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SIDNEI FERRO COSTA

ESTIMULAÇÃO COMBINADA DE IL-12 E IL-15 PROMOVE A RESPOSTA IMUNE CELULAR EM CÃES COM LEISHMANIOSE VIA IFN-y

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SIDNEI FERRO COSTA

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Orientadora: Prof.ª Ass. Valéria Marçal Felix de Lima

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AUTOR: SIDNEI FERRO COSTA
ORIENTADORA: VALERIA MARÇAL FELIX DE LIMA

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

Profa. Dra. VALERIA MARÇAL FELIX DE LIMA
Departamento de Clínica, Cirurgia e Reprodução Animal / Faculdade de Medicina Veterinária - Câmpus de Araçatuba/Unesp

Pesquisadora FLÁVIA LOMBARDI LOPES
Departamento de Apoio, Produção e Saúde Animal / Faculdade de Medicina Veterinária - Câmpus de Araçatuba/Unesp

Profa. Dra. SANDRA HELENA PENHA DE OLIVEIRA
Departamento de Ciências Básicas / Faculdade de Odontologia - Câmpus de Araçatuba/Unesp

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RESUMO

A Leishmaniose Visceral (LV) é causada nas Américas, pelo protozoário intracelular obrigatório *Leishmania infantum* e os cães domésticos são os principais reservatórios urbanos do parasita e em áreas endêmicas, o aumento da LV em humanos tem sido associado ao aumento da infecção canina. Os atuais medicamentos disponíveis para a Leishmaniose Canina (CanL) não são completamente eficientes e meses após o tratamento a maioria dos cães apresentam recidiva, indicando a necessidade de buscar formas alternativas de tratamento. Na CanL, cães desenvolvem uma resposta imune celular (Th1) ineficiente para combater o parasita e a estimulação das vias de citocinas em células de defesa com proteínas recombinantes, tem o potencial de se tornar parte de métodos imunoterapêuticos eficazes. Neste estudo, as citocinas recombinantes caninas (IL-12, IL-2, IL-15 e IL-7) e o receptor solúvel de IL-10R1 (sIL-10R1), com atividade antagonista, foram avaliadas pela primeira vez em combinações (IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-15/IL-7) ou isoladamente (sIL-10R1) quanto à capacidade imunomodulatória em células mononucleares do sangue periférico (sigla em inglês PBMC) de cães com leishmaniose. Todas as combinações de proteínas recombinantes testadas mostraram melhorar a resposta linfoproliferativa. Além disso, as combinações de IL-12/IL-2 e IL-12/IL-15 promoveram a diminuição na expressão da proteína "Programed Cell Death 1" (PD-1) nos linfócitos. Estas mesmas combinações de citocinas e IL-12/sIL-10R1 induziram a produção de IFN-y nas PBMCs. Além disso, a combinação de IL-12/IL-15 aumentou expressão de T-bet nos linfócitos. Esses achados estimulam o uso de IL-12 e IL-15 em futuros estudos *in vivo* com o objetivo de obter a polarização da resposta imune celular em cães com leishmaniose, o que pode contribuir para o desenvolvimento de um tratamento efetivo contra a CanL.

Palavras-chave: Leishmaniose. Cães. Imunologia. Interleucina-12. Interleucina-15

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ABSTRACT

Visceral Leishmaniasis (LV) is caused in the Americas by the obligate intracellular protozoan *Leishmania infantum* and domestic dogs are the major urban reservoirs of the parasite and in endemic areas, and increase LV in humans has been associated with increased canine infection. The current medications available for Canine Leishmaniasis (CanL) are not completely effective and months after treatment most dogs present with relapse, indicating the necessity to looking for alternative forms of treatment. In CanL, dogs develop an ineffective cellular immune response (Th1) to combat the parasite. Then, the stimulation of cytokine pathways in defense cells with recombinant proteins, has the potential to become part of effective immunotherapeutic methods. In this study, the canine recombinant cytokines (IL-12, IL-2, IL-15 and IL-7) and the soluble receptor of IL-10R1 (sIL-10R1) with antagonistic activity, were evaluated for the first time in combinations IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-15/IL-7) or alone (sIL-10R1) for their immunomodulatory capacity in peripheral blood mononuclear cells (PBMC) from dogs with leishmaniasis. All combinations of recombinant proteins tested were shown to improve lymphoproliferative response. Further, combinations of IL-12/IL-2 and IL-12/IL-15 promoted decrease in programmed cell death protein 1 (PD-1) expression in lymphocytes. These same combinations of cytokines and IL-12/casIL-10R1 induced IFN-y production in PBMC. Furthermore, the combination of IL-12/IL-15 led to an increased T-bet expression . These findings encourage the use of IL-12 and IL-15 in future in vivo studies aiming to get polarization of cellular immune response in dogs with leishmaniasis, that may contribute to development of an effective treatment against CanL.

Keywords: Leishmaniasis. Dogs. Immunology. Interleukin-12. Interleukin-15.

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1 INTRODUÇÃO GERAL

1.1 Aspectos clínicos e epidemiológicos da Leishmaniose Visceral

Leishmanioses são doenças causadas por protozoários do gênero *Leishmania,* da ordem *Kinetoplastida* e família *Trypanosomatidae* [1]. O gênero *Leishmania* compreende um grupo importante de parasitas intracelulares obrigatórios que são os agentes causadores das leishmanioses. As formas clínicas da doença no homem se apresentam em três diferentes formas dependendo da espécie de *Leishmania* e da resposta imune do hospedeiro: Leishmaniose Cutânea (LC), é a forma mais ocorrente e caracterizada por lesões ulcerativas na pele; Leishmaniose Mucocutânea, caracteriza pela destruição parcial ou total das mucosas e Leishmaniose Visceral (LV) conhecida também como Kalazar, caracterizada por episódios irregulares de febre, perda substancial de peso, inchaço do baço, fígado e anemia [2].

A LV é a forma clínica mais grave das leishmanioses com uma letalidade de mais de 95% em pacientes que não são submetidos a nenhum tipo de tratamento [3] sendo uma das doenças mais negligenciadas do mundo e classificada entre as seis doenças tropicais mais importantes [4]. As espécies de *Leishmania* que causam a doença visceral pertencem ao subgênero *Leishmania* e incluem *Leishmania (L.) donovani* e *L. infantum* na Europa e *L. chagasi* ou *L. infantum* nas Américas.

A ocorrência da LV é amplamente distribuída no mundo, principalmente em regiões tropicais e subtropicais com aproximadamente 300.000 novas infeções a cada ano e estimativa de 20.000 a 50.000 mortes anualmente em decorrência da doença [4]. O Brasil é uma região endêmica para LV com a maioria dos casos concentrados na região Nordeste (44%) seguida da região Sudeste (22%) e em 2017 foram 4.013 registros da doença com 327 óbitos [5]. Dos casos de LV registrados no Brasil no ano de 2017, 31 foram na região noroeste do estado de São Paulo, com 4 mortes [6]. No estado de São Paulo, a doença foi notificada pela primeira vez no município de Araçatuba no ano de 1999, quando foi confirmado o primeiro caso canino autóctone no município [7] e até o ano de 2013 foram 2.324 casos registrados no estado com mais de 200 óbitos em humanos [8].

A transmissão entre hospedeiros vertebrados no Brasil é feita pela picada da fêmea do flebotomíneo hematófago *Lutzomyia longipalpis* [9], porém outras

espécies já foram identificadas como vetores da doença [10]. Mesmo com a dispersão dos flebotomíneos para quase todas as regiões do Brasil, a ausência do vetor em áreas onde existem casos de LV sugere a existência de outros modos de transmissão da enfermidade [11]. Pulgas [12] e carrapatos [12,13] também já foram incriminados como possíveis transmissores de *L. Infantum*. Já foi descrita também a ocorrência de transmissão venérea [14], vertical [15–17] e por transfusão sanguínea [18].

Os cães domésticos (*Canis familiaris*) são os principais reservatórios de *L. infantum* em áreas urbanas [19] e em áreas endêmicas há uma correlação entre a prevalência de cães soropositivos e o número de casos da doença em humanos [20,21]. Essa relação cães soropositivos versus LV humana acontece devido á proximidade dos cães com os humanos; pelos cães infectados possuírem alto parasitismo na pele, principalmente em regiões ulceradas, o que favorece o contágio de novos vetores e facilita a transmissão da doença [22]; pela alta prevalência de cães infectados em áreas endêmicas e poucos apresentarem a forma sintomática da doença [23] o que dificulta o diagnóstico e consequentemente o controle do parasita no cão infectado [24]. Portanto, os cães são importantes na manutenção do ciclo epidemiológico da LV e o controle da doença no homem não acontece sem um controle efetivo da doença no cão [25].

1.2 Caracterização da Leishmaniose Canina

Nos cães, a infecção por *L. infantum*, tem início após a inoculação de formas promastigotas metacíclicas na pele durante o repasto sanguíneo da fêmea do flebotomíneo. Embora não existam evidências diretas de todas as etapas do processo de instalação da doença no cão, no homem ou mesmo em modelo murino, o cenário geral parece ser o descrito a seguir. As promastigotas de *L. infantum* são fagocitadas por células fagocíticas residentes ou recrutadas como macrófagos e neutrófilos [26]. No interior das células fagocíticas, as promastigotas (que exibem flagelo aparente) se aderem aos fagossomos e transformam-se em formas amastigotas (que possuem flagelo restrito à bolsa flagelar) [27]. Neutrófilos contendo leishmanias podem sofrer apoptose e serem fagocitados por macrófagos, transferindo assim a sua carga parasitária. Os macrófagos produzem óxido nítrico (NO) para eliminação do parasita [28], no entanto, nesse primeiro momento a

ativação de mecanismos leishmanicidas dos macrófagos não é suficiente para evitar a propagação do parasita [29].

Sem uma contenção apropriada, as amastigotas se multiplicam por divisão binária dentro dos macrófagos e são transportadas para o linfonodo de drenagem e a partir daí, no interior dos macrófagos, disseminam-se para diversos órgãos ricos em células do sistema fagocítico mononuclear [30] como o baço, fígado e a medula óssea [26]. Nesses órgãos, as leishmanias continuam a proliferar no interior de macrófagos até ocasionar a ruptura da célula e dessa forma infectar outros macrófagos [31] induzindo uma inflamação crônica, frequentemente granulomatosa, que promove alterações funcionais e estruturais [32,33] e consequentemente, causar a doença.

A Leishmaniose Canina (CanL) é manifestada por lesões e sintomas característicos. Os sinais clínicos mais frequentes da CanL são lesões na pele, linfoadenopatia, onicogrifose, perda de peso, caquexia, febre e doença renal [34]. Além da sintomatologia clássica, o parasita pode eventualmente causar danos ao sistema reprodutor masculino [35] podendo inclusive ser detectado no sêmen [36]. Alterações laboratoriais também são encontradas nos cães doentes, dentre elas anemia não regenerativa, hipoalbuminemia, hiperglobulinemia, trombocitopenia, proteinúria, azotemia renal e aumento de enzimas hepáticas [34]. O número e a intensidade dos sinais clínicos são determinados por um conjunto de fatores que envolvem a cepa do parasita, a genética e principalmente o estado imunológico do hospedeiro [24]. Desta forma, alguns cães são capazes de controlar a infecção por um período de tempo sem o aparecimento de sinais [23,37], e às vezes pode até evoluir para cura espontânea, entretanto, alguns cães apresentam evolução aguda ou curso progressivo que leva à morte, se o manejo e a terapia adequadas não forem adotadas [24].

1.3 Resposta Imunológica na Leishmaniose Canina

A resposta imunológica desenvolvida pelo cão durante a CanL é complexa e determinante na resistência ou susceptibilidade a doença [28,38]. A imunidade inata é a primeira linha de defesa contra *L infantum*. A pele age como primeira barreira de proteção por meio da ativação das vias clássicas e alternativas do sistema complemento [28], no entanto a saliva do flebotomíneo pode inibir a ação

do complemento e consequentemente induzir a falha da resposta imune já no momento da inoculação [39]. Moléculas dos parasitas sobreviventes à ação do complemento são detectadas por receptores da imunidade inata presentes em células dendríticas, células natural killer e macrófagos [40]. Entre esses receptores imunes inatos estão os receptores Toll-like (TLRs), que utilizam proteínas adaptadoras como o MyD88 para desencadear a produção de citocinas pró-inflamatórias, tais como TNF-α, IFN-γ e IL-12, bem como moléculas coestimuladoras que ativam momentaneamente mecanismos leishmanicidas dos macrófagos infectados e induzem a resposta imune adaptativa [41,42].

Os mecanismos inatos desempenham um papel importante nas defesas anti-*Leishmania* [43], no entanto a montagem eficiente de uma resposta imune adaptativa é indispensável para o controle da infecção. A resposta imune adaptativa envolvida no reconhecimento e controle da infeções por patógenos intracelulares, incluindo a infecção causada por *L. infantum*, é a resposta imune celular ou Th1 [44]. Durante essa resposta, células apresentadoras de antígeno, incluindo células dendríticas, capturam leishmanias ou fragmentos delas na pele e migram para região T do linfonodo de drenagem [26,45]. As células dendríticas que amadurecem em condições de estimulação intensa desenvolvem a capacidade de expressar moléculas co-estimulatórias e produzir IL-12 [46]. No linfonodo, após processamento de antígenos do protozoário, células dendríticas que expressam peptídeos da leishmania acoplados a moléculas do complexo principal de histocompatibilidade (classe I e classe II), moléculas co-estimulatórias membros da família B7 como (por exemplo, CD80 e CD86) [47], produzem IL-12 [48] e são capazes de estimular a proliferação e a diferenciação de linfócitos T naïve em linfócitos T efetores (T CD4+ e T CD8+) Th1. Com a diferenciação, as células T CD4+ Th1 produzem INF-γ e TNF-α nos tecidos afetados pela infecção e essas citocinas ativam mecanismos leishmanicidas, incluindo a produção de óxido nítrico e espécies reativas de oxigênio (H_2O_2 e íon superóxido) nos macrófagos infectados pelo protozoário [48–50] e, dessa maneira, eliminam o parasita.

Cães doentes, susceptíveis a CanL e sintomáticos, falham em montar uma resposta imune celular Th1 eficiente contra o parasita concomitante ao aumento da resposta imune humoral ou Th2, que resulta na ativação das células B, produção exacerbada de anticorpos [51,52] e uma incontrolável multiplicação do parasita [53]. O aumento da carga parasitária e a supressão da imunidade celular na

CanL, podem ser acompanhados pelo aumento da expressão da citocina IL-10 [54–57]. Essa citocina é produzida por várias células, incluindo linfócitos T regulatórios (Treg), linfócitos Th2 [58] e células B [59], sendo considerada um regulador da atividade Th1 que mantém o equilíbrio entre as respostas Th1 e Th2 e inibe a atividade microbicida de macrófagos infectados [38,60].

A falha do cão doente em montar uma resposta adaptativa eficiente contra o parasita pode ser também, pelo menos em parte, associada ao aumento da expressão da molécula de co-estimulação negativa PD-1 (programed cell death 1). O PD-1 também conhecido como CD279 é um membro da família B7-CD28 expresso por células imunológicas [61]. Quando associado aos seus ligantes PD-L1 ou PD-L2, PD-1 induz a desativação e apoptose de células T [62]. O aumento da expressão de PD-1 em células T CD4+ de cães infectados com *L. infantum* e sintomáticos, quando submetidos ao estímulo antígeno específico para avaliação da proliferação celular, resultou em redução dessa resposta comparada a animais assintomáticos [63]. Em linfócitos T, B e macrófagos, o aumento da expressão de PD-1 e seus ligantes foi associado à apoptose dos linfócitos T em células esplênicas e mononucleares do sangue periférico de cães doentes [64] e a alta expressão de PD-1 também alterou a produção de citocinas relacionadas com a progressão da CanL [65].

1.4 Estimulação e bloqueio da sinalização de citocinas com proteínas recombinantes na Leishmaniose

A resistência ou susceptibilidade a infecção por *L.infantum* depende muito do tipo de resposta imune montada (Th1 versuus Th2) e a estimulação da sinalização de citocinas que estimula a resposta Th1 (IL-2, IL-12, IL-15) e o bloqueio da sinalização de citocinas que estimulam a resposta Th2 (IL-10) tem sido utilizados em estudos *in vitro* na CanL e na LV humana com o objetivo de desenvolver métodos imunoterápicos para o tratamento da doença [55,66–70].

A IL-2 é uma citocina produzida por várias células do sistema imunológico quando ativado, incluindo células TCD4+, células natural killer (NK), células dendríticas (DCs) e mastócitos [71]. A IL-2 é o principal fator estimulador de células T, sendo um fator de crescimento e ativação para todas as subpopulações de linfócitos T, induzindo ciclo celular para células T não ativadas e a expansão clonal

de células T ativadas [72]. Em pacientes humanos infectados com *L. infantum*, a adição de antígeno solúvel de *Leishmania* (AtgS) combinado com IL-2 recombinante humano (rhuIL-2) e IFN-y recombinante humano (rhuIFN-y) em cultura de PBMC restaurou a resposta linfoproliferativa [73].

A IL-15 é uma citocina sintetizada por diversos tipos celulares, incluindo monócitos/macrófagos, células dendríticas, estroma da medula óssea e células epiteliais do timo, e que exerce um vasto espectro de atividades biológicas, incluindo proliferação e ativação de células NK [74] e proliferação e sobrevivência de linfócitos T de memória [75,76]. A IL-15 é uma das citocinas que induzem as células NK a produzir INF-γ [77] e tem atividade biológica similar a IL-2 [78], aumentando tanto respostas Th1 quanto Th2. Contudo, foi demonstrado que esta citocina pode mudar o equilíbrio entre Th1 e Th2 para resposta Th1 [79]. A adição de IL-15 recombinante humana (rhuIL-15) em cultura de PBMC de pacientes humanos com LV ativa diminuiu a citocina Th2 IL-4 e aumentou a citocina Th1 IL-12 [80].

A IL-12 é uma citocina heterodimérica produzida por células inflamatórias (monócitos, macrófagos, neutrófilos e células dendríticas) em resposta a patógenos intracelulares. IL-12 induz a produção de INF-γ pelas células NK e células T e é importante na indução e manutenção da resposta Th1 [81]. A adição de IL-12 recombinante humana (rhuIL-12) e o bloqueio de IL-10 e IL-4 com anticorpos monoclonais (mAb) em cultura de PBMC de pacientes humanos com LV, restauraram a resposta linfoproliferativa e a produção de IFN-y. Na CanL, a adição de IL-12 recombinante canina (rcaIL-12) em cultura de PBMC de cães naturalmente infectados por *L. infantum* promoveu aumento da expressão de RNA e dos níveis de IFN-y no sobrenadante da cultura [70,82] e melhorou a resposta linfoproliferativa após estimulação com AtgS [70].

A IL-7 é uma citocina produzida por células do estroma da medula óssea e do timo, fibroblastos reticulares em regiões T dos linfonodos e induz diversas atividades biológicas, incluindo reconstituição linfóide e a proliferação e sobrevivência de células T de memória [83]. Não há estudos sobre o papel da IL-7 em infecções por *Leishmania spp.* em humanos ou em cães e seu papel na polarização das células T não foi definido. Entretanto, a IL-7 murina, especialmente quando combinada com IFN-y, demonstrou promover forte efeito leishmanicida em macrófagos de camundongos infectados com *L. major* [84].

Embora na leishmaniose visceral humana e canina, a estimulação de uma única citocina já tenha demonstrado efeitos benéficos, tem sido observado que quando as citocinas são utilizadas em combinações de duas ou mais, podem promover efeitos biológicos mais desejáveis. Em cães, estudo *in vitro* mostrou que rcaIL-12 sozinha foi capaz de induzir a produção de baixas quantidades de IFN-γ por linfócitos de cães infectados com *L. infantum* [70], entretanto, em cães saudáveis, a combinação rcaIL-12 e IL-2 recombinantes canino (rcaIL-2) induziu maior produção de IFN-γ comparado ao efeito isolado dessas citocinas [85]. Em pacientes humanos infectados por *L. infantum*, rhuIL-15 induziu resposta Th1, mas não alterou a produção de IFN-γ *in vitro* [80]. No entanto a IL-15 em sinergia com IL-12 ativou a produção de IFN-γ e induziu a resposta Th1 em infecções por parasitas intracelulares [86]. Portanto, é possível que a estimulação combinada da sinalização de citocinas tenha melhor efeito imunomodulatório na CanL do que a estimulação da sinalização de uma única citocina.

Os atuais tratamentos disponíveis para a CanL promovem redução da carga parasitária, cura clínica, diminuem a infecciosidade, melhoram a qualidade e aumentam a expectativa de vida [24,87,88]. Entretanto, ao final da terapia, a maioria dos cães tratados tem recidiva tornando-se novamente potenciais fontes de transmissão de *L. infantum* para cães saudáveis e humanos [24,88]. Assim, visando o desenvolvimento de métodos imunoterápicos para CanL, citocinas recombinantes caninas (IL-12, IL-2, IL-15 e IL-7) e o receptor solúvel de IL-10R1 (sIL-10R1), com atividade antagonista, foram avaliados pela primeira vez em combinações (IL-12/IL-2, IL-12/IL-15, IL-12/casIL-10R1, IL-15/IL-7) ou isoladamente (sIL-10R1) quanto à capacidade imunomoduladora em PBMC de cães com leishmaniose.

2 CAPÍTULO 1 - COMBINED STIMULATION BY IL-12 AND IL-15 PROMOTES CELLULAR IMMUNE RESPONSE IN DOGS WITH LEISHMANIASIS VIA IFN-Y

Sidnei Ferro Costa[a], Vinícius Oliveira Gomes[a], Marilene Oliveira dos Santos Maciel[a], Larissa Martins Melo[a], Gabriela Lovizutto Venturin[a], Jaqueline Poleto Bragato[a], Gabriela Torres Rebech[a], Catiule de Oliveira Santos[b], Bárbara Maria Nascimento de Oliveira[b], Geraldo Gileno de Sá Oliveira[b¶], Valéria Marçal Felix de Lima[a¶*]

[a]Department of Clinics, Surgery and Animal Reproduction, São Paulo State University (UNESP), School of Veterinary Medicine, Araçatuba

[b]Laboratório de Patologia e Bio-Intervenção LAPEM, Instituto de Pesquisas Gonçalo Moniz, Fundação Oswaldo Cruz, Rua Waldemar Falcão, No. 121, Candeal, Salvador, Bahia, Brazil

[¶]These authors contributed equally to this work.

*Corresponding Author:

Department of Clinical, Surgery and Animal Reproduction, São Paulo State University (UNESP), School of Veterinary Medicine, Araçatuba

793 Clóvis Pestana St. - Jardim Dona Amélia

Zip Code: 16050-680 - São Paulo, Brazil

Phone Number: +55-18-36361422

Email: Sidnei Ferro Costa: sidnei-fcosta@hotmail.com

Valéria Marçal Felix de Lima: valeria.lima@unesp.br

PLOS Neglected Tropical Diseases – ANEXO A

2.1 Abstract

Domestic dogs are the main reservoir of *Leishmania infantum*, a causative agent of visceral leishmaniaisis (VL) in the Americas. There is an association between the number of human disease cases and rate of canine infection. Drugs currently available are not efficient to treat Canine Leishmaniasis (CanL), months after the treatment most dogs show disease relapse, therefore, development of new drugs or new therapeutic strategies should be sought. In CanL, sick dogs lack the ability mount a specific cellular immune response suitable to combat the parasite and manipulation of cytokine signaling pathway has the potential to become part of effective immunotherapeutic methods. In this study, canine recombinant cytokines (IL-12, IL-2, IL-15 and IL-7) and soluble receptor IL-10R1 (sIL-10R1), with antagonistic activity, were evaluated for the first time in combinations (IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-15/IL-7) or isolated (sIL-10R1) for their immunomodulatory capacity in peripheral blood mononuclear cells (PBMC) from dogs with leishmaniasis. All combinations of recombinant proteins tested were shown to improve lymphoproliferative response. Further, combinations of IL-12/IL-2 and IL-12/IL-15 promoted decrease in programmed cell death protein 1 (PD-1) expression in lymphocytes. These same combinations of cytokines and IL-12/sIL-10R1 induced IFN-y production by PBMC. Furthermore, the combination of IL-12/IL-15 led to an increased T-bet expression in lymphocytes. These findings encourage the use of IL-12 and IL-15 in future *in vivo* studies aiming to get polarization of cellular immune response in dogs with leishmaniasis, which may contribute to development of an effective treatment against CanL.

2.2 Author summary

Dogs are the major reservoirs of *Leishmania infantum*, a protozoan parasite that causes a human systemic lethal disease (Visceral Leishmaniasis, VL) and dogs in the Americas. Currently, the disease is often associated with extensive skin lesions and may be named canine leishmaniasis (CanL). The containment canine infection/disease may help controlling human VL. Drugs currently available in the market are not effective in the treatment of CanL, months after the treatment most dogs develop disease relapse, even if they are taken out of the endemic area. Developing of new drugs or alternative therapeutic strategies are necessary for

proper treatment of the canine disease. Dogs that acquire the parasite may develop disease or control the infection depending on the type of immune response that is mounted. Cellular immune responses leads to infection control and prevent the development of the disease. Some proteins called cytokines, which are produced mainly by cells of the immune system, can favor the development of cellular immune responses. In this study, several recombinant cytokines and/or one cytokine blocking protein have been tested, in combinations or isolated, for their ability to promote cellular immune response in dogs with leishmaniasis. Of all combinations of recombinant protein tested, one showed to be the best to favor cellular immune responses. The findings reported here encourage the use of a combination of two recombinant cytokines (IL-12/IL-15) in future immunotherapeutic trials for CanL.

2.3 Introduction

The zoonotic form of visceral leishmaniasis (VL) is caused by the obligate intracellular protozoan *Leishmania infantum* (syn. *L. chagasi*, in the Americas). VL is the most severe form of leishmaniasis and is fatal in 95% of untreated cases [1]. VL is widely distributed worldwide, occurring mainly in tropical and subtropical regions with approximately 300.000 new infections each year and an estimated 20.000 to 50.000 deaths [2]. In Brazil, the numbers of human disease cases has increased annually and, in 2017, 4.013 cases were reported, with 327 deaths [3]. Domestic dogs are considered the main reservoir of the parasite in urban areas [4]. In endemic areas, there is a correlation between prevalence of seropositive dogs and number of human cases of VL [5–7], suggesting that controlling infection and/or disease in dogs (CanL) may contribute to effectively curbing human disease [8].

The current treatments available for CanL have leishmanicide and leshmaniostatic effects [9] and lead to reduction of parasite load, infectiousness, and disappearance of clinical signs [10,11]. However, most dogs remain infected and experience disease relapse months after treatment withdrawal, and become again a source of parasites for other healthy dogs and humans [10]. Frequent disease relapses following currently available therapy suggest that new drugs or therapeutic approaches for CanL, for example, association of existing drugs with immunostimulants, should be sought [12].

Dogs resistant to infection by *L. infantum* develop an efficient cellular immune response (Th1) with simultaneous production of IFN-γ, IL-2, and IL-12 [13–15] and activation of leishmanicidal mechanisms in infected macrophages [16,17]. In contrast, dogs that are susceptible to the infection, mount an exacerbated humoral immune response (Th2) that may be accompanied by increased production of IL-10 [18]. In addition, susceptible dogs present increased expression levels of programmed cell death 1 (PD-1) and PD-1 ligands (PD-L1 and PDL-2), which are part of an immune inhibitory signaling pathway (a inhibitory immune checkpoint), in splenic cells [19]. Such heightened expression of PD-1 and PD-1 ligands may suppress lymphoproliferation and alter the production of Th1 cytokines and contribute to development of the disease [20]. Outcome of infection, caused by *L. infantum* in humans and dogs, relies largely on the type of adaptive immune response mounted (Th1 vs Th2) and cytokines that help to shape such responses [14,15,18,21–24]. Manipulations of certain cytokine signaling pathways may favor control over the parasite in infected individuals. Interestingly, even after mounting an adaptive immune response inefficient to combat *L. infantum*, allowing for the development of the disease, subsequent treatment with antimonials or amphotericin B lead most humans to reprogram their specific immune responses [22,23], keeping the parasite replication under control, and inhibiting disease recurrence.

Peripheral blood mononuclear cells (PBMC) from human patients with VL, stimulated with soluble *leishmania* antigens (SLA) in combination with recombinant human interferon gamma (rhuIFN-γ) and interleukin-2 (rhIL-2), present restoration of lymphoproliferative response [22]. Further, stimulation of PBMC with SLA together with rhuIL-12, or blocking signaling with anti-IL-10 and/or IL-4 antibodies, results in both restoration of lymphoproliferative response and production of IFN-γ [22,23,25]. Addition of rhuIL-15 to PBMC cultured with SLA promotes a decrease of IL-4 and an increase of IL-12 production [26]. Still, in humans with VL, blockade of IL-10 signaling by anti-IL10 antibodies induces increased levels of IFN-y in splenic cells [27]. In sick dogs naturally infected with *L. infantum*, treatment of PBMC with recombinant canine IL-12 (rcaIL-12) generates an increase in IFN-y mRNA expression and protein production [21,28] and a tendency to enhance lymphoproliferative response to SLA [21]. To the best of our knowledge, attempts to evaluate activities of IL-7 in humans or dogs with visceral leishmaniasis, have not yet been carried out. Murine IL-7,

especially when combined with IFN-y, shows strong leishmanicidal effect on autologous macrophages infected with *L. major* [29].

Although interfering with a single cytokine pathway, with agonistic or antagonistic molecules, can drive responses in cells of the immune system, simultaneous intervention in two or more cytokines signaling pathways may elicit stronger responses in these cells, even in low cytokine concentration settings [30–33]. Successful attempts to modify immune responses in human or dog PBMC by the use of combination of cytokines exhibiting additive or synergistic effects have already been performed. For instance, human IL-15 combined with IL-12 promotes higher levels of IFN-γ, as compared to IL-15 or IL-12 individually, and may generate effective responses to infections caused by intracellular parasites [34]. In addition, canine IL-12 and IL-2 together stimulate efficient production of IFN-γ, whereas IL-12 or IL-2 alone fail to induce a significant increase in IFN-y levels [35,36].

In this study, recombinant canine proteins (IL-12, IL-2, IL-15 or IL-7 or soluble IL-10 receptor with antagonistic activity) were evaluated for their capacity to modulate immune responses in PBMC from dogs with leishmaniasis. These recombinant proteins were assessed in combinations or isolated for the first time. Lymphoproliferation, exhibition of PD-1 on lymphocyte surface, production of IFN-γ and IL-10, and expression of T-bet and GATA3 were evaluated in lymphocytes following treatments. Recombinant proteins tested herein, showed activities potentially capable of stimulating macrophages to control replication or destroy *L. infantum,* which could have a positive impact for the development of immunotherapeutic protocols for CanL.

2.4 Methods

2.4.1 Screening of animals and sample collection

This study was approved by the Brazilian Collegiate Board for Ethical Animal Experimentation (COBEA), and received approval from the UNESP Institutional Committee for Animal Care and Use (Universidade Estadual Paulista Júlio de Mesquita Filho, Araçatuba, School of Veterinary Medicine - FMVA/Protocol # 00765-2017).

Five healthy dogs from Araçatuba, São Paulo, with negative results for the detection of *Leishmania* DNA by real time PCR, as well as complete blood counts

and mean serum biochemistry parameters within reference ranges (Table S2 and S3 and S4), were used as negative controls. A total of ten dogs were selected from the Araçatuba Zoonosis Control Center if they showed at least three of the following clinical signs characteristic of CanL: onychogryphosis, cachexia, ear-tip injuries, periocular lesions, alopecia, skin lesions or lymphadenopathy (Table S1).

Blood samples from both groups, healthy controls and diseased dogs, were collected in either tubes without EDTA, to obtain serum for the evaluation of biochemical profiles (Table S2) and to carry out indirect ELISA (Table S1) for the detection of anti-*Leishmania* antibodies [37], or in tubes containing EDTA for complete blood counts (CBC) (Table S3 and S4) and PBMC isolation. Real-time PCR for the detection of *Leishmania* DNA was performed in blood samples from the included dogs in accordance with a previously described protocol using a calibration curve with DNA from 10^2 to 10^7 *Leishmani*a promastigotes [38].

2.4.2 Isolation of peripheral blood mononuclear cells

PBMCs from healthy controls and sick dogs were isolated by gradient centrifugation using Histopaque 1077 (Sigma, USA) in accordance with the manufacturer's recommendations. Isolated cells were then washed three times in phosphate buffered saline (PBS, pH 7.2) and suspended in RPMI 1640 (Sigma, USA) supplemented with inactivated 10% fetal bovine serum (FBS) (Gibco, USA), 0.03% L-glutamine (Sigma, USA), 100 IU/mL penicillin (Sigma, USA) and 100 mg/mL streptomycin (Sigma, USA).

2.4.3 Production of canine recombinant cytokines and soluble IL-10R1 receptor

Under the number of the Instituto Gonçalo Moniz Biosafety Technical Commission CTNbio-CQB # 0111/99, canine recombinant IL-12 was produced using a baculovirus-insect cell system as previously described [36]. Recombinant canine IL-2, IL-7, and the soluble extracytoplasmic domain of the IL-10 receptor alfa chain (sIL-10R1) were also obtained using a baculovirus-insect cell system. Briefly, DNA constructs encoding in tandem the signal peptide of GP64, present in AcMNPV, either mature IL-2 [39] or IL-7 (Genebank, accession DQ845341), or the sIL-10R1 (Genebank, accession XM_005620306.1) polypeptide, followed by a spacer (only in IL-2 and IL-7 constructs) and His-tag. All DNA constructs were synthetized by GenScript using codons optimized for translation in *Trichoplusia ni* (Piscataway,

USA). Bacmids (AcBacΔCC-GP64-IL-2E6H, AcBacΔCC-GP64-IL-7E6H, and AcBacΔCC-GP64-sIL-10R16H) were generated and purified from the DH10BacΔCC *Escherichia coli* strain [36]. The human IL-10R1 extracytoplasmic domain (Genebank, accession NP_001549) was used as a model to identify the canine sIL-10R1 polypeptide chain using a freely available online tool (http://www.sbc.su.se/~miklos/DAS/). Viral stocks (AcBacΔCC-GP64-IL-2E6H, AcBacΔCC-GP64-IL-7E6H and AcBacΔCC-GP64-sIL-10R16H) were titrated using an end-point method in Sf-9 cells (Invitrogen, Carlsbad, USA), and recombinant protein production and purification was performed as previously described [36]. Recombinant canine IL-15 was generated in the BL21(DE3) pLysS *E. coli* strain (Invitrogen) transformed by a plasmid DNA construct (*pRSET-mcaIL-15-opt-3S*) following the manufacturer's instructions, and subsequently purified by affinity chromatography [35] then refolded in 100 mM Tris-HCl, 500 mM glycine, 1 mM oxidized glutathione and 10 mM reduced glutathione (pH 8.0). To produce the protein, a DNA construct was synthetized encoding mature canine IL-15 (Genebank, accession XM_844053) using codons optimized for translation in *E. coli* (GenScript). In addition, endotoxin concentrations from all purified recombinant proteins were determined using Limulus Amebocyte Lysate (Gel-clot Method, Pyrotell, USA) [40], with resultantly low levels of endotoxins (<0.03 EU of endotoxin per mg of protein). All purified recombinant proteins were confirmed by Western blot assays using anti-his antibodies. Biological activity was verified as follows: both IL-2 and IL-15 were found to induce CTLL-2 cell proliferation [36]; IL-12 in combination with IL-2 promoted IFN-γ production in canine PBMCs [35]; IL-7 and IL-15 both stimulated the proliferation of canine PBMCs. Finally, sIL-10R1 was also shown to inhibit the proliferation of canine IL-10-treated MC/9 murine mast cells (ATCC CRL 8306) [41].

2.4.4 Lymphoproliferation assay

To assess the proliferation of lymphocytes, PBMCs were stained with carboxyfluorescein diacetate succinimidyl ester (2.5 μM) (CFSE, CellTrace, Invitrogen, UK) for 10 min at 37°C, in accordance with the manufacturer's recommendations and a previously published protocol [42]. Stained PBMCs were cultured on sterile 96-well plates (1×10^6/mL) in RPMI 1640 medium (Sigma, USA), either alone (negative control) or with the following canine recombinant proteins: rcaIL-2 (IL-2) (2 ng/mL), rcaIL-7 (IL-7) (40 ng/mL), rcaIL-12 (IL-12) (20 ng/mL), rcaIL-

15 (IL-15) (20 ng/mL), rcasIL-10R1 (sIL-10R1) (4 µg/mL). These recombinant proteins were tested in the following combinations: IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-7/IL-15, or sIL-10R1 alone. Culturing was performed in the presence or absence of 20 µg/mL of soluble *L. infantum* antigens (SLA) (MHOM/BR/00/MERO2), as described by Lima et al., 2010 [43]. Cultures containing the mitogen phytohemagglutinin-M (PHA-M, 5 µL/mL) were used as positive controls. CFSE-unmarked PBMCs were used to verify CFSE staining. PBMCs were cultured for 5 days at 37°C under 5% CO2. Ten thousand events were acquired in a flow cytometer (BD C5 Accuri Flow Cytometer, USA) and data analysis was performed using BD Accuri C6 software, version 1.0 (BD Biosciences, CA, USA). A gate (R) was defined in the analysis to select cell populations with size and complexity similar to those of lymphocyte population and gate (M) for positive labeling (Fig. S1).

The assays were repeated in duplicate under the same conditions without CFSE staining. Supernatants were used to determine IFN-y and IL-10 levels by capture ELISA (R&D Systems, USA) following manufacturers recommendations. Pelleted cells were used to determine the expression of PD-1 and T-bet and GATA3 transcription factors by flow cytometry (described below).

2.4.5 Flow cytometric analysis for labeling PD-1, T-bet and GATA3 in PBMC

To detect PD-1 expression, PBMC were suspended in PBS containing 1% bovine serum albumin, 0.1% azide and 20% fetal bovine serum, for Fc receptor blockade (FcR). The cells were then mixed with PE-conjugated monoclonal antibodies anti-Human CD279 (PD-1) [19,20] or control isotype (BD Pharmigen, USA) following the manufacturer's instructions. Ten thousand events were acquired on channel FL2 of a flow cytometer and data analysis was performed as described in the lymphoproliferation assay (Fig. S2).

To evaluate T-bet and GATA3 expression, PBMC were fixed and permeabilized with commercial buffer (eBioscience Bioscience, USA) according to manufacturer's instructions. Cells were mixed with FITC conjugated anti-human monoclonal antibody T-bet (R&D Systems) and with PE conjugated anti-human monoclonal GATA3 (R&D Systems) or control isotypes (R&D Systems), following the manufacturer's instructions. The similarity of human (GenBank, accession #NP_037483 and CAA38877) and canine (XP_548164 and XP_005617214) T-bet and GATA3 proteins was 93 and 96% respectively. Ten thousand events were

acquired on channel FL1 and FL2 and cytometric analysis was performed as described in the lymphoproliferation assay (Fig. S3).

2.4.6 Statistical analysis

Statistical analysis was performed using the GraphPad Prism 6 software (GraphPad Software, Inc., La Jolla, CA, USA). Data was tested for normality (Shapiro-Wilk test). To compare values corresponding to lymphoproliferation, expression of PD-1 of T-bet and GATA3 and levels concentration of IL-10 and IFN-y within groups, Friedman test with Dunn's post-test was used. Results were considered significant when $p < 0.05$.

2.5 Results

2.5.1 Description of clinical and laboratory findings of the animals used in the study

Dogs selected from Araçatuba Zoonosis Control Center exhibited at least three signs compatible with CanL which included onychogryphosis and skin lesions (in 7 out of 10 animals), lymphadenopathy (6 out of 10), periocular lesions and cachexia (5 out of 10), alopecia (4 out of 10), and ear-tip lesions (3 out of 10). Negative control animals showed no clinical manifestations (Table S1). In each one of the 10 sick dogs and none of 5 negative control dogs, anti-*Leishmania* antibodies (ELISA OD, mean ± standard deviation, sick dogs: 0.88 ± 0.38 and negative control dogs: 0.10 ± 0.05, Table S1) (cut-off value: 0.27) and *Leishmania* DNA (real-time PCR median CT value: 27.0) (*Leishmania* DNA calibration curve CT values range: 13.2 to 33.7). Moreover, sick dogs presented a statistically significant reduction in RBC counts, hematocrit, hemoglobin concentration, serum albumin concentration, albumin/globulin ratio, and an increase in serum globulin concentration, as compared to negative controls dogs (Tables S2 and S3). Based on clinical signs and laboratory findings, sick dogs manifested leishmaniasis in clinical stage II, according to Solano-Gallego and collaborators [44].

2.5.2 Combinations of canine recombinant cytokines and/or soluble IL-10R1 receptor induce lymphoproliferation in naturally infected sick dogs

In CanL, dogs lack the ability to mount lymphoproliferative responses after stimulation of PBMC with *Leishmania* antigens [14,45,46]. In the attempt to develop protocols to restore lymphoproliferative responses in such dogs, combinations of IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-15/IL-7 or sIL-10R1 alone were tested. PBMC from healthy dogs or dogs with leishmaniasis were cultured in the absence or presence of SLA in combination or not with the recombinant proteins or just with PHA for 5 days and the MFI of CFSE-labeled lymphocytes was determined under each condition. Reduction in CFSE-fluorescence in lymphocytes kept in each condition was used to determine cell proliferation, as compared fluorescence in lymphocytes maintained in culture medium alone [42]. In healthy dogs, lymphoproliferation occurred when PBMC were cultured with PHA or the combination of IL-12/IL-15 in absence or presence of SLA (Fig 1A). In *Leishmania*-infected sick dogs, although CFSE-labeled lymphocytes cultured with PHA showed some reduction in MFI, which was not statistically significant (Fig 1B). Interestingly, lymphocytes from sick dogs displayed proliferative response when cultured in each one of the combinations of recombinant proteins tested (IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-15/IL-7) or sIL-10R1 alone, independently of presence of SLA in culture (Fig 1B).

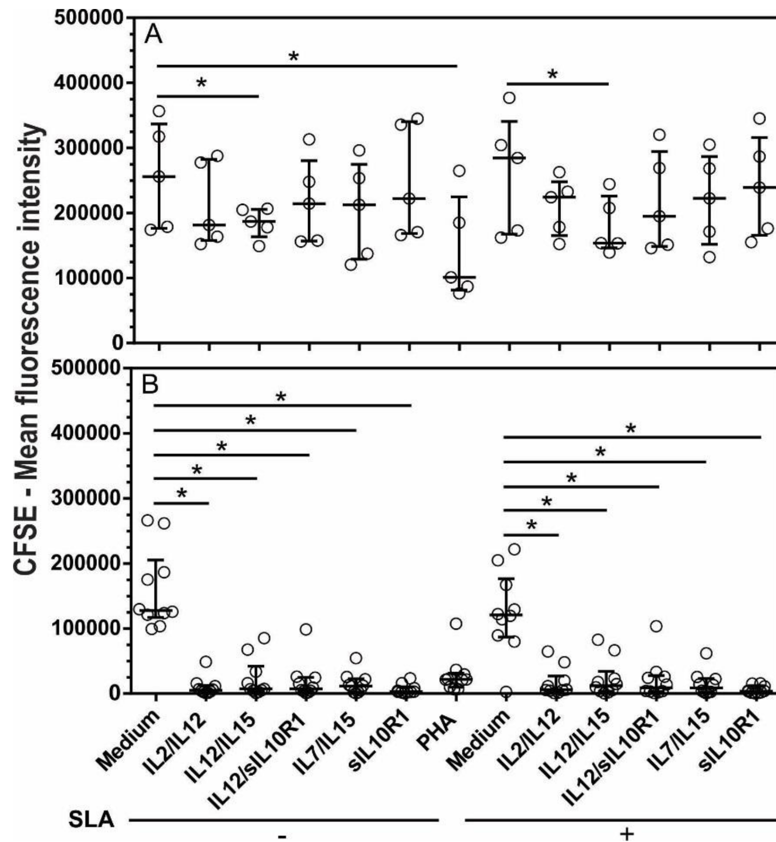

Fig 1. Evaluation of lymphoproliferation in healthy control dogs or dogs with leishmaniasis following stimulation with canine recombinant proteins. CFSE-labeled PBMC were cultured for 5 days with medium alone (Medium) or medium with Soluble Leishmania Antigens (SLA) or phytohemagglutinin (PHA). In addition, PBMC cultured with medium alone or with SLA were stimulated with recombinant canine proteins, in combinations, IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-15/IL-7, or sIL-10R1 isolated. CFSE-labeled lymphocyte mean fluorescence intensities were assessed by flow cytometry. Lymphocytes from healthy dogs (n=5) **(A)** and dogs with leishmaniasis (n=10) **(B)**. Horizontal bars represent median and interquartile 25 and 75 values of MFI. Each symbol represents datum of a single dog. Asterisk indicates significant differences (Friedman test with Dunn's multiple comparison, * $p < 0.05$).

2.5.3 Combination of IL-12/IL-2 or IL-12/IL-15 promotes decrease in lymphocyte PD-1 expression

Inability of lymphocytes from dogs with leishmaniasis to proliferate and produce Th1 cytokines, may be associated, at least in part, with an increase in PD-1 expression, which promotes apoptosis, during a progression of infection [19,20]. To assess if an interference with cytokine signaling could lead to reduction in expression of inhibitory molecules of the immune system in sick dogs, PBMC were cultured with or without SLA and/or combinations of recombinant canine cytokines, IL-12/sIL-10R1 or sIL-10R1 alone, and lymphocyte PD-1 expression was evaluated by flow cytometry. Lymphocytes from healthy dogs did not change PD-1 expression regardless of the addition of canine recombinant proteins to cultures with or without SLA (Fig 2A). However, lymphocytes from sick dogs exhibited a significant decrease

in PD-1 expression when the combinations of IL-12/IL-2 or IL-12/IL-15, in the absence or presence of SLA, were used in the cultures (Fig 2B). There was also some decrease in PD-1 expression while using IL-12/sIL-10R1, with or without SLA, but this was not statistically significant (Fig 2B).

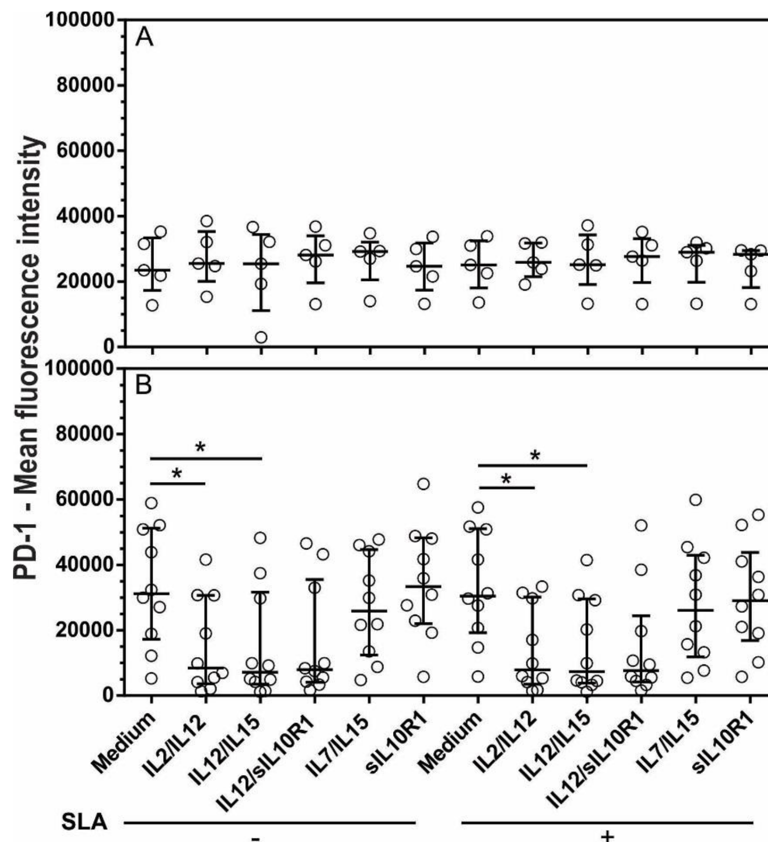

Fig 2. Evaluation of PD-1 expression in lymphocytes from healthy control dogs and dogs with leishmaniasis after culturing the cells with canine recombinant proteins. PBMC were cultured for 5 days without any treatment (Medium) and in the presence of immunomodulatory canine recombinant proteins, in combination or not with Soluble Leishmania Antigens (SLA) and after this, labeled with PE-conjugated monoclonal antibodies Anti-Human CD279 (PD-1) and the mean fluorescence intensity was assessed by flow cytometry.**(A)** Lymphocytes of healthy dogs (n=5). **(B)** Lymphocytes of dogs with leishmaniasis (n=10). Horizontal bars represent median and interquartile 25 and 75 values of MFI. PBMC of the groups of dogs cultured in certain condition and symbols represent data of a single dog. Asterisk indicates significant differences (Friedman test with Dunn's multiple comparison, * $p < 0.05$).

2.5.4 Combination of IL-12/IL-15 induces an increase in T-bet without altering GATA-3 expression

Progression of leishmaniasis in dogs is associated with an incapacity to establish an effective cellular immune response (Th1) and by setting up an exacerbated humoral immune response (Th2) and/or development of an immunosuppressive state [8,47]. Immune system programming to generate Th1 or Th2 cell subsets involves expression of the master transcription factors T-bet and GATA3, respectively [48,49]. To identify conditions capable of modifying T helper cell

differentiation programming in dogs with leishmaniasis, PBMC were cultured with recombinant canine proteins with or without SLA and lymphocyte expression of T-bet and GATA3 were assessed by flow cytometry.

In PBMC from healthy dogs, the combinations of IL-12/IL-2 or IL-12/IL-15 without SLA generated a significant increase in lymphocyte T-bet expression that was inhibited by addition of SLA to the cultures (Fig 3A). On the other hand, in PMBC from sick dogs, IL-12/IL-15 induced a significant increase in lymphocytes T-bet expression, both in the absence or presence of SLA in the cultures (Fig. 3B). The other recombinant proteins tested, in combination or isolated, did not affect T-bet expression in the lymphocyte. None of the recombinant proteins evaluated, in combination with SLA or not, significantly altered the expression of GATA3 in lymphocytes of healthy or sick dogs (Fig 3C and 3D).

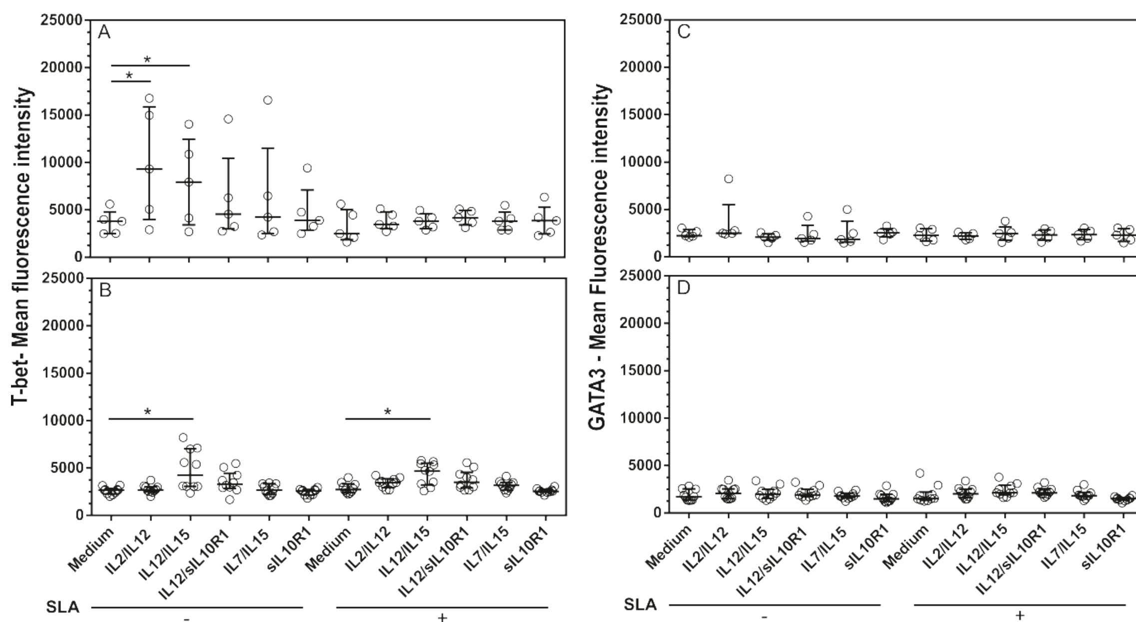

Fig 3. Effect of immunoregulatory canine recombinant proteins in combination (IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-15/IL-7) or isolated (sIL-10R1) on the expression of the transcription factors T-bet and GATA3 of healthy dogs and with leishmaniasis. PBMC were cultured for 5 days without any treatment (Medium) and in the presence of immunomodulatory canine recombinant proteins in combination or not with Soluble Leishmania Antigens (SLA) and after this, labeled with FITC conjugated anti-human monoclonal antibody T-bet and with conjugated PE anti-human monoclonal GATA3 and the mean fluorescence intensity was assessed by flow cytometry. **(A and C)** Expression of T-bet and GATA3 respectively in lymphocytes of healthy dogs (n=5). **(B and D)** expression of T-bet and GATA3 respectively in lymphocytes of dogs with leishmaniasis (n=10). Horizontal bars represent median and interquartile 25 and 75 values of MFI. PBMC of the groups of dogs cultured in certain condition and symbols represent data of a single dog. Asterisk indicates significant differences (Friedman test with Dunn's multiple comparison, * $p < 0.05$).

2.5.5 Combinations of IL-12/IL-2, IL-12/IL15 or IL-12/sIL-10R1 provoke an increase in IFN-γ without altering IL-10 production

Driving long-term specific Th1 and preventing Th2 immune responses and/or an immunosuppressive state, may be useful in the treatment of CanL [8,18,47]. To determine if the recombinant proteins assessed herein show an impact in the production of cytokines mediating Th1 or immunosuppression in dogs with leishmaniasis, PBMC were cultured with combinations of IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-15/IL-7 or sIL-10R1 alone, in the absence or the presence of SLA, and levels of IFN-γ and IL-10 in culture supernatants were measured by capture ELISA. The combinations of IL-12/IL-2 and IL-12/IL-15 induced a significant increase in IFN-γ levels in PBMC from healthy dogs (Fig 4A) and dogs with leishmaniasis (Fig 4B), both in the absence or presence of SLA. In addition, in sick dogs, the combination of IL-12/ sIL-10R1 promoted a significant increase in IFN-γ production, only in the presence of SLA (Fig 4D).

In PBMC from healthy dogs or sick dogs, none of the combinations IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-15/IL-7 or sIL-10R1 alone, without or with SLA, caused a significant change in the levels of IL-10 (Fig 4C and D). Interestingly, there was a tendency for an increase in IL-10 levels in cell culture supernatant of sick dogs, especially in the presence of SLA in the cultures (Fig 4D). Finally, in PBMC from healthy dogs, detection of IL-10 was minimal when sIL-10R1 was used in culture in combination or not with IL-12, though this was not statistically significant

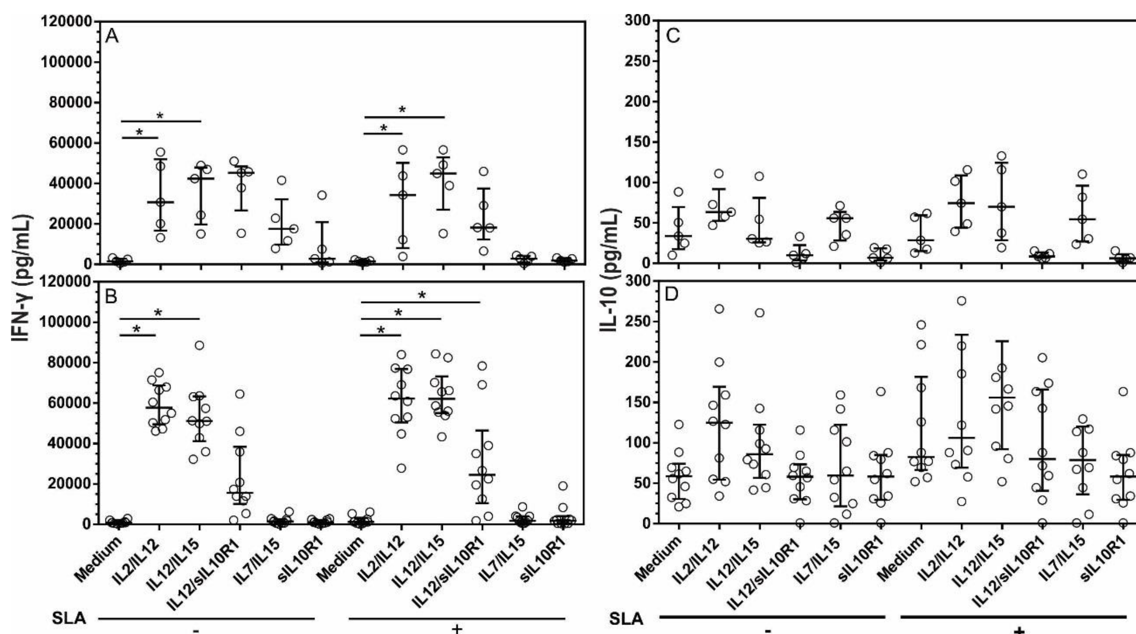

Fig 4. Effect of immunoregulatory canine recombinant proteins in combination (IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-15/IL-7) or isolated (sIL-10R1) on the levels of IFN-γ and IL-10 in the culture supernatant from PBMC of healthy dogs and with leishmaniasis. PBMC were cultured for 5 days without any treatment (Medium) and in the presence of immunomodulatory canine recombinant proteins, in combination or not with Soluble Leishmania Antigens (SLA) and after this, IFN-γ and IL-10 concentration in the culture supernatant of the PBMC was determined by capture ELISA. **(A and C)** Production of IFN-γ and IL-10 respectively by the PBMC of healthy dogs (n=5). **(B and D)** Production of IFN-γ and IL-10 respectively by the PBMC of dogs with leishmaniasis (n=10). Horizontal bars represent median and interquartile 25 and 75 levels in pg/mL. PBMC of the groups of dogs cultured in certain condition and symbols represent data of a single dog. Asterisk indicates significant differences (Friedman test with Dunn's multiple comparison, * $p < 0.05$).

2.6 Discussion

Dogs naturally infected with *Leishmania infantum* exhibit an inability to mount a specific effective adaptive immune response, the so-called Th1 immune response with long-term memory. This is true in animals that progress in disease development, as well as in those that, after being treated with currently available drugs, exhibit disease relapse. In order to develop immunotherapeutic protocols, the present study evaluated a set of canine recombinant proteins capable of interfering with cytokine signaling pathways to determine the modulation of cellular responses in dogs with CanL.

Dogs with CanL develop T cell exhaustion, involving both $CD4^+$ and $CD8^+$ T lymphocytes [50], and thereby lose the ability to perform effector functions. In these animals, the capacity for lymphocytes to proliferate intensely under antigenic stimulation is one of the first functions lost [51]. To date, few authors have investigated the conditions capable of promoting lymphoproliferative responses against *Leishmania* antigens in CanL dogs [21,50]. In this study, several different combinations of recombinant proteins (IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-15/IL-7 or sIL-10R1 alone) were shown to promote lymphoproliferation in dogs naturally infected with *L. infantum*. Lymphoproliferation was found to occur following incubation with IL-12/IL-2, IL-12/IL-15, IL-12/sIL-10R1, IL-15/IL-7, or sIL-10R1 alone, regardless of whether SLA was added to cultures or not. A probable explanation for this phenomenon could be the presence of *Leishmania* parasites within the PBMCs used in experimentation [52], more precisely in antigen-presenting cells (APCs), which does not require the addition of SLA to obtain an anti-parasitic proliferative response. In fact, *Leishmania* parasites were detectable in every blood sample from each diseased dog (Table S1).

Previously, some authors reported a significant increase or a tendency to increase lymphoproliferative response in dogs naturally infected by *L. infantum* by addition of IL-12 [21] or anti-IL-10 antibodies (antagonistic antibodies) and LSA to cultures of PBMC [50]. The data presented here also indicate that IL-12 and IL-10 blockade (by sIL-10R1) may contribute to the generation of lymphoproliferative response in CanL dogs. Human cytokines homologous to canine cytokines evaluated herein are capable of stimulating the proliferation of resting and/or activated peripheral blood lymphocytes (PBLs) in healthy individuals. Depending on circumstances, such as cell activation status, cytokines concentration and microenvironment, each of these cytokines preferentially stimulates certain subpopulations of lymphocytes. Human IL-12, IL-2, IL-15 and IL-7, tend to stimulate proliferation of: a) NK, CD4 and $CD8^+$ T cells, b) T and NK cells, c) NK cells, naïve, effector memory (TEM) and central memory (TCM) T cells, and d) naïve T cells, TCM and TEM cells, respectively [53–58]. In future experiments, the subpopulations of lymphocytes stimulated to expand by the recombinant canine proteins tested here will be determined.

T cell exhaustion state results from inhibitory signals received by these cells. PD-1 cell surface receptor and its PD-L1 and PD-L2 ligands constitute main signaling pathway of this process. PD-1 binding to its ligands leads to recruitment of SHP-2 phosphatase, dephosphorylation of molecules such as PI3K and Zap70, and cell inactivation [59]. PD-1 protein expression becomes progressively higher as the exhaustion process intensifies in T cells [51]. Interestingly, none of the combinations of recombinant proteins or sIL-10R1 caused significant alteration in PD1 expression of lymphocytes from the healthy negative control dogs. However, the IL-12/IL-2 and IL-12/IL-15 elicited a significant decrease in PD-1 protein expression in lymphocytes from dogs with CanL. In these animals, IL-12/sIL-10R1 and sIL-10R1, provoked a reducing trend or no changing in expression of PD-1, respectively. These data suggest that, under the conditions in which recombinant proteins were tested, IL-12 was responsible for promoting the reduction in PD-1 expression. In a previous study, stimulation with IL-12 was shown to cause reduction in PD-1 and increase in T-bet expression, and arouse effector function in $CD8^+$ T lymphocytes, rescuing these cells from exhaustion in human patients infected with hepatitis B virus [60]. IL-12 probably modulates PD-1 expression through induction in T-bet transcription factor [61,62].

Depending upon combinations of several stimuli, including from TCR signaling, cytokines, costimulatory, co-inhibitory, adhesion molecules, and/or chemokines, naïve T lymphocytes may initiate one of various gene expression programs, resulting in cell differentiation and acquisition of certain functional capacities [63,64]. The differentiation genetic programs of Th1 and Th2 lymphocytes involves the expression of the master transcription factors T-bet and GATA3 [65,66] respectively. These transcription factors negatively modulate one another [67]. To date, there are a limited number of reports on the expression of T-bet or GATA-3 in dogs. To the best of our knowledge, this is the first study to describe expression of T-bet and GATA-3 proteins in CanL dogs. Interestingly, IL-12/IL-2 and IL-12/IL-15 promoted an increase in T-bet expression in healthy negative control dog lymphocytes, which was inhibited by the addition of SLA, suggesting some suppressive SLA activity.

In lymphocytes from dogs with CanL, only IL-12/IL-15 induced an increase in T-bet expression, independently of addition of SLA in the cultures. IL-15 may have elicited an enhancement in IL-12Rβ1 expression [68] resulting a higher level of IL-12 signaling, and as consequence, increased production T-bet. None of the recombinant canine or sIL-10R1 protein combinations modified the expression of GATA-3 in healthy control dogs or dogs with leishmaniasis. Expression of T-bet and GATA-3 mRNA was previously evaluated in spleen [69] and skin [70] from dogs with leishmaniasis. In these animals, expression of T-bet and GATA-3 mRNAs in spleen and skin was shown to be increased or unchanged, respectively [69,70] compared to uninfected animals. In our study, no significant differences were found in T-bet and GATA-3 protein expression in PBLs between dogs with CanL and healthy negative controls.

IFN-γ and IL-10 are able to stimulate or attenuate defense mechanisms in macrophages and contribute to control or favor infection in dogs naturally inoculated with *L. infantum* [18]. Several cells of the innate or adaptive immune system can secrete IFN-γ or IL-10 [71,72]. Among the cells of the adaptive immune system, Th1 and CD8 T lymphocytes are the IFN-γ-producing cells [71]. On the other hand, several subpopulations of lymphocytes, including Th1, Th2 and Th1y cells can produce IL-10 [72]. The negative control dogs exhibited significant increase in IFN-γ production when PBMC were stimulated with IL-12/IL-2 or IL-12/IL-15 in the absence and presence of SLA, respectively. However, PMBCs from these animals also

showed a tendency to increase IFN-γ production following incubation with: a) IL-12/sIL-10R1 along to addition of SLA or not, or b) IL-7/IL-15 without addition of LSA, though not statistically significant.

Interestingly, dogs with CanL also exhibited significant increase in IFN-γ production by PBMCs cultured with IL-12/IL-2 or IL-12/IL-15 combination with addition of LSA or not. Further, PBMCs of these animals produced higher levels of IFN-γ after stimulation with IL-12/sIL-10R1 and addition of SLA in the cultures. Our findings suggest that IL-12 associated with an additional stimulus may lead to efficient production of IFN-γ in dogs [35,36] including with CanL, and is consistent with the data reported by Ayali et al., 2005 [21], that showed significant increase in IFN-γ production in CanL dog PBMCs by stimulation with IL-12 and SLA. Moreover, the data showing that IL-12/sIL-10R1 or sIL-10R1 have only limited effect and no effect in promoting increase in IFN-γ production, respectively, corroborate the findings of Esch et al., 2014, who reported that blockade of IL-10 signaling does not boost IFN-γ synthesis in either $CD4^+$ or $CD8^+$ T lymphocytes in dogs with leishmaniasis [50].

Healthy negative controls and dogs with leishmaniasis revealed an increase in IL-10 production, though statistically insignificant, after stimulation with either IL-12/IL-2, IL-12/IL-15 in cultures in which SLA were added or not. Curiously, sIL-10R1 alone or IL-12/sIL-10R1 virtually prevented detection of IL-10 in cultures from negative control dogs. It is possible that sIL-10R1 has bound to IL-10 and, by doing so, prevented the cytokine binding to antibodies in the detection assay. In future experiments, evaluation of production of IL-10 will be carried out by intracellular detection using flow cytometry.

In conclusion, among the various combinations of canine recombinant proteins and casIL-10R1 alone capable of interfering with cytokine signaling pathways tested, IL-12/IL-15 proteins have shown to promote significant lymphoproliferative response, increase in T-bet without altering GATA-3 expression, increase in IFN-γ without changing IL-10 production. These data suggest that IL-12/IL-15 may enhance cellular immune responses and contribute to reprogramming of immune responses, being potentially useful developing effective treatments to CanL.

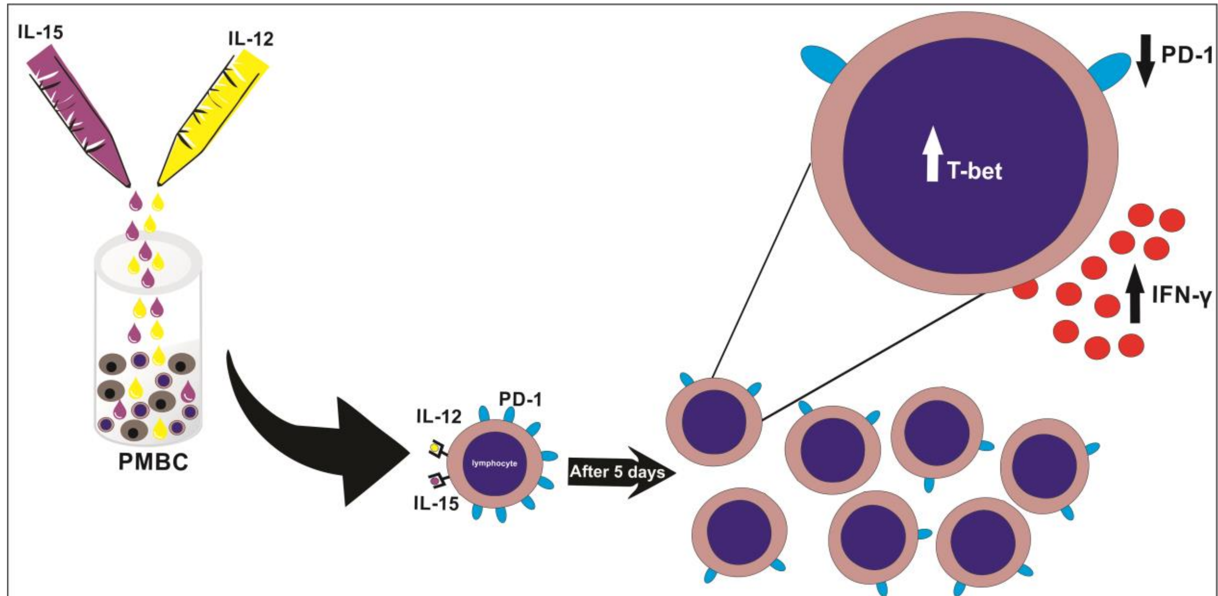

Fig 5. Graphical abstract. Effects of combined stimulation of IL-12 and IL-15 restores lymphoproliferative response, decreases PD-1 expression and increases T-bet expression and IFN-γ production.

2.7 Acknowledgment

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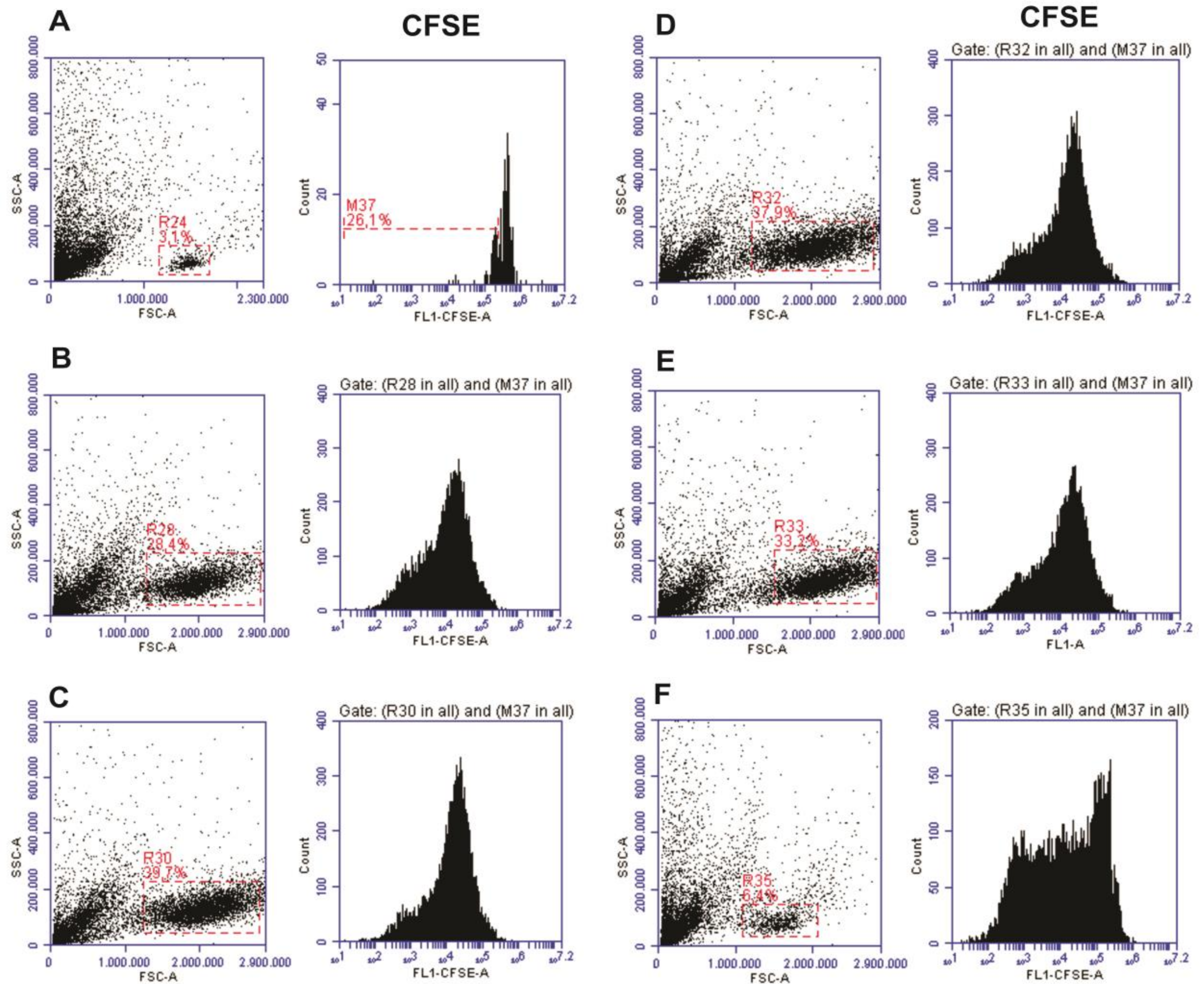
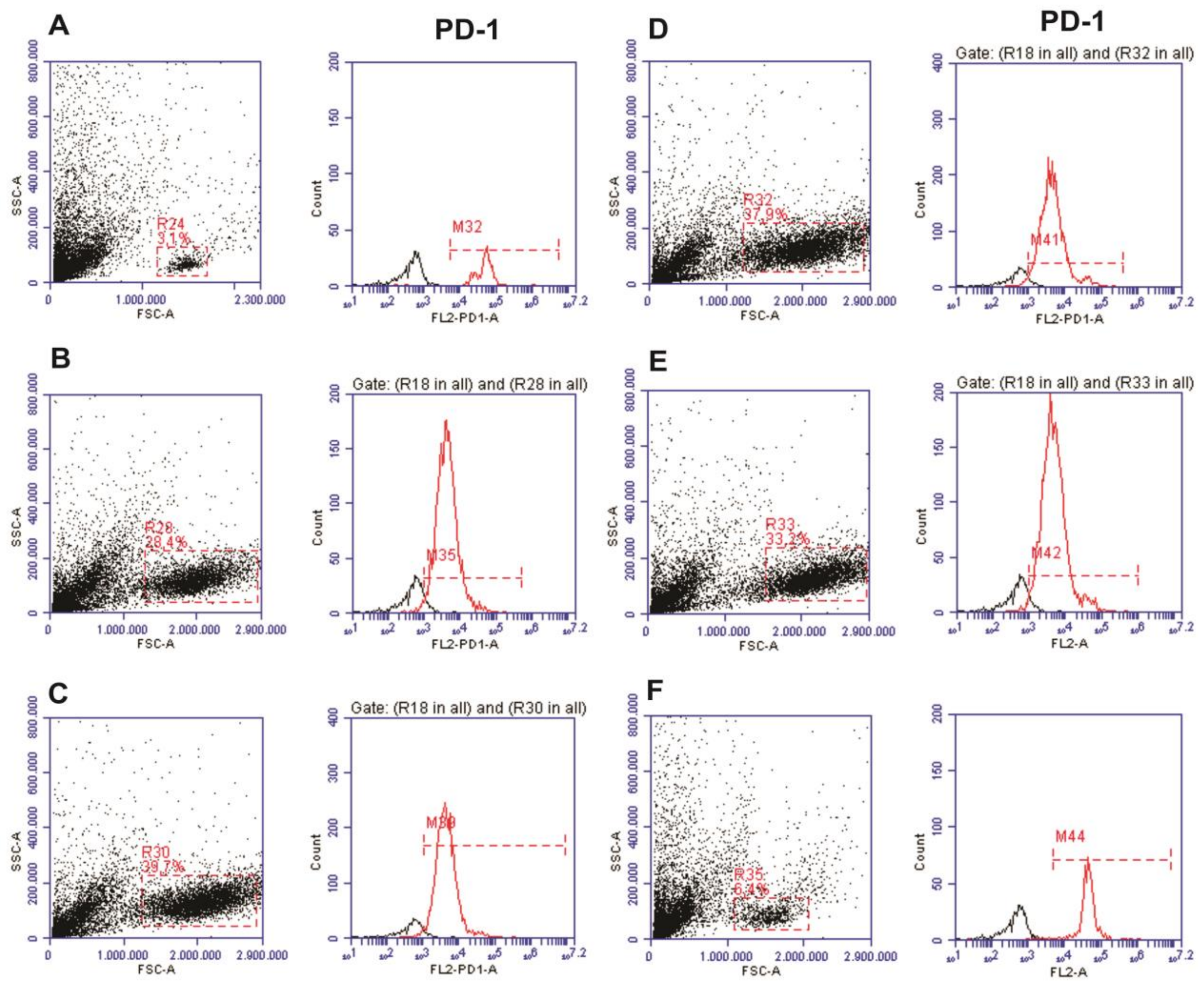
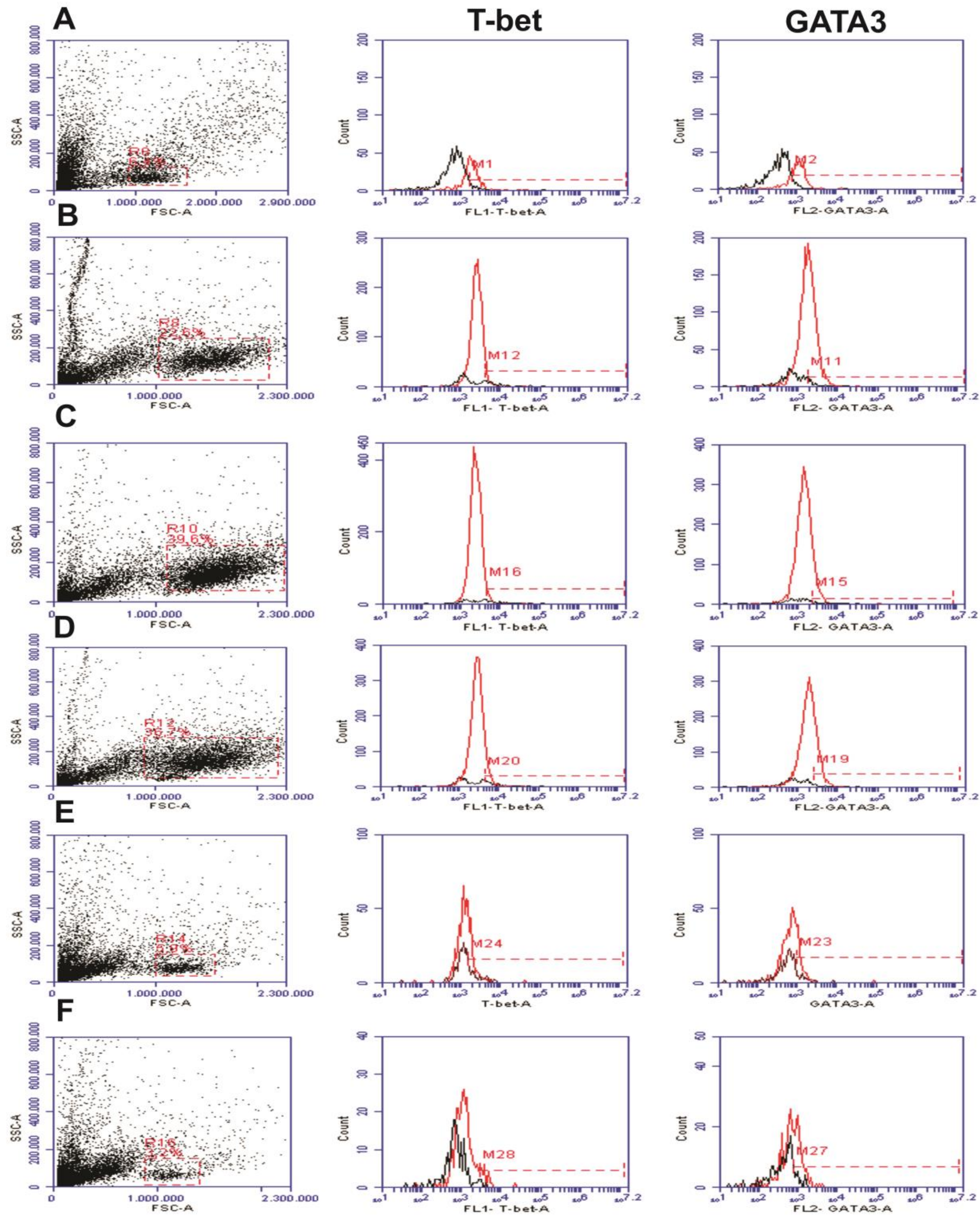
2.8 Support information



S1 Fig. Histogram representative of flow cytometric analysis of lymphoproliferative response. PBMC were cultured for 5 days without any treatment (Medium) **(A)**, in the presence of the phytohemagglutinin mitogen (PHA) or combined immunomodulatory recombinant proteins IL-2/IL-12 **(B)**, IL-12/IL15 **(C)**, IL-12/sIL-10R1 **(D)**, IL-7/IL-15 **(E)** and alone sIL-10R1 **(F)** in combination or not with Soluble Leishmania Antigen (SLA) and, after this, mean intensity fluorescence of CFSE was assessed by flow cytometry. Gate in R marking of lymphoid cells, gate in M marking of lymphoproliferation and PD1, peak red PD1 positive and peak black Iso-Control. All experiments included 15 dogs, 5 healthy dogs and 10 dogs with leishmaniasis.



S2 Fig. Histogram representative of the flow cytometric analysis of the labeling of PD-1. PBMC were cultured for 5 days without any treatment (Medium) **(A)**, in the presence of the combined immunomodulatory recombinant proteins IL-2/IL-12 **(B)**, IL-12/IL15 **(C)**, IL-12/sIL-10R1 **(D)**, IL-7/IL-15 **(E)** and isolated sIL-10R1 **(F)** in combination or not with Soluble Leishmania Antigens (SLA) and after this, labeled with PE-conjugated monoclonal antibodies: Anti-Human CD279 (PD-1) and the mean intensity fluorescence was assessed by flow cytometry. Gate in R marking of lymphoid cells, gate in M marking of lymphoproliferation and PD1, peak red PD1 positive and peak black Iso-Control. All experiments included 15 dogs, 5 healthy dogs and 10 dogs with leishmaniasis.



S3 Fig. Histogram representative of the flow cytometric analysis of the labeling of T-Bet and GATA3 transcription factors. PBMC were cultured for 5 days without any treatment (Medium) **(A)**, in the presence of the combined immunomodulatory recombinant proteins IL-2/IL-12 **(B)**, IL-12/IL15 **(C)**, IL-12/sIL-10R1 **(D)**, IL-7/IL-**15 (E)** and isolated sIL-10R1 (**F)** in combination or not with Soluble Leishmania Antigens (SLA) and after this, labeled with FITC conjugated anti-human monoclonal antibody T-bet and with PE conjugated anti-human monoclonal GATA-3 and the mean intensity fluorescence was assessed by flow cytometry. Gate in R marking of lymphoid cells, gate in M marking of lymphoproliferation and PD1, peak red PD1 positive and peak black Iso-Control. All experiments included 15 dogs, 5 healthy dogs and 10 dogs with leishmaniasis.

Table S1. Clinical findings, detection of anti-*Leishmania* antibodies and *Leishmania* DNA.

Dog	CanL			Control		
	Clinical findings	**ELISA OD values***	**Real-time PCR CT values****	**Clinical findings**	**ELISA OD values***	**Real-time PCR CT values****
1	Alopecia, lymphadenopathy, periocular lesions, onychogryphosis	0.956	27.89	None	0.143	BCT
2	Cachexia, periocular lesions, onychogryphosis,skin lesions	0.506	26.38	None	0.129	BCT
3	Cachexia, ear-tip injuries, periocular lesions, onychogryphosis	1.131	32.11	None	0.055	BCT
4	Alopecia, skin lesions, lymphadenopathy	1.238	29.34	None	0.041	BCT
5	Cachexia, onychogryphosis periocular lesions, skin lesions	0.355	26.83	None	0.121	BCT
6	Alopecia, cachexia, skin lesions, lymphadenopathy	0.711	27.11	-	-	-
7	Cachexia, lymphadenopathy, onychogryphosis, skin lesions	1.315	24.23	-	-	-
8	Ear-tip injuries, lymphadenopathy, onychogryphosis, skin lesions	0.929	26.46	-	-	-
9	Lymphadenopathy, onychogryphosis, skin lesions	0.368	25.04	-	-	-
10	Alopecia, onychogryphosis, ear-tip injuries, periocular lesions	1.323	31.78	-	-	-
Mean± SD	**-**	**0.883± 0.379**	**27.72± 2.63**	**-**	**0.098± 0.046**	**-**

CanL: canine leishmaniasis. Control: healthy negative control. OD: optical density. *ELISA cut-off value: OD 0.270. CT: threshold cycle. BCT: below CT value after 40 amplification cycles. **Real-time PCR calibration curve carried out with DNA from 10^2 to 10^7 *Leishmania* promastigotes gave CT values from 13.23 to 33.74. Real-time PCR amplification specificity was confirmed by determination of melting point in each reaction.

Table S2. Sera biochemical profile.

Dogs #		Albumin	Globulin	Total protein	Uric Acid	Creatinine	Urea	ALT	AST	GGT
	Ref. values	**26-33 g/L**	**24-44 g/L**	**54-71 g/L**	**0-2 mg/dl**	**0.5-1.5 mg/dL**	**1.67-8.33 mmol/L**	**21-102 UI/L**	**23-66 UI/L**	**1.2-6.4 UI/L**
CanL 1		16.4	68.7	85.1	0.6	0.9	3.8	63.0	63.2	2.3
CanL 2		8.6	43.2	51.9	2.4	0.5	6.6	54.1	65.6	7.9
CanL 3		15.6	55.2	70.9	0.5	0.7	5.3	84.3	66.7	1.0
CanL 4		20.9	62.8	83.7	0.7	1.1	5.0	36.6	65.3	0.7
CanL 5		8.8	65.9	74.7	0.8	0.7	7.3	120.6	116.3	0.7
CanL 6		11.8	84.4	96.2	3.7	0.8	10.6	192.1	195.6	0.2
CanL 7		13.5	67.2	80.7	2.0	1.1	10.0	81.0	69.3	0.5
CanL 8		14.1	65.7	79.8	5.3	0.5	5.4	38.8	42.6	4.3
CanL 9		9.9	32.9	42.8	2.2	0.7	3.1	31.5	40.7	8.6
CanL 10		12.4	62.6	75.0	1.7	0.6	2.7	24.1	44.2	4.2
	Mean± SD	**13.2± 3.8[a]**	**60.9± 14.3[a]**	**74.1± 15.9[a]**	**2.0± 1.5[a]**	**0.8± 0.2[a]**	**6.0± 2.7[a]**	**72.6± 51.3[a]**	**77.0± 46.9[a]**	**3.0± 3.1[a]**
Control 1		29.3	30.6	60.0	2.0	1.4	9.2	77.4	46.0	2.5
Control 2		33.7	29.3	63.0	1.5	1.1	6.0	47.8	45.3	4.5
Control 3		29.1	23.9	53.0	0.9	1.1	4.2	52.3	29.1	2.4
Control 4		21.6	39.4	61.1	1.6	1.0	5.2	63.5	58.1	2.5
Control 5		30.1	30.6	60.7	1.6	1.3	7.6	20.1	43.1	2.8
	Mean± SD	**28.8± 4.4[b]**	**30.8± 5.6[b]**	**59.6± 3.8[b]**	**1.5± 0.4[a]**	**1.2± 0.2[a]**	**6.4± 2.0[a]**	**52.2± 21.3[a]**	**44.3± 10.3[a]**	**2.9± 0.9[a]**

CanL: canine leishmaniasis. Control: healthy negative control. ALT: alanine aminotransferase, AST: aspartate aminotransferase, GGT: gamma glutamyl transferase. a,b Equal letters in the same colum indicate no statistical difference using unpaired t-test

Table S3. Red blood cells parameters.

Dog #		RBC	Ht	Hemoglobin	MCV	MCHC
	Referenc e values	5.5-8.5 x10^{6}/μL	37-55 %	12-18 g/dL	60-77 fL	32-36 %
CanL 1		3.75	24.0	7.5	64.2	31.2
CanL 2		4.08	24.5	7.7	60.1	31.4
CanL 3		3.20	24.7	7.1	77.2	28.7
CanL 4		5.94	38.5	12.6	64.9	32.7
CanL 5		5.01	35.0	11.8	69.9	33.7
CanL 6		2.49	16.7	5.2	67.1	31.1
CanL 7		2.37	18.5	6.0	78.1	32.4
CanL 8		3.74	17.0	8.5	64.2	35.4
CanL 9		3.62	19.9	7.2	55.1	36.1
CanL 10		4.30	28.8	10.0	67.0	34.7
	Mean±SD	**3.85±1.08[a]**	**24.8±7.4[a]**	**8.4±2.4[a]**	**66.8±7.0[a]**	**32.7±2.3[a]**
Control 1		8.26	55.0	18.1	66.5	32.7
Control 2		7.21	53.8	18.8	74.7	34.9
Control 3		5.32	41.2	12.9	77.5	31.3
Control 4		7.43	52.7	15.4	76.4	35.9
Control 5		7.96	53.9	16.8	75.3	34.7
	Mean±SD	**7.24±1.15[b]**	**51.3±5.7[b]**	**16.4±2.3[b]**	**74.1±4.4[a]**	**33.9±1.9[a]**

CanL: canine leishmaniasis. Control: healthy negative control. RBC: red blood cells, Ht: hematocrit, MCHC: mean corpuscular hemoglobin concentration, MCV: mean corpuscular volume. a,b Equal letters in the same colum indicate no statistical difference using unpaired t-test

Table S4. White blood cells and platelets counts.

Dog #		Leukocytes	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Platelets
	Ref. values	**6-17 x10³/ µL**	**3,000-11,000/µL**	**1,000-4,800/µL**	**150-1,350/µL**	**150-1,250/µL**	**Rares/ µL**	**160-430 x10³/µL**
CanL 1		9.8	6.272	2.156	392	980	0	145
CanL 2		23.9	13.076	2.760	478	478	0	165
CanL 3		10.2	7.752	1.836	510	102	0	82
CanL 4		6.3	4.158	1.071	756	63	0	159
CanL 5		12.5	9.500	1.375	1.125	500	0	420
CanL 6		14.9	9.238	3.427	1.043	1.192	0	277
CanL 7		12.5	10.000	1.750	375	375	0	331
CanL 8		14.8	6.808	5.032	2.220	740	0	168
CanL 9		8.7	6.264	2.001	174	261	0	95
CanL 10		11.8	10.738	0.708	354	200	0	60
	Mean± SD	**12.5± 4.8[a]**	**8.381± 2.621[a]**	**2.212± 1.265[a]**	**743± 603[a]**	**489± 377[a]**	**0±0[a]**	**190± 116[a]**
Control 1		10.2	6.324	3.468	102	306	0	341
Control 2		8.8	6.160	1.408	880	352	0	296
Control 3		13.1	6.419	6.668	174	261	0	341
Control 4		8.4	4.480	1.024	512	384	0	211
Control 5		10.6	7.844	1.060	424	1.250	0	228
	Mean± SD	**10.2± 1.9a**	**6.245± 1.195a**	**2.726± 2.424[a]**	**418± 309[a]**	**261± 152[a]**	**0±0[a]**	**283± 61[a]**

CanL: canine leishmaniasis. Control: healthy negative control. a,b Equal letters in the same colum indicate no statistical difference using unpaired t-test

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ANEXO

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

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