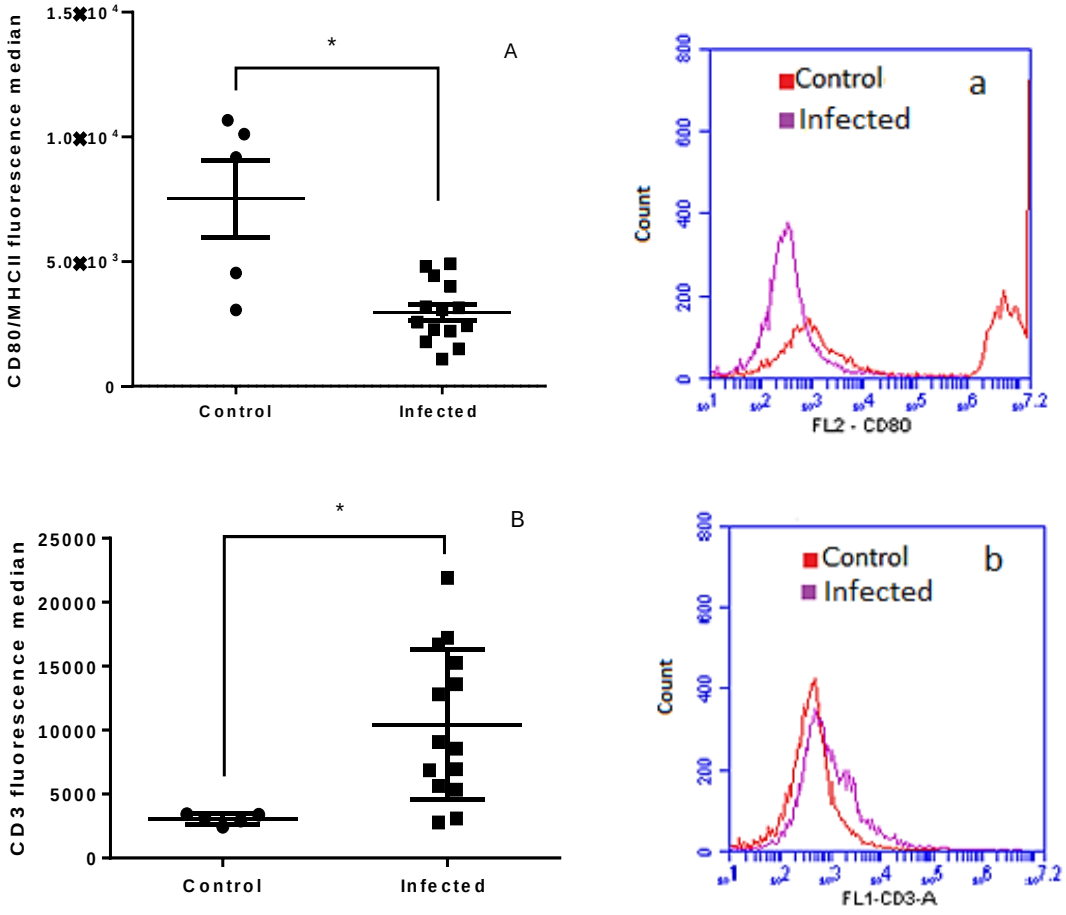


Figure 1. Expression of miR-148a. miR-148a expression was quantified by real-time PCR in SL of CanL and control dogs. Asterisks represent significant difference ($p<0.05$) following Mann-Whitney test. Mean values of miRNA expression +/- SEM.

2.5.2 Canine Leishmaniasis regulates CD80, CD3 and GATA-3 expression in SL culture

Considering immune response regulation during CanL [12], expression of MHCII, CD80, iNOS, CD3, T-bet and GATA-3, was analyzed after SL culture from control (healthy dogs) and infected (dogs with CanL) groups, without any treatment. Expression of CD80 protein was lower in MHCII+ cells in the infected group (figure 2A), whereas, expression of CD3 (figure 2B) and transcription factor GATA-3 in TCD3+ lymphocytes (figure 2C) was higher in infected group when compared to control. Expression of MHCII, iNOS in MHCII+ leukocytes and transcription factor T-bet in TCD3+ lymphocytes showed no significant differences between groups (data not shown). Figure 2 contains representative histogram of overlay proteins expression analysis that showing significant differences CD80/MHC (2a), CD3 (2b) and GATA-3/CD3 (2c) between control (red) and infected (purple) groups.



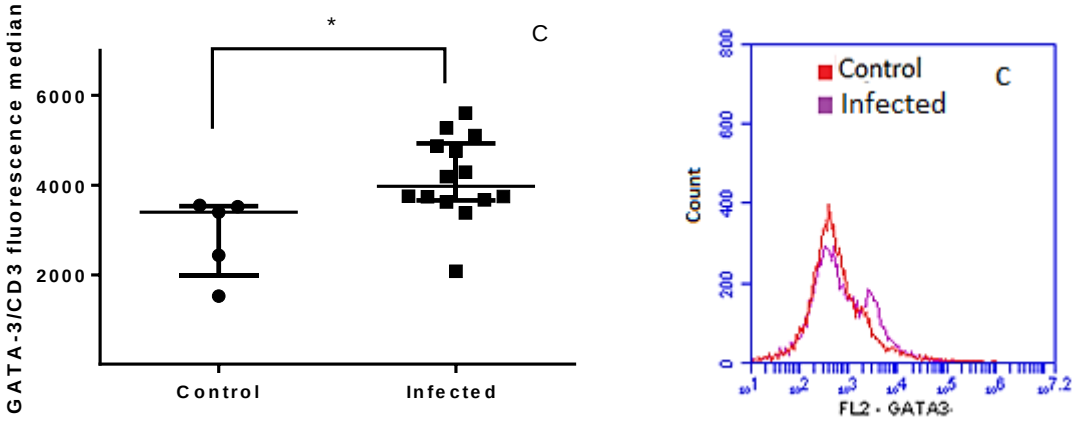
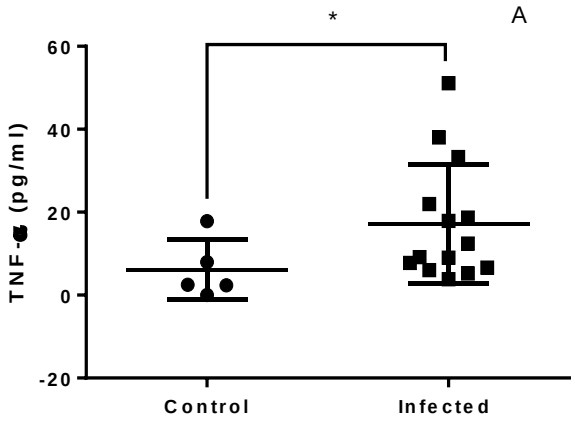


Figure 2. Expression of CD80/MHCII, CD3 and GATA-3/CD3. Expression of CD80/MHCII (A), CD3 (B) and GATA-3/CD3 (C) protein in splenic leukocytes cultures of dogs from control (without *L. infantum* infection) and infected groups (naturally infected by *L. infantum)*, maintained in culture for 48h at 37°C and 5% CO_2, without any treatment (Medium). Graphical data is presented as mean ± SD. Asterisk indicates significant difference (Mann-Whitney test, * $p<0.05$). Representative histogram of overlay, proteins expression analysis that showed significant differences: CD80/MHC (a), CD3 (b) and GATA-3/CD3 (c) between control (red) and infected (lilac) groups.

2.5.3 Canine leishmaniasis regulates TNF-α and IL-1β expression in the supernatant of cultured SL.

We compared TNF-α, IL-6, IL-12 and IL-1β quantification in cultured SL supernatant, obtained from dogs of control and infected groups, to investigate immune response modulation in CanL. Indeed, we observed that *L. infantum* infection increases TNF-α (figure 3A) and IL-1β (figure 3B), whereas IL-6 and IL-12 did not differ between groups (data not shown).



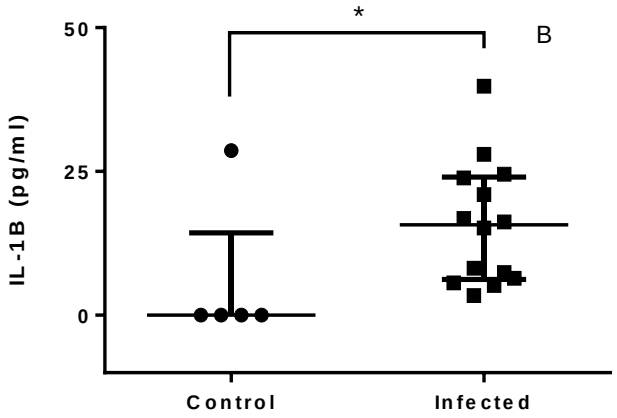


Figure 3. Concentration of TNF-α and IL-1β. TNF-α (A) and lL-1β (B) was quantified in supernatants from splenic leukocytes cultures of dogs from control (without *L. infantum* infection) and infected groups (naturally infected by *L. infantum)*, by capture ELISA and maintained in culture for 48 hours at 37ºC and 5% CO_2, without treatment. Graphical data represents mean ± SD. Asterisk indicates significant difference (Mann-Whitney test, * $p < 0.05$).

2.5.4 MicroRNA-148a mimic transfection decreases iNOS expression in CanL.

Following confirmation that miR-148a was increased in SL from CanL, we further investigated the role of this miR through transfection with a mimic and an inhibitor of miR-148a. Transfected SL were cultured at 37°C and 5% CO2 for 48 hours, when the expression of target proteins, MHCII, CD80, iNOS, CD3, T-bet and GATA-3 was evaluated by flow cytometry. We did not find significant differences in the control group following treatment with mimic or inhibitor of miR-148a (data not shown). In contrast, in the infected group, miR-148a mimic reduced iNOS when compared to SL in the presence of scrambled (SCR) oligonucleotides, used as a transfection control (figure 4), albeit MHCII, CD80, CD3, T-bet and GATA-3 expression on SL did not vary between treatments in the infected group (data not shown).

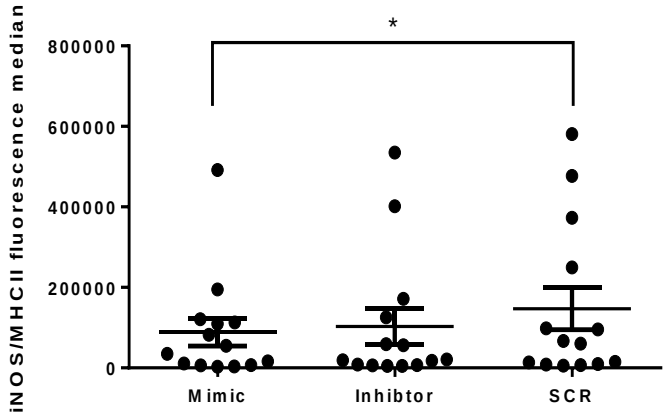


Figure 4. Expression of iNOS in the infected group. Expression of iNOS protein in MHCII^{+} cells in CanL group. Splenic leukocytes of dogs naturally infected by *L. infantum* were transfected with miR-148a, mimic and miR-148a inhibitor and Scrambled (SCR) control transfection control, all in presence of Hiperfect, following 48 h in culture at 37 ºC and 5% CO_2. Splenic leukocytes were using with monoclonal antibodies conjugated to fluorochromes and analyzed by flow cytometry. Graphical data presented as mean ± SD. Friedman test with Dunn's multiple comparison test. Graphical data presented as mean ± SD. Asterisk indicates significant difference (Mann-Whitney test, * $p < 0.05$).

2.5.5 Mir-148a mimic transfection decreases TNF-α, IL-6 and IL-12 concentration.

To investigate whether miR-148a regulates TNF-α, IL-6, IL-12 and IL-1β production, we compared these cytokines in SL culture supernatants from control and infected groups. Splenic leukocytes were transfected with miR-148a mimic and inhibitor. We did not find significant differences in the control group regardless of treatment (data not shown). However, in the infected group, TNF-α (figure 5A), IL-6 (figure 5B) and IL-12 (figure 5C) concentrations were lower in the presence of miR-148a mimic when compared to the SCR transfection control. IL-1β production did not vary significantly between transfection conditions in the infected group as well (data not shown).

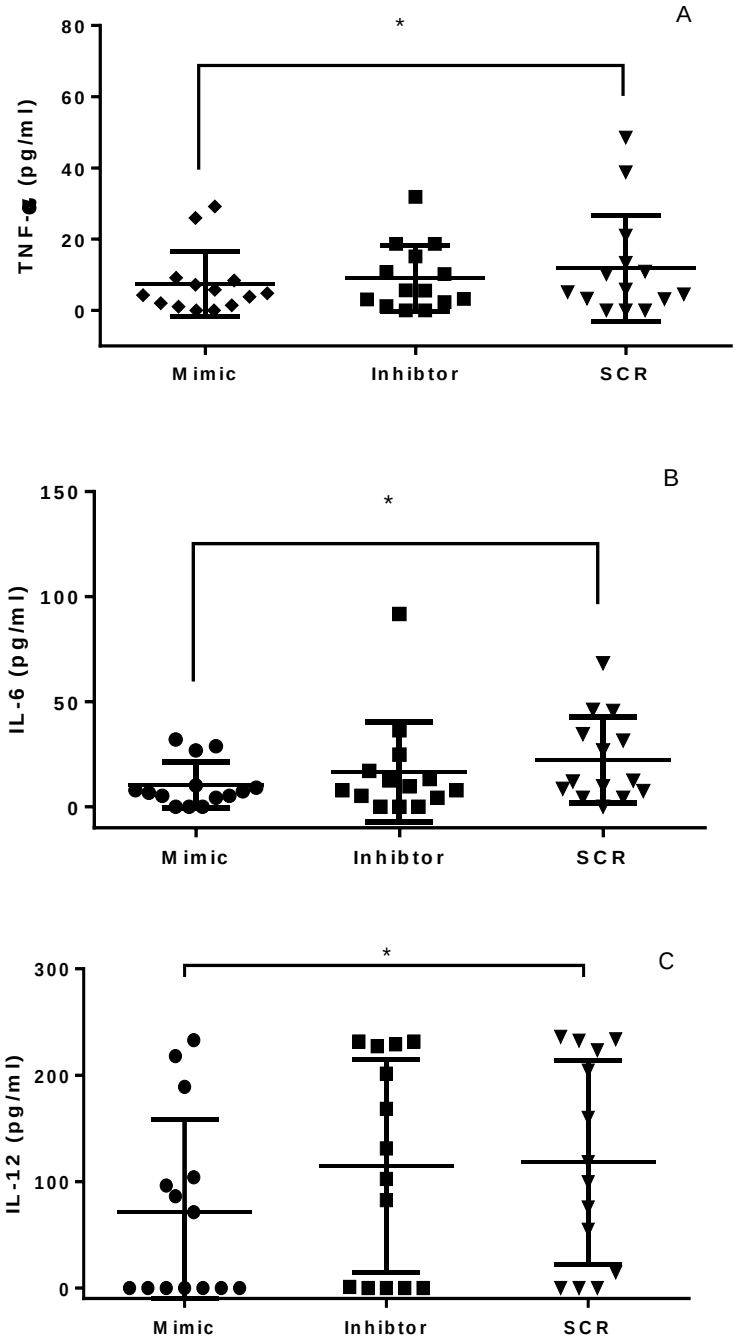


Figure 5. Concentration of TNF-α, IL-6 and IL-12 after transfection. TNF-α (A), IL-6 (B) and IL-12 (C) was quantified in supernatants from splenic leukocytes cultures of dogs naturally infected by *L. infantum* and transfected with miR-148a mimic, miR-148a inhibitor and Scrambled (SCR) transfection control, all in the presence of Hiperfect, following 48 h in culture at 37°C and 5% CO_2. Graphical data presented as mean ± SD. Asterisk indicates significant difference (Friedman test with Dunn's multiple comparison test, * $p < 0.05$).

2.5.6 Inhibition of miR-148a reduces parasite load in SL culture from dogs with CanL

To confirm whether miR-148a plays a role in regulating *L. infantum* infection, we verified the parasitic load by flow citometry in SL transfected with miR-148a mimic and inhibitor. We found that inhibition of miR-148a reduced parasite load (figure 6).

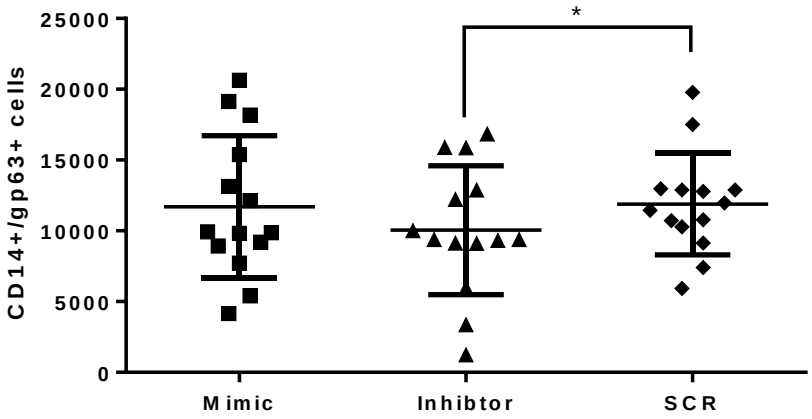


Figure 6. Parasitic load of SL. Parasitic load was quantified in splenic leukocytes cultures of dogs naturally infected by *L. infantum* and transfected with, miR-148a mimic, miR-148a inhibitor and Scrambled (SCR) transfection control all in the presence of Hiperfect, following 48 h in culturein at 37°C and 5% CO_2. Graphical data is presented as mean ± SD. Asterisk indicates significant difference (Friedman test with Dunn's multiple comparison test, * p <0.05).

2.6 Discussion

Immune response observed in naturally infected dogs with symptomatic *Leishmania (L.) infantum* is characterized by effective cellular response suppression, which may be related to an increase in microRNA (miR)s, such as miR-148a. To investigate the role of miR-148a in canine leishmaniasis (CanL), we analyzed target proteins before and after transfection of splenic leukocytes (SL) from naturally infected dogs by *L. infantum*, with mimic and inhibitor of this miR. *In silico* analysis allowed for the identification of miR-148a targets, such as CD80, iNOS, T-bet, GATA-3 and pro-inflammatory cytokines [24]. In our study, we observed reduced expression of CD80 and an increase of CD3, GATA-3, TNF-α and IL-1β in dogs with CanL (infected group) when compared to uninfected dogs (control group). Following transfection, increased

action of miR-148a, with the help of mimics, reduced iNOS expression in LE and TNF-α, IL-6 and IL-12 cytokines concentration in the supernatant of cultured SL from infected Group. Further, its inhibition resulted in parasite load reduction, strongly suggesting a regulatory role for this miR in immune response to CanL.

We did not observe changes in MHCII expression in SL, collected in basal culture conditions, when comparing infected cells to control. Similar results were observed in canine macrophages [34]. The MHCII molecule plays a crucial role in adaptive immune response development through antigenic peptide presentation to T lymphocytes [35], however; MHCII expression does not seem to be affected in CanL.

In our study, we observed a reduction in CD80+/MHCII+ expression in dogs from infected group compared to control group. CD80 is a positive coestimulatory molecule that determines adaptive immune response induction [36] and its absence results in T lymphocyte anergy or apoptosis [34,37]. Another study indicated that CD80 mRNA expression is unaffected in monocyte-derived macrophages from symptomatic dogs with CanL [38]. Thus, our result suggests a post-transcriptional regulation, impairing the antigen presentation process by suppressing CD80 as an evasion mechanism used by *Leishmania*, resulting in disease progression.

Induced nitric oxide synthase (iNOS) showed a tendency towards decrease in SL without transfection of CanL group, albeit without statistical significance. In CanL, iNOS expressed by macrophages in spleen is negatively associated with parasite load, suggesting a possible deregulation of this molecule in this disease, ensuring the permanence of the protozoan in infected cells [15].

Decrease of iNOS in SL from the infected group following transfection with mimic, suggests that miR-148a participates in the regulation of iNOS expression. Our study is the first to report miR-148a participation in iNOS regulation in CanL, showing that this miR can interfere with dog's immune response to *Leishmania*, as previously observed with miR-21 [24].

In our study, SL from the infected group showed increased expression of CD3 when compared to the control group. A similar result was observed in other tissues of dogs severely affected by leishmaniasis [39,40]. The observed increase in CD3 suggests that immune response to infection is being mounted with possible down-regulation of key proteins for inducing cell-type response, contributing to disease progression in dogs.

Expression of the transcription factor T-bet in CD3+ lymphocytes, did not vary between groups in our work, unlike the increase of this transcription factor that was previously reported in the spleen of dogs with CanL [41]. T-bet is related to Th1 type response development [40] and associated with disease resistance [41,42]. However, there is an imbalance between Th1/Th2 responses, already evidenced in CanL [12], which may explain the lack of difference in T-bet expression we observed between, reinforcing the idea of effective immune response suppression in CanL [43].

On the other hand, we observed an increase of GATA-3 expression in CD3+ lymphocytes from the infected group. GATA-3 mRNA expression is observed in the spleen of dogs from the first month following experimental infection with *L. infantum*, together with Th2 type cytokine mRNA detection, such as IL-4 mRNA [44]. Transcription factor GATA-3, precursor of Th2 type response, is responsible for anti-inflammatory cytokines production, which can lead to control of clinical signs, not necessarily accompanied by disease control [42], being therefore associated with disease progression [45]. Our results reinforces the modulation hypothesis of immune response in CanL [12,45]. Increase or decrease of miR-148a did not alter T-bet and GATA-3 expression in CD3+ lymphocytes from infected group, suggesting that these factors may not be directly regulated by miR-148a.

In our study, the increase in TNF-α observed in the infected SL supernatant confirms the involviment of this cytokine, already evidenced in the disease [19], which is associated with host resistance t in CanL [20,43]. However, TNF- α decreased in the transfected SL culture supernatant after the increase in miR-148a, by means of mimic transfection, in the infected group. Our study is the first to show the regulation of TNF-α expression by modulation of miR-148a in CanL. A similar result was observed in mouse dendritic cells LPS stimulated [26], confirming the participation role of miR-148a in the down-regulation of TNF-α.

The disease in dogs did not alter IL-6 levels in SL culture supernatant, as reported in previous studies [46], however, high IL-6 mRNA expression was found in the spleen of dogs [18]. Taken together, these results suggest that post- transcriptional regulation of this cytokine in leishmaniasis is likely at play. Interleukin 6 is a pro-inflammatory cytokine that plays a role in immune response, parasite control and tissue repair [47]. Supernatant concentration of IL-6 decreased in SL transfected with mimic miR-148a, similarly to a previous report in mouse dendritic cells [26]. Thus, we suggest that miR-148a can negatively regulate IL-6 in CanL.

Interleukin 12 in SL supernatant did not differ between groups in our study. In fact, IL-12 mRNA was absent in spleen of naturally infected dogs [44]. Interleukin 12 is an important cytokine for differentiation of Th1 lymphocytes [12] and in dogs with CanL it is negatively related to parasite load and can be suppressed by the excess of parasites [14]. In our study, IL-12 decreased in SL transfected with miR-148a mimic in the infected group. This result suggests that IL-12 mRNA is a target of miR-148a. There are no previous studies reporting the relationship between IL-12 and miR-148a in CanL, however, it has been observed that inhibition of miR21 in CanL leads to restoration of the concentration of this cytokine and reduction in parasite load [24] showing that more than one miR could modulate this cytokine.

In our study IL-1beta (IL-1β) increased in the supernatant of SL from naturally infected dogs, confirming previous results [46], and its role in CanL. Interleukin 1β is an essential pro-inflammatory cytokine during infection, however, its exacerbated production can result in tissue damage [48,49]. Transfection with miR-148a in SL did not alter IL-1β concentrations in supernatant, suggesting that miR-148a not a regulator of this cytokine.

Interestingly, inhibition of miR-148a resulted in a decrease in parasite load in SL from the infected group in our study. The same result was observed with the inhibition of miR21 [24]. This suggests that parasite load control is multifactorial and appears to be regulated by more than one miRNA in the species.

Decrease of iNOS expression and TNF-α, IL-6 and IL-12 cytokines concentration after miR-148a mimic transfection in SL, suggests a negative regulatory role of this miR in immune response to infection by *L. infantum*. In an attempt to elicit the biological function of down-regulated cytokines in CanL, it would be interesting to performed neutralization of, TNF-α, IL-6 and IL-12, and observe if it will influencing in parasite load.

Decrease in parasite load in SL following the use of miR-148a inhibtor, observed in our study, suggests a key role for this miR in the development of microbicidal activity in canine leishmaniasis. We conclude that miR-148a can regulate immune response evidenced by reduction of iNOS, TNF-α, IL-6 and IL-12 after miR increase and plays an important role in adaptive response demonstrated by the reduction in parasite load following its inhibition.

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2.8 Supporting Information

2.8.1 Data not shown

2.8.1.1 Expression of MHCII, iNOS/MHCII+ and transcription factor T-bet in TCD3+ lymphocytes without any treatment (Medium), showed no significant differences between the groups.

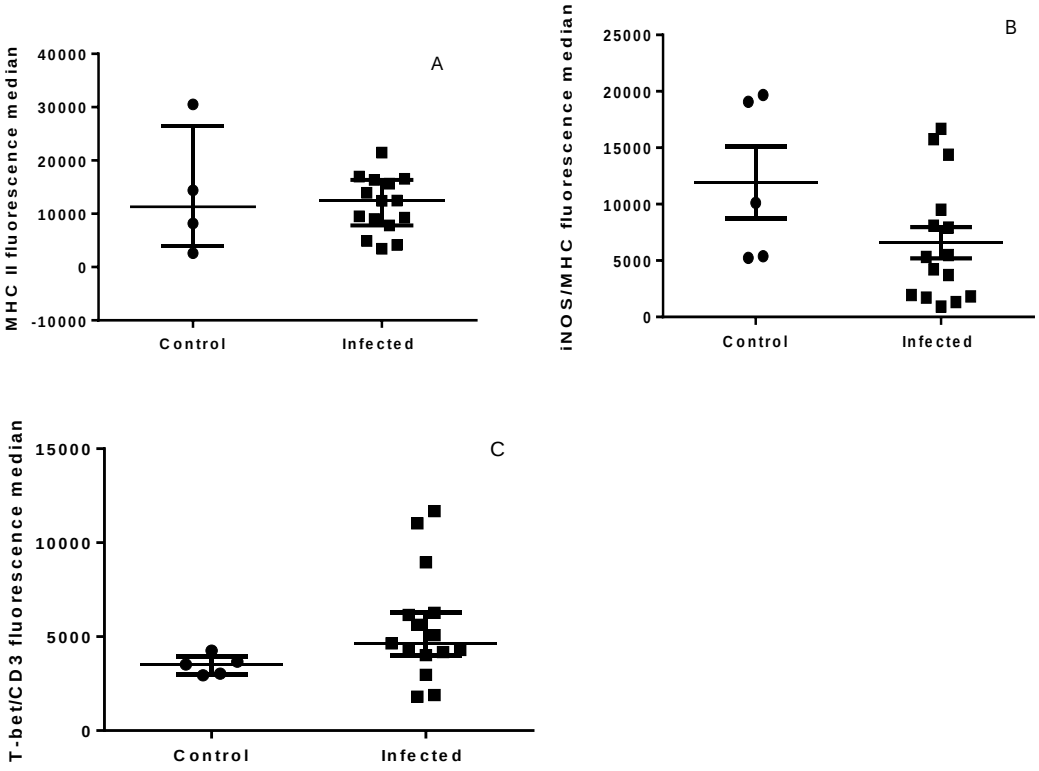


Figure 1. Expression of MHCII (A), iNOS/MHCII^{+} (B) and T-bet/CD3^{+} (C) of control and infected group, in splenic leukocytes maintained in culture for 48h at 37°C and 5% CO_2, without any treatment (Medium). Graphical data presented as mean ± SD. Mann-Whitney test. There is no significant difference.

2.8.1.2 Concentration of IL-6 and IL-12 in SL supernatant culture without any treatment (Medium), showed no significant difference between the groups.

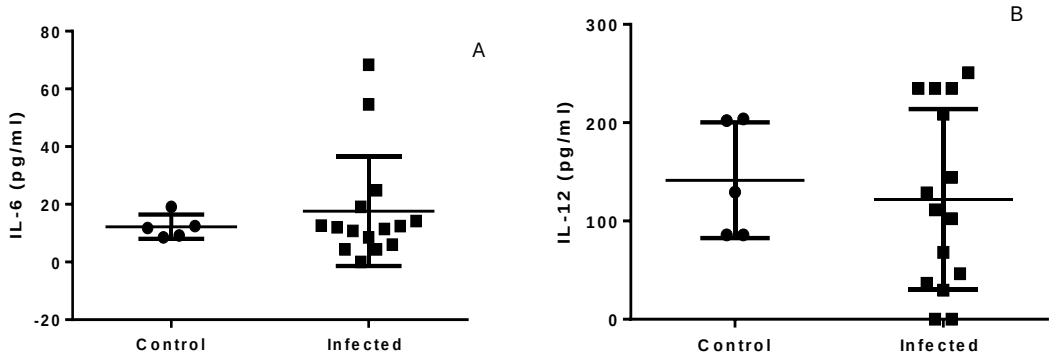
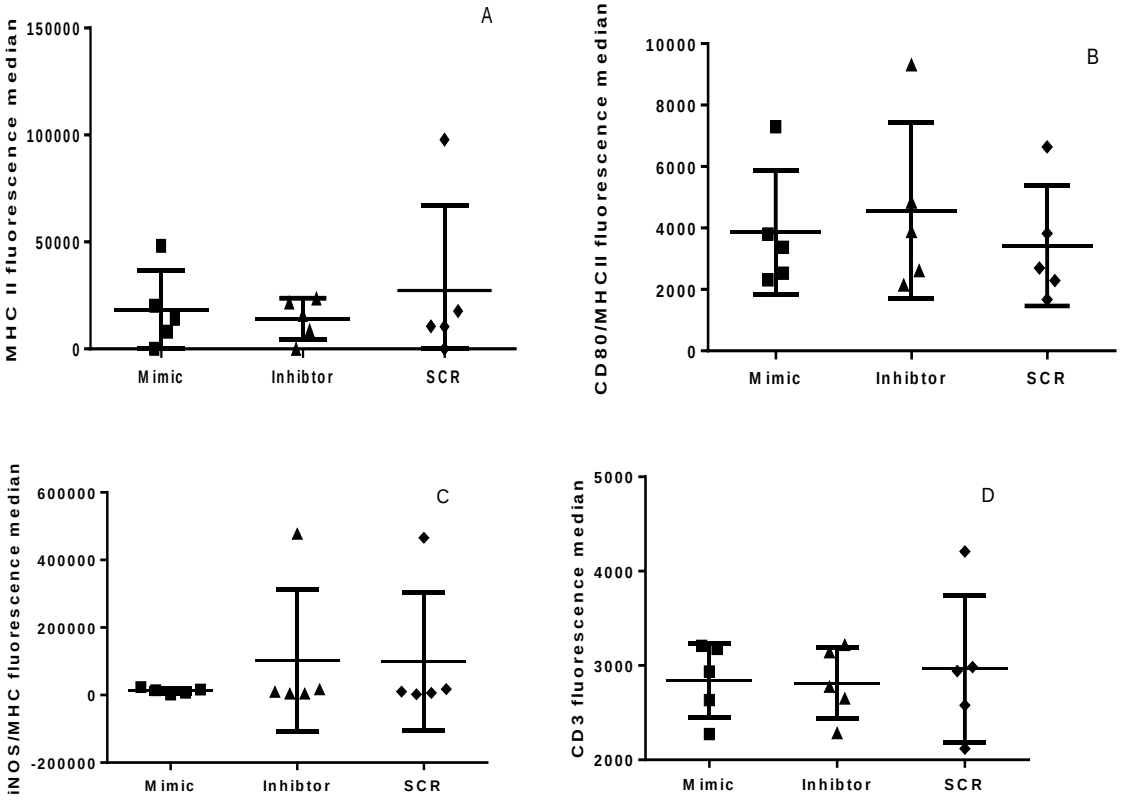


Figure 2. Concentration of IL-6 (A) and IL-12 (B) in cultured SL supernatant, by capture ELISA, from control and infected groups, maintained in culture for 48 hours at 37ºC and 5% CO2, without treatment (medium). Graphical data represents mean ± SD. Mann-Whitney test. There is no significant difference.

2.8.1.3 MicroRNA-148a mimic and inhibitor transfection did not change MHCII, CD80 in MHCII+ leukocytes, CD3 and transcription factors T-bet and Gata3 in TCD3+ expression in control group.



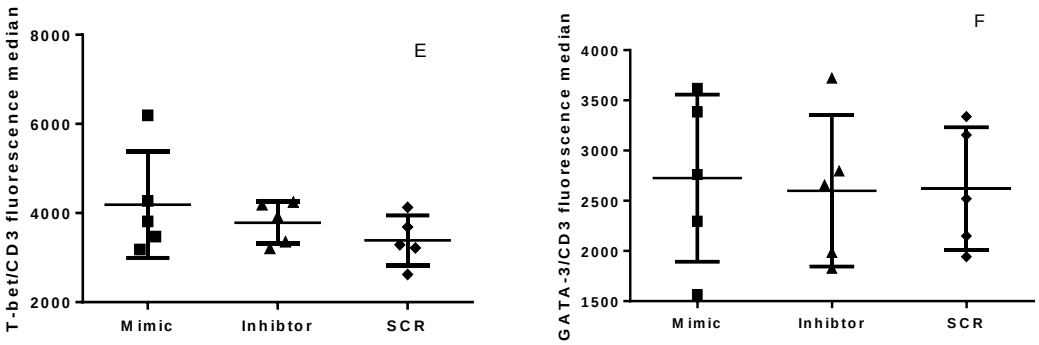
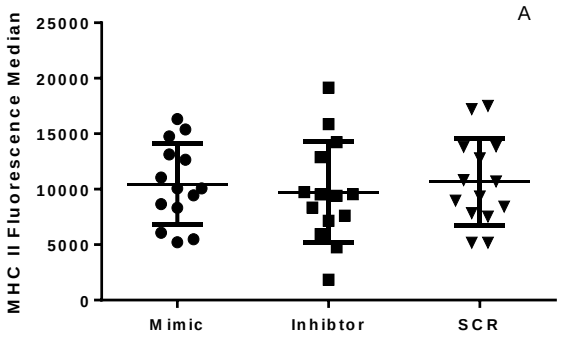


Figure 3. Median expression of MHCII (A), CD80/MHCII$^+$ (B), iNOS/MHCII$^+$ (C), CD3 (D), T-bet/CD3$^+$ (E), Gata-3/CD3$^+$ (F) in SL cultured from control group. Splenic leukocytes maintained in culture for 48h at 37°C and 5% CO_2, in presence of mimic or inhibitor of miR-148a, or scrambled (SCR). Splenic leukocytes were stained with monoclonal antibodies conjugated to fluorochromes and analyzed by flow cytometry. Graphical data presented as mean ± SD. Friedman test with Dunn's multiple comparison test. Graphical data presented as mean ± SD. Friedman test with Dunn's multiple comparison test. There is no significant difference.

2.8.1.4 MicroRNA-148a mimic and inhibitor transfection did not change MHCII, CD80/MHCII$^+$ leukocytes, CD3 and transcription factors T-bet/ CD3$^+$ and Gata3/CD3$^+$ expression in CanL.



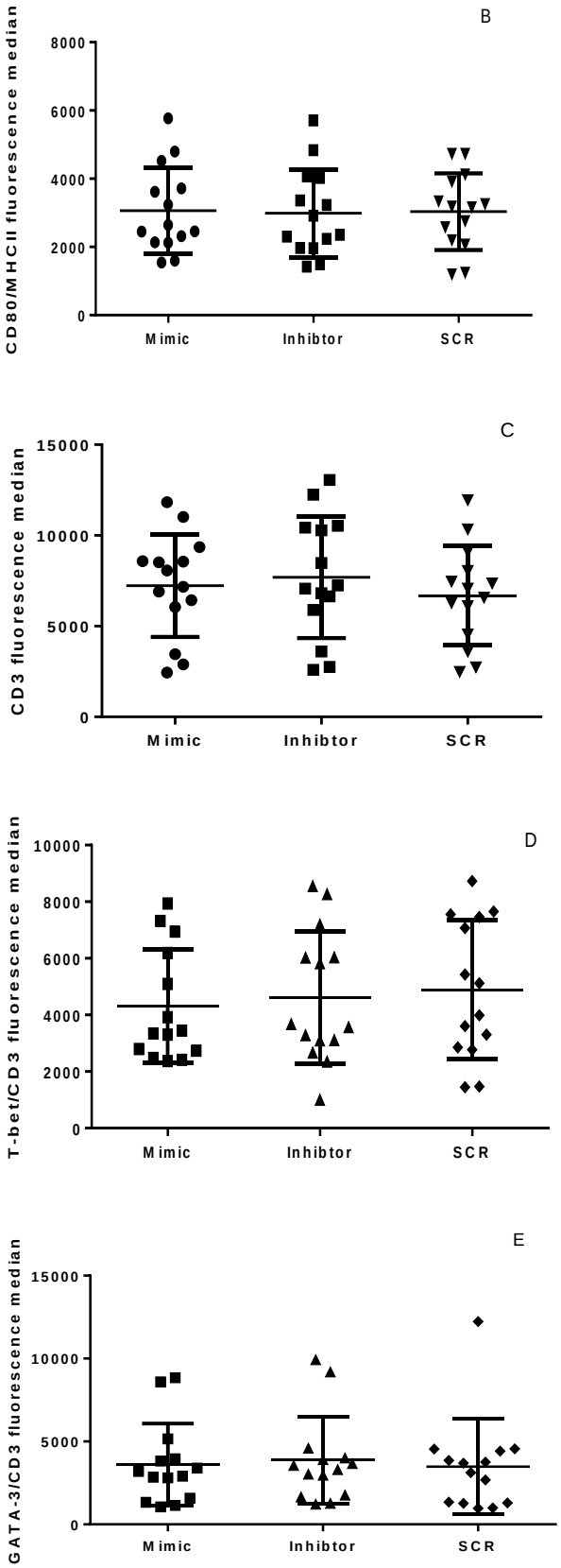


Figure 4. Median expression of MHCII (A), CD80/MHCII^{+} (B), CD3 (C), T-bet/CD3^{+} (D), Gata 3/CD3^{+} (E) in infected group. Splenic leukocytes maintained in culture for 48h at 37°C and 5% CO2, in the presence of mimic and inhibitor of miR-148a, and

scrambled (SCR). Splenic leukocytes were stained with monoclonal antibodies conjugated to fluorochromes and analyzed by flow cytometry. Graphical data presented as mean ± SD. Friedman test with Dunn's multiple comparison test. Graphical data presented as mean ± SD. Friedman test with Dunn's multiple comparison test. There is no significant difference.

2.8.1.5 Concentration of TNF-α, IL-6, IL-12 and IL-1β in SL supernatant culture of control dogs did not change after miR-148a mimic and inhibtor transfection.

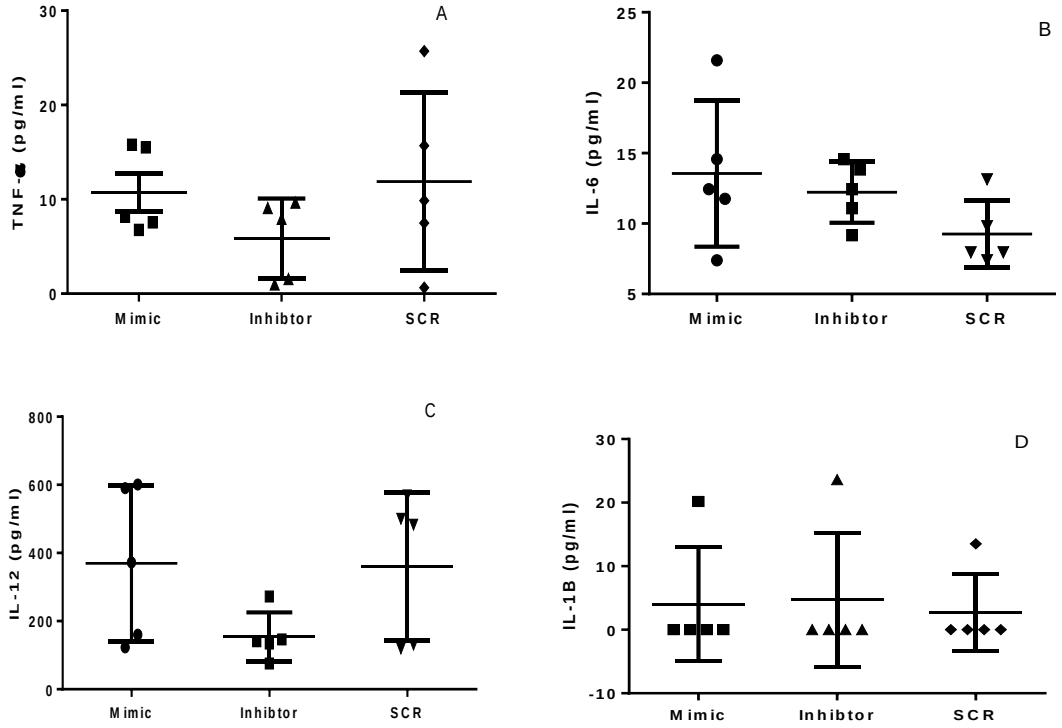


Figure 5. Concentration of TNF-α (A), IL-6 (B), IL-12 (C) and IL-1β (D) in SL supernatant culture, by capture ELISA, from control group, maintained in culture for 48 hours at 37ºC and 5% CO2, without treatment (medium). Graphical data represents mean ± SD. Friedman test with Dunn's multiple comparison test. There is no significant difference.

2.8.1.6 Concentration of IL-1β in SL supernatant culture from infected group did not change after miR-148a mimic and inhibtor transfection.

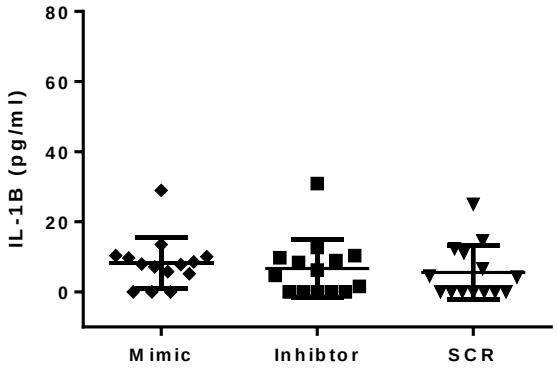


Figure 6. Concentration of IL-1β in SL supernatant culture, by capture ELISA, from infected group, maintained in culture for 48 hours at 37ºC and 5% CO2, without treatment (medium). Graphical data represents mean ± SD. Friedman test with Dunn's multiple comparison test. There is no significant difference.

S1 Table. Screening of dogs. Optical density on ELISA and clinical signs of CanL and control groups.

Animal	O.D ELISA	Sex	Clinical Signs	PCR	DPP
1	1,1325	F	Onychogryphosis, skin lesions, cachexia, seborrhea	+	+
2	1,027	F	Onychogryphosis, cachexia, skin lesions, seborrhea, alopecia, periocular lesion	+	+
3	1,060	M	Lymphadenomegaly, onychogryphosis, cachexia and skin lesions	+	+
4	0,920	M	Cachexia, seborrhea and skin lesions	+	+
5	0,637	M	Lymphadenomegaly, cachexia, skin lesions, periocular lesion	+	+
6	0,473	M	Lymphadenomegaly, skin lesions, hepatosplenomegaly	+	+
7	1373,5	F	Onychogryphosis, cachexia, alopecia, skin lesions	+	+
8	1207,5	M	Lymphadenomegaly, onychogryphosis, cachexia, alopecia, foot injuries	+	+
9	1,267	F	Lymphadenomegaly, onychogryphosis, seborrhea, alopecia, periocular lesion, hepatosplenomegaly	+	+

10	1048	F	Lymphadenomegaly, onychogryphosis, periocular lesion, hepatosplenomegaly	+	+
11	0,968	F	Lymphadenomegaly, onychogryphosis, cachexia	+	+
12	1,049	F	Lymphadenomegaly, onychogryphosis, seborrhea	+	+
13	0,705	M	Lymphadenomegaly, cachexia, skin lesions and muzzle, hepatosplenomegaly	+	+
14	0,552	M	Onychogryphosis, Cachexia, Seborrhea, Snout Lesions, Periocular Lesions	+	+
Control 1	0,062	F	No clinical signs	-	-
Control 2	0,026	M	No clinical signs	-	-
Control 3	0,147	F	No clinical signs	-	-
Control 4	0,028	M	No clinical signs	-	-
Control 5	0,071	F	No clinical signs	-	-

S2 Table. Biochemical profile of CanL and control groups.

Animal	Albumin (g/dL)	Total Protein (g/dL)	Globulin (g/dL)	ALT U/L	Alkaline phosphatase (U/L)	GGT mg/dL	Creatinine mg/dL	Ureia mg/dL
Infected 1	1,5	7,1	5,6	37	22	2,9	0,5	30
Infected 2	1,1	6,5	5,4	31	57	1,9	0,6	22
Infected 3	1,9	8,7	6,8	92	92	1	0,7	49
Infected 4	2,2	6,8	4,6	70	70	1,5	0,5	35
Infected 5	1	8,4	7,4	25	135	1,9	1,5	87
Infected 6	1,2	8,8	7,6	30	42	1,4	1,5	120
Infected 7	1,4	12,2	10,8	19	76	1,9	1	60
Infected 8	2,7	11,5	8,8	159	51	1,7	0,5	28
Infected 9	1,6	10,6	9	20	17	1,9	0,8	63
Infected 10	1,6	8,4	6,8	22	166	3,4	0,6	51
Infected 11	1,9	10,8	8,9	25	107	1,1	0,5	25
Infected 12	1,9	7	5,1	27	135	1,4	0,5	23
Infected 13	1,7	9	7,3	22	23	2,1	0,7	33

Infected 14	1,6	8,8	7,2	23	26	3,2	0,8	16
Control 1	3,3	7	3,7	80	74	2,6	0,9	25
Control 2	2,9	6,7	3,8	105	148	5,9	0,7	30
Control 3	3,4	6,9	3,5	40	36	2,9	1	27
Control 4	2,9	7	4,1	32	33	1,2	1	50
Control 5	2,7	6,6	3,9	50	85	1,9	1,1	21

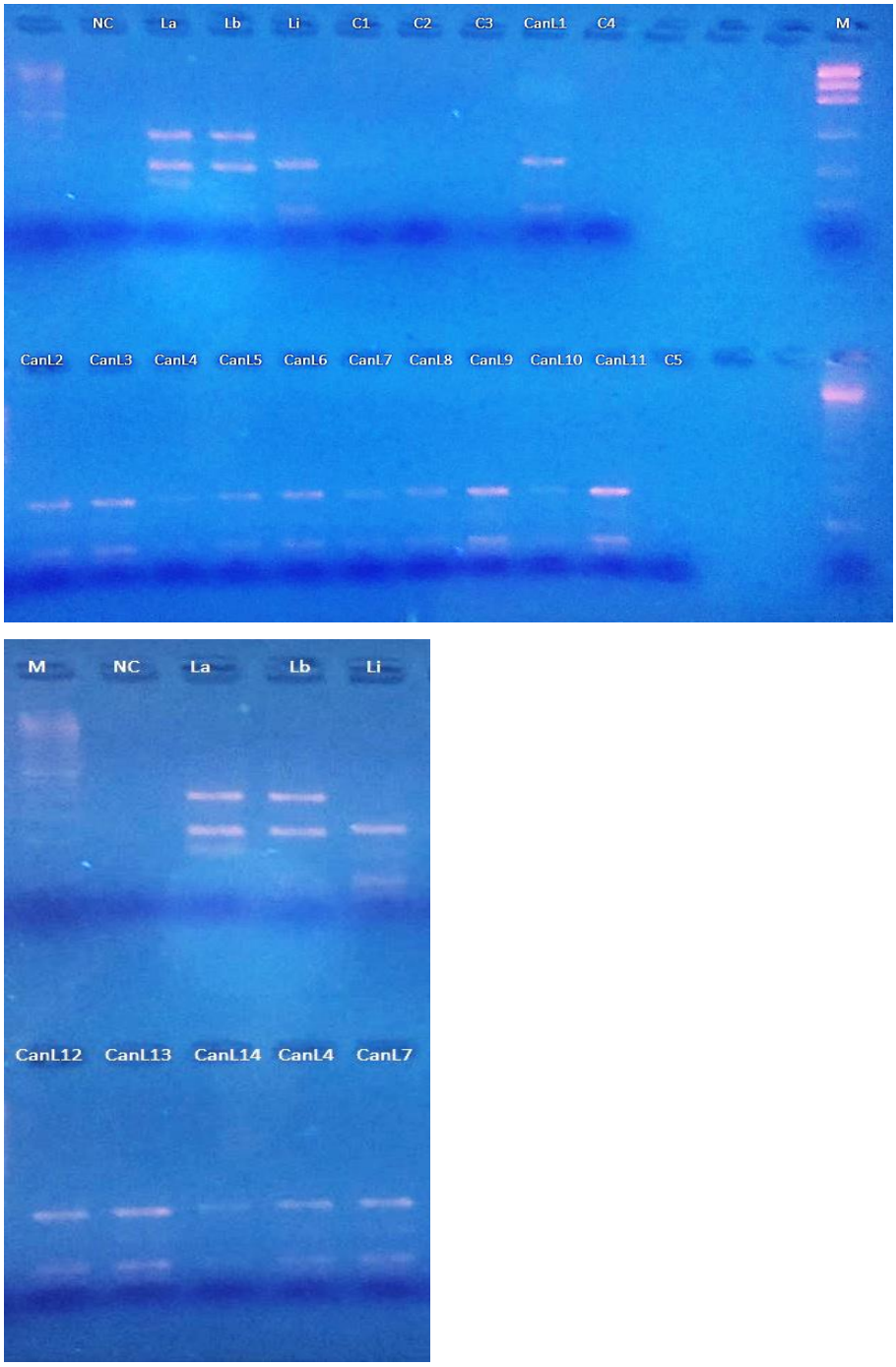
¶ALT: alanine aminotransferase; GGT: gamma glutamyl transferase

S3 Table. Complete Blood Count of CanL and control groups.

Animal	Red blood cells (x10 ^{12}/L)	Hemoglobin (g/dL)	GV (%)	MCV (fL)	MCHC (%)	Leukocytes (x10 9 /L)	Neutrophils (x106 /L)	Lymphocytes (x106 /L)	Monocytes (x10 6 /L)	Eosinophils (x10 6 /L)	Basophils (x10 6 /L)	Platelets (x10 00)	PP (g/dL)
Reference	5,5-8,5	12,0 - 18,0	37 - 55	60-77	32-36	6,0-17	3000 - 11500	1000-4800	150-1350	150-1250	rare	160 - 440	6,0-8,0
infectado 1	4,05	7,8	25	61,73	31,2	9,9	6831	2475	396	198	0	220	11
infectado 2	3,84	9,1	25	65,1	36,4	17,9	13783	2506	1253	358	0	400	8,4
infectado 3	5,38	11	31	57,62	35,48	16,2	11178	3402	1296	324	0	300	7
infectado 4	2,93	6,7	22	75,09	30,45	7,1	4686	2130	284	0	0	300	9,8
infectado 5	2,95	6,5	18	61,02	36,11	9,3	6417	1860	837	186	0	280	8,2
infectado 6	4,3	9,5	27	62,79	35,19	7,2	4320	1800	288	72	0	140	7
infectado 7	3,34	6,2	18	53,89	34,44	8,1	5022	2997	81	0	0	180	7,4
infectado 8	2,11	3,6	11	52,13	32,73	9,8	7350	2156	294	0	0	60	7,8
infectado 9	4,06	10	28	68,97	35,71	8,5	6035	2040	425	0	0	200	10
infectado 10	2,1	4,1	12	57,14	34,17	3	2100	780	30	90	0	220	7
infectado 11	2,82	6,6	20	70,92	33	12,4	9176	2976	248	0	0	160	8,8

infectado 12	3,81	8,7	25	65,62	34,8	14,6	10950	3358	146	146	0	280	9
infectado 13	3,44	7,3	21	61,05	34,76	8,3	6142	1826	249	83	0	140	12
infectado 14	4,05	10,2	30	74,07	74,07	6	3.780	1800	180	240	0	280	12
controle 1	7,89	17,8	53	67,17	33,58	16,2	10692	2754	1496	1250	0	200	8
controle 2	6,78	16,9	49	72,27	34,49	15,7	10048	2983	628	2041	0	300	7,8
controle 3	7,89	17,8	52	65,91	34,23	12,2	6588	4800	610	122	0	320	7,8
controle 4	6,78	17,3	50	73,75	34,6	16,2	10854	3726	324	1296	0	220	8
controle 5	7,44	17,1	51	68,55	33,53	10,3	6077	3605	515	103	0	220	6,6

¶GV: globular volume; MCV: mean corpuscular volume; MCHC: mean corpuscular hemoglobin concentration; PP: plasma protein



S1 Fig. PCR-RFLP. Restriction fragment length polymorphism (RFLP) analysis of ITS1-PCR fragments amplified from DNA samples by using Hae III enzyme. NC: Negative control (water); M: molecular marker (123 bp); La: *Leishmania amazonensis* (IOC / L0575-MHOM / BR / 1967 / PH8); Lb: *Leishmania braziliensis* (IOC / L0566-MHOM / BR / 1975 / M2903); Li: *Leishmania infantum* (IOC / L0575-MHOM / BR / 2002 / LPC-RPV); C1 to C5: control group; CanL 1 to CanL14: CanL group. CanL samples profile were identical to *L. infantum*.

APÊNDICE A- REFERÊNCIAS DA INTRODUÇÃO GERAL

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ANEXO A: NORMAS DA REVISTA PLOS ONE

Manuscript Organization

Manuscripts should be organized as follows.

Beginning section	*The following elements are required, in order:* • Title page: List title, authors, and affiliations as first page of the manuscript • Abstract • Introduction
Middle section	*The following elements can be renamed as needed and presented in any order:* • Materials and Methods • Results • Discussion • Conclusions (optional)
Ending section	*The following elements are required, in order:* • Acknowledgments • References • Supporting information captions (if applicable)
Other elements	• Figure captions are inserted immediately after the first paragraph in which the figure is cited. Figure files are uploaded separately. • Tables are inserted immediately after the first paragraph in which they are cited. • Supporting information files are uploaded separately.

Stile format	Manuscript files can be in the following formats: DOC, DOCX, or RTF. Microsoft Word documents should not be locked or protected. LaTeX manuscripts must be submitted as PDFs. Read the LaTeX guidelines.
Length	Manuscripts can be any length. There are no restrictions on word count, number of figures, or amount of supporting information. We encourage you to present and discuss your findings concisely.
Font	Use a standard font size and any standard font, except for the font named “Symbol”. To add symbols to the manuscript, use the Insert → Symbol function in your word processor or paste in the appropriate Unicode character.
Headings	Limit manuscript sections and sub-sections to 3 heading levels. Make sure heading levels are clearly indicated in the manuscript text.
Layout and spacing	Manuscript text should be double-spaced. Do not format text in multiple columns.
Page and line numbers	Include page numbers and line numbers in the manuscript file. Use continuous line numbers (do not restart the numbering on each page).
Footnotes	Footnotes are not permitted. If your manuscript contains footnotes, move the information into the main text or the reference list, depending on the content.
Language	Manuscripts must be submitted in English. You may submit translations of the manuscript or abstract as supporting information. Read the supporting information guidelines.
Abbreviations	Define abbreviations upon first appearance in the text. Do not use non-standard abbreviations unless they appear at least three times in the text. Keep abbreviations to a minimum.
Reference style	PLOS uses “Vancouver” style, as outlined in the ICMJE sample references.

Parts of a Submission

Title

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

Title	Length	Guidelines	Examples
Full title	250 characters	Specific, descriptive, concise, and comprehensible to readers outside the field	Impact of cigarette smoke exposure on innate immunity: A *Caenorhabditis elegans* model Solar drinking water disinfection (SODIS) to reduce childhood diarrhoea in rural Bolivia: A cluster-randomized, controlled trial
Short title	100 characters	State the topic of the study	Cigarette smoke exposure and innate immunity SODIS and childhood diarrhoea

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

Author names and affiliations

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

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

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

ANEXO B – CERTIFICADO DO COMITÊ DE ÉTICA

unesp **UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"**

ceua unesp araçatuba

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 **"miR 21 e miR148a na regulação da resposta imunológica de leucócitos esplênicos na Leishmaniose Visceral Canina"**, Processo FOA nº 00624-2018, 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 14 de Agosto de 2018.

VALIDADE DESTE CERTIFICADO: 01 de Novembro de 2021.
DATA DA SUBMISSÃO DO RELATÓRIO FINAL: até 01 Dezembro de 2021.

CERTIFICATE

We certify that the study entitled **"miR 21 e miR 148a in regulation of immune response of splenic leukocytes in Canine Visceral Leishmaniasis"**, Protocol FOA nº 00624-2018, 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 August 14, 2018.

VALIDITY OF THIS CERTIFICATE: November 01, 2021.
DATE OF SUBMISSION OF THE FINAL REPORT: December 01, 2021.

Prof. Ass. Dr. Leonardo Perez Faverani
Coordenador da CEUA
CEUA Coordinator

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