UNIVERSIDADE FEDERAL DE MINAS GERAIS
INSTITUTO DE CIÊNCIAS BIOLÓGICAS
DEPARTAMENTO DE BIOQUÍMICA E IMUNOLOGIA

AVALIAÇÃO DE PROTEÍNAS RECOMBINANTES DE *Leishmania* NO DIAGNÓSTICO SOROLÓGICO E COMO CANDIDATAS A COMPOR UMA VACINA CONTRA AS LEISHMANIOSES

VIVIAN TAMIETTI MARTINS

BELO HORIZONTE
2016

VIVIAN TAMIETTI MARTINS

AVALIAÇÃO DE PROTEÍNAS RECOMBINANTES DE *Leishmania* NO DIAGNÓSTICO SOROLÓGICO E COMO CANDIDATAS A COMPOR UMA VACINA CONTRA AS LEISHMANIOSES

Tese apresentada ao Programa de Pós-Graduação em Bioquímica e Imunologia do Instituto de Ciências Biológicas da UFMG, como requisito parcial para obtenção do Título de Doutora pelo referido Programa.

Orientador: Dr. Eduardo Antonio Ferraz Coelho
Coorientador: Dr. Carlos Alberto Pereira Tavares

BELO HORIZONTE
2016

AGRADECIMENTOS

Agradeço ao Professor Doutor Eduardo A. F. Coelho por abrir as portas do seu laboratório para mim quando eu ainda era apenas uma aluna de iniciação científica. Por me ensinar como realizar cada experimento e também a ter responsabilidade e empenho em cada trabalho executado. Por me orientar e me ajudar em cada momento de dúvida. Por todas as oportunidades dadas a mim e pela confiança. Agradeço por seu exemplo como pesquisador e não poderia deixar de dizer obrigada pela amizade e pelas boas risadas nos momentos de descontração!

Ao Professor Doutor Carlos Alberto P. Tavares, pela orientação, exemplo, por todos os ensinamentos, pelo apoio e encorajamento, pela constante presença e pela confiança. Muito obrigada Beto!

Aos amigos que passaram pelo laboratório e resolveram triar outros caminhos... Obrigada Belinha, Pedro e Lelê por sempre estarem dispostos a ajudar enquanto estivemos trabalhando juntos e pela amizade e companheirismo! Adoro vocês!

A todos os colegas de laboratório!!! Obrigada pela ajuda no desenvolvimento do trabalho, discussões sobre novas ideias, pela amizade e pelas diversões fora do ambiente de trabalho! Vocês são nota 10!

À minha amiga e companheira de trabalho Dani... Muito obrigada por estar sempre presente e disposta a ajudar em tudo! Obrigada por me escutar e ser minha confidente em vários momentos! Obrigada pela confiança e transparência! Amo você!

Aos Professores Ronaldo Nagem, Santuza Teixeira, Ana Paula Fernandes, Maria Norma de Melo e Wanderson da Rocha pela colaboração e auxílio neste trabalho.

Ao amigo Jamil Silvano de Oliveira, que sempre me recebeu de braços abertos e esteve bem disposto a ajudar em vários experimentos!

A toda minha família pela paciência, confiança, aconchego e apoio! Amo vocês!

A todos que de alguma forma colaboraram para que este trabalho pudesse ser concluído, o meu muito obrigada!

SUMÁRIO

LISTA DE FIGURAS E TABELAS

LISTA DE ABREVIATURAS E SIGLAS

® **ou** **™** **-** Marca registrada
Abs - Absorbância
ANOVA - Análise de variância
BM - Medula óssea
BSA - Albumina bovina sérica
CPB - Cisteíno proteinase B
CVL - Leishmaniose visceral canina
DAT - Teste de aglutinação direta
dLN - Linfonodo drenante
dNTPs - Desoxirribonucleotídeos fosfatados
DO - Densidade ótica
DPP - Teste imunocromatográfico *Dual Path Platform*
elF-2 - Fator de alongamento 2
ELISA - Teste de imunoadsorção enzimática
FML - Ligante fucose-manose
FPLC - Cromatografia líquida rápida de proteínas
GM-CSF - Fator estimulador de colônias de macrófagos e granulócitos
gp63 - glicoproteína de superfície de 63 kDa
HASPB1 - Proteína acilada hidrofílica B1 de superfície
HbR - Receptor de hemoglobina
HRF - Fator de liberação de histamina
ICT - Teste de imunocromatografia rápida
IDRM - Intradermoreação de Montenegro
IFAT - Teste de imunofluorescência indireta
IFN-ɣ - Interferon-gama
IgG - Imunoglobulina G
IL - Interleucina
iNOS - Óxido nítrico sintetase induzido
KMP-11 - Proteína de membrana de 11kDa dos cinetoplastídeos
LACK - Homólogo do receptor para cinase C ativada de *Leishmania*
LB - Linfócito B
LC - Leishmaniose cutânea
LCD - Leishmaniose cutâneo-difusa
LelF - Fator de iniciação e alongamento de *Leishmania*
LiHyp - Proteína hipotética de *Leishmania infantum*
LMC - Leishmaniose muco-cutânea
LmSTI-1 - Proteína 1 de *L. major* induzida por stress
LPG - Lipofosfoglicano
LT - Leishmaniose tegumentar
LT - Linfócito T
LV - Leishmaniose visceral
LVC - Leishmaniose visceral canina
Meio LB - meio Luria Bertani
MHC - Complexo principal de histocompatibilidade
MPL - Monofosforil lipídio A
MPL-SE - Monofosforil lipídio A e esqualeno
n - Número amostral

NCBI - *National Center for Biotechnology Information*
NO - Óxido nítrico
OG - oxoglutarato
ORF - Fase de leitura aberta
pb - Pares de bases
PBMC - Células mononucleares de sangue periférico
PCR - Reação em cadeia da polimerase
PCR *Real Time* - Reação em cadeia da polimerase em tempo real
PDI - Proteína dissulfeto isomerase
PPG - Proteofosfoglicano
PpSP15 - Proteína salivar 15 do *Phlebotomus papatasi*
Sap - saponina
SDS-PAGE - Gel de poliacrilamida desnaturante
SFB - Soro fetal bovino
SLA - Extrato antigênico solúvel de *Leishmania* spp.
SLA*Li* - Extrato antigênico solúvel de *Leishmania infantum*
SMT - esterol 24-c-metiltransferase
Syn. - Sinônimo
r – Recombinante
ROC - Característica de operação do receptor
RPMI - *Roswell Park Memorial Institute*
TCTP - Proteína translacional de controle de tumor
TGF-β - Fator de transformação de crescimento beta
Th - Linfócitos T auxiliares
TNF-α - Fator de necrose tumoral alfa
TPI - Triose fosfato isomerase
TPR - Tripanotiona redutase
TSA - Antioxidante específico de tiol
UV - Ultra-violeta

RESUMO

A proteção contra a reinfecção por algumas espécies de *Leishmania* indica a possibilidade do desenvolvimento de uma vacina profilática contra as leishmanioses. Na busca por selecionar novos antígenos candidatos a compor uma vacina contra as leishmanioses, visceral e tegumentar, no presente trabalho quatro proteínas de *Leishmania infantum*, denominadas LiHyp1, LiHyp6, HRF e LiHyV, dois epitopos de células T $CD8^+$ extraídos da LiHyV e uma proteína quimérica composta por regiões imunodominantes ricas em epitopos de células T $CD4^+$ e $CD8^+$ presentes nestas quatro proteínas, foram avaliadas. Tais proteínas foram recentemente identificadas nas formas amastigotas e/ou promastigotas do parasito e utilizadas na forma recombinante, individualmente, associadas em uma vacina polipeptídica ou fusionadas em uma proteína quimérica. Os dois peptídeos de células T $CD8^+$ selecionados na proteína LiHyV foram sintetizados e administrados separadamente como antígenos vacinais. A imunogenicidade dos candidatos vacinais, todos associados à saponina, foi testada em camundongos BALB/c e a eficácia foi avaliada após o desafio com formas promastigotas de *L. infantum ou L. amazonensis*. Os esplenócitos dos animais vacinados com as proteínas recombinantes apresentaram uma produção elevada de IFN-ɣ, IL-12 e GM-CSF, aliados a uma baixa produção de IL-4 e IL-10, e quando os animais foram comparados aos grupos controle (salina e saponina), apresentaram uma redução significativa da carga parasitária em todos os órgãos analisados. Esta proteção foi relacionada com uma elevada produção de IFN-ɣ, que ocorreu principalmente por meio de linfócitos T $CD4^+$. As proteínas recombinantes (LiHyp1, LiHyp6, HRF e LiHyV) foram também avaliadas em sua antigenicidade para o sorodiagnóstico da leishmaniose visceral canina (LVC). Tais antígenos foram reconhecidos por anticorpos presentes em soros de cães com LV assintomática e sintomática, e não apresentaram reatividade cruzada com anticorpos presentes em soros de cães com doença de Chagas, erliquiose, babesiose ou de cães saudáveis e/ou vacinados com a vacina Leish-Tec®. O presente trabalho apresenta novas proteínas recombinantes que podem ser utilizadas para o sorodiagnóstico da LVC e, quando combinadas à saponina, podem ser usadas para compor uma vacina efetiva contra as leishmanioses visceral e tegumentar.

Palavras-chave: *Leishmania infantum*, *Leishmania amazonensis*, proteínas recombinantes, epitopos, quimera, vacinas, diagnóstico laboratorial.

ABSTRACT

The protection against reinfection by some *Leishmania* species indicates the possibility of development of a prophylactic vaccine against leishmaniasis. In the persuit for the selection of canditade antigens for composing a vaccine against VL and CL, in the present work, four *Leishmania infantum* proteins, named LiHyp1, LiHyp6, HRF and LiHyV, two $CD8^+$ T cells epitopes extracted from LiHyV as well as a chimeric protein composed by immunodominant regions rich in $CD4^+$ and $CD8^+$ T cells epitopes present in these four proteins, were evaluated. The proteins were identified recently in amastigotes and/or promastigotes parasite forms and used in a recombinant form, individually, combined in a polypeptide vaccine or fused in a chimeric protein. The $CD8^+$ T cell peptides selected from LiHyV protein were synthesized and administered separately as vaccine antigens. The immunogenicity of cadidates, associated with saponin, was tested in BALB/c mice and the vaccine efficacy was evaluated after the challenge with *L. infantum* or *L. amazonensis* promastigotes. The splenocytes of mice vaccinated with the recombinant proteins showed a high IFN-ɣ, IL-12 and GM-CSF production, together with a low production of IL-4 and IL-10, and when the animals were compared to control groups (saline and saponin), they showed a significant reduction in the parasite load in all the organs analysed. This protection was related to a high production of IFN-ɣ, which was mainly derived from T $CD4^+$ cells. Also, the recombinant proteins (LiHyp1, LiHyp6, HRF e LiHyV) were assessed in their antigenicity for the serodiagnosis of canine visceral leishmaniasis (CVL). The proteins were recognized by antibodies present in sera from dogs with asymptomatic and symptomatic VL, and did not show cross-reactivity with antibodies present in dog sera with Chagas' disease, ehrlichiosis, babesiosis or health dogs and/or vaccinated with the Leish-Tec® vaccine. This study showed new recombinant proteins that can be used for CVL serodiagnosis tests and, when associated with saponin, can compose an effective vaccine against visceral and tegumentar leishmaniasis.

Keywords: *Leishmania infantum*, *Leishmania amazonensis*, recombinant proteins, epitopes, chimera, vaccine, laboratorial diagnosis.

1. JUSTIFICATIVA

As leishmanioses são doenças causadas por parasitos protozoários do gênero *Leishmania* e afetam alguns milhões de pessoas em todo o mundo, podendo levar a uma elevada morbidade e letalidade. O Brasil é responsável por aproximadamente 95% e 40% dos casos registrados de leishmaniose visceral (LV) e tegumentar (LT), respectivamente, no Continente Americano, o que torna as leishmanioses um importante problema de Saúde Pública no país e que requer, dessa forma, atenção especial pelas autoridades de saúde competentes (Alvar *et al*., 2012).

Devido à ineficiência ou não adesão às medidas de controle disponíveis, da dificuldade para o diagnóstico correto e dos problemas encontrados no tratamento da doença, como por exemplo, os diversos efeitos tóxicos causados aos pacientes, a ocorrência de resistência dos parasitos aos fármacos convencionais e/ou seu elevado custo (Gontijo & Melo, 2004; Minodier & Parola, 2007, Mondal *et al.,* 2010); o desenvolvimento de vacinas que sejam capazes de prevenir e controlar a dispersão das leishmanioses torna-se desejável como uma medida alternativa de controle da doença (Palatnik-de-Sousa, 2008).

A leishmaniose visceral canina (LVC) é uma zoonose endêmica em diversos países da América Central, América do Sul (ocorrendo principalmente no Brasil), bacia do Mediterrâneo e Ásia (Gramiccia e Gradoni, 2005; Baneth *et al.*, 2008). Uma vez que os cães são os principais hospedeiros domésticos dos parasitos e apresentam-se como potenciais reservatórios da doença, o diagnóstico precoce destes animais é de extrema importância como uma possível medida de controle. As autoridades competentes no Brasil determinam que o diagnóstico imunológico para a doença deva ser baseado em testes sorológicos como o *Dual Path Platform* (DPP) e o ensaio imunoenzimático (ELISA), entretanto, problemas relacionados à sensibilidade e/ou especificidade dos mesmos podem ocorrer. Sabe-se que animais infectados podem apresentar uma baixa produção de anticorpos anti-*Leishmania*, podendo acusar resultados falso-negativos nos testes sorológicos utilizados (Coelho *et al.,* 2009). Da mesma forma, animais infectados por outros patógenos, como *Trypanosoma cruzi* (Trocarelli *et al*., 2009; Viol *et al*., 2012), *Babesia canis* ou *Ehrlichia canis* (Oliveira *et al*., 2008), dentre outros; ou ainda aqueles vacinados com a Leish-Tec® (vacina comercializada no Brasil), podem apresentar anticorpos que reagem com proteínas de *Leishmania* spp. e, dessa forma, acusarem resultados falso-positivos nos exames sorológicos. Deste modo, a busca por

um diagnóstico que apresente elevada sensibilidade e especificidade poderia também ser considerado importante para o controle da doença (Gomes *et al*., 2008).

Uma vez que a cura contra a infecção por algumas espécies de *Leishmania* spp. é capaz de induzir proteção contra a reinfecção pelo parasito, a busca por vacinas protetoras tem sido constante (Grimaldi & Tesh, 1993). Preferencialmente, uma vacina protetora contra as leishmanioses deve conter imunógenos conservados em várias espécies de *Leishmania* spp., induzir uma resposta imune Th1, além de ser segura e apresentar um custo acessível à população (Grimaldi & Tesh, 1993; Coelho *et al*., 2003; WHO, 2010). Cabe destacar que a maioria dos estudos realizados utiliza antígenos expressos nas formas promastigotas do parasito, entretanto, antígenos presentes nas formas amastigotas devem ser também considerados como alvos alternativos para o desenvolvimento de uma formulação vacinal contra as leishmanioses, haja vista que essa é a forma biológica que o parasito mantém contato com o sistema imune do hospedeiro mamífero durante a infecção crônica (Wenzel *et al.*, 2012; Fernandes *et al.*, 2012).

A utilização de novas ferramentas biotecnológicas, como a imunoproteômica, tem levado à identificação de diversas proteínas com potencial para o emprego na prevenção e/ou diagnóstico de várias doenças. Esta técnica foi recentemente utilizada por nosso grupo de pesquisa para identificar proteínas de *L. infantum*, as quais foram reconhecidas por anticorpos presentes em amostras de soros de cães com leishmaniose visceral sintomática e/ou assintomática; sendo que a espécie do parasito foi escolhida devido à capacidade de infectar e desencadear a doença visceral tanto em cães quanto em humanos (Coelho *et al.*, 2012). Diversas proteínas foram identificadas, dentre elas algumas já descritas na literatura como candidatos vacinais, alvos diagnósticos e/ou agentes terapêuticos contra as leishmanioses visceral e/ou tegumentar. Uma destas proteínas, denominada LiHyp1, quando utilizada sob a forma recombinante e associada ao adjuvante saponina, foi capaz de induzir proteção significativa contra a infecção experimental por *L. infantum* em camundongos BALB/c, além de apresentar potencial para aplicação no diagnóstico da leishmaniose visceral (Martins *et al.*, 2013). Atualmente, proteínas recombinantes são muito estudadas com a finalidade de se desenvolver uma vacina segura, passível de padronização, pura e com custo acessível à população (Joshi *et al*., 2014). A Organização Mundial de Saúde tem preconizado o desenvolvimento de uma vacina multiprotéica ou polipeptídica para as leishmanioses, uma vez que esta estratégia possibilitaria englobar diferentes regiões imunogênicas de várias proteínas do parasito em uma única vacina (WHO, 2010).

Neste trabalho, foram utilizadas quatro proteínas identificadas pelo estudo proteômico previamente citado (Coelho *et al.*, 2012). As proteínas LiHyp6 (XP_001568689.1) e HRF (CAJ05086.1) são encontradas na forma promastigota do parasito e foram reconhecidas por anticorpos presentes nos soros de cães sintomáticos para a doença; A proteína LiHyp1 (XP_001468941.1) é expressa na forma amastigota, enquanto a LiHyV (XP_888524.1) encontra-se tanto na forma amastigota quanto na promastigota dos parasitos, sendo que ambas foram reconhecidas por soros de animais sintomáticos e assintomáticos. Estas quatro proteínas foram selecionadas para que os genes ortólogos em *L. infantum* fossem clonados e as proteínas pudessem ser expressas e purificadas em suas formas recombinantes. As proteínas foram testadas tanto individualmente quanto em associação (no caso, entre LiHyp1, LiHyp6 e HRF) como candidatas vacinais em camundongos BALB/c desafiados com *L. infantum*, bem como utilizadas para o sorodiagnóstico da LVC, a fim de que a eficácia entre elas fosse comparada. Além das proteínas, dois epitopos lineares específicos para LT $CD8^+$ (AGQSVPNTL e VGIAKSTAALFVLNATAI) presentes na proteína LiHyV, foram selecionados com a finalidade de se comparar a eficiência vacinal de uma proteína recombinante frente aquela induzida por epitopos sintéticos. Uma quimera polipeptídica composta por regiões imunodominantes ricas em epitopos de LT $CD4^+$ e $CD8^+$ presentes nas quatro proteínas selecionadas, também foi construída, objetivando a avaliação da proteção vacinal utilizando esta estratégia contra LV e LT.

Sendo assim, o objetivo deste trabalho foi avaliar a eficácia de proteínas recombinantes administradas na sua forma isolada ou em associação, de peptídeos sintéticos específicos de LT e ainda de uma quimera polipeptídica como candidatos vacinais contra as leishmanioses; bem como avaliá-las como marcadores para o sorodiagnóstico da LVC, uma vez que a identificação prévia de tais antígenos ocorreu em um estudo envolvendo soros de cães com LV sintomática e assintomática.

2. REVISÃO DE LITERATURA

2.1. Epidemiologia das leishmanioses

As leishmanioses são doenças infecto-parasitárias endêmicas em 98 países e 3 territórios, distribuídas em regiões tropicais e subtropicais do mundo. Cerca de 90% dos casos de LV ocorrem em 6 países: Índia, Bangladesh, Sudão, Sudão do Sul, Etiópia e Brasil; enquanto que cerca de 70 a 75% da incidência global de LT encontra-se principalmente no Afeganistão, Argélia, Colômbia, Brasil, Irã, Síria, Etiópia, Sudão do Norte, Costa Rica e Peru (Alvar *et al.*, 2012).

Estima-se que 310 milhões de pessoas encontram-se expostas ao risco de contrair a doença e que haja uma incidência mundial aproximada de 0,2 a 0,4 milhões e de 0,7 a 1,2 milhões de novos casos de LV e LT, respectivamente, por ano. No Continente Americano, calcula-se que o Brasil seja responsável por aproximadamente 95% dos casos de LV e 40% dos casos de LT, o que torna a doença um importante problema de Saúde Pública no país (Alvar *et al*., 2012; WHO, 2015).

Nas últimas décadas, alguns fatores têm determinado o aumento da incidência de casos de leishmaniose na Europa, África, América e Ásia, como por exemplo: a migração de pessoas que levam seus cães infectados para áreas onde exista a presença de vetores transmissores; a expansão no *habitat* dos flebotomíneos devido ao aquecimento global; o aumento no número de cães de áreas não endêmicas que viajam acompanhando seus donos para locais endêmicos para a doença e acabam por se infectarem; o aumento do desflorestamento devido à urbanização crescente, dentre outros (Baneth *et al*., 2008; Palatnik de Souza, 2012). A infecção canina merece atenção especial, uma vez que apresenta uma maior soroprevalência quando comparada à doença humana e pelo fato de que muitos animais doentes, ainda que assintomáticos, podem atuar na manutenção do ciclo do parasito (Marzochi *et al.*, 1985; Oliveira *et al.*, 2001).

2.2. A etiologia e o ciclo biológico do parasito *Leishmania* spp.

As leishmanioses são doenças causadas por parasitos protozoários pertencentes ao gênero *Leishmania*, Sub-Reino Protozoa, Filo Sarcomastigophora, Ordem Kinetoplastida e Família *Trypanosomatidae*. O vetor transmissor é um inseto da Ordem Diptera, Família Psychodidae, Sub-Família Phlebotominae, pertencente aos gêneros *Phlebotomus* em países do Velho Mundo e *Lutzomyia* nas Américas, sendo que apenas as fêmeas possuem hábitos

hematófagos e, assim, são capazes de transmitir o parasito aos hospedeiros mamíferos (Grimaldi & Tesh, 1993). O parasito *Leishmania* sp. pode se apresentar sob duas formas morfológicas principais: promastigota ou amastigota. As formas promastigotas são alongadas, afiladas, com um único flagelo que lhes conferem motilidade, cinetoplasto localizado entre a porção anterior e o núcleo e são encontradas no vetor transmissor. As formas amastigotas são arredondadas, com flagelo rudimentar, cinetoplasto em forma de bastão e se multiplicam nos vacúolos fagocíticos no interior de células do sistema fagocítico-mononuclear dos hospedeiros mamíferos (Sacks & Kamhawi, 2001).

A transmissão do parasito ocorre no momento em que o vetor flebotomínio fêmea realiza o repasto sanguíneo em um hospedeiro mamífero infectado, podendo ingerir, juntamente com a alíquota de sangue, fagócitos infectados por formas amastigotas de *Leishmania* spp. Essas células, ao alcançarem o intestino do vetor, se rompem e liberam os parasitos, que se transformam rapidamente em formas promastigotas procíclicas e migram para o trato digestório médio e anterior. Os parasitos iniciam um processo de multiplicação e assumem a forma paramastigota, seguida por diferenciação em formas promastigotas metacíclicas e migração para a parte anterior do aparelho bucal do inseto vetor. Quando um novo repasto sanguíneo ocorre com o vetor infectado, formas promastigotas metacíclicas, que apresentam elevada motilidade e capacidade migratória, juntamente com a saliva, penetram na derme do hospedeiro mamífero não infectado. Em seguida, as células do sistema fagocítico-mononuclear do hospedeiro, dentre as quais se destacam os monócitos e macrófagos, fagocitam as formas promastigotas dos parasitos que, no interior dos fagolisossomos formados, se diferenciam em formas amastigotas. Os parasitos passam a se multiplicar até que ocorra a lise da célula, quando então há liberação das formas amastigotas no organismo do hospedeiro. Os parasitos liberados podem ser fagocitados por novas células imunes, dando continuidade à infecção no hospedeiro mamífero, ou podem ser ingeridos por um novo inseto vetor, completando assim o ciclo biológico do parasito (Pessoa & Martins, 1988; Medeiros *et al.*, 2005).

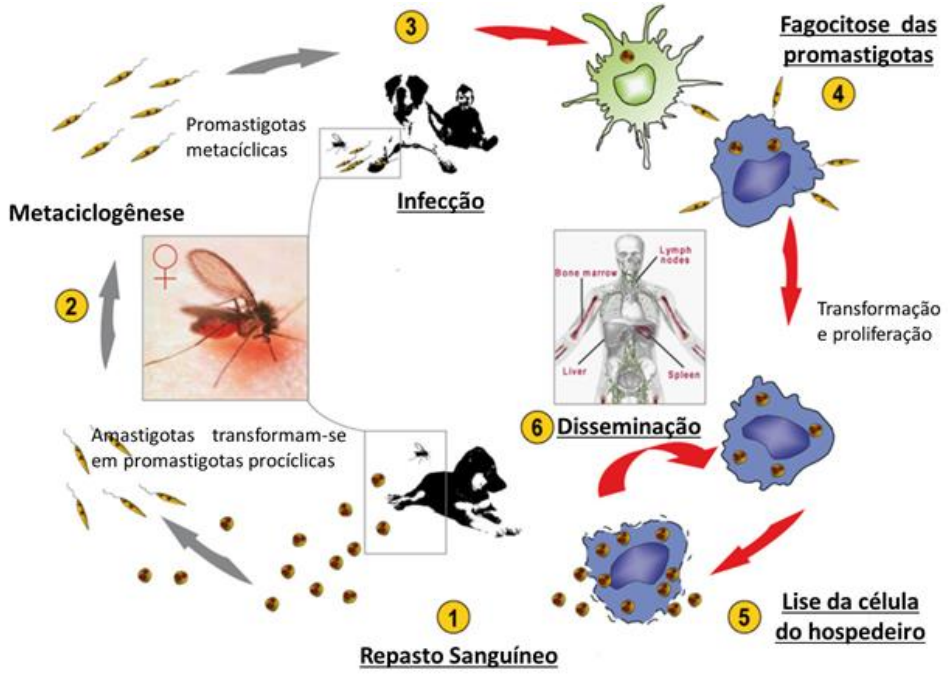


Figura 1: Ciclo biológico do parasito *Leishmania spp.* 1. Infecção do inseto vetor fêmea no momento em que realiza o repasto sanguíneo em um hospedeiro infectado (ingestão de amastigotas). 2. Transformação das formas amastigotas em promastigotas metacíclicas no interior do vetor. 3. Penetração passiva das formas promastigotas metacíclicas e infecção do hospedeiro mamífero. 4. Fagocitose dos parasitos por macrófagos, onde ocorre a transformação em amastigotas e proliferação. 5. Lise da célula hospedeira com liberação das amastigotas. 6. Disseminação dos parasitos para órgãos e tecidos do hospedeiro mamífero e possibilidade de um novo repasto sanguíneo pelo inseto vetor. Adaptado de Nieto *et al.* (2011).

2.3. Manifestações clínicas da doença

A patogenia das leishmanioses pode ser determinada pelas interações entre as características genéticas e a resposta imune do hospedeiro, pela virulência do parasito infectante e por fatores relacionados ao inseto vetor, como os componentes salivares que portam os parasitos quando da infecção no homem. O resultado desta interação pode levar desde a resistência à infecção, passando pelo desenvolvimento da forma assintomática da doença, até a ocorrência da doença tegumentar ou visceral aguda (Kane & Mosser, 2000).

A leishmaniose cutânea (LC) é a forma clínica mais comum, ocorrendo em cerca de 90% dos pacientes diagnosticados com a doença tegumentar. Normalmente, a doença se manifesta em partes mais expostas do corpo, como face, braços e pernas. Apresenta-se como um nódulo no local da picada que evolui formando uma úlcera granulosa contornada por uma borda elevada. Normalmente, a lesão é única, mas nódulos satélites podem ocorrer nas proximidades da lesão inicial. Quando as úlceras são curadas, deixam cicatrizes permanentes, as quais podem levar a uma condição de morbidade (Stebut, 2014).

Casos de leishmaniose muco-cutânea (LMC) ocorrem em diversos países no mundo e, normalmente, manifestam-se como lesões de caráter infiltrante que podem ocasionar destruição parcial ou total da mucosa da cavidade do nariz, boca, faringe, laringe e traquéia. Esta manifestação clínica da doença pode gerar uma elevada rejeição do paciente perante a sociedade, uma vez que gera uma grande deformidade física no local da lesão (WHO, 2015).

A leishmaniose cutâneo-difusa (LCD) é uma forma da doença na qual as lesões apresentam-se como nódulos, de forma anérgica e que raramente ulceram. As lesões espalham-se por todo o corpo e este quadro pode estar relacionado à ineficiência ou ausência de resposta celular por parte do sistema imune do hospedeiro (Desjeux, 2004). A LCD, embora rara, não apresenta cura espontânea. Devido às frequentes recidivas, torna-se um grave problema de Saúde Pública, pois os pacientes apresentam lesões desfigurantes e incapacitantes, excluindo-os do seu meio de vida social (Gontijo & Carvalho, 2003; Desjeux, 2004).

Na LV, a infecção parasitária acomete principalmente órgãos como baço, fígado, órgãos linfóides e medula óssea. Após um período de incubação, que geralmente varia entre dois a seis meses, indivíduos sintomáticos apresentam sinais de uma infecção sistêmica e persistente, incluindo febre, fadiga, fraqueza, perda de peso e evidências sugestivas da presença dos parasitos, como por exemplo, hepatoesplenomegalia e inchaço dos gânglios linfáticos (Chappuis *et al*., 2007). A intensidade das manifestações clínicas é variável e indivíduos podem permanecer assintomáticos por longos períodos de tempo, dificultando seu diagnóstico clínico, embora cerca de 20% dos pacientes infectados desenvolvam a forma aguda da doença. Os sintomas são progressivos e as complicações decorrentes da evolução da infecção são responsáveis pelos óbitos (Badaró *et al*., 1986; Gama *et al*., 2004).

As principais espécies responsáveis pelas variadas manifestações clínicas e o local de ocorrência da doença podem ser visualizados na tabela 1.

Tabela 1: Principais espécies responsáveis pelas manifestações clínicas das leishmanioses. Adaptado de Stebut (2014).

Manifestação Clínica	**Espécie envolvida**	
	Velho Mundo	**Novo Mundo**
Leishmaniose cutânea	*L. major* *L. tropica* *L. infantum*	*L. mexicana* *L. braziliensis* *L. guyanensis* *L. panamensis* *L. amazonensis*

Tabela 2: Continuação

Manifestação Clínica	Espécie envolvida	
	Velho Mundo	**Novo Mundo**
Leishmaniose muco-cutânea	-	*L. peruviana* *L. braziliensis* *L. guyanensis* *L. panamensis*
Leishmaniose cutâneo-difusa	*L. aethiopica* *L. major*	*L. mexicana* *L. braziliensis* *L. guyanensis* *L. amazonensis*
Leishmaniose visceral	*L. donovani* *L. infantum* *L. tropica*	*L. chagasi* (*syn. L. infantum*)

Na LVC, o cão infectado pode desenvolver manifestações clínicas diversas, sendo que as mais comuns são as alterações dermatológicas, linfadenopatia, hepatoesplenomegalia, emagrecimento acentuado, uveíte, onicogrifose, alopécia e ceratoconjutivite (Ettinger & Feldman, 2004). O período de incubação da doença pode variar de alguns meses a vários anos, entretanto, cães infectados podem permanecer assintomáticos por longos períodos de tempo, ainda que permaneçam infectivos (Lanotte *et al*., 1979). A susceptibilidade e/ou a resistência à doença é dependente principalmente da resposta imune celular do animal infectado. Parâmetros como a carga parasitária, a expressão de citocinas como IL-10 e TGF-β, a imunomodulação de células específicas à *Leishmania* spp. (como LT $CD4^+$ e LT $CD8^+$) ou a presença de títulos elevados de anticorpos específicos ao parasito, normalmente, são condições associadas com a progressão clínica da doença. Em contrapartida, parâmetros relacionados ao aumento e proliferação de células mononucleares do sangue periférico (PBMCs) após o estímulo com antígenos de *Leishmania* spp., a expressão de níveis elevados de IFN-ɣ e TNF-α e a proliferação de LT $CD4^+$ e LT $CD8^+$, encontram-se relacionados com a resistência contra a infecção visceral no cão (Reis *et al*, 2010).

Normalmente, cães assintomáticos apresentam uma resposta imune celular predominante, com a presença de elevados níveis de IL-2 e TNF-α e uma resposta mista Th1/Th2, com prevalência da resposta Th1, mediada principalmente pelas citocinas IL-12, IL-18 e IFN-ɣ. Em contrapartida, animais sintomáticos apresentam uma fraca resposta celular,

com baixos níveis de IFN-ɣ e uma resposta humoral elevada frente aos antígenos do parasito (Palatnik-de-Sousa, 2012).

2.4. Diagnóstico das leishmanioses

O diagnóstico das leishmanioses é realizado com base na avaliação das manifestações clínicas da doença em conjunto com dados epidemiológicos e exames laboratoriais (Tesh, 1995). Testes parasitológicos normalmente são realizados por meio de aspirados dos linfonodos ou da medula óssea e, embora invasivos, mostram-se conclusivos na identificação dos parasitos através de análises microscópicas em lâminas e/ou culturas *in vitro* dos materiais coletados (Reithinger *et al*., 2007).

O diagnóstico imunológico pode ser realizado através do teste de intradermorreação de Montenegro (IDRM), que se baseia na resposta celular induzida pela memória imunológica de células T que são ativadas em indivíduos que tiveram contato prévio com o parasito. O IDRM foi o primeiro método de diagnóstico laboratorial utilizado em diversas regiões no mundo, entretanto, o teste pode apresentar reduzida especificidade, o que leva indivíduos não infectados de áreas endêmicas para leishmaniose, curados desta doença ou com outras doenças, como: esporotricose, doença de Chagas, hanseníase ou tuberculose; a acusar resultados falso-positivos para leishmaniose. Outra desvantagem do IDRM é que indivíduos imunossuprimidos, com a forma difusa da doença ou ainda com lesões recentes, podem ser diagnosticados como negativos (Weigle *et al*., 1991; de Paiva-Cavalcanti *et al.*, 2015).

Testes utilizando métodos moleculares, como a Reação em Cadeia da Polimerase (PCR) e a PCR *Real Time,* têm sido utilizados para a detecção de ácidos nucleicos específicos do parasito. Entretanto, estas técnicas podem apresentar problemas relacionados com uma variação na sensibilidade do teste devido às diferenças referentes às porções específicas do material coletado, que podem não abrigar os parasitos e levar a um resultado falso-negativo. A necessidade de treinamento técnico especializado, a possibilidade de contaminação com agentes externos levando a resultados falso-positivos e o elevado custo, também são algumas das desvantagens destes métodos (Sundar & Rai, 2002; Srivastava *et al*., 2011; de Paiva-Cavalcanti *et al.*, 2015).

Testes sorológicos, tais como o ensaio imunoenzimático (ELISA), ensaio de imunofluorescência indireta (RIFI), teste de aglutinação direta (DAT), Western-blot e o teste imunocromatográfico (ICT) também são utilizados no diagnóstico laboratorial das leishmanioses e baseiam-se na detecção de anticorpos e/ou antígenos específicos dos parasitos

em amostras de soro ou plasma dos indivíduos, entretanto, também podem apresentar problemas de sensibilidade e/ou especificidade (Marzochi & Marzochi, 1994; Tavares *et al*., 2003; de Paiva-Cavalcanti *et al.,* 2015).

A detecção sorológica de cães com LV assintomática é considerada crítica para um correto diagnóstico laboratorial, para a realização de estudos epidemiológicos e para um controle mais efetivo contra a propagação da doença, uma vez que tais animais permanecem como potenciais transmissores dos parasitos (Molina *et al.,* 1994; Costa-Val *et al.,* 2007; Michalsky *et al.,* 2007). A técnica de ELISA utilizando extratos proteicos obtidos de formas promastigotas de *Leishmania* spp. não apresenta sensibilidade satisfatória para a detecção dos casos de LVC assintomática (Porrozzi *et al.,* 2007; Miro *et al.,* 2008). A especificidade deste teste também não é satisfatória, uma vez que pode gerar resultados falso-positivos devido à presença de anticorpos nos soros de cães com doença de Chagas, erliquiose, babesiose ou toxoplasmose, os quais podem reagir de forma cruzada com antígenos de *Leishmania* spp. (Kar, 1995; Ferreira *et al.,* 2007; Porrozzi *et al.,* 2007).

No Brasil, até o ano de 2011, o teste de ELISA era utilizado como sendo de triagem, enquanto o RIFI era o teste confirmatório para LVC. Entretanto, por meio de uma Nota Técnica Conjunta, em 01/2011, o Ministério da Saúde substituiu o protocolo de diagnóstico da doença, passando a recomendar a utilização do teste rápido DPP® (Dual-Path Platform – Biomanguinhos) como método diagnóstico de triagem e o ELISA como confirmatório (Faria & Andrade, 2012). O DPP® é um teste imunocromatográfico composto pelos antígenos recombinantes de *Leishmania* sp. rK39 e rK26, os quais conferem uma elevada sensibilidade e especificidade ao teste na detecção de cães sintomáticos para leishmaniose. Entretanto, ao avaliar a capacidade de detecção de animais assintomáticos, o teste apresenta uma variação em sua eficácia, com uma grave redução na sensibilidade (Grimaldi *et al*., 2012). O teste confirmatório de ELISA utilizado é composto por antígenos solúveis de *Leishmania* spp. e apresenta uma elevada sensibilidade em identificar cães doentes, entretanto, a especificidade do teste pode ser reduzida, uma vez que reações cruzadas podem ocorrer e acusar resultados falso-positivos (Faria & Andrade, 2012). Devido à variação na eficiência dos testes diagnósticos utilizados para detectar cães com leishmaniose, graves problemas podem ocorrer, tais como a questão de diversos animais saudáveis com resultados falso-positivos para leishmaniose serem sacrificados diariamente devido a erros dos testes aplicados; ou ainda a baixa sensibilidade e a detecção de casos falso-negativos em manter, em meio à

sociedade, animais reservatórios com elevado potencial para transmitir a doença (Werneck, 2014).

No Brasil, existe atualmente uma vacina licenciada para a LVC, denominada Leish-Tec® (Fernandes *et al.*, 2008), e postula-se que a especificidade do teste diagnóstico deva ser mantida quando da utilização de amostras de soros de cães vacinados, a fim de que estes cães se diferenciem dos animais infectados. Por esta razão, proteínas recombinantes dos parasitos vêm sendo testadas como antígenos para ELISA, tendo em vista o desenvolvimento de um teste de diagnóstico mais sensível e específico para LVC (Kubar & Fragaki, 2005). A utilização de combinações de antígenos também tem tornado uma boa alternativa (Porrozzi *et al.*, 2007; Goto *et al.*, 2009), e podem melhorar o desempenho dos testes de ELISA.

Desta forma, a busca por antígenos que possam conferir uma elevada sensibilidade e especificidade em animais sintomáticos e assintomáticos é de grande relevância para compor um novo método diagnóstico que possua uma excelente eficácia.

2.5. Tratamento das leishmanioses

O tratamento clínico das leishmanioses é realizado com a finalidade de se evitar a mortalidade causada pela LV e reduzir a morbidade provocada pelas lesões desfigurantes nas formas mais graves de LT. Pacientes com lesões severas, com infecções recorrentes, disseminadas ou com a forma visceral da doença, devem ser tratados sistemicamente. Entretanto, pacientes com lesões locais e menos complexas devem ser tratados apenas com métodos locais, a fim de se evitar os efeitos colaterais gerados pela maioria dos medicamentos utilizados. Recomenda-se ainda que pacientes tratados façam um acompanhamento que pode variar de meses a anos, no intuito de acompanhar a ocorrência de uma possível recidiva da doença (WHO, 2010; Stebut, 2014).

O tratamento das leishmanioses é baseado no uso dos antimoniais pentavalentes. O antimoniato de N-metil meglumina, produzido com o nome comercial de Glucantime® pela empresa Rhône Poulenc Rorer (França) e o estibogluconato de sódio, produzido com o nome comercial de Pentostan® pela Wellcome Foundation (Inglaterra) são os fármacos mais utilizados, entretanto, podem causar diversos efeitos colaterais e tóxicos aos pacientes (Figueiredo *et al*., 1999; Balasegaram *et al*., 2012). Doses diárias elevadas, necessárias ao curso do tratamento, podem causar fadiga, artralgias, mialgias, além de toxicidade renal, hepática e cardíaca. A dificuldade no transporte até os centros de saúde especializados para a administração intramuscular ou endovenosa dos medicamentos, o período relativamente longo

de tratamento (até 40 dias) e a via de administração dolorosa, acabam por dificultar a adesão dos pacientes ao tratamento, podendo levar à resistência dos parasitos aos produtos utilizados (Grogl *et al.,* 1991; Carvalho *et al.,* 2000; Tavares *et al.,* 2003).

A anfotericina B aparece como uma droga antileishmanial eficiente, entretanto, pode causar febre, cefaleias, náuseas, vômitos, diarreia, anorexia, mal-estar geral, mialgias, artralgias, flebite no local da infusão, anemia hemolítica e nefrotoxicidade durante a administração. Com o intuito de reduzir seus efeitos colaterais, a anfotericina B lipossomal (AmBisome^{TM}) foi desenvolvida. A formulação é capaz de reduzir a toxicidade do produto, entretanto, é um medicamento de custo elevado e necessita ser administrado em ambiente hospitalar (Balasegaram *et al*., 2012).

A miltefosina, com o objetivo de tornar o tratamento mais acessível ao paciente, foi o primeiro medicamento oral utilizado para a LV, entretanto, distúrbios gastrointestinais, nefro e hepatotoxicidade e possível teratogenicidade foram descritos, além de apresentar custo elevado (Balasegaram *et al*., 2012).

A LT é tratada de acordo com a severidade da lesão, da espécie etiológica causadora e da possível evolução da doença para as formas mucosa e disseminada, entretanto, para lesões mais simples, deve-se usar aplicação local de pomadas à base de paramomicina ou de antimoniais pentavalentes (WHO, 2010). A combinação de medicamentos tem apresentado algumas vantagens para o tratamento das leishmanioses, tais como a redução no período de tratamento, a diminuição da concentração do medicamento administrado e dos efeitos tóxicos gerados, um custo mais acessível, o aumento na adesão dos pacientes e uma menor probabilidade do parasito tornar-se resistente. Tais fatos têm levado à adoção deste método terapêutico pelas autoridades de Saúde competentes (WHO, 2010).

2.6. Medidas de prevenção e controle das leishmanioses

A transmissão das leishmanioses é mantida devido a um complexo sistema biológico e social que envolve o hospedeiro mamífero e o inseto vetor. Normalmente, o controle da disseminação da doença não é eficaz apenas com simples intervenções, fazendo-se necessária uma combinação de gestão estratégica integrada ao controle do vetor e do animal reservatório, de acordo com cada contexto (WHO, 2010).

As medidas de prevenção e controle para as leishmanioses podem objetivar a interrupção do ciclo biológico do parasito, entretanto, o número elevado de espécies de *Leishmania* spp., o caráter zoonótico da doença e a manutenção do seu ciclo silvestre

dificultam a adoção de medidas eficazes de controle (Tesh, 1995). Para a leishmaniose humana, a estratégia de controle utilizada se apoia na detecção dos casos, no diagnóstico correto e no tratamento efetivo dos pacientes, acompanhada de medidas de prevenção à reinfecção, como o uso de repelentes individuais e telas de proteção nas casas. Tais medidas podem reduzir a transmissão da doença, mas não geram um impacto significativo (Grimaldi & Tesh, 1993; WHO, 2010).

No que se refere à LVC, o cão, como um importante reservatório doméstico dos parasitos, representa uma importante fonte de infecção para o vetor transmissor, agindo como um elo na manutenção da transmissão entre o vetor e o homem. Para o controle da doença nos cães, órgãos de Saúde Pública devem realizar a triagem dos animais através da detecção de sinais clínicos e de testes sorológicos, sendo que tanto os animais sintomáticos, quanto os assintomáticos, devam ser eutanasiados com a finalidade de se tentar reduzir a disseminação do parasito. Esta medida de controle tem apresentado falhas devido a determinados donos recusarem a realização do teste em seus animais de estimação, ou ainda, devido a não autorização do processo de eutanásia em animais doentes. Junto a estas falhas, há o problema de rápida reposição de animais em áreas endêmicas ou a escolha de se tratar os animais, o que, na maioria dos casos, mostra-se ineficiente para a redução do número de casos naquele ambiente (Gontijo & Melo, 2004; WHO, 2010). A utilização de inseticidas tópicos ou impregnados em coleiras e a vacinação poderiam ser estratégias preventivas com algum impacto no controle da LVC (Werneck, 2014). Em relação aos hospedeiros selvagens, a eliminação não é uma medida executável ou ecologicamente correta. Além disso, existe a possibilidade de adaptação do parasito a outros reservatórios existentes em ambientes rurais (Grimaldi & Tesh, 1993; Gramiccia & Gradoni, 2005).

O controle do inseto vetor tem por objetivo interromper a transmissão da doença e pode ser feito através da administração de inseticidas em ambientes domésticos, entretanto, a eficácia depende da classe de inseticida utilizada, da susceptibilidade do inseto vetor, do tipo de superfície ao qual o mesmo é aplicado e da dosagem e método de aplicação. Para que esta medida de controle seja eficiente, é necessário que se tenha procedimentos operacionais padrão, gerenciamento, logística, supervisão, monitoramento e avaliação da eficácia em determinados períodos de tempo. Algumas classes de inseticidas utilizadas são os organoclorados, organofosfatados, carbamatos e piretróides, e estes devem ser trocados em determinados períodos de tempo para que se evite o aparecimento de insetos vetores resistentes (WHO, 2010). No Brasil, os produtos mais utilizados são as cipermetrinas e

deltametrinas, pertencentes à classe dos piretróides, os quais apresentam eficácia durante três meses após sua aplicação (Brasil, 2006).

De acordo com o Ministério da Saúde, além de medidas preventivas relacionadas diretamente ao controle do vetor e do hospedeiro reservatório, investimentos em programas de saneamento, educação social e conscientização ambiental também são de extrema importância em auxiliar o controle das leishmanioses.

Devido às dificuldades encontradas na execução dos métodos profiláticos, a problemas de sensibilidade e especificidade dos testes diagnósticos e aos relacionados aos tratamentos, o desenvolvimento de novas medidas profiláticas, tais como a busca por vacinas, torna-se uma alternativa atrativa e vem sendo muito pesquisada por diferentes grupos; entretanto, essa é uma tarefa complexa, embora seja considerada uma solução real e com melhor custo-benefício para o controle efetivo e prevenção da doença (Grimaldi & Tesh, 1993; Gonzalo *et al*., 2002; Ramiro *et al*., 2003; Sukumaran *et al*., 2003; Desjeux, 2004; Palatnik-de-Sousa *et al.*, 2012).

2.7. Resposta imune na leishmaniose murina

Dentre os vários modelos experimentais em animais, o murino, utilizando camundongos, são os mais estudados para a investigação da eficácia de antígenos vacinais e de novos medicamentos contra as leishmanioses. A linhagem de camundongo a ser utilizada pode variar de acordo com a espécie de *Leishmania* sp. e o tipo de infecção a ser avaliada. Camundongos C57BL/6 ou C3H normalmente são utilizados para avaliar um perfil de resistência à infecção com *L. major*, entretanto, quando infectados com *L. amazonensis* podem desenvolver lesões crônicas. Por outro lado, camundongos BALB/c são altamente susceptíveis à infecção por *L. major*, *L. amazonensis* ou *L. infantum*, entretanto, desenvolvem lesões limitadas quando infectados por *L. brasiliensis* (de Luca & Macedo, 2016).

A resistência ou a susceptibilidade dos camundongos à infecção deve-se principalmente a fatores genéticos dos animais. Camundongos geneticamente resistentes à LV, como os CBA, apresentam o gene *Slc1 1a1* funcional, o qual confere imunidade inata contra o parasito e possibilita o controle da infecção. Camundongos susceptíveis à LV, como o BALB/c, apresentam uma mutação neste gene, o que permite a replicação de parasitos no fígado durante as primeiras semanas de infecção (Das & Ali, 2012; Kumar & Nylén, 2012).

O controle da infecção por hospedeiros mamíferos é mediado via imunidade inata (monócitos, macrófagos, células dendríticas e neutrófilos) e adaptativa (células T), sendo que

as citocinas são as principais moléculas relacionadas a uma rede de interações que induz e controla o perfil da resposta imune. As células T $CD4^+$ são consideradas essenciais na resposta às leishmanioses e quando diferenciadas em células Th1 produzem citocinas pró-inflamatórias que possuem um papel importante na resposta protetora durante a infecção (Kedzierski e Evans, 2014). As células T $CD8^+$ também são responsáveis por um importante papel na resposta protetora, contribuindo principalmente na geração de uma resposta imune de memória (Stager *et al*., 2000).

Estudos científicos utilizando camundongos como modelos experimentais para leishmaniose deram origem ao paradigma Th1/Th2 de resistência e susceptibilidade associado à infecção celular. Este perfil dicotômico da resposta imune encontra-se mais relacionado à LT, causada pelo parasito *L. major*, entretanto, não é bem definido para LV (Kedzierski e Evans, 2014). A resposta protetora na LV murina depende, principalmente, da produção de IL-12, a qual induz uma resposta do tipo Th1, mediada pela produção de IL-2 e IFN-γ. A ativação da enzima iNOS por meio da ação do IFN-ɣ leva à produção de NO pelos macrófagos do baço e fígado, o que permite o controle da multiplicação dos parasitos nestes órgãos (Green *et al*., 1990; Blackwell, 1996). Citocinas como a IL-17 e IL-22 complementam o papel de proteção na LV, porém, os mecanismos de atuação que lhes são inerentes permanecem ainda desconhecidos (Faleiro *et al*., 2014).

Elevadas concentrações de citocinas TGF-β, IL-4, IL-10 e IL-13, normalmente, encontram-se relacionadas à supressão da resposta imune Th1, direcionando para o desenvolvimento de uma resposta Th2 e à susceptibilidade do hospedeiro à infecção (Wilson *et al*., 2005). Entretanto, baixas concentrações de IL-4 melhoram a proteção induzida por uma vacina, aumentando indiretamente a produção de IFN-ɣ por LT e impedindo uma exacerbação da infecção por *L. donovani* (Stager *et al.*, 2003).

De acordo com Oliveira e colaboradores (2012), o perfil da resposta imune em camundongos BALB/c infectados experimentalmente pode variar de acordo com o inóculo de parasitos, a via e o tempo de infecção. Animais infectados por via subcutânea com 10^5 ou 10^7 parasitos apresentam produção elevada de IL-4 e IL-10, 60 dias após a infecção. A baixa produção de IFN-ɣ e a ativação da via alternativa de macrófagos foram também responsáveis pela incapacidade de controle da doença por estes animais e pelo aumento da carga parasitária nos órgãos analisados. Ao contrário, animais inoculados com 10^3 parasitos, pela mesma via, apresentaram uma redução na produção de IL-4 e IL-10 e um ligeiro aumento na produção de

IFN-ɣ, o que gerou uma redução na carga parasitária entre 45 e 60 dias após a infecção (Oliveira *et al.,* 2012).

Em camundongos, a depuração de carga parasitária hepática ocorre cerca de duas semanas após a infecção, devido à formação de granulomas resultantes da interação de células T com células dendríticas e macrófagos parasitados por *Leishmania* spp. A eficácia de uma vacina ou a progressão da doença pode ser predita baseando-se no grau de maturação dos granulomas hepáticos juntamente com a imunidade mediada por células nos animais desafiados (Murray *et al*., 1992; Carrión *et al*., 2006). Em contraste com a resposta ocorrida no fígado, o baço e a medula óssea agem como locais de persistência dos parasitos, resultando em infecção crônica. A ausência de reação granulomatosa no baço e a produção reduzida de IL-12 pelos macrófagos locais direcionam para um aumento da carga parasitária neste órgão (Nieto *et al*., 2011).

Apesar de modelos murinos para estudos vacinais na LV não se assemelharem clinicamente com a LV humana, a utilização de inóculos contendo um elevado número de parasitos pode gerar alterações patológicas que aumentam a semelhança da doença entre os dois mamíferos (Carrión *et al*., 2006). Estudos demonstraram que camundongos BALB/c inoculados com uma cepa de *L. donovani* apresentaram manifestações clínicas como hepatoesplenomegalia, observada até seis meses após a infecção (Mazumder *et al*., 2004; Bhowmick *et al*, 2009). A progressão da doença neste modelo encontra-se associada a uma supressão da resposta Th1 devido à produção reduzida de IFN-ɣ e IL-12 pelos esplenócitos dos animais, sendo também observado um aumento na produção de IL-4 e IL-10 por tais células. Camundongos infectados apresentaram níveis elevados de TGF-β e níveis reduzidos de TNF-α, quatro meses após a infecção, apresentando também uma redução na produção de NO mediado por macrófagos, o que resultou na progressão da doença (Bhowmick *et al*., 2009; Das & Ali, 2012).

No que se refere à resposta humoral, alguns autores consideram que os anticorpos não desempenham papel de proteção contra a infecção por *Leishmania* spp., podendo inclusive opsonizar parasitos e contribuir para a progressão da doença em animais susceptíveis (Bohme *et al*., 1986; Coelho et al., 2003; Kedzierski e Evans, 2014). Todavia, verifica-se uma tendência para o perfil de resistência quando há uma elevação na produção de anticorpos do isotipo IgG2a; enquanto que a progressão da doença se relaciona com o predomínio de anticorpos do isotipo IgG1 específicos aos antígenos dos parasitos (Martins *et al*., 2013; Costa *et al*., 2014).

2.8. Desenvolvimento de vacinas utilizando antígenos definidos

A pesquisa por vacinas contra as leishmanioses provém do fato de que indivíduos curados de lesões cutâneas desenvolvem uma resposta imune frente aos parasitos e tornam-se protegidos contra a reinfecção pela espécie infectante (Grimaldi & Tesh, 1993; Handman, 2001). Ao longo dos anos, diversas preparações foram propostas e estudos têm sido conduzidos com a finalidade de se desenvolver uma vacina que apresente resultados satisfatórios na proteção contra a infecção por *Leishmania* spp.

A leishmanização foi uma das primeiras estratégias vacinais utilizadas a partir de 1908 em combate às leishmanioses no Oriente Médio, União Soviética e Israel. Esta técnica consiste na inoculação de uma pequena quantidade de parasitos vivos em uma região não exposta do corpo, com o objetivo de que uma lesão cutânea local se desenvolva e cure espontaneamente, conferindo ao indivíduo uma imunidade protetora contra a reinfecção. Contudo, esta técnica foi abandonada por muitos países devido ao risco de se causar uma infecção crônica, levando o indivíduo a necessitar de tratamento; tendo dado lugar à utilização de parasitos mortos como estratégia vacinal (Handman, 2001; Kumar & Engwerda, 2014).

A utilização de parasitos irradiados ou autoclavados para compor uma vacina foi descontinuada devido aos resultados conflitantes de efetividade obtidos na década de 1940 (Grimaldi & Tesh, 1993; Handman, 2001). Enquanto no Oriente Médio a vacinação com organismos mortos falhou em proteger os indivíduos, no Brasil, os ensaios clínicos mostraram uma excelente proteção. Esta instabilidade na eficácia de uma vacina com parasitos mortos também foi percebida em estudos realizados com camundongos na década de 1980. Os ensaios clínicos demonstraram que havia uma diferença na eficácia vacinal dependente da via de administração utilizada. Animais inoculados por via intravenosa ou intraperitoneal apresentaram uma excelente proteção contra a doença, em contrapartida, esta proteção não foi observada quando a via subcutânea foi utilizada (Handman, 2001).

Devido aos resultados obtidos em diversos experimentos realizados com parasitos mortos, foi observado que a persistência do patógeno no organismo do indivíduo tivesse certa importância para se obter uma resposta imune protetora e assim, foi sugerido que vacinas de parasitos vivos atenuados tornassem uma nova alternativa (Selvapandiyan *et al*., 2014).

Vacinas com parasitos vivos atenuados podem ser obtidas através de culturas *in vitro* com meios modificados, alterações de temperatura, irradiação gama ou mutações genéticas (Foroughi-Parvar & Hatam, 2014). Este tipo de vacina vem sendo testada em alguns modelos animais, demonstrando que os parasitos modificados são reconhecidos pelas células do

hospedeiro da mesma forma que os parasitos virulentos e que conseguem permanecer no organismo por algum tempo, sem se replicarem. A presença deste parasito permite que o sistema imune do hospedeiro monte uma resposta específica contra os antígenos e que células efetoras e de memória geradas venham conferir proteção. Uma das preocupações com a utilização desta vacina é a possível reversão da virulência dos parasitos, entretanto, uma alternativa para a resolução deste problema seria a eliminação destes genes de virulência, permitindo que tais candidatos vacinais ainda sejam estudados. A utilização de parasitos mutantes sensíveis a determinadas drogas, bem como a utilização de espécies não patogênicas, como *L. tarentolae*, seriam também possíveis alternativas, porém, ainda deve-se priorizar a segurança da vacina e atentar para a possibilidade de que parasitos vivos estão susceptíveis a mutações espontâneas, além de se considerar a dificuldade de produção e análise de qualidade em larga escala (Kumar & Engwerda, 2014).

Como alternativa vacinal mais segura, frações proteicas de *Leishmania* spp. têm demonstrado excelentes propriedades imunoprotetoras em modelos experimentais (Das & Ali, 2012). Vacinas utilizando extratos brutos dos parasitos, proteínas recombinantes purificadas, peptídeos sintéticos e frações de DNA inseridos em plasmídeos, vêm sendo testadas com diferentes sistemas de entrega.

Uma formulação vacinal baseada em extrato bruto de *L. brasiliensis* adicionada de saponina, denominada de LBSap, foi administrada em cães e demonstrou ser capaz de induzir uma resposta humoral com anticorpos específicos anti-*Leishmania*, além de induzir um perfil celular baseado em LT $CD4^+$ e LT $CD8^+$. Uma elevada produção de IFN-ɣ e IL-12 e a redução na carga parasitária do baço demonstraram a imunogenicidade e proteção contra a infecção experimental por *L. infantum* (Roatt *et al.*, 2012; Resende *et al*., 2013). Uma vacina muito parecida foi também testada com extrato bruto de *L. amazonensis* e BCG, nomeada de Leishvaccine. Esta formulação foi capaz de estimular uma ativação inicial de LT $CD4^+$ e uma ativação tardia de LT $CD8^+$ e LB, demonstrando um elevado potencial imunogênico contra as leishmanioses (Araújo *et al*., 2008).

Na França, a utilização de proteínas de *L. infantum* secretadas e purificadas do sobrenadante da cultura também estão sendo aplicadas no desenvolvimento de vacinas. A formulação denominada LiESAP-MDP recebeu o nome comercial de CaniLeish® e apresentou eficácia de 92% nos animais vacinados (Lemesre *et al.*, 2007). Experimentos utilizando o composto ligante fucose-manose (FML) de formas promastigotas de *L. donovani* e adjuvante indutor de resposta Th1 apresentou resultados de imunogenicidade,

imunoprofilaxia e imunoterapêutica em camundongos, hamsters e cães (Palatnik-de-Sousa *et al*., 1994; Santos *et al*., 2002, 2003, 2007; Borja-Cabrera *et al*., 2004, 2008, 2010), além de dar origem a uma vacina denominada Leishmune®, composta por FML-saponina, que foi capaz de induzir proteção na maioria dos animais vacinados (Palatnik-de-Sousa, 2012).

Vacinas com antígenos recombinantes podem ser consideradas em relação à eficiência, segurança e custo (Foroughi-Parvar & Hatam, 2014). Com o avanço na tecnologia do DNA recombinante, moléculas específicas de determinada espécie ou fase de vida do parasito estão sendo extensivamente estudadas, originando vários candidatos à vacina (Joshi *et al.,* 2014). O primeiro antígeno recombinante utilizado para imunização contra as leishmanioses foi a glicoproteína gp63, considerada uma proteína de virulência dos parasitos e encontrada conservada em todas as espécies de *Leishmania* spp. Tanto a proteína nativa quanto a recombinante foram capazes de estimular uma resposta protetora em camundongos, demonstrando que esta imunogenicidade está relacionada ao processamento e apresentação de peptídeos e não apenas da estrutura conformacional nativa (Das & Ali, 2012).

Proteínas de *Leishmania* spp. como LCR1, A2, HASPB1 foram expressas em sua forma recombinante e testadas contra LV. A LCR1 é uma proteína específica da forma amastigota de *L. infantum* capaz de estimular a produção de IFN-ɣ e de proteger parcialmente camundongos BALB/c contra a infecção por esta espécie (Wilson *et al*., 1995). Stager e colaboradores (2000) confirmaram que a proteína rHASPB1 é altamente imunogênica e que gerou proteção significativa em camundongos desafiados com *L. donovani.* Fernandes e colaboradores (2008) investigaram a imunidade protetora da proteína recombinante A2 em associação com a saponina contra a LVC. Esta formulação tornou-se uma vacina comercial denominada Leish-Tec® e provou induzir um aumento significativo de IFN-ɣ nos cães vacinados, além de reduzida produção de IL-10, entretanto, a escala de produção não tem sido suficiente para suprir a necessidade do mercado brasileiro.

Proteínas específicas da forma promastigota dos parasitos têm demonstrado ser potentes estimuladoras de uma resposta imune do tipo Th1, como foi visto com a PDI, TPI, eIF-2, aldolase, enolase, P45 e TPR, as quais levaram à proteção variada de 60 a 90% em hamsters dourados (Joshi *et al*., 2014). Outras proteínas recombinantes ainda foram testadas e demonstraram ser boas candidatas à vacina, como a F14 e as proteínas ribossomais de *Leishmania* spp., uma vez que ambas forneceram proteção parcial em hamsters e camundongos BALB/c, respectivamente (Bhardwaj *et al.*, 2009; Chávez-Fumagalli *et al*., 2010).

Antígenos expressos nas formas amastigotas dos parasitos têm sido menos explorados como candidatos à vacina contra as leishmanioses, haja vista a dificuldade de cultivo destas formas. Assim, a maioria dos estudos em relação às vacinas têm centrado em antígenos expressos nas formas promastigotas de *Leishmania* spp. No entanto, as formas amastigotas parecem ser um alvo apropriado para o sistema imune dos hospedeiros, uma vez que poucas horas após a infecção inicial e durante a doença ativa, esta fase morfológica do parasito encontra-se em contato com as células do sistema imune dos hospedeiros. Além disso, as formas amastigotas residem no interior das células fagocíticas e são alvos para LT $CD4^+$ e LT $CD8^+$, elementos envolvidos na imunidade protetora contra *Leishmania* spp. (Fernandes *et al.*, 2012).

Uma nova estratégia, denominada imunoproteômica, vem sendo utilizada para identificar novas proteínas, consideradas ainda como hipotéticas, mas que podem se apresentar como potenciais candidatas à vacina (Coelho *et al*, 2012). Uma destas proteínas, LiHyp1, foi identificada na forma amastigota de *L. infantum* por anticorpos de cães com LV ativa e demonstrou ter um potencial vacinal em camundongos BALB/c, induzindo uma elevada produção das citocinas IFN-ɣ, IL-12 e GM-CSF, e uma produção reduzida de IL-4 e IL-10. A resposta imune frente à proteína foi capaz de induzir uma redução significativa da carga parasitária nos animais desafiados, tendo sido primada pela participação de LT $CD4^+$ (Martins *et al*, 2013). Uma segunda proteína, conservada dentre as espécies de *Leishmania* spp. e também identificada por esta ferramenta, foi nomeada de LiHyT, sendo um antígeno capaz de proteger camundongos BALB/c contra as formas tegumentar e visceral das leishmanioses. Neste caso, animais imunizados com a proteína recombinante conjugada ao adjuvante saponina e desafiados com as espécies *L. infantum, L. major* ou *L. brasiliensis* foram capazes de desenvolver um perfil de resposta imune protetor, polarizada Th1, demonstrando uma elevada eficiência deste imunógeno (Martins *et al*, 2016'; 2016").

O desenvolvimento de vacinas a partir de proteínas recombinantes ou mesmo de peptídeos sintéticos apresentam a vantagem de permitir a utilização de epitopos determinados e já caracterizados, uma vez que é possível modificar tais moléculas e evitar os inconvenientes de epitopos desnecessários para o desenvolvimento de uma imunidade protetora (Handman, 2001; Skeiky *et al.*, 2002). Para o desenvolvimento de uma vacina peptídica, o estudo dos epitopos através de ferramentas de bioinformática e o conhecimento em relação à ativação de LT e LB são de grande importância. As vacinas com peptídeos sintéticos podem apresentar algumas vantagens em relação aos outros compostos vacinais

como, por exemplo, a ausência de qualquer material potencialmente infeccioso, possibilidade de inclusão de diversos e determinados epitopos, diminuição na quantidade de antígenos utilizados, dentre outras (Joshi *et al*., 2014). Entretanto, os peptídeos podem também apresentar desvantagens, como a incapacidade de determinados indivíduos a responderem ao estímulo ofertado. Vacinas com peptídeos isolados normalmente são incapazes de induzir uma resposta protetora, sendo necessária uma mistura a outros antígenos para que possam estimular esta resposta e/ou o uso de adjuvantes e diferentes sistemas de entrega (Handman, 2001). Peptídeos extraídos de proteínas muito estudadas, como a gp63, KMP-11, A2, LPG, cisteíno-proteinases, dentre outras, vêm sendo utilizados para compor vacinas, uma vez que têm apresentado resultados satisfatórios em camundongos (Joshi *et al*., 2014). Mais recentemente, a utilização da técnica de phage diplay selecionou mimotopos de *Leishmania* spp. em clones de bacteriófagos, os quais foram capazes de induzir uma resposta imunogênica com elevada concentração de IFN-ɣ, produzido principalmente por LT $CD8^+$ e pela reduzida produção de IL-4, tendo sido capaz de proteger camundongos BALB/c contra a infecção experimental por *L. infantum* (Costa *et al*., 2014).

Devido aos polimorfismos genéticos encontrados no sistema imune de mamíferos, uma vacina multiantigênica, composta por antígenos definidos e que leve a uma resposta imune do tipo Th1, pode apresentar uma melhor imunogenicidade e proteção do que os imunógenos utilizados isoladamente (Goto *et al*., 2011; Martins *et al.*, 2015). Algumas preparações multiproteicas vêm sendo testadas e têm apresentado bons resultados, como a proteína Q (Lip2a, Lip2b, H2A e P0) (Molano *et al*., 2003), a Leish111f – MPL – SE (TSA, LmSTI-1, LeIF) (Coler *et al.*, 2007) e a KSAC (KMP11, SMT, A2 e CPB)(Goto *et al*., 2011).

Em se tratando da idéia de que diversos antígenos compondo uma única vacina seriam capazem de melhorar a estimulação do sistema imune, a busca por uma quimera composta por epitopos de células T $CD4^+$ e T $CD8^+$ presentes em diferentes proteínas poderia ser uma boa estratégia a ser empregada em uma vacina. Além da possibilidade de juntar diferentes regiões imunogênicas, tanto de proteínas presentes nas formas amastigotas, quanto nas formas promastigotas dos parasitos, estes antígenos estariam presentes em um único produto final, o que poderia impactar em uma redução de custo de produção.

Vacinas de DNA também são atrativas em compor uma estratégia profilática contra as leishmanioses. Este método pode apresentar vantagens, como o custo reduzido, facilidade na produção e armazenamento, estabilidade do material, expressão de antígenos relevantes e eficiente indução de uma resposta imune efetora e de memória (Handman, 2001; Kumar &

Engwerda, 2014). Questões relacionadas à segurança das vacinas de DNA são discutidas, como a possibilidade da integração do DNA ao genoma de mamíferos e a indução ao desenvolvimento de doenças autoimune, entretanto, nada foi comprovado até o momento. Vacinas de DNA contendo genes que expressam a proteína A2 (Zanin *et al.*, 2007), PPG (Samant *et al.*, 2009) ou HbR (Guha *et al.*, 2013) provaram estimular uma resposta do tipo Th1, com níveis elevados de IFN-ɣ e reduzida produção de IL-4; resultando na proteção de camundongos e hamsters imunizados contra infecções experimentais com diferentes espécies de *Leishmania* spp.

Devido ao fato de que alguns indivíduos não infectados, mas expostos ao inseto vetor, apresentarem uma resposta protetora contra a infecção pelo parasito, vacinas utilizando proteínas salivares de *Phlebotomus* spp. ou *Lutzomyia* spp., como PpSP15, LJM17 ou LJM143 vêm sendo estudadas como potentes imunógenos, sendo capazes de estimular infiltração de linfócitos, macrófagos e uma produção aumentada de IFN-ɣ (Morris *et al*., 2001; Joshi *et al*., 2014).

Estratégias vacinais contendo diferentes veículos de entrega, como lipossomas (Firouzmand *et al*., 2013), nanopartículas (Danesh-Bahreini *et al*., 2011), vetores virais (Guha *et al*., 2013), células dendríticas (Matos *et al*., 2013) e bactérias (Hugentobler *et al.*, 2012) têm resultado em graus variados de eficiência; assim como a variação na utilização dos diferentes adjuvantes vacinais (Vitoriano-Souza *et al*., 2012; Pereira *et al.*, 2015; Thakur *et al.*, 2015). Desta forma, a escolha dos antígenos, do veículo de entrega, do adjuvante e da via de administração da vacina são importantes ao se definir a estratégia vacinal a ser utilizada.

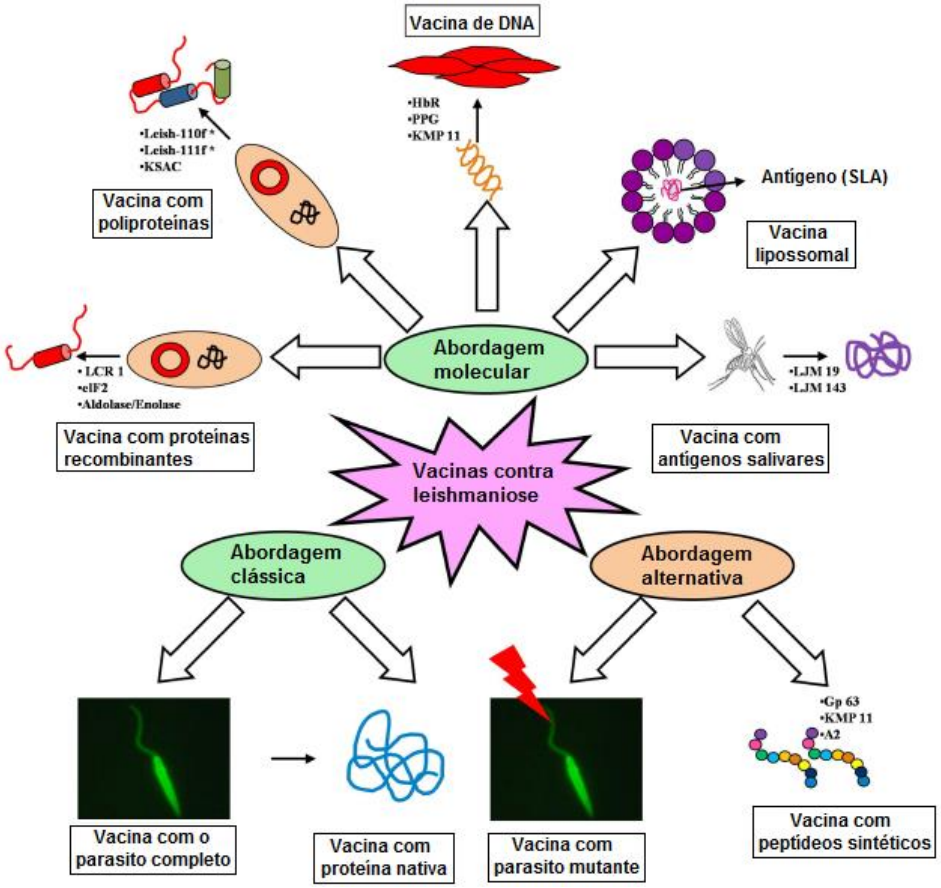


Figura 2: Estratégias utilizadas para a formulação de uma vacina contra as leishmanioses. Adaptado de Joshi *et al*. (2014).

2.9. Antígenos utilizados neste trabalho

As proteínas LiHyp1 (XP_001468941.1), LiHyp6 (XP_001568689.1), HRF (CAJ05086.1) e LiHyV (XP_888524.1), utilizadas neste trabalho, foram recentemente identificadas por Coelho *et al*. (2012), tendo sido reconhecidas, através da técnica de imunoproteômica, por anticorpos presentes em soros de cães com leishmaniose. As proteínas foram identificadas nos genomas de *L. infantum* (LiHyp1 – LinJ.35.1290), *L. brasiliensis* (LiHyp6 – LbrM.35.0640) e *L. major* (HRF – LmjF.24.1510 e LiHyV- LmjF.04.0130), entretanto, genes ortólogos de *L. infantum* foram identificados para LiHyp6 (LinJ.36.0580), HRF (LinJ.24.1560) e LiHyV (LinJ.04.0160).

O gene responsável pela codificação da proteína LiHyp1 (Proteína hipotética de *L. infantum* 1) foi identificado no cromossomo 35 e apresenta 957 pares de bases (pb). A sequência de aminoácidos contém 318 resíduos linearizados, totalizando em um peso molecular de aproximadamente 36,6 kiloDaltons (kDa). É uma proteína que não apresenta peptídeo sinal e que se encontra expressa na forma amastigota dos parasitos, sendo

reconhecida por anticorpos presentes no soro de cães com LV assintomática e sintomática. A proteína pertence à superfamília das oxigenases dependentes de 2-oxoglutarato (2-OG) e Fe^{+2}. Normalmente, as enzimas pertencentes a esta superfamília catalisam a oxidação de substratos orgânicos através de uma molécula de oxigênio, íons de ferro atuando como cofatores enzimáticos e o 2-OG como um cosubstrato, o qual é decarboxilado e liberado como succinato e gás carbônico. Enzimas pertencentes a esta superfamília podem apresentar diversas funções. Em metazoários, as prolil hidroxilases atuam catalisando resíduos de prolina em hidroxiprolina, assim como as lisina-hidroxilases catalisam a conversão de lisina em hidroxilisina, sendo que ambas as reações encontram-se relacionadas à obtenção de moléculas formadoras de colágeno e outras proteínas. Nas plantas, algumas destas enzimas podem catalisar reações que levam à produção dos hormônios e pigmentos. Em bactérias e fungos, estas enzimas participam na biossíntese de antibióticos, como a penicilina (isopenicilina sintase) e cefalosporinas (deacetocefalosporina C sintase), e tanto nos eucariotos como nos procariotos a proteína AlkB, também pertencente a esta superfamília, apresenta-se envolvida em reações de reparo de DNA através de excisões de grupos alquila.

O gene responsável pela codificação da proteína LiHyp6 (Proteína hipotética de *L. infantum* 6) em *L. infantum* é encontrado no cromossomo 36 e apresenta 633 pb. A proteína possui uma sequência de 210 aminoácidos, os quais conferem um peso molecular de aproximadamente 23,8 kDa. Não se sabe muito a respeito da proteína LiHyp6, entretanto, através de análises por bioinformática, foi encontrado que além de apresentar epitopos para LT $CD4^{+}$ e LT $CD8^{+}$ murino, ela também apresenta epitopos específicos para tais células no homem, o que a torna atrativa visando-se a busca por proteínas imunoestimuladoras e com potencial desenvolvimento para vacinas humanas. Sabe-se ainda que esta proteína encontra-se presente nas formas promastigotas dos parasitos e foi reconhecida por soros de cães com LV sintomática.

A proteína HRF (*IgE-dependent histamine-releasing fator*) é codificada por um gene que apresenta 513 pb e encontra-se presente no cromossomo 24. Possui uma sequência de 170 aminoácidos, peso molecular de 19,4 kDa e foi identificada como uma TCTP (*Translationally controlled tumor protein*). É conservada dentre alguns eucariotos e está envolvida em uma variedade de atividades celulares, incluindo estabilização de estrutura celular, ligação de cálcio, apoptose, inibição e prevenção de estresse e morte celular, além de estar presente em células durante o estágio inicial de crescimento de alguns tumores. A proteína possui diversos epitopos reconhecidos pelas células T do homem e de modelos murinos, foi identificada na

fase promastigota dos parasitos e é reconhecida por anticorpos presentes nos soros de cães com LV sintomática.

A LiHyV (Proteína hipotética de *L. infantum* V) é uma proteína que possui uma sequência de 528 aminoácidos, os quais conferem à mesma um peso molecular de aproximadamente 53,7 kDa. O gene responsável por sua codificação encontra-se presente no cromossomo 4 e possui 1.587 pb. É uma proteína de superfície que está presente principalmente no estágio amastigota dos parasitos e foi reconhecida por anticorpos presentes nos soros de cães com LV sintomática e assintomática. Assim como as outras proteínas, apresenta epitopos específicos para células T $CD4^+$ e $CD8^+$, sendo que dois dos epitopos preditos para LT $CD8^+$ (AGQSVPNTL e VGIAKSTAALFVLNATAI) foram selecionados e utilizados neste trabalho como candidatos vacinais comparativos a esta proteína recombinante.

Para construção de uma quimera polipeptídica utilizada como antígeno vacinal, epitopos de células T $CD4^+$ (tabela 2) e T $CD8^+$ (tabela 3) das proteínas LiHyp1, LiHyp6, HRF e LiHyV foram avaliados em análises de bioinformática frente ao reconhecimento por MHC de classe I e II, em humanos e camundongos. Cinco regiões gênicas ricas nestes epitopos foram selecionadas com a finalidade de serem sintetizadas em sequência, objetivando a expressão de uma única proteína polipeptídica recombinante (figura 3). A proteína recombinante quimérica apresenta 279 aminoácidos em sua sequência, um peso molecular de 31,6kDa e ponto isoelétrico de aproximadamente 9,6.

Tabela 2: Predição *in silico* de MHC de classe II humano e de camundongo BALB/c restrito à epitopos de proteínas presentes em *L. infantum.*

MHC de classe II – humano			
Proteína	**Número**	**Sequência peptídica**	**% de alelos ligantes**
LiHyp6 (XP_001568689.1)	1	52-LLYRSTFRHAMLLRV-66	38,46
	2	53-LYRSTFRHAMLLRVQ-67	38,46
	3	54-YRSTFRHAMLLRVQR-68	38,46
	4	55-RSTFRHAMLLRVQRE-69	42,31
	5	56-STFRHAMLLRVQRET-70	38,46
HRF (CAJ05086.1)	6	108-RKAFQTNAAAFVKKV-122	30,77

MHC de classe II – camundongo				
Proteína	**Número**	**Sequência peptídica**	**Alelo**	**Net MHCII Score**
LiHyp1 (XP_001468941.1)	1	169-ARYVYFHMVLPVEAQ-183	H-2-IAd	394,4
	2	170-RYVYFHMVLPVEAQR-184	H-2-IAd	446,9
	3	171-YVYFHMVLPVEAQRF-185	H-2-IAd	161,2
	4	172-VYFHMVLPVEAQRFS-186	H-2-IAd	160,5
	5	173-YFHMVLPVEAQRFSL-187	H-2-IAd	169,2
	6	174-FHMVLPVEAQRFSLV-188	H-2-IAd	255,3
LiHyp6 (XP_001568689.1)	7	55-RSTFRHAMLLRVQRE-69	H-2-IAd	204,8
	8	56-STFRHAMLLRVQRET-70	H-2-IAd	182,5
	9	57-TFRHAMLLRVQRETR-71	H-2-IAd	221,1
	10	58-FRHAMLLRVQRETRF-72	H-2-IAd	252
	11	59-RHAMLLRVQRETRFD-73	H-2-IAd	484,1
LiHypV (XP_888524.1)	12	83-TPSSARLSMSMAITT-97	H-2-IAd	411,1
	13	84-PSSARLSMSMAITTV-98	H-2-IAd	347,9
	14	85-SSARLSMSMAITTVA-99	H-2-IAd	193,3
	15	86-SARLSMSMAITTVAQ-100	H-2-IAd	145,8
	16	87-ARLSMSMAITTVAQS-101	H-2-IAd	113,9
	17	88-RLSMSMAITTVAQSA-102	H-2-IAd	93,5
	18	89-LSMSMAITTVAQSAI-103	H-2-IAd	165,8
	19	90-SMSMAITTVAQSAIT-104	H-2-IAd	231,5
	20	97-TVAQSAITLSGVMPA-111	H-2-IAd	382,5
	21	98-VAQSAITLSGVMPAN-112	H-2-IAd	391

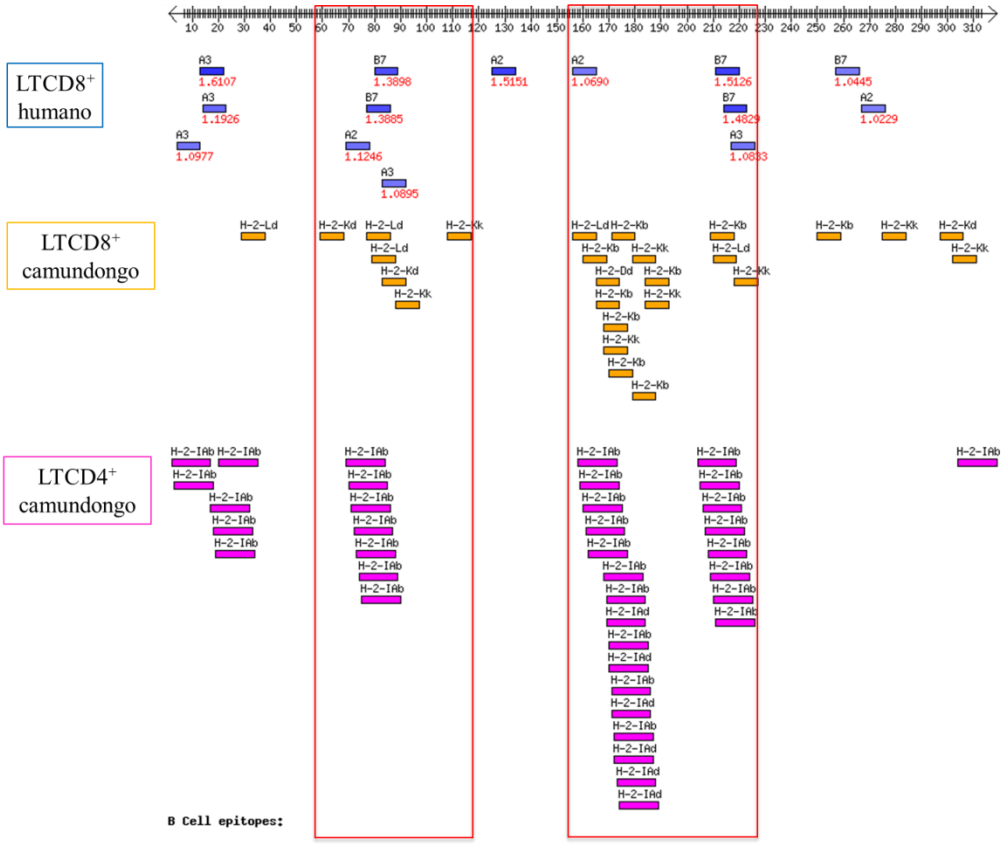
Tabela 3: Predição *in silico* de MHC de classe I humano e de camundongo BALB/c restrito à epitopos de proteínas presentes em *L. infantum.*

MHC de classe I – humano				
Proteína	**Número**	**Sequência peptídica**	**Alelo**	**Net CTL Score**
LiHyp1 (XP_001468941.1)	1	69-ILNDGRFQL-77	A2	1,008
	2	156-MVPDRSVYI-164	A2	0,748
	3	83-ASFMPLLER-91	A3	0,754
	4	77-LPPLPPASF-85	B7	0,801
	5	80-LPPASFMPL-88	B7	0,995
LiHyp6 (XP_001568689.1)	6	37-SLATAFGLV-45	A2	0,85392
	7	52-LLYRSTFRH-60	A3	0,69486
HRF (CAJ05086.1)	8	87-YMAHIRSYM-95	A2	0,78478
	9	111-FQTNAAAFV-119	A2	0,76162
	10	88-MAHIRSYMK-96	A3	0,68151
	11	112-QTNAAAFVK-120	A3	0,6143
	12	115-AAAFVKKVL-123	B7	0,4089
LiHypV (XP_888524.1)	13	90-SMSMAITTV-98	A2	0,94397
	14	69-VSGNGLTIK-77	A3	0,5355
	15	83-TPSSARLSM-91	B7	0,97149
	16	97-TVAQSAITL-105	B7	0,50925
	17	109-MPANSDIRI-117	B7	0,72604
	18	116-RIVATTSSL-124	B7	0,78072
	19	125-APAQSLFDF-133	B7	0,74818

MHC de classe I – camundongo BALB/c				
Proteína	**Número**	**Sequência**	**Alelo**	**Net CTL Score**
LiHyp1 (XP_001468941.1)	1	77-LPPLPPASF-85	H-2-Ld	0.27186
	2	79-PLPPASFMP-87	H-2-Ld	0.08248
	3	165-MSGPARYVY-173	H-2-Dd	0.23280
	4	59-DVYTRASDR-67	H-2-Kd	0.07271
	5	83-ASFMPLLER-91	H-2-Kd	0.20710
	6	156-MVPDRSVYI-164	H-2-Ld	0.23140
LiHyp6 (XP_001568689.1)	7	29-LTYAETVVS-37	H-2-Kd	-
LiHyp4 (XP_888524.1)	8	82-STPSSARLS-90	H-2-Ld	0.11316
	9	124-LAPAQSLFD-132	H-2-Ld	-

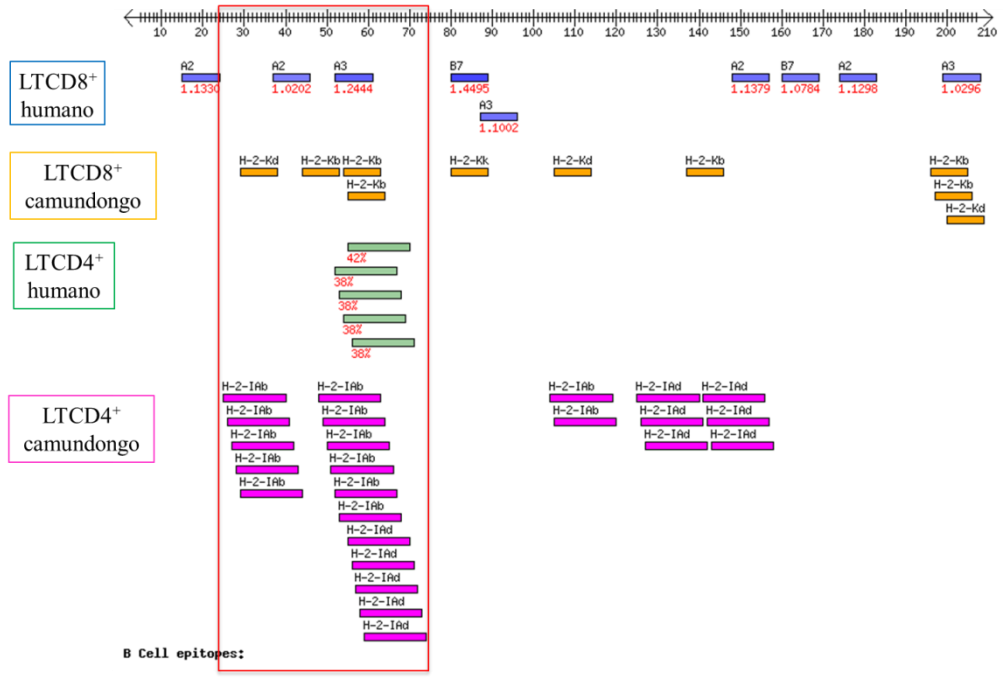
A.

LiHyp1 (XP_001468941.1)

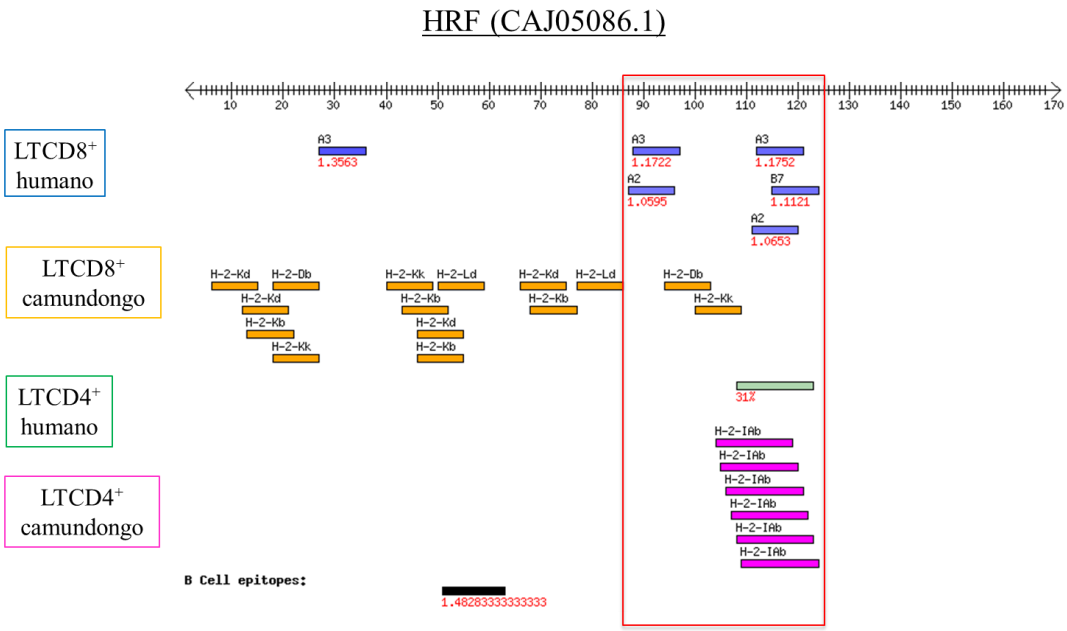


B.

LiHyp6 (XP_001568689.1)



C.


HRF (CAJ05086.1)
LTCD8+ humano
LTCD8+ camundongo
LTCD4+ humano
LTCD4+ camundongo
B Cell epitopes:

D.

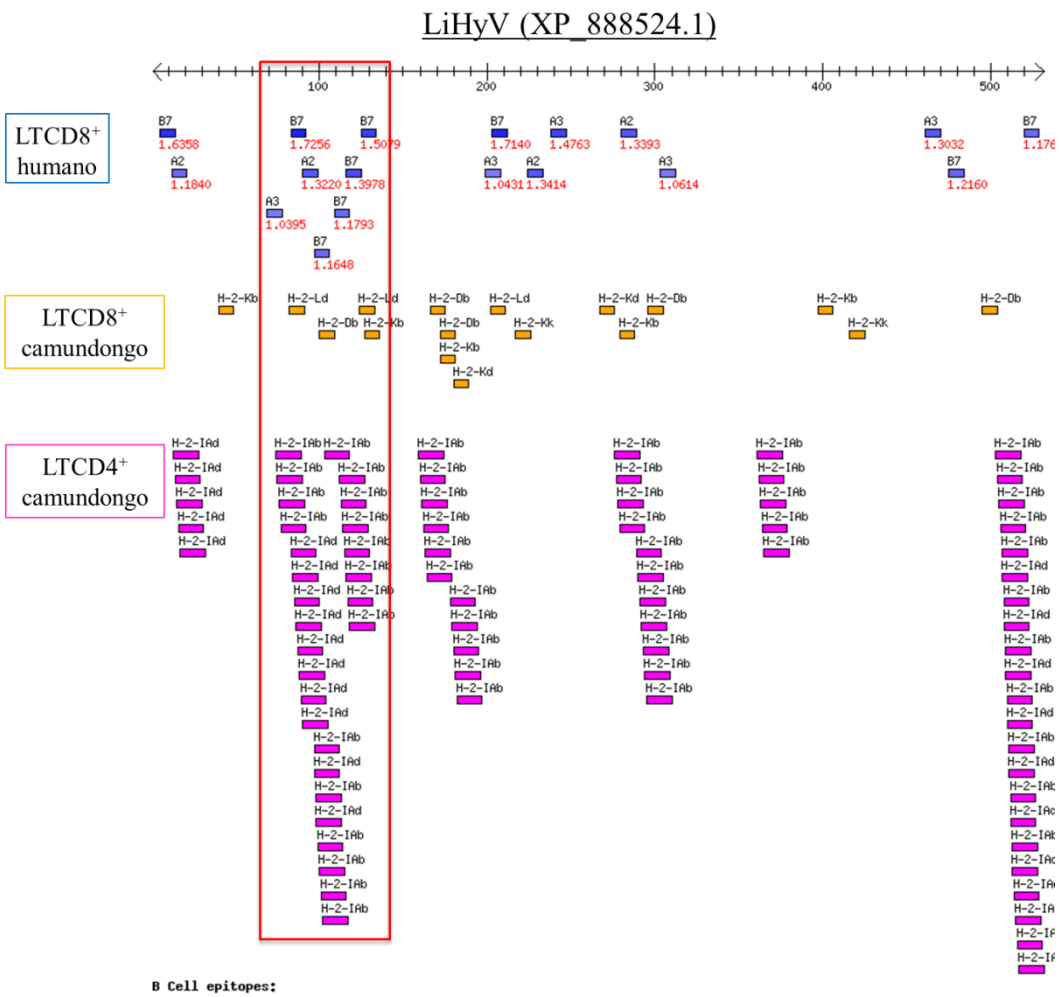

LiHyV (XP_888524.1)
LTCD8+ humano
LTCD8+ camundongo
LTCD4+ camundongo
B Cell epitopes:

Figura 3: Seleção das regiões imunogênicas presentes nas proteínas LiHyp1, LiHyp6, HRF e LiHyV para construção da proteína quimérica polipeptídica. As regiões compostas por uma elevada densidade de epitopos (circuladas por um retângulo vermelho) foram selecionadas em cada uma das proteínas em estudo e então os genes responsáveis por tais regiões foram sintetizados em sequência para que pudessem compor, quando expressos, uma única proteína quimérica recombinante. Os números dentro dos suportes representam posições de aminoácidos dentro das proteínas e cada retângulo colorido se refere a um epitopo potencial. Retângulos azuis são referentes aos epitopos de células T $CD8^+$ humanos; os amarelos se referem aos epitopos de células T $CD8^+$ murinos; os retângulos verdes são referentes aos epitopos de células T $CD4^+$ humanos; os de cor rosa referem-se aos epitopos de células T $CD4^+$ murinos; e por fim, os retângulos pretos são referentes aos epitopos de células B.

2.10. Saponina como adjuvante de resposta imune

Adjuvantes são substâncias capazes de iniciar uma resposta imune em relação a um dado antígeno ou auxiliar no desenvolvimento de uma resposta já existente (Claassen & Boersma, 1992). Vacinas de subunidades, muitas vezes, necessitam de auxílio para ativarem eficientemente o sistema imune inato e adaptativo; desta forma, a associação de adjuvantes de resposta imune se faz necessária (Rueckert & Guzmán, 2012). Estas substâncias apresentam a capacidade de aumentar a imunogenicidade de antígenos fracos, reduzir a quantidade de antígenos necessária nos protocolos de imunização e o número de doses administradas, induzir a um fenótipo de citocinas favorável para uma resposta imune benéfica ao hospedeiro, além de prolongar a resposta imune gerada (Voguel, 1998; Vitoriano-Souza *et al*., 2012).

Saponinas são glicosídeos tensoativos que contém um núcleo hidrofóbico de estrutura triterpenóide, com cadeias de carboidratos ligadas a ele (Rajput *et al*. 2007). São obtidas da casca da árvore *Quillaja saponária* e apresentam-se ideais para utilização em experimentos vacinais, como adjuvantes de resposta imune (Liu *et al.*, 2002; Ravindran & Ali, 2004). A saponina apresenta uma formulação simples, custo reduzido e é considerada segura para uso veterinário (Vitoriano-Souza *et al*., 2012), entretanto, seu uso não é permitido em humanos por possuir efeitos citotóxicos, principalmente hemolíticos, que podem ser reduzidos através de remoção química de porções glicídicas da molécula (Palatnik-de-Sousa *et al.*, 2004).

Da mesma forma que outros adjuvantes, a saponina induz a um rápido recrutamento de células inflamatórias em seu local de administração e esta resposta pode ser mantida por dias após o estímulo (Taylor, 1995). Após administrada, a saponina induz uma migração diferencial de neutrófilos, monócitos e linfócitos para o sítio de aplicação, além de induzir a produção das citocinas IL-2, IFN-ɣ e IL-6 (Vitoriano-Souza *et al*., 2012).

Além dos estudos em modelos murinos (Palatnik de Sousa *et al.*, 2004; Aguilar-Be *at al.*, 2005; Paraguai de Sousa *et al.*, 2001), a utilização da saponina na proteção contra a LVC

foi também avaliada. Quando a proteína recombinante A2 foi administrada em cães juntamente com a saponina, pôde-se observar uma elevação nos níveis de IFN-ɣ nos animais imunizados, que se apresentaram protegidos contra a infecção experimental por *L. infantum* (Fernandes *et al.*, 2008).

Desta forma, o presente trabalho objetivou a utilização das proteínas recombinantes LiHyp1, LiHyp6, HRF e LiHyV, dois peptídeos da proteína LiHyV, bem como uma proteína recombinante quimérica composta por epitopos advindos das proteínas selecionadas, todos associados ao adjuvante saponina, a fim de testar e comparar seu efeito protetor contra a infecção experimental de camundongos BALB/c com formas promastigotas estacionárias de *Leishmania* spp.

3. OBJETIVOS

3.1. Objetivo geral

⇒ Avaliar as proteínas recombinantes rLiHyp1, rLiHyp6, rHRF, rLiHyV, isoladas ou em associação, como candidatas ao sorodiagnóstico da leishmaniose visceral canina; bem como avaliar e comparar o potencial imunogênico e a eficácia dos antígenos de *Leishmania* spp. em diferentes estratégias vacinais contra a infecção experimental com *L. infantum* ou *L. amazonensis*.

3.2. Objetivos específicos

⇒ Avaliar a sequência nucleotídica e de aminoácidos das proteínas LiHyp1, LiHyp6, HRF, LiHyV e selecionar epitopos de células T $CD4^+$ e T $CD8^+$ destas proteínas com a finalidade de utilizá-los em forma de peptídeos sintéticos ou em uma quimera polipeptídica.

⇒ Avaliar a capacidade diagnóstica das proteínas recombinantes isoladas (rLiHyp1, rLiHyp6, rHRF ou rLiHyV) ou de uma mistura entre elas (rLiHyp1, rLiHyp6 e rHRF) utilizando amostras de soros de cães com leishmaniose visceral ativa; de cães não infectados por *Leishmania* spp., de cães vacinados com a vacina Leish-Tec® e de cães infectados com *Trypanosoma cruzi*, *Ehrlichia canis* ou *Babesia canis*.

⇒ Avaliar o perfil da resposta imune gerada em camundongos BALB/c imunizados com dois peptídeos sintéticos individualizados, com as proteínas recombinantes isoladas (rLiHyp1, rLiHyp6, rHRF ou rLiHyV) ou em conjunto (rLiHyp1, rLiHyp6 e rHRF) ou ainda com uma quimera polipeptídica composta por epitopos de células T $CD4^+$ e T $CD8^+$ presentes nas proteínas recombinantes em análise, sendo todos os antígenos adicionados ao adjuvante saponina.

⇒ Avaliar o perfil da resposta celular e humoral geradas nos camundongos imunizados e desafiados com *L. infantum* ou *L. amazonensis*.

⇒ Avaliar o grau de proteção nos camundongos imunizados após a infecção com *L. infantum* ou *L. amazonensis.*

⇒ Avaliar e comparar a eficácia das estratégias vacinais utilizando peptídeos sintéticos, proteínas recombinantes isoladas ou associadas e a proteína quimérica recombinante.

4. MATERIAIS E MÉTODOS

4.1. Animais de experimentação

Camundongos BALB/c fêmeas, de 8 semanas de idade, foram utilizados. Os animais foram adquiridos no Biotério do Instituto de Ciências Biológicas (ICB) da UFMG e mantidos em estante apropriada no Biotério do Departamento de Patologia Clínica do COLTEC, UFMG, sob as devidas condições de manejo. Este projeto foi aprovado junto ao Comitê de Ética em Experimentação Animal (CEUA) da UFMG, com o protocolo número 43/2011 (ANEXO 1).

4.2. Parasitos

Parasitos da cepa MHOM/BR/1970/BH46 de *Leishmania infantum* ou da cepa IFLA/BR/1967/PH-8 de *L. amazonensis* foram cultivados em meio de cultura Schneider's (Schneider's Insect Medium, Sigma) completo, constituído por meio Schneider's acrescido com 20% de soro fetal bovino (SFB) inativado (Sigma), 20 mM de L-glutamina, 200 unidades por mililitro (U/mL) de penicilina e 100 microgramas por mililitro (µg/mL) de estreptomicina, pH 7,4. Os parasitos foram cultivados a 24°C e repiques das culturas foram efetuados de cinco em cinco dias, período que os parasitos apresentavam-se em fase logarítmica de crescimento. Estoques de parasitos foram mantidos em nitrogênio líquido, a -196ºC.

4.3. Amostras de soros

Para a avaliação da antigenicidade da proteína rLiHyV, 73 amostras de soro de cães (*Canis familiaris*), machos e fêmeas e de diferentes raças e idade, foram utilizadas. Os soros positivos para LVC foram selecionados com base em dois testes sorológicos da Biomanguinhos (Fiocruz, Brasil): IFAT (IFAT-LVC Bio-Manguinhos kit) e ELISA (EIE-LVC Bio-Manguinhos kit) e pelo teste molecular de PCR. Soros com titulação < 1/40 no IFAT e identificados abaixo do valor do ponto de corte indicado pelo fabricante foram considerados negativos. Animais com titulação de IFAT > 1/40 e valor de absorbância acima do ponto de corte foram considerados positivos. Os animais com LV sintomática (n=16) foram positivos nos testes IFAT, ELISA e PCR, e apresentaram três ou mais sinais clínicos da doença, como perda de peso, alopecia, adenopatia, onicogrifose, hepatomegalia, conjuntivite e dermatite esfoliativa no focinho, cauda ou pontas das orelhas. Soros de animais não infectados

por *Leishmania* spp. (n=20) apresentaram resultados sorológico, molecular e clínico negativos. Soros de animais não infectados, mantidos isolados em canil e imunizados com a vacina Leish-Tec® (n=10) e de animais experimentalmente infectados com *T. cruzi* (n=13), *E. canis* (n=7) e *B. canis* (n=7) também foram utilizados nos ensaios sorológicos.

Além dos testes realizados com a proteína recombinante LiHyV, as proteínas rLiHyp1, rLiHyp6 e rHRF, isoladas ou conjugadas, também foram utilizadas para o sorodiagnóstico da LVC. Para a avaliação da antigenicidade destas proteínas foram utilizados soros de animais com LV sintomática (n=15) e assintomática (n=9). Os animais assintomáticos, assim como os sintomáticos, apresentaram testes de sorologia e PCR positivos, entretanto, não apresentavam sinais clínicos da doença. Cães não infectados com *Leishmania* spp. (n=15) foram selecionados de área endêmica (Belo Horizonte, Minas Gerais, Brasil) e não endêmica (n=15) (Poços de Caldas, Minas Gerais, Brasil) para as leishmanioses e apresentaram resultados sorológico, molecular e clínico negativos. Soros de animais não infectados, mantidos isolados em canil e imunizados com a vacina Leish-Tec® também foram utilizados nos ensaios (n=12), além dos soros de cães experimentalmente infectados por *Trypanosoma cruzi* (n=12) ou *Ehrlichia canis* (n=8), os quais somados totalizaram 86 amostras.

4.4. Preparo do extrato antigênico de *Leishmania infantum* e de *Leishmania amazonensis* (SLA*Li*/SLA*La*)

O extrato solúvel antigênico (SLA) de *L. infantum* ou *L. amazonensis* foi preparado como descrito previamente por Coelho *et al.* (2003). Uma estimativa da concentração de proteínas foi avaliada conforme descrito por Bradford (1976).

4.5. Análise das proteínas por bioinformática e construção da quimera polipeptídica

As sequências de aminoácidos das proteínas LiHyp1 (XP_001468941.1), LiHyp6 (XP_001568689.1), HRF (CAJ05086.1) e LiHyV (XP_888524.1) foram obtidas no banco de dados do *National Center of Biotechnology Information* (NCBI; http//www.ncbi.nlm.nih.gov) (Figura 4A) e para analisar a conservação das proteínas dentre o genoma de outros organismos foi realizado um alinhamento através da ferramenta de bioinformática BLAST.

Partindo das sequências gênicas conservadas e codificadoras das proteínas selecionadas, pares de iniciadores foram desenhados para permitir a amplificação de cada gene de interesse, por meio da técnica de PCR. Os primers *Forward* e *Reverse* (Eurofins

mwg/operon), descritos abaixo, foram desenhados de acordo com a sequência da região ORF do DNA genômico para *L. infantum*.

LiHyp1 (LinJ.35.1290)

F → (5'-GAAGGATCCAGCATGTCTATCGTGTCGAG-3')
R → (5'-GGAAAGCTTCGCTTGCGGCGTCACGTGAGC-3')

LiHyp6 (LinJ.36.0580)

F → (5'-TTTGCTAGCATGAGCTTCTTTGACTTCTCA-3')
R → (5'-TTTAAGCTTTCATTGCAGAACTTTGAGTACA-3')

HRF (LinJ.24.1560)

F → (5´-GGATCCATGAAGATCTTCAAGGATGTG-3´)
R → (5´-AAGCTTAGACGCGCTCGCCCTTCAG-3´)

LiHyV (LinJ.04.0160)

F → (5´-GACGGATCCATGTCGGACGCATCATTC-3')
R → (5´-GCACAAGCTTAAGGGCGTAGAAAGCGGC-3')

Peptídeos (Peptídio 1: AGQSVPNTL e Peptídeo 2: VGIAKSTAALFVLNATAI) presentes na proteína LiHyV foram selecionados por meio de predição de epitopos de células T $CD8^+$ através do programa Bimas (*Bioinformatics and Molecular Analysis Section*) e utilizados para os experimentos de imunização conjugados à saponina.

Com a finalidade de identificar regiões imunogênicas ricas em epitopos de células T humanas e murinas para o desenvolvimento de uma quimera polipeptídica, as proteínas selecionadas por imunoproteômica e previamente citadas foram analisadas. Para a seleção de epitopos de células T $CD8^+$ que se liguem ao MHC de classe I foi utilizado o programa Net CTL Pan. Quando a análise foi realizada para MHC de classe I humano, o programa foi adequado aos parâmetros padrões e aos alelos A2, A3 e B7, os quais juntos representam uma conservação gênica em mais de 90% da população de qualquer etnia. Para avaliar os epitopos que se ligam ao MHC de classe I murino, o programa foi adequado aos alelos H-2-Kd, H-2-Ld e H-2-Dd, conservados em camundongos BALB/c.

Para análise dos epitopos que se liguem ao MHC de classe II humano, o programa NetMHCII 2.2 foi utilizado e pôde predizer a afinidade de ligação de peptídeos de 15 aminoácidos a 26 alelos diferentes (Nielsen *et al.*, 2007; Nielsen & Lund, 2009). Epitopos com capacidade de se ligar com uma afinidade predita menor que 500nM a pelo menos 30% dos alelos foram selecionados. O programa também foi utilizado com parâmetros padrões e

ajustado para identificar potenciais peptídeos que se liguem a alelos de MHC de classe II I-Ad e I-Ed de camundongos BALB/c.

Além das avaliações de epitopos de linfócitos T, análises de regiões contendo epítopos lineares de célula B preditas com 9 ou mais aminoácidos e valor de predição acima de 1.3, foram realizadas pelo programa Bepipred, sendo que estas regiões foram excluídas por não serem de interesse ao desenvolvimento de uma vacina.

A quimera polipeptídica construída com a sequência de epitopos selecionados (Figura 4B) foi submetida à análise de códons preferenciais para expressão em bactéria *Escherichia coli* através da ferramenta *Web Codon Optimization* disponibilizada pela *Integrated DNA Technologies* (https://www.idtdna.com/CodonOpt) e otimizada para redução da presença de interações intramoleculares do RNA mensageiro calculados pelo programa mfold (Zuker, 2003). A quimera desenhada foi então enviada para a empresa GenScript para síntese genética em plasmídeo de clonagem pET28a-c+.

4A.

LiHyp1 (XP_001468941.1)

MSIVSRFIGGAKHLLKGGSMKYLAAGEPYCPFGEAFGLTILPEYILEDDASNLRKGYV**DVYTRASDRIIL NDGRFQLPPLPPASFMPLLERLEQDNVVPKNWLNNQTANLYEPGD**FIRAHIDNLFVYDDIFAICSLG SNCLLRFVHVQNGEELDV**MVPDRSVYIMSGPARYVYFHMVLPVEAQRFSLVFRRSIMESDGGFRPV KTPFKEIMPYRATQ**ILNALYSKQVGGVRVSVDDDFLESANIGAFDTSRWVKRLHPLRDWSLLRQLDE DEARVEELREKRFIDVDFSWRYRELRSYYKAMEESLVSPHVAHVTPQA

LiHyp6 (XP_001568689.1)

MGFLGFSGDSSESYSLSDNSSKVD**DRIKLTYAETVVSLATAFGLVIIVLLDLLYRSTFRHAMLLRVQ RETRFD**GAYILGKPEGSRARIILENGLKTHAFRISDPPQYSRVVPAVVSESRDAKKDCVEMLSKVRTILA KSYGRCAELMSMRCCLACVTGALPAEQSERFLRIYERVMFCSHRVNGDEKLVTSDDIRYMHAFFYNNV LKVLQ

HRF (CAJ05086.1)

MKIFKDVLTGSEVVCDNDCPFDVEGDIMYVVNGRYIDVGGEDYGISANVDEDAAEGATGEVAEGKER VVDVVYNNRYTETSYDKAS**YMAHIRSYMKQLLEKIENEEERKAFQTNAAAFVKKVL**KDIDEYQFFI PEGNDEDPDNGMIVLCRWDGETPRFYFWKDGLKGERV

LiHyV (XP_888524.1)

MGPFAPRCCALALLCSVLVLAAVLVRAESFTVTRDVTMSSTSFDDYTMVLDLSSSSADVVTVQLINSQ**V SGNGLTIKNARGSTPSSARLSMSMAITTVAQSAITLSGVMPANSDIRIVATTSSLAPAQSLFDFSG**LA LDSNATVMVENTAVTWPKDSINTGSIVLISAGSNAVGIKNTAALFVLNATAINGASVVRVDTQSSFPISK GAALAVDYGRCERCSSALVSINVPLVVDASSLFRVANCKVVGASNGLLTSAGSITVSDKSAYLIYDSAV ESGALFSFPAGLEDASEAYPFAVSGGSTVSFLNLKGSSTGVAAGQSVPNSLEQSNAIGGGCVISDKELRV ASEYRSHGLSVETVVDSQGASSGTCANAKCIPGNTKPGATVSGTELCTCQCSSTKHHSPFCTSVVDPMQ NYDPNVWCTVPDCITCDRLDPSNRCTECDTGYSLTSDYQCKAITTITTTTTTTKAPTCTAPHCSVCAAGS GSICSSCRSPYTLSNGVCVANTNIAAGAHTATLAAAVCVAAALYAL

4B. **Quimera Polipeptídica**

MVPDRSVYIMSGPARYVYFHMVLPVEAQRFSLVFRRSIMESDGGFRPVKTPFKEIMPYRATQDVY TRASDRIILNDGRFQLPPLPPASFMPLLERLEQDNVVPKNWLNNQTANLYEPGDFVSGNGLTIKNA RGSTPSSARLSMSMAITTVAQSAITLSGVMPANSDIRIVATTSSLAPAQSLFDFSGDRIKLTYAETVV SLATAFGLVIIVLLDLLYRSTFRHAMLLRVQRETRFDYMAHIRSYMKQLLEKIENEEERKAFQTN AAAFVKKVLHHHHHH-

Figura 4: Sequência de aminoácidos das proteínas recombinantes e construção da quimera polipeptídica. As sequências de LiHyp1, LiHyp6, HRF e LiHyV foram analisadas e seus respectivos epitopos de células T foram selecionados (A) com a finalidade de darem origem a uma quimera polipeptídica (B).

4.6. Clonagem das proteínas LiHyp1, LiHyp6, HRF e LiHyV

4.6.1. Extração do DNA genômico dos parasitos

A cepa MHOM/BR/1970/BH46 de *L. infantum* foi cultivada de acordo com protocolo descrito por Coelho *et al.* (2003). O DNA genômico foi extraído conforme descrito por Piarroux *et al.* (1993). Para verificar a qualidade e a quantidade do material obtido, foi realizada uma eletroforese em gel de agarose 1% peso por volume (p/v) (Sigma) em tampão TAE (constituído de Tris 40mM, ácido acético 20 mM e EDTA 1 mM). O gel foi submetido a 80 volts (V) e corado com brometo de etídio (10 μg/mL). A dosagem foi realizada em espectrofotômetro nos comprimentos de onda de 260 e 280 nm, e a amostra foi armazenada a -80°C.

4.6.2. Amplificação das sequências gênicas por PCR

A região codificadora das proteínas foram amplificadas por PCR, utilizando-se oligonucleotídeos iniciadores complementares aos fragmentos. Para as proteínas LiHyp1, HRF e LiHyV foram inseridos sítios de restrição para as enzimas *Bam*HI e *Hin*dIII enquanto que para LiHyp6 foram inseridas as enzimas *Nhe*I e *Hin*dIII. A reação de amplificação dos genes de interesse foi realizada utilizando o kit GoTaq® DNA Polymerase, de acordo com as instruções do fabricante (Promega). A termociclagem foi padronizada em um ciclador térmico (Tonegen Palm, Tonederm), utilizando-se o seguinte programa:

Passo 1 – Desnaturação a 95°C por 5 min.
Passo 2 – Desnaturação a 95°C por 45 seg;
Anelamento a 55°C por 45 seg; } 30 ciclos
Extensão a 72°C por 120 seg.
Passo 3 – Extensão final a 72°C por 5 min.

Após a reação, os produtos da PCR foram aplicados em um gel de agarose 1% e corados com brometo de etídio para serem identificados. Os produtos da PCR foram quantificados utilizando o comprimento de onda 260/280 nm no aparelho NanoDrop® 2000 (Thermo scientific).

4.6.3. Ligação dos genes em pGEM®-T *Easy Vector Systems*

Os produtos das amplificações dos genes LiHyp1, LiHyp6, HRF e LiHyV foram ligados individualmente ao vetor de clonagem pGEM®-T *Easy Vector Systems* de acordo com as instruções do fabricante (Promega). A ligação de cada inserto ao vetor ocorreu a 4°C, durante 16 h.

4.6.4. Preparo e transformação de bactérias *Escherichia coli* XL1*blue*

Células competentes *E. coli* XL1*blue* foram preparadas através da técnica de cloreto de cálcio, segundo Sambrook *et al*. (1989). A transformação das bactérias XL1 blue foi realizada através de choque térmico com os plasmídeos pGEM-LiHyp1, pGEM-LiHyp6, pGEM-HRF e pGEM-LiHyV e plaqueadas como sugerido pelo fabricante (*Agilent Technologies*).

4.6.5. Extração do DNA plasmidial

Após a incubação das placas por 16 h à 37ºC, clones positivos foram selecionados a fim de se realizar a extração dos seus DNAs plasmideais. As colônias selecionadas foram replicadas em meio LB e ampicilina (100 μg/mL) e seus DNAs plasmidiais extraídos através do kit *Wizard® Plus SV Minipreps DNA Purification System* (Promega). A digestão dos plasmídeos foi realizada com as endonucleases de restrição *Hin*dIII e *Bam*HI (Promega) para LiHyp1, HRF e LiHyV, e *Nhe*I e *Eco*RI para LiHyp6 de acordo com protocolo do fabricante (Promega). Os produtos foram confirmados em gel de agarose 1%, corado com brometo de etídio e as respectivas bandas obtidas foram excisadas e purificadas com o *kit Invisorb® Fragment CleanUp* (Invitek).

4.6.6. Sequenciamento dos genes

Após a clonagem em vetor pGEM-T®, amostras dos plasmídeos purificados foram separadas para a realização do sequenciamento automático de DNA de alta qualidade e para a confirmação das identidades dos insertos (LiHyp1, LiHyp6, HRF e LiHyV) e do vetor

(pGEM-T). Os sequenciamentos foram realizados em *MegaBACE 1000 DNA Sequencing System* (GE Healthcare) e analisados pelo Núcleo de Análise de Genoma (NAGE), do Departamento de Bioquímica e Imunologia do ICB, UFMG.

4.6.7. Clonagem de cada gene em vetor de expressão específico

Os insertos e os plasmídeos de expressão específicos [LiHyp1 e LiHyV - pET21a (Novagen), LiHyp6 - pET28a-c+ (Addgene) e HRF - pQE30 (Qiagen)] foram digeridos com as enzimas de restrição descritas no item 4.6.5 de acordo com o protocolo sugerido pelo fabricante. As amostras foram confirmadas em gel de agarose 1% e purificadas com o *kit Invisorb® Fragment CleanUp* (Invitek). As ligações foram realizadas com a enzima T4 ligase, como sugerido pelo fabricante (Promega). Para a transformação por choque térmico, bactérias *E. coli* BL21 (pET21a-LiHyp1; pET21a-LiHyV), BL21AI (pET28a-LiHyp6), M15 (pQE30-HRF) e *Artic express* (pET28a-quimera) foram utilizadas, seguindo o protocolo sugerido pelos fabricantes e plaqueadas em meio de cultura LB sólido acrescido de antibióticos específicos. As colônias crescidas na placa foram selecionadas e replicadas em meio de cultura LB, com os devidos antibióticos. Para confirmar a presença dos insertos ligados, os plasmídeos foram extraídos pelo método de lise alcalina e os DNAs das colônias recombinantes foram submetidos à digestão enzimática com as endonucleases específicas, sendo que um novo gel de agarose a 1% foi realizado para a confirmação dos insertos. Alíquotas das bactérias transformadas positivas foram estocadas em glicerol 16%, à -80°C.

4.7. Expressão e purificação das proteínas recombinantes

Para verificar a cinética de expressão das proteínas, foi realizado um experimento piloto utilizando 20 mL de cultura de cada bactéria transformada. Após a densidade ótica (DO) da cultura alcançar aproximadamente uma leitura de 0,5, em comprimento de onda de 600nm, uma alíquota de 1mL foi removida, sendo correspondente ao tempo não induzido. As culturas foram induzidas com 1mM de IPTG e 1, 2, 3 e 4 h após a indução, alíquotas de 1 mL foram retiradas para confirmação da expressão em gel SDS-PAGE. As proteínas foram expressas em maiores concentrações após um período de 3 h de indução, em temperatura de 37°C.

Para a purificação em larga escala, a expressão das proteínas foi realizada em 2L de cultura, dentro das condições padronizadas, com excessão da proteína quimérica, que foi expressa a 12ºC durante 24h. A lise das bactérias foi realizada em ultrassom (5 pulsos de

90MHz), as proteínas foram purificadas por cromatografia de afinidade em colunas de níquel e as amostras foram dialisadas frente a PBS 1x. As proteínas foram submetidas à purificação por gel filtração (Superdex^{TM} 200) e em seguida, passadas em uma coluna de agarose-polimixina (Sigma), para remoção de qualquer endotoxina residual de bactéria.

As proteínas purificadas foram dosadas pelo método de Bradford, aplicadas em gel SDS-PAGE a 12% para confirmação do peso molecular e estocadas à -80°C, até o momento do uso.

4.8. Eletroforese em gel SDS-PAGE

As eletroforeses de proteínas em gel desnaturante (SDS-PAGE) foram realizadas em sistema de Minigel da BioRad (Hercules, CA, USA) para géis de poliacrilamida descontínuos, segundo Laemmli (1970).

4.9. Western-blot

Os experimentos de Western-Blot foram realizados como descrito por Towbin *et al.* (1979), com algumas modificações. Após o preparo do gel SDS-PAGE, 10 μg dos SLAs e das proteínas recombinantes foram aplicados e a corrida eletroforética foi realizada a 200 V. As proteínas foram transferidas do gel para uma membrana de nitrocelulose (NC, poro de 0,2 μm, Millipore) em tampão de transferência (0,192 M glicina, 0,025 M tris e metanol 20%), a 100 V por 1 h e 30 min. As membranas foram bloqueadas com PBS-T 1x (PBS 1x, pH 7,4 e Tween 20 a 0,05% v/v), e albumina bovina a 5%, por 1 h, e posteriormente, lavadas com PBS-T 1x. As membranas foram incubadas individualmente frente a um *pool de* soros de cães com LV ou não infectados (diluídos 1:200 e 1:100, respectivamente, em solução PBS-T 1x), ou ainda frente a soros de camundongos imunizados com as proteínas rLiHyp1, rLiHyp6, rHRF, rLiHyV e rQuimera, saudáveis ou infectados com *L. infantum* ou *L. amazonensis* (diluídos 1:50 em solução PBS-T 1x). Em seguida, as membranas foram lavadas e incubadas com anticorpo secundário específico (anti-IgG de cão ou camundongo) conjugado à enzima peroxidase (Sigma-Aldrich), ambos diluídos em PBS-T 1x (1:10.000). Após realização das lavagens com PBS-T 1x, seguida por PBS 1x, o substrato (cloronaftol 12,5 mg, metanol 4%, diaminobenzidina 25 mg, 20 μL de peróxido de hidrogênio (H_2O_2) 20 vol. e PBS 1x para um volume final de 50 mL) foi acrescentado para a revelação da reação. A reação foi interrompida com água destilada e as membranas foram secas em papel de filtro.

4.10. ELISA para o sorodiagnóstico da LVC

A avaliação da antigenicidade das proteínas rLiHyp1, rLiHyp6, rHRF, LiHyV e do mix rLiHyp1/rLiHyp6/rHRF, foi realizada de acordo com Martins *et al*. (2013) com algumas modificações. Placas de microtitulação para ELISA de 96 poços (Jet-Biofil®) foram sensibilizadas com: rLiHyp1 ou rLiHyp6 → 1,5 μg; LiHyV ou SLA*Li* → 1,0 μg; A2 ou rHRF → 0,5 μg; ou ainda com um mix rLiHyp1, rLiHyp6 e rHRF → 0,5 μg de cada uma das proteínas por poço; diluídos em 100 μL de tampão de sensibilização (carbonato de sódio 0,1M e bicarbonato de sódio 0,05 M, pH 9,6). As placas foram lavadas e bloqueadas por 2 h a 37°C. Após serem lavadas, as placas foram incubadas com os soros de cães individuais, em duplicata, tendo sido diluídos a 1:200 em PBS-T. As placas foram lavadas novamente e o anticorpo anti-IgG de cão (Sigma) conjugado à peroxidase foi utilizado em uma diluição de 1:10,000 em PBS-T 1x. As placas foram lavadas e incubadas com a solução reveladora [10 mL de tampão citrato-fosfato (Na_2HPO_4 24 mM e ácido cítrico 17,5 mM para 1 L de água destilada, pH 5,0); 2 mg de orto-fenilenodiamina (OPD) e 2 μL de H_2O_2] durante 30 min e ao abrigo da luz. A reação de revelação foi parada e a leitura ocorreu ao comprimento de onda de 492 nm em espectrofotômetro. Controles positivos e negativos foram utilizados em todas as placas.

4.11. Imunização de camundongos BALB/c

Grupos de camundongos BALB/c fêmeas (n=8, por grupo) foram imunizados pela via subcutânea no coxim plantar esquerdo, com três doses em intervalos de 15 dias entre as mesmas. Os animais foram divididos em grupos dentro de quatro experimentos:

Primeiro experimento (*infecção L. infantum*):

1) Grupo inoculado com salina;
2) Grupo imunizado com 25 μg de saponina (*Quillaja saponaria bark saponin*, Sigma);
3) Grupo imunizado com 25 μg de Peptídeo1 + 25 μg de saponina;
4) Grupo imunizado com 25 μg de Peptídeo2 + 25 μg de saponina;
5) Grupo imunizado com 25 μg de rLiHyV + 25 μg de saponina.

Segundo experimento (*infecção L. infantum*):

1) Grupo inoculado com salina;
2) Grupo imunizado com 25 μg de saponina (*Quillaja saponaria bark saponin*, Sigma);
3) Grupo imunizado com 25 μg de rLiHyp1 + 25 μg de saponina;
4) Grupo imunizado com 25 μg de rLiHyp6 + 25 μg de saponina;
5) Grupo imunizado com 25 μg de rHRF + 25 μg de saponina;

6) Grupo imunizado com 25 μg rLiHyp1 + 25 μg rLiHyp6 + 25 μg rHRF + 25 μg de saponina.

Terceiro experimento (*infecção L. infantum*):

1) Grupo inoculado com salina;
2) Grupo imunizado com 25 μg de saponina (*Quillaja saponaria bark saponin*, Sigma);
3) Grupo imunizado com 25 μg de rQuimera + 25 μg de saponina;

Quarto experimento (*infecção L. amazonensis*):

1) Grupo inoculado com salina;
2) Grupo imunizado com 25 μg de saponina (*Quillaja saponaria bark saponin*, Sigma);
3) Grupo imunizado com 25 μg de rQuimera + 25 μg de saponina;

As concentrações dos imunógenos e do adjuvante foram determinadas com base em experimentos vacinais padronizados por nosso grupo de pesquisa.

4.12. Infecção desafio

As infecções desafio foram realizadas 30 dias após a última dose da vacina, quando também ocorreu a eutanásia de metade do número dos animais de cada grupo para a realização de experimentos de imunogenicidade. Para a infecção, $1x10^7$ promastigotas em fase estacionária de crescimento de *L. infantum* (primeiro, segundo e terceiro experimento) ou $1x10^6$ promastigotas em fase estacionária de crescimento de *L. amazonensis* (quarto experimento) foram inoculadas em 20 μL de PBS 1x, no coxim plantar direito de cada animal. Os camundongos infectados foram monitorados durante 10 semanas após o desafio e então eutanasiados para realização dos experimentos de imunogenicidade e determinação da carga parasitária. Animais desafiados com a espécie *L. amazonensis* foram submetidos à medição da lesão causada na pata infectada semanalmente, durante um período de 6 semanas, com auxílio de um paquímetro (L.S. Starrett S.A., SP, Brasil).

4.13. Isolamento e cultura dos esplenócitos

Camundongos imunizados ou imunizados e infectados foram submetidos à eutanásia para a coleta do baço, isolamento, cultura e estimulação dos esplenócitos de acordo com Martins *et al*. (2013), com modificações. Para tal, $1x10^6$ células/mL foram incubadas em meio de cultura RPMI 1640 completo na presença de estímulos específicos (peptídeos, proteínas recombinantes ou SLA) ou não (controle negativo). As placas foram incubadas em estufa com 5% de CO_2 durante 48 h à 37°C, e os sobrenadantes foram coletados para a dosagem das

citocinas. A avaliação da participação de $LTCD4^+$ e $LTCD8^+$ na produção de IFN-ɣ, pelos esplenócitos dos animais imunizados e desafiados, foi realizada com a utilização dos anticorpos monoclonais anti-$CD4^+$ (GK 1.5), anti-$CD8^+$ (53-6.7) ou anti-IL12 (C17.8) como recomendado pelo fabricante (todos da Pharmingen, San Diego, CA, USA).

4.14. Dosagem das citocinas

As citocinas IFN-ɣ, IL-4, IL-10, IL-12 e GM-CSF foram avaliadas. A dosagem das citocinas foi realizada através da técnica de ELISA de captura, utilizando-se os kits comerciais *BD OptEIA* ^{TM} *Set Mouse IFN-ɣ, IL-4, IL-10, IL-12* e *GM-CSF* [número de catálogo: 555138, 555232 , 555167, 555256 e 555252 respectivamente (Pharmingen, San Diego, CA, USA)], seguindo as instruções dos fabricantes.

4.15. Citometria de fluxo

Esplenócitos foram coletados de animais do grupo controle ou vacinados com a proteína quimérica e infectados com *L. infantum* para cultivo *in vitro* como descrito por Vieira *et al*. (2012), com algumas modificações. Para isto, o baço dos animais foi retirado e colocado em uma placa de Petri para maceração. Os fragmentos de tecido foram filtrados e as células obtidas foram lavadas duas vezes com meio RPMI 1640. Os esplenócitos foram ressuspendidos com 1×10^7 células por mL e incubados em tubos de polipropileno (Falcon, BD Pharmingen) em 1 mL de meio RPMI 1640. Os tubos foram separados em quatro grupos de acordo com o antígeno utilizado para estímulo. O primeiro grupo de células foi estimulado com 25 µg/mL de SLA*Li;* o segundo grupo foi estimulado com 20 µg/mL de proteína recombinante quimérica; o terceiro grupo foi composto apenas por células em contato com o meio RPMI 1640 e no quarto e último grupo, as células foram incubadas com PMA 25ng/mL (Phorbol-12-miristato-13-acetato) e ionomicina 1µg/mL em meio RPMI 1640. Todas as culturas foram deixadas por 48h a 37ºC e 5% de CO_2. Após este período de incubação foram adicionados 10µg/mL de Brefeldina A (BFA, Sigma, St Louis, MO, USA) por tubo e as culturas celulares foram deixadas por mais 4h nas mesmas condições. Passado o período de incubação, 200µL de EDTA (Sigma) foram adicionados em cada tubo em uma concentração final de 2mM. As culturas celulares foram lavadas com tampão FACS (PBS1x, 0,5% BSA e 0,1% azida sódica), centrifugadas e ressuspendidas em meio RPMI 1640. As células foram separadas e marcadas com FITC anti-CD4 ou anti-CD8 (Caltag, Burlingame, CA, USA) durante 30 min a temperatura ambiente e ao abrigo de luz. Após a marcação, os esplenócitos

foram permeabilizados com um tampão FACS-perm (tampão FACS adicionado de 0,5% de saponina) e incubados com anti-citocinas (IFN-γ, TNF-α e IL-10; Serotec e Caltag, respectivamente) marcadas com ficoeritrina durante 30 min à temperatura ambiente e ausência de luz. Após a marcação das citocinas citoplasmáticas as células foram lavadas com tampão FACS, fixadas com uma solução de fixação FACS-fix (10 g/L de paraformaldeído, 10,2 g/L de cacodilato de sódio e 6.63 g/L de cloreto de sódio, pH 7.2) e armazenadas a 4ºC até a realização da leitura. A citometria de fluxo foi realizada em um FACScalibur® (Becton Dickson - BD, USA) utilizando o pacote de software Cell-Quest™ (Franklin Lakes, NJ, USA) e foi baseada em 30.000 eventos por amostra. Para avaliar as ligações não específicas, reagentes isotípicos marcados com fluorocromo foram utilizados e a autofluorescência foi monitorada pela utilização de um controle negativo (suspensão celular incubada na ausência de anticorpos marcados com fluorocromo, mas na presença dos tampões). A frequência de células T $CD4^+$ e T $CD8^+$, que expressam citocinas citoplasmáticas (IFN-γ, TNF-α e IL-10), foi determinada seguindo uma estratégia de análise convencional. O tamanho (*forward laser scatter* – FSC) e granulosidade (*side laser scatter* - SSC) para as células de interesse foram definidos e a região R1 foi selecionada para análise. Os gráficos de densidade celular de $CD4^+$/ ou $CD8^+$/FL1 *versus* IFN- γ /, TNF-α/ e IL-10/FL2 foram definidos para determinar a porcentagem de IFN-γ, TNF-α e IL-10 presentes no interior de células T pré-selecionadas. Os resultados foram expressos como *indexes*, obtidos quando se divide o percentual de células T $CD4^+$ ou T $CD8^+$ em uma cultura estimulada pela observada na cultura controle não estimulada.

4.16. Avaliação da produção de anticorpos IgG1 e IgG2a

O perfil da resposta humoral nos animais imunizados ou imunizados e desafiados foi avaliado através da produção de anticorpos IgG1 e IgG2a específicos aos SLAs de *L. infantum* ou *L. amazonensis*. Amostras de sangue dos animais foram retiradas 1 dia antes e 10 semanas após a infecção, incubadas por 10 min a 37°C e centrifugadas a 5.000 x g por 10 min, para a obtenção de soro. Os ensaios de ELISA foram realizados em placas de 96 poços (Jet-Biofil®), sendo que para o primeiro experimento (rLiHyV x *L. infantum*) 2μg/poço de SLA*Li* foram utilizados em 100 μL de tampão de sensibilização; para o segundo (LiHyp1,LiHyp6,HRF x *L. infantum*) foram utilizados 1μg/poço de rLiHyp1ou rLiHyp6; 0,5 μg/poço de rHRF; 0,5μg/poço de cada proteína para o mix ou 2 μg/poço de SLA*Li*; para o terceiro (rQuimera x *L. infantum*) foram utilizados 1μg/poço de rLiHyp1, rLiHyp6 ou

rQuimera; 0,5 μg/poço de rHRF ou LiHyV e 2 μg/poço de SLA*Li* e para o quarto (rQuimera x *L. amazonensis*) foram definidas as mesmas concentrações do terceiro, com a diferença de que o SLA utilizado foi de *L. amazonensis* e em concentração de 1μg/poço. As placas foram incubadas a 4°C por 18 h e após este período foram lavadas com PBS-T 1x e bloqueadas. Após uma nova lavagem, as amostras de soro foram aplicadas em duplicatas, diluídas 1:50 em PBS-T 1x e caseína 0,25% de acordo com cada experimento. Após incubação, as placas foram lavadas e os conjugados anti-IgG1 ou anti-IgG2a de camundongo, ligados à peroxidase (Sigma), foram acrescentados na diluição de 1:5000 em PBS-T 1x e caseína 0,25%. As amostras foram reveladas e a leitura das absorbâncias foi realizada em leitor de ELISA, com comprimento de onda de 492 nm. Em todos os ensaios, controles positivos e negativos foram utilizados.

4.17. Avaliação da carga parasitária

Para realizar a quantificação de parasitos, baço, fígado, linfonodo drenante, medula óssea e lesão da pata infectada (quando pertinente) foram removidos 10 semanas após a infecção. Os órgãos foram macerados em meio de Schneider's incompleto na proporção de 1 mL de meio de cultura a cada 100 mg de tecido. Diluições seriadas foram realizadas utilizando meio de Schneider's completo, partindo de uma diluição de 10^{-1} até uma diluição máxima de 10^{-12}. As placas de cultura celular de 96 poços (Nunc) foram incubadas em estufa a 24ºC durante 7 dias e os resultados foram representados pela maior diluição na qual os parasitos puderam ser visualizados utilizando um microscópio trinocular invertido (Axiovert 25, Zeiss), de acordo com Coelho *et al.* (2003).

4.18. Avaliação da carga parasitária dos animais imunizados com rQuimera e infectados por *L. infantum* ou *L. amazonensis* através da técnica de PCR *Real Time*

A carga parasitária do baço (terceiro experimento) ou da pata infectada (quarto experimento) também foi avaliada pelo teste de PCR *Real Time*. Para isto, os DNAs das amostras foram extraídos através do método de fenol-clorofórmio, como descrito por Ferreira *et al*. (2012), e ressuspendidos em 100 μL de água ultrapura. As reações de PCR foram realizadas com 2x SYBR® Green PCR Master Mix, utilizando 5 μL do mix disponibilizado no kit, 2mM de cada primer (*Forward* - CCTATTTTACACCAACCCCCAGT e *Reverse* - GGGTAGGGGCGTTCTGCGAAA) e 20ng de cada DNA. Uma curva padrão foi obtida do DNA extraído de $1x10^8$ parasitos para comparação e análise dos resultados finais. A PCR foi

realizada no aparelho StepOne^{TM} (Life Technologies, USA), as amostras foram incubadas a 95ºC durante 10 min e sucessivamente foram submetidas a 40 ciclos de 95ºC por 15 seg e 60ºC por 1 min. Durante cada ciclo os sinais de fluorescência foram coletados para análise e após terminada a reação, a quantificação de parasitos de cada amostra foi calculada de acordo com a curva padrão. O experimento foi realizado em duplicata e os resultados expressos como o número de parasitos por DNA total (Fiuza *et al*., 2015). A comparação da carga parasitária entre os grupos experimentais também foi calculada (em percentual).

4.19. Análise estatística

As análises estatísticas foram realizadas no *software* Graph Pad Prism (versão 5.0 para Windows). Curvas ROC (*Receiver Operating Characteristic*) foram preparadas para análise dos dados de antigenicidade, assim como os testes de análise de variância (ANOVA), seguidos pelo pós-teste de Tukey's. Para a estatística da imunogenicidade, os valores encontrados para os diferentes grupos experimentais foram analisados pelo teste ANOVA, seguido pelo pós-teste de Tukey's, enquanto que os animais imunizados com o peptídeo1/sap, com o peptídeo2/sap e com a rLiHyV/sap foram analisados pelo teste ANOVA seguido pelo pós-teste de Bonferroni's. Para os valores encontrados nos experimentos de imunização com a rQuimera/Sap, ambos os testes, Tukey's e Bonferroni's, foram utilizados. Diferenças foram consideradas significativas quando o valor de $P < 0.05$.

5. RESULTADOS E DISCUSSÃO

Conforme as normas que regem a redação das dissertações de mestrado e teses de doutorado do Programa de Pós-Graduação, as seções de Resultados e Discussão desta Tese serão apresentadas sob a forma dos quatro artigos científicos.

5.1. Artigo 1 – Breve introdução e Objetivo

O primeiro artigo a ser apresentado foi intitulado "*Uma proteína hipotética específica de Leishmania expressa nas fases amastigota e promastigota de Leishmania infantum empregada para o sorodiagnóstico e como candidata vacinal contra a leishmaniose visceral*" e publicado pela revista internacional *Parasite & Vectors.*

O presente estudo avaliou as propriedades antigênicas e imunoprofiláticas de uma proteína específica de *Leishmania* identificada previamente na espécie de *L. infantum* através da técnica de imunoproteômica. A proteína, nomeada LiHyV e com número de acesso XP_888524.1, foi produzida em sua forma recombinante para avaliação do potencial como marcador diagnóstico para LVC. Devido a realização de um estudo de epitopos de células T $CD4^+$ e $CD8^+$ e a confirmação da presença destes peptídeos dentre a sequência da proteína, a LiHyV foi também avaliada como uma candidata a vacina para LV murina. Dois epitopos de célula T $CD8^+$ foram também selecionados e sintetizados para serem testados como imunógenos em camundongos BALB/c desafiados com *L. infantum*. Sendo assim, o objetivo deste trabalho foi avaliar o potencial da rLiHyV como marcador diagnóstico da LVC, bem como, avaliar e comparar a imunogenicidade e o grau de proteção vacinal em camundongos BALB/c imunizados com o peptídeo 1/saponina ou com o peptídeo 2/saponina em relação aos imunizados com a proteína recombinante LiHyV quando infectados por *L. infantum*.

Martins et al. Parasites & Vectors (2015) 8:363
DOI 10.1186/s13071-015-0964-5

Parasites & Vectors

RESEARCH Open Access

A *Leishmania*-specific hypothetical protein expressed in both promastigote and amastigote stages of *Leishmania infantum* employed for the serodiagnosis of, and as a vaccine candidate against, visceral leishmaniasis

Vivian T. Martins[1], Mariana C. Duarte[2,3], Miguel A. Chávez-Fumagalli[2], Daniel Menezes-Souza[3,4], Cecília S. P. Coelho[5], Danielle F. de Magalhães-Soares[6], Ana Paula Fernandes[7], Manuel Soto[8], Carlos A. P. Tavares[1] and Eduardo A. F. Coelho[2,3,9*]

Abstract

Background: LiHyV is an antigenic hypothetical protein present in both promastigote and amastigote stages of *Leishmania infantum*, which was recently identified by an immunoproteomic approach. A recombinant version of this protein (rLiHyV) was evaluated as a diagnostic marker for canine VL (CVL). In addition, the prophylactic efficacy of the rLiHyV protein, and two of its $CD8^+$ T cell epitopes, has been analyzed in a murine model of visceral leishmaniasis (VL).

Methods: Initially, the rLiHyV protein was evaluated by an ELISA technique for the serodiagnosis of CVL. Secondly, vaccines composed of the recombinant protein and both chemically synthesized peptides, combined with saponin as an adjuvant; were administered subcutaneously into BALB/c mice. The cellular and humoral responses generated by vaccination were evaluated. In addition, the parasite burden and immune response were studied 10 weeks after *L. infantum* infection.

Results: The rLiHyV protein was recognized by antibodies of VL dogs. No cross-reactivity was obtained with sera from dogs vaccinated with a Brazilian commercial vaccine, with sera from animals infected with *Trypanosoma cruzi*, *Babesia canis* and *Ehrlichia canis*, or those from non-infected animals living in an endemic area for leishmaniasis. After challenge with *L. infantum*, spleen cells of BALB/c mice vaccinated with rLiHyV/saponin stimulated with parasite antigens showed a higher production of IFN-γ, IL-12 and GM-CSF, than the same cells obtained from mice vaccinated with the individual peptides, or mice from control (inoculated with saline or saponin) groups. This Th1-type cellular response observed in rLiHyV/saponin vaccinated mice was accompanied by the induction of parasite-specific IgG2a isotype antibodies. Animals immunized with rLiHyV/saponin showed significant reductions in the parasite burden in the liver, spleen, bone marrow and in the lymph nodes draining the paws relative to control mice.

Conclusions: The present study showed for the first time that the *L. infantum* LiHyV protein could be considered as a vaccine candidate against *L. infantum* infection, as well as a diagnostic marker for CVL.

Keywords: *Leishmania spp*, Hypothetical proteins, BALB/c mice, Vaccine, Serodiagnosis, Canine visceral leishmaniasis

* Correspondence: eduardoferrazcoelho@yahoo.com.br
[2]Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
[3]Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
Full list of author information is available at the end of the article

Background

Leishmaniasis comprises a spectrum of diseases caused by protozoan parasites of the genus *Leishmania*, which presents a high morbidity and mortality in the world [1]. About 350 million people are at risk of contracting the infection in 98 countries, where 1.0 to 1.5 million new cases of cutaneous leishmaniasis (CL), and 200,000 to 500,000 new cases of visceral leishmaniasis (VL) are registered annually [2]. The treatment of disease is based on the parenteral administration of pentavalent antimonials, however, increased parasite resistance and side effects observed in the patients have been important problems [3, 4]. Other drugs, such as amphotericin B and its liposomal formulations, pentamidine and miltefosine are encouraging, however, their toxicity and/or high cost had limited their use [5, 6].

Canine visceral leishmaniasis (CVL) due to *Leishmania infantum* is a major global zoonosis, which is endemic in approximately 70 countries worldwide [7, 8]. It cannot only be considered as a veterinary disease, since infected dogs are the main domestic reservoirs of parasites for human disease [7]. In this context, and aiming to reduce the transmission of parasites between dogs and humans, one could suggest that it is necessary to diagnose CVL as early as possible. The Brazilian Public Health authorities determine that, to a precise immunological diagnosis of CVL, serological tests, such as IFAT and ELISA, should be employed for the effective serodiagnosis of disease [9]. However, ELISA is hampered by some factors, such as by variable specificity when different antigenic preparations are employed, which can lead to the occurrence of false-positive results caused by related pathogens, such as *Trypanosoma cruzi* [10, 11], *Ehrlichia canis* and *Babesia canis* [12]; or in vaccinated dogs [13]. In addition, its sensitivity presents problems, mainly related to the fact that a high percentage of infected dogs present low antileishmanial serology, and they are classified as false-negative in the serological assays performed; depending on the nature of the antigen employed in the assay [13]. Therefore, the development of new strategies to diagnose leishmaniasis has become a priority.

Studies to evaluate vaccine candidates against leishmaniasis have demonstrated the need to develop a Th1 cell-mediated immune response, which is based on the production of cytokines, such as IFN-γ, IL-12, and others [14–19]. The induction of $CD4^+$ Th1 cells specific to parasite proteins is crucial in controlling the infection caused by *Leishmania spp.* Cytokines such as IFN-γ are able to induce the production of nitric oxide and other compounds by infected phagocytic cells, thereby assisting in the control of parasites' multiplication [20, 21]. By opposite, cytokines such as TGF-β, IL-4, IL-10, and IL-13 represent important disease promoting substances, leading to the suppression of Th1 response [22, 23]. Concomitantly to the role of $CD4^+$ T cells, the cytotoxic activity performed by $CD8^+$ T cells also contributes to protection against VL. These cells has been shown to protect against re-infection, as well as having a significant role in controlling the primary infection against *Leishmania spp.*, by increasing the Th1 immune response [19, 24–26].

Protozoan parasites of the genus *Leishmania* have a dimorphic asexual life cycle consisting of extracellular promastigotes, which multiply and develop within the alimentary tract of the sand fly vector, and intracellular amastigotes that multiply within the phagolysosomes of the macrophages of the mammal hosts [27]. In addition, most of the studies on *Leishmania spp.* vaccines have focused in the use of promastigote proteins [28]. Amastigote antigens have been far less evaluated as vaccine candidates against disease. However, these forms seem to be the more appropriate targets for the immune responses elicited by a vaccine, since after a few hours of initial infection and during the active disease, only the amastigote stage is present in the infected host tissues. In addition, in opposition to promastigotes, amastigotes reside inside host cells, and can be targets for $CD8^+$ T cells [29, 30].

In the present study, the antigenic and immunoprophylactic properties of a recently described parasite protein have been studied. The *Leishmania*-specific hypothetical protein, LiHyV (NCBI accession number: XP_888524.1), was described as an antigen present in both promastigote and amastigote stages of *L. infantum* by an immunoproteomic approach [31]. A recombinant version of the LiHyV protein (rLiHyV) was evaluated as a diagnostic marker for CVL. In addition, the rLiHyV protein and two peptides containing two of its $CD8^+$ T cells predicted epitopes were evaluated as vaccine candidates in a murine model of VL.

Methods

Ethics statement

All technical protocols using mice and dogs' sera in the present study were performed in compliance with the National Guidelines of the Institutional Animal Care and Committee on the Ethical Handling of Research Animals (CEUA) from the Federal University of Minas Gerais (UFMG), which approved this study with the code number 043/2011.

Mice and parasites

Female BALB/c mice (8 weeks age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences (ICB), Federal University of Minas Gerais (UFMG); and were maintained under specific pathogen-free conditions. *Leishmania infantum* (MHOM/BR/1970/BH46) strain was used. Parasites were grown at 24 °C in Schneider's

medium (Sigma, St. Louis, MO, USA), which was supplemented by 20 % heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4. The soluble *L. infantum* antigenic extract (SLA) was prepared from stationary-phase promastigotes of parasites, as previously described [32].

Canine sera

The sample size of dogs' sera consisted of 73 domestic animals (*Canis familiaris*), made up of males and females of different breeds and ages. Sera of CVL were selected on the basis of two *Leishmania spp.* serological tests (IFAT [IFAT- LVC Bio-Manguinhos kit] and ELISA [EIE-LVC Bio-Manguinhos kit], both from Biomanguinhos, Fiocruz, Brazil). Dogs with an IFAT titer < 1/40 or ELISA reactivity below the cut-off value indicated by the manufacturer were considered to be serologically seronegative. Animals with an IFAT titer > 1/40 and an ELISA value over the cut-off were considered to be serologically seropositive. Thus, symptomatic VL dogs ($n = 16$) were those positive by IFAT and ELISA, and presenting three or more of the following clinical symptoms: weight loss, alopecia, adenopathy, onychogryposis, hepatomegaly, conjunctivitis and exfoliative dermatitis on the nose, tail, and ear tips. In addition, all dogs presented positive parasitological results (PCR detection of *L. infantum* DNA). Non-infected dogs ($n = 20$) were selected from an endemic area of leishmaniasis (Belo Horizonte, Minas Gerais, Brazil), but presenting negative serological and parasitological results, as well as were free of any clinical suspicion or signals of leishmaniasis. Also, sera samples of non-infected animals immunized with a Brazilian commercial vaccine (Leish-Tec®) ($n = 10$), which were isolated in kennels to prevent their contact with transmitting vectors of leishmaniasis, were used. In addition, sera of dogs experimentally infected with *T. cruzi* ($n = 13$), *Ehrlichia canis* ($n = 7$) or *Babesia canis* ($n = 7$) were also used in the ELISA assays, in order to evaluate the cross-reactivity. These sera samples were obtained from previous projects, which evaluated the immune response in the infected animals [15, 33, 34]. Serum samples were provided by Prof. Maria Norma Mello (Department of Parasitology, UFMG, Belo Horizonte, Brazil), and Prof. Ana Paula Fernandes (Department of Clinical and Toxicological Analysis, UFMG, Belo Horizonte, Brazil).

Cloning of DNA sequence coding for *L. infantum* hypothetical protein, LiHyV

The LiHyV (XP_888524.1) nucleotide and amino acid sequences were obtained from the National Center for Biotechnology Information (http//www.ncbi.nlm.nih.gov). The local alignment of the protein sequence against the available complete genomes of other organisms was performed by BLAST, which verified a high homology of amino acid sequence (>85 %) of this protein among the *L. major*, *L. amazonensis* and *L. infantum* species. The recombinant protein was obtained after cloning a *L. infantum* (MHOM/BR/1970/BH46) DNA fragment containing the protein-coding region. Initially, genomic DNA was extracted using a phenol:chloroform extraction, and this was used as a template. The *forward* (5′-GACGGATCCATGTCGGACGCATCATTC-3′) and *reverse* (5′-GCACAAGCTTAAGGGCGTAGAAAGCGGC-3′) primers were designed according to the DNA sequence of the \ORF described in the *L. infantum* genome sequence database (LinJ. 04.0160). The PCR product was cloned into the pGEM-T *easy* vector confirmed by sequencing and transferred to the pET21a expression vector (Novagen), using the *Bam*HI and *Hin*dIII restriction enzymes included in the primers for this purpose (underlined). Recombinant plasmid was transformed into *Escherichia coli* BL21 (DE3). The recombinant protein expression was performed by adding 1.0 mM IPTG (isopropyl-β-D-thiogalactopyranoside, Promega, Montreal, Canada), for 3 h at 37 °C, when cells were lysed by a homogenizer after five passages through the apparatus. The product was centrifuged at 13,000 × *g* for 20 min at 4 °C, while the rLiHyV protein, containing a tag of 6x residues of histidine, was purified under non-denaturing conditions, using a 5 mL HIS-Trap column (GE Healthcare Life Science) attached to an FPLC (GE Healthcare Life Science) system. After purification, the recombinant protein content was passed through a polymyxin-agarose column (Sigma), in order to remove residual endotoxins. The recombinant protein A2 was cloned and purified as previously described [32].

In silico prediction of T-cell epitopes

The linear $CD8^+$ T cells epitopes of LiHyV protein were predicted using BIMAS software (www-bimas.cit.nih.gov/molbio/hla_bind/), where peptides that presented a score higher than 100.0 were selected to be evaluated as vaccine candidates against VL. Two amino acid sequences were synthesized to be employed in the vaccination experiments: AGQSVPNSL (Peptide1) and VGIKNTAALFVLNATAI (Peptide2). The second peptide was originally composed of two different peptides (VGIKNTAAL and LFVLNATAI), which presented the best results of *in silico* prediction of $CD8^+$ T cell epitopes in the analysis, besides a sequential position in the primary sequence of amino acids of the LiHyV protein. So, the peptides were commercially synthesized (Genscript®, USA)".

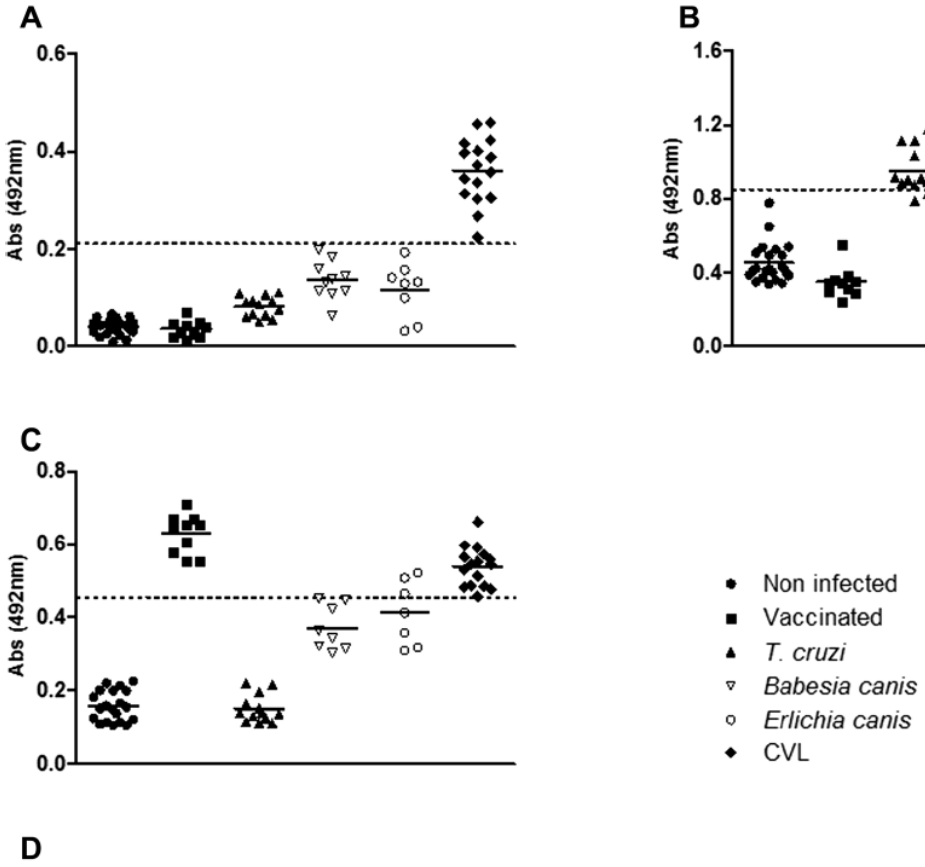
ELISA for the serodiagnosis of CVL

Previous titration curves were performed to determine the most appropriate antigen concentration and antibody dilution to be used. The recombinant LiHyV and A2 proteins, as well as SLA *L. infantum*-specific IgG antibodies were evaluated. For this, microtiter immunoassay plates (Falcon)

were coated with rLiHyV, rA2 or SLA (1.0, 0.5 and 1.0 μg per well, respectively), diluted in 100 μL of coating buffer (50 mM carbonate buffer pH 9.6), for 18 h at 4 °C. The experimental protocol used in the ELISA assays was developed as previously described [21]. In addition, all sera samples were evaluated in the same day using the same reagents (lots, dilutions, etc.), aiming to reduce or eliminate the possible experimental variations and the interference in the interpretation of data that could occur if the ELISA assays had been performed on different days.

Immunization and challenge infection

Mice ($n = 8$, per group) were vaccinated subcutaneously in their left hind footpad with 25 μg of rLiHyV, Peptide1 or Peptide2, all associated with 25 μg of saponin (*Quillaja saponaria* bark saponin, Sigma); or received saponin or saline. Three doses were administered at two-week intervals. Four weeks after the third and last immunization, animals ($n = 4$, per group) were euthanized to analyze the immune response elicited by vaccination. At the same time, the remaining animals were infected



D

Negatives vs Positives				
	Sensitivity (%)	Se (CI95%)	Specificity (%)	Sp (CI95%)
rLiHyV	100,0	78.20% to 100.0%	100,0	94.31% to 100.0%
SLA	100,0	79.41% to 100.0%	81,67	69.56% to 90.48%
A2	100,0	79.41% to 100.0%	77,59	64.73% to 87.49%

Fig. 1 Diagnostic performance of LiHyV protein. ELISA assays were performed using sera samples of dogs with visceral leishmaniasis ($n = 16$), non-infected dogs living in an endemic area of leishmaniasis ($n = 20$), Leish-Tec®-vaccinated animals ($n = 10$) and animals experimentally infected by *Trypanosoma cruzi* ($n = 13$), *Ehrlichia canis* ($n = 7$) or *Babesia canis* ($n = 7$). The rLiHyV (**a**), SLA *L. infantum* (**b**), and rA2 (**c**) were used as antigens. The optical density (O.D.) values of each individual serum are shown. The cut-off values (*dotted line*) for negative and positive sample discrimination were calculated using the mean ± three times the standard deviation of all negative samples. The sensitivity and specificity values of the antigens were calculated and are also showed (**d**)

subcutaneously in the right hind footpad with 1×10^7 stationary-phase promastigotes of *L. infantum*. Ten weeks after infection, animals were euthanized and the spleen, liver, bone marrow (BM), and lymph nodes draining the paws (dLN) of the animals were collected to evaluate the immune response and parasite burden. Vaccination experiments were repeated twice and presented similar results.

Estimation of parasite burden

The liver, spleen, BM, and dLN were collected for parasite quantification, following a limiting-dilution technique, as previously described [19].

Cytokine production before and after *L. infantum* infection

Splenocyte cultures and cytokine assays were performed after the last immunization and before infection, as well as at the 10th week after challenge. For this, single-cell suspensions were plated in duplicate in 24-well plates (Nunc), at 1×10^6 cells per mL. Cells were incubated in RPMI 1640 medium (negative control), which was supplemented with 10 % FBS, 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4; or separately stimulated with rLiHyV, Peptide1, Peptide2 or *L. infantum* SLA (20, 25, 25, or 25 μg mL^{-1}, respectively); for 48 h at 37 °C in 5 % CO_2. IFN-γ, IL-4, IL-10, IL-12, and GM-CSF levels were assessed in the supernatants by a sandwich ELISA provided in commercial kits (BD OptEIA TM set mouse IFN-γ, IL-4, IL-10, IL-12, and GM-CSF; all obtained from Pharmingen, San Diego, CA, USA); following manufacturer's instructions. In order to block IL-12, $CD4^+$, and $CD8^+$ mediated T cell cytokine release, spleen cells of mice vaccinated with rLiHyV/saponin and challenged with *L. infantum* were *in vitro* stimulated with SLA (25 μg mL^{-1}), and incubated in the presence of 5 μg mL^{-1} of monoclonal antibodies (mAb) against mouse IL-12 (C17.8), CD4 (GK 1.5), or CD8 (53–6.7). Appropriate isotype-matched controls – rat IgG2a (R35-95) and

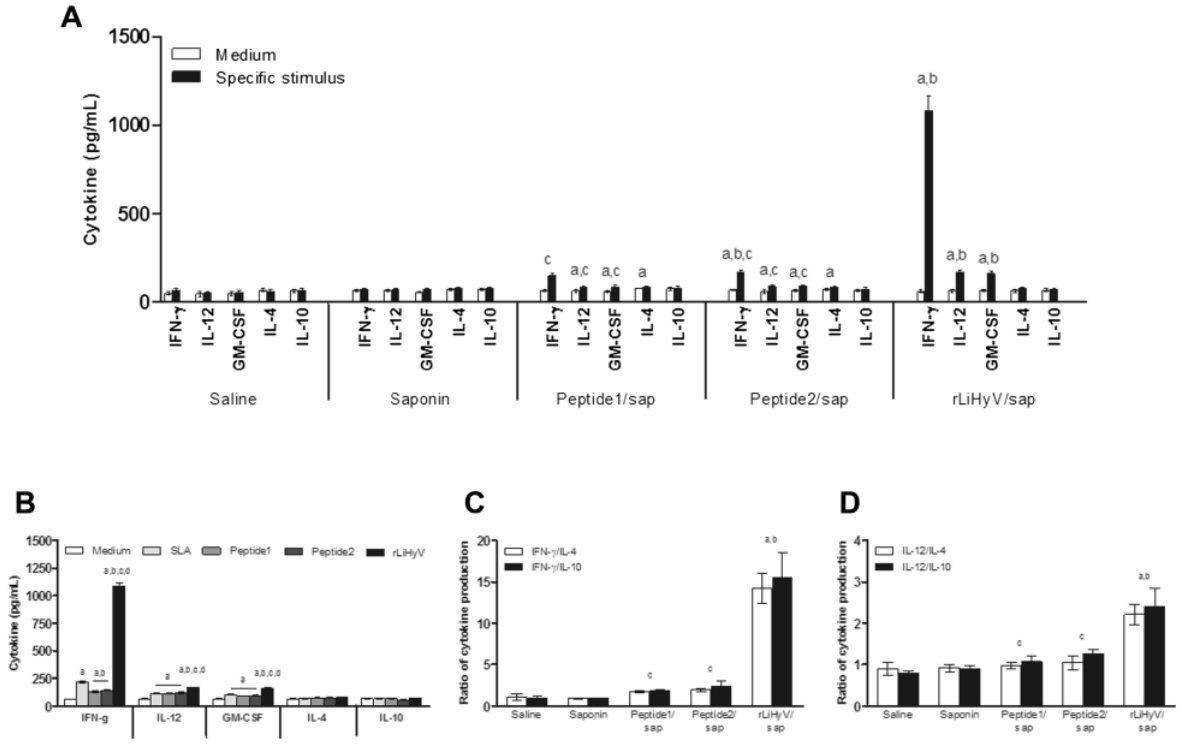


Fig. 2 Immune response induced by immunization with rLiHyV protein plus saponin. Single cells suspensions were obtained from the spleen of mice, four weeks after the last immunization and before infection. Cells were unstimulated (*negative control*), or separately stimulated with rLiHyV (rLiHyV/saponin group, 20 μg/mL), Peptide1 (Peptide1/saponin group, 20 μg/mL) or Peptide2 (Peptide2/saponin group, 25 μg/mL). Saline and saponin groups were stimulated with SLA (25 μg/mL). IFN-γ, IL-12, GM-CSF, IL-4, and IL-10 levels were measured by a capture ELISA (**a**). Cytokine levels of spleen cells derived from animals immunized with rLiHyV/saponin and stimulated with rLiHyV, Peptide1, Peptide2 or SLA (**b**). Statistically significant differences ($P < 0.001$) were observed between negative control (*medium*), SLA, Peptide1 and Peptide2 (*a, b, c,* and *d,* respectively) and in ratios of IFN-γ/IL-10 and IFN-γ/IL-4 (**c**), as well as between IL-12/IL-10 and IL-12/IL-4 (**d**). Bars represent the mean ± standard deviation of the groups. Statistically significant differences ($P < 0.05$) in relation to the saline, saponin or rLiHyV/saponin groups were observed (*a, b* and *c* respectively)

rat IgG2b (95–1) – were employed in the assays. Antibodies (no azide/low endotoxin™) were purchased from BD (Pharmingen).

Evaluation of the humoral response

The antibody production was evaluated after the third and last immunization and before infection, as well as at the 10th week after challenge. The parasite-specific IgG1 and IgG2a isotype antibody levels were measured by an ELISA technique, as previously described [19].

Statistical analysis

The results were entered into Microsoft Excel (version 10.0) spreadsheets, and analyzed using GraphPad Prism™ (version 6.0 for Windows). The mean optical density (O.D.) values were calculated by subtracting the mean blank O.D. from the mean value for each sample by using specific data obtained in the ELISA assays. The lower limits of positivity (cut-off) for rLiHyV and A2 proteins, as well as to SLA *L. infantum*, were established for optimal sensitivity and specificity using the Receiver Operator Curve (ROC curves). The accuracy was evaluated according to the area under the curve (AUC) relative to the ROC curve, with a 95 % confidence interval (CI95%). The ROC curves were plotted with the values from serum samples from dogs presenting CVL, when compared to those from the control groups (*T. cruzi*-, *E. canis*- or *B. canis*-infected, Leish-Tec®-immunized animals, and non-infected dogs), according to a sick/non-sick rating method. Statistical analysis of the data from vaccinated and/or infected animals was performed by one-way analysis of variance (ANOVA), following Bonferroni's post-test for multiple comparisons between the groups. Results are shown as dotted or bars graphs, where mean ± standard deviation (SD) of each experimental group is shown. Differences were considered significant when $P < 0.05$.

Results and discussion

Serodiagnosis of CVL using the rLiHyV protein

Serological tests are currently recommended for the laboratorial diagnosis of CVL. Some of them, such as IFAT and ELISA, can present low sensitivity to detect cases from animals presenting low levels of antileishmanial antibodies, also their specificity can be hampered due tocross-reaction with antibodies in sera of animals developing other pathologies, such as Chagas' disease, ehrlichiose, and babesiosis [35, 36]. In addition, in Brazil, there are two commercial vaccines against CVL, Leishmune® [37] and Leish-Tec® [15] and, although protective, they can induce seroconversion in the immunized animals, causing them to be classified as false-positive in the serological assays performed [9, 38].

In the present study, a non-described *Leishmania*-specific protein was fused as a recombinant protein to an N-

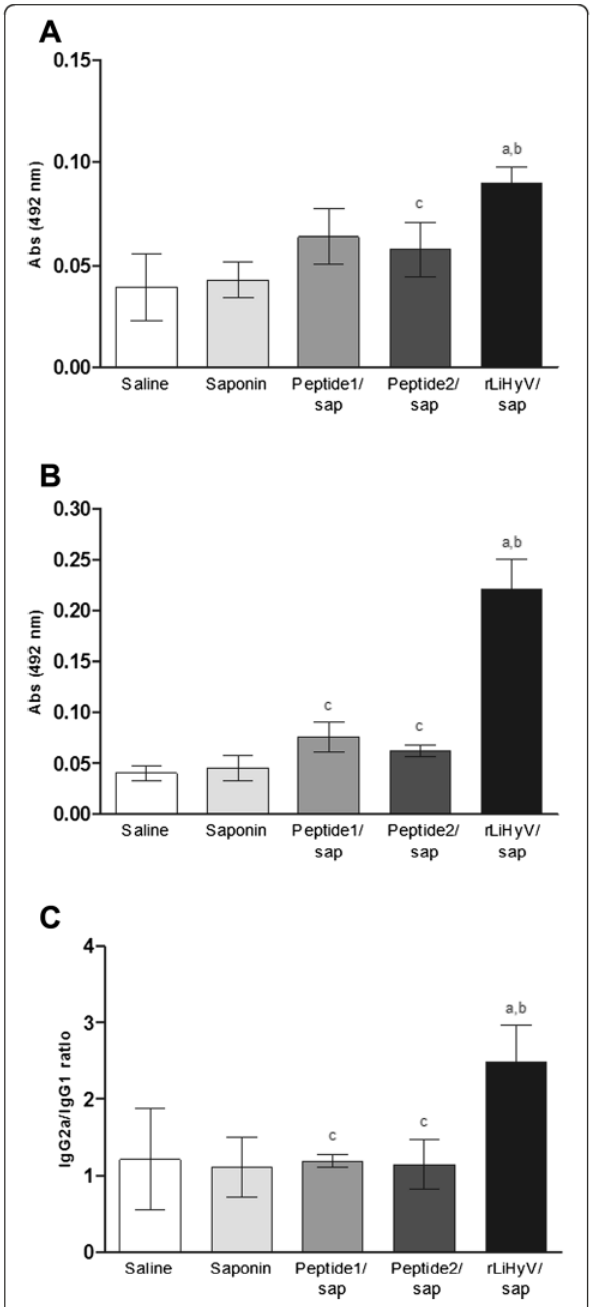


Fig. 3 Humoral response induced by vaccination with rLiHyV protein plus saponin. Sera samples were collected from animals four weeks after the third and last immunization, and before infection. The parasite-specific IgG1 (**a**) and IgG2a (**b**) isotype levels were determined, and the bars represent the mean ± standard deviation of the groups. The ratios between IgG2a and IgG1 levels were also calculated (**c**). Statistically significant differences in relation to the saline, saponin or rLiHyV/saponin groups were observed (*a*, *b* and *c* letters, respectively; $P < 0.05$)

terminal 6x histidine-tag, and expressed in *E. coli*. The rLiHyV protein was purified and evaluated for the serodiagnosis of CVL. In the results, it was possible to verify that rLiHyV was recognized by all sera from VL dogs. In contrast, antibodies from *T. cruzi*-, *E. canis*- or *B. canis*-infected dogs, from Leish-Tec®-vaccinated animals or those of non-infected dogs; did not react with the recombinant protein (Fig. 1a). The SLA *L. infantum* and rA2 protein were used as comparative antigens. Using SLA, although all VL dogs' sera had been recognized, a significant cross-reactivity was observed when sera from *T. cruzi*-infected animals were evaluated (Fig. 1b). Using the A2 protein as a diagnosis marker [39–41], a cross-reaction was also observed when sera of dogs experimentally infected with *E. canis* or *B. canis* were evaluated (Fig. 1c). To determine the diagnostic performance of the rLiHyV protein, ROC curves were constructed, and it was possible to observe that this antigen presented sensitivity and specificity values of 100 %; whereas using SLA, these values were of 100 and 81.67 %, respectively; and with the A2 protein, they were of 100 and 77.59 %, respectively (Fig. 1d). The number and variety of sera used in the present study could be considered a limiting factor. In this context, data here presented should be taken as a proof-of-concept of the efficacy of the proposed antigen to be employed in the serodiagnosis of CVL, and may well serve as a reference for further assays. On the other hand, we believe that, after an adequate validation, the rLiHyV protein may be promptly applied for a sensitive and specific serodiagnosis of CVL.

Immunogenicity of LiHyV protein in BALB/c mice

In this study, the immunogenicity of rLiHyV protein, as well as two synthetic peptides (Peptide1 and Peptide2) covering two putative $CD8^+$ T cell epitopes present in the primary structure of the protein was evaluated in BALB/c mice. Four weeks after the last vaccine dose and before infection, the immune response elicited by the vaccine was evaluated. Following the *in vitro* stimulation with the rLiHyV protein, spleen cells derived from mice vaccinated with rLiHyV/saponin produced significantly higher levels of IFN-γ, IL-12, and GM-CSF after rLiHyV stimulation, when compared to the negative control (background medium). Although the Peptide1/saponin and Peptide2/saponin groups had presented a high IFN-

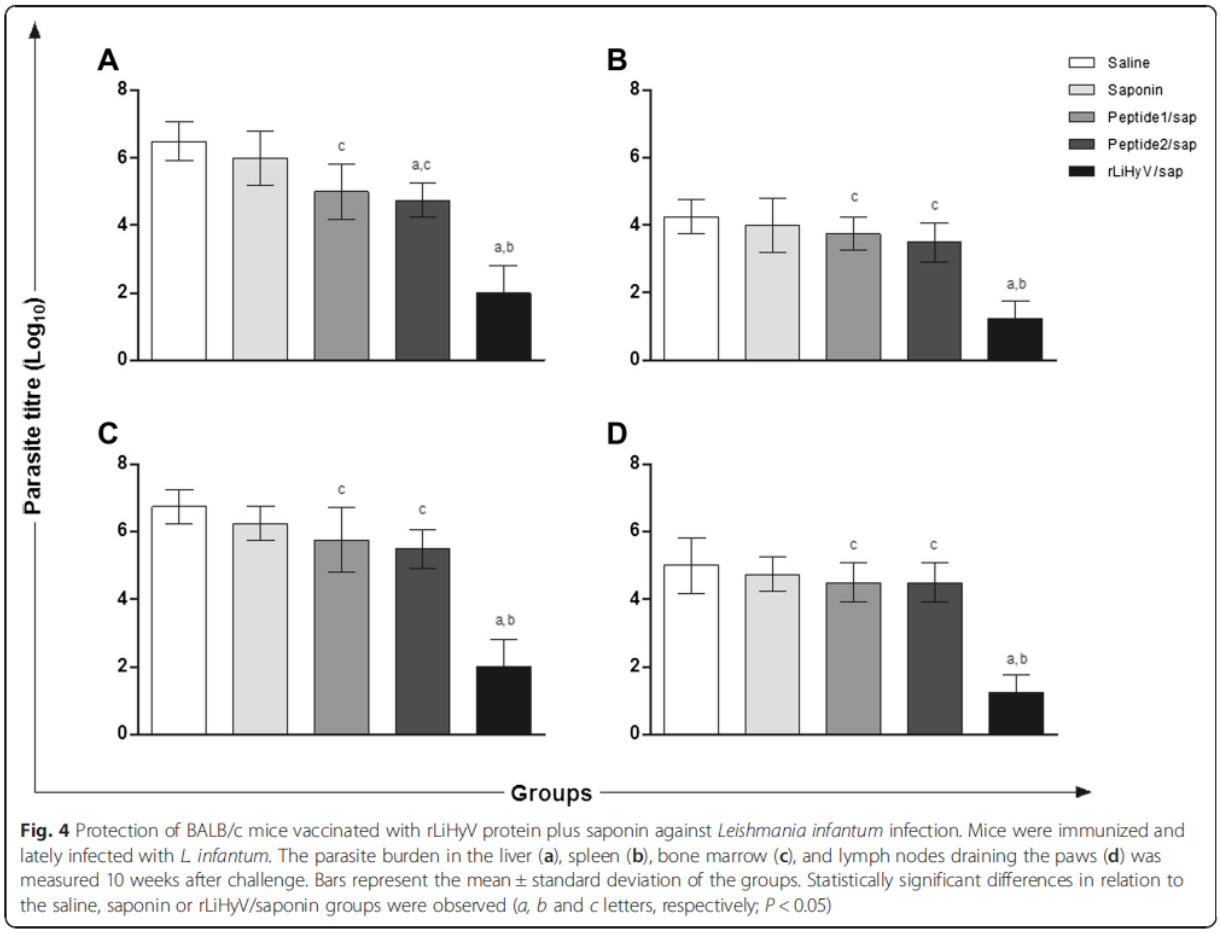


Fig. 4 Protection of BALB/c mice vaccinated with rLiHyV protein plus saponin against *Leishmania infantum* infection. Mice were immunized and lately infected with *L. infantum*. The parasite burden in the liver (**a**), spleen (**b**), bone marrow (**c**), and lymph nodes draining the paws (**d**) was measured 10 weeks after challenge. Bars represent the mean ± standard deviation of the groups. Statistically significant differences in relation to the saline, saponin or rLiHyV/saponin groups were observed (*a, b* and *c* letters, respectively; $P < 0.05$)

γ production after the specific stimulus, this production was comparatively lower in relation to that observed in the rLiHyV/saponin group. In addition, a low production of IL-4 and IL-10 was observed in all experimental groups (Fig. 2a).

Using the different stimulations (rLiHyV, Peptide1, Peptide2 and SLA) in the spleen cells of mice of the rLiHyV/saponin group; it was observed that both Peptide1 and Peptide2 were able to induce a similar IFN-γ production, but that was significantly lower when compared to the levels found of this cytokine using the recombinant protein stimulus (Fig. 2b). Using the data collected from the specific stimulus performed in each experimental group, the ratios between IFN-γ/IL-4 and IFN-γ/IL-10 levels (Fig. 2c), as well as between IL-12/IL-4 and IL-12/IL-10 levels (Fig. 2d) were calculated, and the results showed that mice vaccinated with rLiHyV/saponin mounted a more pronounced Th1 response before infection, when compared to the other groups. In the evaluation of humoral response, mice vaccinated with rLiHyV/saponin produced higher levels of parasite-specific IgG1 (Fig. 3a) and IgG2a (Fig. 3b) isotype antibodies, when compared to the control groups. The ratio between the IgG2a and IgG1 levels was also calculated, and the results showed that mice vaccinated with rLiHyV/saponin presented a higher predominance of the IgG2a isotype in relation to its IgG1 levels (Fig. 3c). In this context, it could be concluded that the immunization with rLiHyV/saponin induced a higher *in vitro* IFN-γ, IL-12 and GM-CSF production, as well as low levels of IL-4, IL-10 and parasite-specific IgG1 antibodies in the vaccinated animals.

Protective efficacy against *L. infantum* infection

To evaluate the protective efficacy of rLiHyV protein and its two peptides in BALB/c mice against *L. infantum* infection, the parasite load was estimated in the liver, spleen, BM, and dLN of the vaccinated and infected animals,

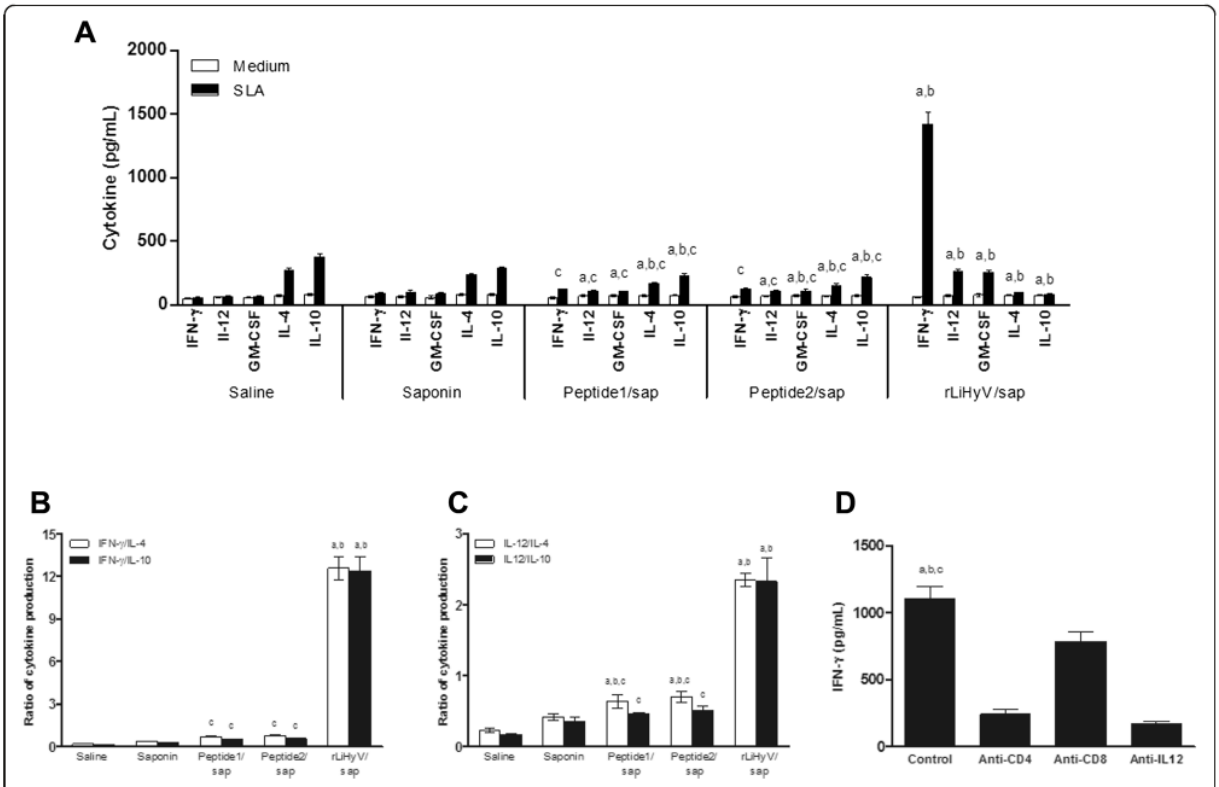


Fig. 5 Analysis of cellular response and involvement of $CD4^+$ and $CD8^+$ T cells in the IFN-γ production after *L. infantum* infection. Spleen cells of mice were obtained 10 weeks after challenge, and were *in vitro* stimulated with SLA (25 μg per mL), for 48 h at 37 °C in 5 % CO_2. IFN-γ, IL-12, GM-CSF, IL-4 and IL-10 levels were measured by a capture ELISA (**a**). The ratios between IFN-γ/IL-10 and IFN-γ/IL-4 levels (**b**), as well as between IL-12/IL-10 and IL-12/IL-4 levels (**c**) were calculated and also shown. Statistically significant differences in relation to the saline, saponin or rLiHyV/saponin groups were observed (*a, b* and *c* letters, respectively; $P < 0.05$). The involvement of $CD4^+$ and $CD8^+$ T cells in the IFN-γ production in the rLiHyV/saponin group was evaluated (**d**). Statistically significant differences in relation to the use of anti-IL-12, anti-CD4 or anti-CD8 monoclonal antibodies were observed (*a, b* and *c* letters, respectively; $P < 0.05$). Bars represent the mean ± standard deviation of the groups

10 weeks after challenge. In the results, significant reductions in the parasite burden were observed in all organs of mice vaccinated with rLiHyV/saponin, when compared to the other groups (Fig. 4). In this context, mice vaccinated with rLiHyV/saponin presented reductions in the parasite load in the liver (3.4- and 3.2-log reductions, Fig. 4a), spleen (3.3- and 3.0-log reductions, Fig. 4b), BM (4.0- and 3.8-log reductions, Fig. 4c), and dLN (3.4- and 3.3-log reductions, Fig. 4d), when compared to the saline and saponin groups, respectively. Comparing the protective efficacy of the recombinant protein and its synthetic peptides, it was possible to verify that mice immunized with rLiHyV/saponin presented significant reductions in the parasite load in the liver (3.0- and 2.8-log reductions, Fig. 4a), spleen (2.5- and 2.4-log reductions, Fig. 4b), BM (3.6- and 3.6-log reductions, Fig. 4c), and dLN (2.9- and 2.8-log reductions, Fig. 4d), when compared to the Peptide1/saponin and Peptide2/saponin groups, respectively. These data were demonstrating that the inoculation of the recombinant protein was able to induce better protection than the peptide-derived vaccines.

With the advancement in recombinant DNA technology, *Leishmania spp.* proteins, either species or stage-specific of the parasites, had been evaluated as vaccine candidates against leishmaniasis. The major advantages associated to the use of recombinant proteins have been based in terms of standardization, purity and yield of production [24]. On the other hand, although whole proteins had been well-studied [15, 18, 21, 42], the protective immunity is known to be triggered by their $CD4^+$ and/or $CD8^+$ T cells epitopes [43–45]. More recent bioinformatics approaches utilize a number of algorithms for predicting epitopes, HLA-binding, transporter of antigen processing affinity, and proteasome cleavage to explore the use of these peptides with the higher probability to be immunogenic, and inducing protection against intracellular pathogens [46, 47]. In the present study, a comparison of the protective efficacy between the recombinant LiHyV protein and two of its putative $CD8^+$ T cells-specific epitopes, Peptide1 and Peptide2, was performed. The choice to study $CD8^+$ T cells epitopes was based on the fact that rLiHyV protein is also a protein expressed in the amastigote stage of *L. infantum*. In the results, it was obtained that the peptide-based vaccine was also able to reduce the parasite burden in the inoculated animals, when compared to the saponin and saline inoculated mice groups. These data may indicate the possibility of designing peptide-based vaccines. However, the protective characteristic of these vaccines should be improved, such as increasing the number of doses, the amount of peptide in each dose, and even using some carrier molecules [48–50]. On the other hand, employing combined vaccines containing different peptides could optimize the protective efficacy, when compared to the use of individual peptides [14, 24, 25, 51].

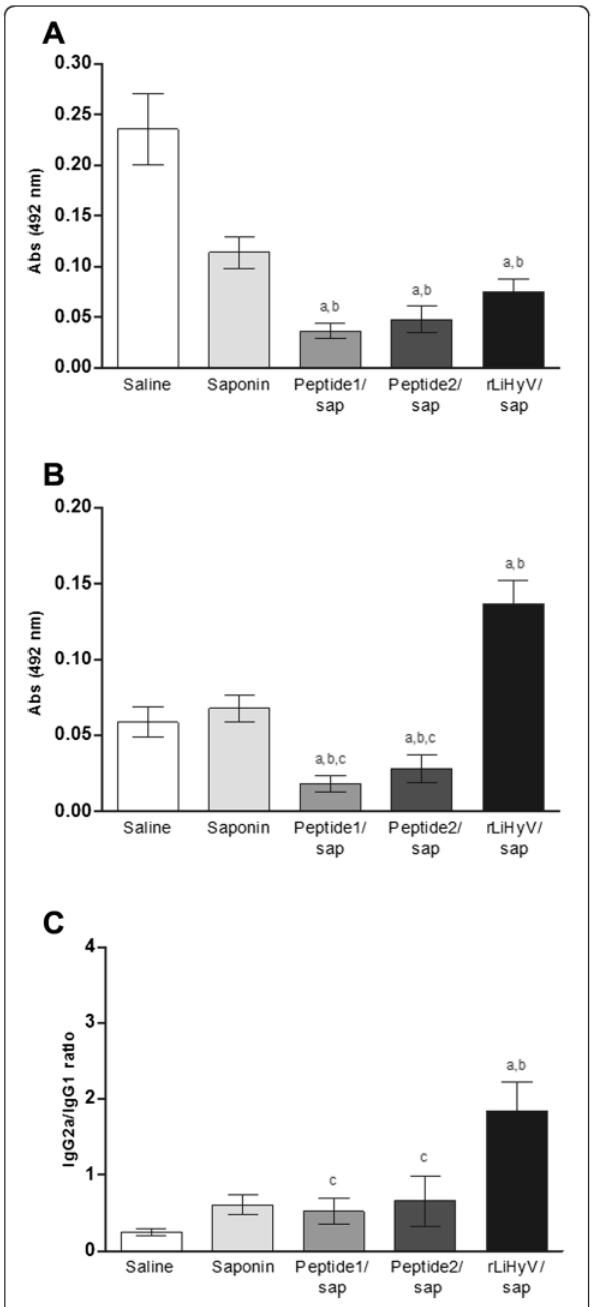


Fig. 6 Analysis of humoral response after *L. infantum* infection. Sera samples were collected of animals, 10 weeks after infection, when the parasite-specific IgG1 (**a**) and IgG2a (**b**) isotype levels were evaluated. The ratios between the IgG2a and IgG1 levels were calculated, and the bars represent the mean ± standard deviation of the groups (**c**). Statistically significant differences in relation to the saline, saponin or rLiHyV/saponin groups were observed (*a*, *b* and *c* letters, respectively; $P < 0.05$)

Immunological parameters related with protection against infection

The production of cytokines in the culture supernatants of spleen cells was also evaluated 10 weeks after infection. In the results, spleen cells of mice vaccinated with rLiHyV/saponin produced higher levels of parasite-specific IFN-γ, IL-12 and GM-CSF cytokines, than those secreted by spleen cells of the other groups (Fig. 5a). In contrast, in mice vaccinated with the rLiHyV/saponin, the SLA-driven production of IL-4 and IL-10 was lower when compared to animals that received saline or saponin (Fig. 5a). In addition, the ratios between IFN-γ/IL-4 and IFN-γ/IL-10 levels (Fig. 5b), as well as between IL-12/IL-4 and IL-12/IL-10 (Fig. 5c) levels demonstrated that mice vaccinated with rLiHyV/saponin mounted a more pronounced parasite-derived Th1 response after infection. The contribution of $CD4^+$ and $CD8^+$ T cells and the dependence of IL-12 production in the SLA-specific IFN-γ production of mice immunized with rLiHyV/saponin after infection was evaluated (Fig. 5d). The addition of anti-CD4 antibodies to the cultures was able to significantly decrease the parasite-specific IFN-γ production. A slight reduction was also observed when the anti-CD8 monoclonal antibody was used. These results indicate that the main production of IFN-γ is due to the induction of a parasite-specific $CD4^+$ T cells response. These cells have proved to be an important source of IFN-γ in mice vaccinated with recombinant proteins. It is expected that these products are taken by phagocytic cells, processed and presented associated with MHC class II molecules, inducing a predominant $CD4^+$ T cell response [50]. These results do not exclude the possibility of the existence of $CD8^+$ epitopes in this protein, as expected by the protection observed in mice vaccinated with Peptide1 and Peptide2. For instance, it is possible to suggest that using the LiHyV sequence as a DNA vaccine, both $CD4^+$ and $CD8^+$ T cells responses could be generating, improving the vaccine protection based on a similar contribution of both T cells subtypes [52].

Spleen cells derived from BALB/c mice vaccinated with rLiHyV/saponin, when compared to the other groups, produced higher levels of SLA-specific GM-CSF, a cytokine related to macrophage activation and resistance against *L. major* [53], *L. donovani* [54], and *L. infantum* [16, 19, 21]. In addition, this cytokine has also played an important role in the activation, maturation, and function of dendritic cells [55]. The present study also showed that immunization using rLiHyV/saponin induced a low production of IL-4 and IL-10 after infection. Very low levels of parasite-specific IL-10 were detected after stimulation of spleen cells derived from vaccinated and infected mice, although spleen cells obtained from saline and saponin groups had showed a significantly higher production of these cytokines. Indeed, the control of parasite-mediated IL-10 response in mice may be critical for protection against murine VL, since this cytokine is considered to be the most important factor for inhibiting the disease progression in IL-10 deficient mice [56, 57], as well as in animals treated with an anti-IL-10 receptor antibody [58].

In BALB/c mice, IL-4-dependent production of IgG1 antibodies is associated with disease progression caused by *L. amazonensis* [32, 43, 59] and *L. infantum* [19, 21, 60]. For instance, BALB/c mice vaccinated with the recombinant A2 protein [15, 32] or *Leishmania* ribosomal proteins [16] develop low levels of parasite-specific IgG1 antibodies, and this fact could contribute to protect the animals against *Leishmania spp.* infection. In the present study, mice that received saline or saponin presented higher parasite-specific IgG1 antibody levels (Fig. 6a), when compared to the other groups. On the other hand, animals vaccinated with rLiHyV/saponin showed higher levels of specific IgG2a antibodies (Fig. 6b), which possibly contributed to the protective response. These animals also presented a higher ratio between the IgG2a and IgG1 levels (Fig. 6c), demonstrating that the recombinant protein was able to mount a more pronounced Th1 response in the vaccinated animals, which was maintained after *L. infantum* infection.

Conclusions

In the present study, a non-described *Leishmania*-specific protein, LiHyV, which was found to be expressed in both promastigote and amastigote stages of *L. infantum*, was successfully evaluated as a vaccine candidate against VL. Also, the recombinant protein demonstrated to be an effective marker for the serodiagnosis of CVL, and may well be employed in future studies aiming both immunological applications on leishmaniasis.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

VTM, MCD, LEC, MCST, DPL, LMD, JSO carried out the experimental procedures. MACF, EAFC, CSPC, MS, CAPT, DFMS, APF conceived the research, contributed with data analysis and revised the manuscript. MACF, EAFC, MS, CAPT wrote the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgments

We would like to thank Míriam C. Souza-Testasicca, Jamil S. Oliveira, Leonardo M. Damasceno, Daniela P. Lage, and Lourena E. Costa for their scientific and technical assistance. This work was supported by grants from Instituto Nacional de Ciência e Tecnologia em Nanobiofarmacêutica (INCT-Nanobiofar), FAPEMIG (CBB-APQ-00819-12), and CNPq (APQ-472090/2011-9, RHAE-456287/2012-4, APQ-482976/2012-8, and APQ-488237/2013-0). MACF is a grant recipient of FAPEMIG/CAPES. EAFC and APF are grant recipient of CNPq.

Author details

[1]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. [2]Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. [3]Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. [4]Departamento de Parasitologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.

[5]RW Bioprospection Ltda, Belo Horizonte, Minas Gerais, Brazil. [6]Departamento de Medicina Veterinária Preventiva, Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. [7]Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. [8]Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Departamento de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain. [9]Laboratório de Biotecnologia Aplicada ao Estudo das Leishmanioses, Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Avenida Antônio Carlos 6627, 31.270-901 Belo Horizonte, Minas Gerais, Brazil.

Received: 15 April 2015 Accepted: 23 June 2015
Published online: 11 July 2015

References

1. World Health Organization. Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, World Health Organization Tech Rep Ser 949. Geneva: WHO; 2010.
2. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012;7:e35671.
3. Lemos EM, Gomes IT, Carvalho SF, Rocha RD, Pissinate JF, Martins-Filho OA, et al. Detection of anti-*leishmania* (*Leishmania*) *chagasi* immunoglobulin G by flow cytometry for cure assessment following chemotherapeutic treatment of American visceral leishmaniasis. Clin Vaccine Immunol. 2007;14:569–76.
4. Mondal S, Bhattacharya P, Ali N. Current diagnosis and treatment of visceral leishmaniasis. Expert Rev Anti Infect Ther. 2010;8:919–44.
5. Croft SL, Coombs GH. Leishmaniasis-current chemotherapy and recent advances in the search for novel drugs. Trends Parasitol. 2003;19:502–8.
6. Bern C, Adler-Moore J, Berenguer J, Boelaert M, den Boer M, Davidson RN, et al. Liposomal amphotericin B for the treatment of visceral leishmaniasis. Clin Infect Dis. 2006;43:917–24.
7. Gramiccia M, Gradoni L. The current status of zoonotic leishmaniases and approaches to disease control. Int J Parasitol. 2005;35:1169–80.
8. Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L. Canine leishmaniosis - new concepts and insights on an expanding zoonosis: part one. Trends Parasitol. 2008;24:324–30.
9. Figueiredo FB, Madeira MF, Nascimento LD, Abrantes TR, Mouta-Confort E, Passos SRL, et al. Canine visceral leishmaniasis: study of methods for the detection of IgG in serum and eluate samples. Rev Inst Med Trop. 2010;52:193–6.
10. Zampoli M, Giantomassi J, Baldini S, Langoni H. *Leishmania* spp. and/or *Trypanosoma cruzi* diagnosis in dogs from endemic and nonendemic areas for canine visceral leishmaniasis. Vet Parasitol. 2009;164:118–23.
11. Viol MA, Lima VMF, Aquino MCC, Gallo IG, Gallo IP, Alves D, et al. Detection of cross infections by *Leishmania spp*. and *Trypanosoma spp*. in dogs using indirect immunoenzyme assay, indirect fluorescent antibody test and polymerase chain reaction. Parasitol Res. 2012;111:1607–13.
12. Oliveira TM, Furuta PI, Carvalho D, Machado RZ. A study of cross-reactivity in serum samples from dogs positive for *Leishmania* sp., *Babesia canis* and *Ehrlichia canis* in enzyme-linked immunosorbent assay and indirect fluorescent antibody test. Rev Bras Parasitol Vet. 2008;17:7–11.
13. Coelho EA, Ramírez L, Costa MA, Coelho VT, Martins VT, Chávez-Fumagalli MA, et al. Specific serodiagnosis of canine visceral leishmaniasis using *Leishmania* species ribosomal protein extracts. Clin Vaccine Immunol. 2009;16:1774–80.
14. Noazin S, Modabber F, Khamesipour A, Smith PG, Moulton LH, Nasseri K, et al. First generation leishmaniasis vaccines: a review of field efficacy trials. Vaccine. 2008;26:6759–67.
15. Fernandes AP, Costa MM, Coelho EA, Michalick MS, de Freitas E, Melo MN, et al. Protective immunity against challenge with *Leishmania (Leishmania) chagasi* in beagle dogs vaccinated with recombinant A2 protein. Vaccine. 2008;26:5888–95.
16. Chávez-Fumagalli MA, Costa MA, Oliveira DM, Ramírez L, Costa LE, Duarte MC, et al. Vaccination with the *Leishmania infantum* ribosomal proteins induces protection in BALB/c mice against *Leishmania chagasi* and *Leishmania amazonensis* challenge. Microbes Infect. 2010;12:967–77.
17. Das A, Ali N. Vaccine prospects of killed but metabolically active *Leishmania* against visceral leishmaniasis. Expert Rev Vaccines. 2012;11:783–5.
18. Ramírez L, Santos DM, Souza AP, Coelho EA, Barral A, Alonso C, et al. Evaluation of immune responses and analysis of the effect of vaccination of the *Leishmania major* recombinant ribosomal proteins L3 or L5 in two different murine models of cutaneous leishmaniasis. Vaccine. 2013;3:1312–9.
19. Costa LE, Goulart LR, Pereira NC, Lima MI, Duarte MC, Martins VT, et al. Mimotope-based vaccines of *Leishmania infantum* antigens and their protective efficacy against visceral leishmaniasis. PLoS One. 2014;9:e110014.
20. Green SJ, Mellouk S, Hoffman SL, Meltzer MS, Nacy CA. Cellular mechanisms of nonspecific immunity to intracellular infection: cytokine-induced synthesis of toxic nitrogen oxides from L-arginine by macrophages and hepatocytes. Immunol Lett. 1990;25:15–9.
21. Martins VT, Chávez-Fumagalli MA, Costa LE, Canavaci AM, Lage PS, Lage DP, et al. Antigenicity and protective efficacy of a *Leishmania* amastigote-specific protein, member of the super-oxygenase family, against visceral leishmaniasis. PLoS Negl Trop Dis. 2013;7:e2148.
22. Stäger S, Alexander J, Carter KC, Brombacher F, Kaye PM. Both interleukin-4 (IL-4) and IL-4 receptor alpha signaling contribute to the development of hepatic granulomas with optimal antileishmanial activity. Infect Immun. 2003;71:4804–7.
23. Wilson ME, Jeronimo SM, Pearson RD. Immunopathogenesis of infection with the visceralizing *Leishmania* species. Microb Pathog. 2005;38:147–60.
24. Joshi J, Kaur S. Studies on the protective efficacy of second-generation vaccine along with standard antileishmanial drug in *Leishmania donovani* infected BALB/c mice. Parasitology. 2014;141:554–62.
25. Kharazmi A, Kemp K, Ismail A, Gasim S, Gaafar A, Kurtzhals JA. T-cell response in human leishmaniasis. Immunol Lett. 1999;65:105–8.
26. Stäger S, Smith DF, Kaye PM. Immunization with a recombinant stage-regulated surface protein from *Leishmania donovani* induces protection against visceral leishmaniasis. J Immunol. 2000;165:7064–71.
27. Tibayrenc M, Kjellberg F, Ayala FJ. A clonal theory of parasitic protozoa: the population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas*, and *Trypanosoma* and their medical and taxonomical consequences. Proc Natl Acad Sci USA. 1990;87:2414–8.
28. Peters NC, Kimblin N, Secundino N, Kamhawi S, Lawyer P, Sacks DL. Vector transmission of *Leishmania* abrogates vaccine-induced protective immunity. PLoS Pathog. 2009;5:e1000484.
29. Fernandes AP, Coelho EAF, Machado-Coelho GLL, Grimaldi JG, Gazzinelli RT. Making an anti-amastigote vaccine for visceral leishmaniasis: rational, update and perspectives. Curr Opin Microbiol. 2012;15:1–10.
30. Wenzel UA, Bank E, Florian C, Forster S, Zimara N, Steinacker J, et al. *Leishmania major* parasite stage-dependent host cell invasion and immune evasion. Faseb J. 2012;26:29–39.
31. Coelho VT, Oliveira JS, Valadares DG, Chávez-Fumagalli MA, Duarte MC, Lage PS, et al. Identification of proteins in promastigote and amastigote-like *Leishmania* using an immunoproteomic approach. PLoS Negl Trop Dis. 2012;6:e1430.
32. Coelho EA, Tavares CAP, Carvalho FAA, Chaves KF, Teixeira KN, Rodrigues RC, et al. Immune responses induced by the *Leishmania (Leishmania) donovani* A2 antigen, but not by the LACK antigen, are protective against experimental *Leishmania (Leishmania) amazonensis* infection. Infect Immun. 2003;71:3988–94.
33. Bahia MT, Tafuri WL, Caliari MV, Veloso VM, Carneiro CM, Coelho GL, et al. Comparison of *Trypanosoma cruzi* infection in dogs inoculated with blood or metacyclic trypomastigotes of Berenice-62 and Berenice-78 strains via intraperitoneal and conjunctival routes. Rev Soc Bras Med Trop. 2002;35:339–45.
34. Carneiro CM, Martins-Filho OA, Reis AB, Veloso VM, Araújo FM, Bahia MT, et al. Differential impact of metacyclic and blood trypomastigotes on parasitological, serological and phenotypic features triggered during acute *Trypanosoma cruzi* infection in dogs. Acta Trop. 2007;101:120–9.
35. Courtenay O, Quinnell RJ, Garcez LM, Shaw JJ, Dye C. Infectiousness in a cohort of Brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. J Infect Dis. 2002;186:1314–20.
36. Reis AB, Teixeira-Carvalho A, Vale AM, Marques MJ, Giunchetti RC, Mayrink W, et al. Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite density in Brazilian dogs naturally infected by *Leishmania (Leishmania) chagasi*. Vet Immunol Immunopathol. 2006;112:102–16.
37. Palatnik-de-Sousa CB, Barbosa AF, Oliveira SM, Nico D, Bernardo RR, Santos WR, et al. FML vaccine against canine visceral leishmaniasis: from second-generation to synthetic vaccine. Expert Rev Vaccines. 2008;7:833–51.
38. Mettler M, Grimm F, Capelli G, Camp H, Deplazes D. Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests (immunochromatographic-dipstick and gel tests) for serological diagnosis of

symptomatic and asymptomatic *Leishmania* infections in dogs. J Clin Microbiol. 2005;43:5515–9.
39. Carvalho FAA, Charest H, Tavares CAP, Matlashewski G, Valente EP, Rabello A, et al. Diagnosis of American visceral leishmaniasis in humans and dogs using the recombinant *Leishmania donovani* A2 antigen. Diagn Microbiol Infect Dis. 2002;43:289–95.
40. Costa MM, Penido M, Santos MS, Doro D, Freitas E, Michalick MS. Improved canine and human visceral leishmaniasis immunodiagnosis using combinations of synthetic peptides in enzyme-linked immunosorbent assay. PLoS Negl Trop Dis. 2012;6:e1622.
41. Akhoundi B, Mohebali M, Shojaee S, Jalali M, Kazemi B, Bandehpour M, et al. Rapid detection of human and canine visceral leishmaniasis: assessment of a latex agglutination test based on the A2 antigen from amastigote forms of *Leishmania infantum*. Exp Parasitol. 2013;133:307–13.
42. Ramirez L, Villen LC, Duarte MC, Chávez-Fumagalli MA, Valadares DG, Santos DM, et al. Cross-protective effect of a combined L5 plus L3 *Leishmania major* ribosomal protein based vaccine combined with a Th1 adjuvant in murine cutaneous and visceral leishmaniasis. Parasites Vectors. 2014;2:3–10.
43. Mou Z, Liu D, Okwor I, Jia P, Orihara K, Uzonna JE. MHC class II restricted innate-like double negative T cells contribute to optimal primary and secondary immunity to Leishmania major. PLoS Pathog. 2014;10:e1004396.
44. Nico D, Gomes DC, Alves-Silva MV, Freitas EO, Morrot A, Bahia D, et al. Cross-protective immunity to *Leishmania amazonensis* is mediated by $CD4^+$ and $CD8^+$ epitopes of *Leishmania donovani* nucleoside hydrolase terminal domains. Front Immunol. 2014;5:189.
45. Santos SC, Brodskyn CI. The role of CD4+ and CD8+ T cells in human cutaneous leishmaniasis. Front Public Health. 2014;2:165.
46. Nielsen M, Lundegaard C, Lund O, Keşmir C. The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. Immunogenetics. 2005;57:33–41.
47. Tenzer S, Peters B, Bulik S, Schoor O, Lemmel C, Schatz MM, et al. Modeling the MHC class I pathway by combining predictions of proteasomal cleavage, TAP transport and MHC class I binding. Cell Mol Life Sci. 2005;62:1025–37.
48. Frankenburg S, Axelrod O, Kutner S, Greenblatt CL, Klaus SN, Pirak EA, et al. Effective immunization of mice against cutaneous leishmaniasis using an intrinsically adjuvanted synthetic lipopeptide vaccine. Vaccine. 1996;14:923–9.
49. Topuzogullari M, Cakir Koc R, Dincer Isoglu S, Bagirova M, Akdeste Z, Elcicek S, et al. Conjugation, characterization and toxicity of lipophosphoglycan-polyacrylic acid conjugate for vaccination against leishmaniasis. J Biomed Sci. 2013;20:35.
50. Agallou M, Athanasiou E, Koutsoni O, Dotsika E, Karagouni E. Experimental validation of multi-epitope peptides including promising MHC class I- and II-restricted epitopes of four known Leishmania infantum proteins. Front Immunol. 2014;5:268.
51. Seyed N, Taheri T, Vauchy C, Dosset M, Godet Y, Eslamifar A, et al. Immunogenicity evaluation of a rationally designed polytope construct encoding HLA-A*0201 restricted epitopes derived from Leishmania major related proteins in HLA-A2/DR1 transgenic mice: steps toward polytope vaccine. PLoS One. 2014;9:e108848.
52. Coelho EA, Tavares CA, Lima KM, Silva CL, Rodrigues Jr JM, Fernandes AP. Mycobacterium hsp65 DNA entrapped into TDM-loaded PLGA microspheres induces protection in mice against *Leishmania (Leishmania) major* infection. Parasitol Res. 2006;98:568–75.
53. Dumas C, Muyombwe A, Roy G, Matte C, Ouellette M, Olivier M, et al. Recombinant *Leishmania major* secreting biologically active granulocyte-macrophage colony-stimulating factor survives poorly in macrophages *in vitro* and delays disease development in mice. Infect Immun. 2003;71:6499–509.
54. Murray HW, Cervia JS, Hariprashad J, Taylor AP, Stoeckle MY, Hockman H. Effect of granulocyte-macrophage colony-stimulating factor in experimental visceral leishmaniasis. J Clin Invest. 1995;95:1183–92.
55. Bayih AG, Daifalla NS, Gedamu L. DNA-protein immunization using *Leishmania* peroxidoxin-1 induces a strong $CD4^+$ T cell response and partially protects mice from cutaneous leishmaniasis: role of fusion murine granulocyte-macrophage colony-stimulating factor DNA adjuvant. PLoS Negl Trop Dis. 2014;8:e3391.
56. Murphy ML, Wille U, Villegas EN, Hunter CA, Farrell JP. IL-10 mediates susceptibility to *Leishmania donovani* infection. Eur J Immunol. 2001;31:2848–56.
57. Awasthi A, Mathur RK, Saha B. Immune response to *Leishmania* infection. Indian J Med Res. 2004;119:238–58.
58. Murray HW, Lu CM, Mauze S, Freeman S, Moreira AL, Kaplan G, et al. Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. Infect Immun. 2002;70:6284–93.
59. Pereira BA, Alves CR. Immunological characteristics of experimental murine infection with *Leishmania (Leishmania) amazonensis*. Vet Parasitol. 2008;158:239–55.
60. Zanin FH, Coelho EA, Tavares CA, Marques-da-Silva EA, Costa MMS, Rezende SA, et al. Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against *Leishmania chagasi* and *Leishmania amazonensis* experimental infections. Microbes Infect. 2007;9:1070–7.

5.2. Artigo 1 – Conclusão

A proteína LiHyV, identificada tanto nas formas amastigotas, quanto nas promastigotas de *L. infantum*, demonstrou ser um marcador efetivo para o sorodiagnóstico da LVC, bem como foi capaz de induzir um perfil de resposta imune Th1 em camundongos BALB/c. Entretanto, quando os animais foram imunizados com os peptídeos sintéticos 1 ou 2, derivados da proteína recombinante, e conjugados à saponina, esta reposta imune protetora não foi observada. A proteína LiHyV demonstrou ainda ser capaz de induzir uma maior redução da carga parasitária nos órgãos analisados, apresentando uma maior eficicácia vacinal em relação ao uso de peptídeos sintéticos como imunógenos.

5.3. Artigo 2 – Breve introdução e objetivo

O segundo artigo a ser apresentado foi intitulado "*Antigenicidade, imunogenicidade e proteção vacinal de três proteínas expressas nas formas amastigotas e promastigotas de Leishmania infantum contra a leishmaniose visceral*" e publicado pela revista internacional *Plos One.*

O presente trabalho avaliou duas proteínas hipotéticas de *L. infantum* identificadas nas formas amastigotas (LiHyp1 e LiHyp6) e uma, denominada fator de liberação de histamina dependente de IgE – HRF, identificada nas formas promastigotas do parasito. As proteínas recombinantes foram utilizadas tanto isoladas, quanto em associação, com o objetivo de testar o potencial como marcadoras para o sorodiagnóstico da LVC.

Estudos de epitopos de linfócitos T $CD4^+$ e $CD8^+$ foram realizados para as três proteínas acima citadas, com a finalidade de selecioná-las ou não para possível uso em vacinas profiláticas para as leishmanioses. Devido à presença de diversos epitopos para células T nestas proteínas, as capacidades imunogênicas foram analisadas, sendo que o objetivo principal deste estudo foi avaliar e comparar a proteção vacinal em camundongos BALB/c imunizados com rLiHyp1/saponina, rLiHyp6/saponina, rHRF/saponina e com um mix composto por todas estas proteínas (LiHyp1+LiHyp6+HRF+saponina) contra infecção por *L. infantum*.

PLOS ONE

RESEARCH ARTICLE

Antigenicity, Immunogenicity and Protective Efficacy of Three Proteins Expressed in the Promastigote and Amastigote Stages of *Leishmania infantum* against Visceral Leishmaniasis

Vivian Tamietti Martins[1], Miguel Angel Chávez-Fumagalli[2], Daniela Pagliara Lage[3], Mariana Costa Duarte[2,3], Esther Garde[4], Lourena Emanuele Costa[2], Viviane Gomes da Silva[1], Jamil Silvano Oliveira[1], Danielle Ferreira de Magalhães-Soares[5], Santuza Maria Ribeiro Teixeira[1], Ana Paula Fernandes[6], Manuel Soto[4], Carlos Alberto Pereira Tavares[1], Eduardo Antonio Ferraz Coelho[2,3]*

1 Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **2** Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **3** Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **4** Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Departamento de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain, **5** Departamento de Medicina Veterinária Preventiva, Escola de Veterinária, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, **6** Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

* eduardoferrazcoelho@yahoo.com.br

OPEN ACCESS

Citation: Martins VT, Chávez-Fumagalli MA, Lage DP, Duarte MC, Garde E, Costa LE, et al. (2015) Antigenicity, Immunogenicity and Protective Efficacy of Three Proteins Expressed in the Promastigote and Amastigote Stages of *Leishmania infantum* against Visceral Leishmaniasis. PLoS ONE 10(9): e0137683. doi:10.1371/journal.pone.0137683

Editor: Jude Marek Przyborski, Philipps-University Marburg, GERMANY

Received: April 10, 2015

Accepted: August 19, 2015

Published: September 14, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: This work was supported by grants from Pró-Reitoria de Pesquisa from UFMG (Edital 01/2014), Instituto Nacional de Ciência e Tecnologia em Nanobiofarmacêutica (INCT-Nanobiofar), FAPEMIG (CBB-APQ-00819-12), and CNPq (APQ-472090/2011-9, APQ-482976/2012-8, APQ-488237/2013-0). MACF is a grant recipient of FAPEMIG/CAPES. EAFC, APF and SMRT are grant recipient of CNPq. This study was also, in part, supported in Spain by

Abstract

In the present study, two *Leishmania infantum* hypothetical proteins present in the amastigote stage, LiHyp1 and LiHyp6, were combined with a promastigote protein, IgE-dependent histamine-releasing factor (HRF); to compose a polyproteins vaccine to be evaluated against *L. infantum* infection. Also, the antigenicity of the three proteins was analyzed, and their use for the serodiagnosis of canine visceral leishmaniasis (CVL) was evaluated. The LiHyp1, LiHyp6, and HRF DNA coding sequences were cloned in prokaryotic expression vectors and the recombinant proteins were purified. When employed in ELISA assays, all proteins were recognized by sera from visceral leishmaniasis (VL) dogs, and presented no cross-reactivity with either sera from dogs vaccinated with a Brazilian commercial vaccine, or sera of *Trypanosoma cruzi*-infected or *Ehrlichia canis*-infected animals. In addition, the antigens were not recognized by antibodies from non-infected animals living in endemic or non-endemic areas for leishmaniasis. The immunogenicity and protective efficacy of the three proteins administered in the presence of saponin, individually or in combination (composing a polyproteins vaccine), were evaluated in a VL murine model: BALB/c mice infected with *L. infantum*. Spleen cells from mice inoculated with the individual proteins or with the polyproteins vaccine plus saponin showed a protein-specific production of IFN-γ, IL-12, and GM-CSF after an *in vitro* stimulation, which was maintained after infection. These animals

grants from Ministerio de Ciencia e Innovación (FIS PI14/00366). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

presented significant reductions in the parasite burden in different evaluated organs, when compared to mice inoculated with saline or saponin. The decrease in parasite burden was associated with an IL-12-dependent production of IFN-γ against parasite total extracts (produced mainly by $CD4^+$ T cells), correlated to the induction of parasite proteins-driven NO production. Mice inoculated with the recombinant protein-based vaccines showed also high levels of parasite-specific IgG2a antibodies. The polyproteins vaccine administration induced a more pronounced Th1 response before and after challenge infection than individual vaccines, which was correlated to a higher control of parasite dissemination to internal organs.

Introduction

Visceral leishmaniasis (VL) represents an important disease in the world, leading to nearly 50,000 deaths annually [1]. The primary choice for the treatment of disease is based on the parenteral administration of pentavalent antimonials; however, parasites' increased resistance and side effects have been registered in the patients as important problems [2,3]. Other drugs, such as amphotericin B and its liposomal formulations, as well as paramomycin and miltefosine, have shown encouraging results; however, their use is commonly related to toxicity and/or high cost [4]. Therefore, the development of new strategies to prevent VL has become a priority [5].

Canine visceral leishmaniasis (CVL) caused by *Leishmania infantum* is a major global zoonosis. Upon infection, dogs can develop distinct clinical manifestations of the disease: asymptomatic, oligosymptomatic, or symptomatic stages [6,7,8]. Symptomatic CVL usually results in death, and the clinical manifestations are varied, ranging from cutaneous alterations to neurological disorders [7,9,10]. Infected dogs can also remain asymptomatic, and even be classified as false-negative in both clinical evaluations and serological trials performed [8]. This is an important problem, since infected dogs (even asymptomatic ones) are important domestic reservoirs of parasites, and can further contribute to transmission between sand flies and humans [11]. In this context, a precise and early diagnosis of CVL is of utmost importance [12].

As described in detail previously [13], in active VL, the cell-mediated immune response is absent and in the patients that are cured, the Th1 type response is increased, leading to long time immunity [14]. This provides a rationale that Th1 response play a major role in prevention and/or cure of VL. Therefore, proteins that stimulate the Th1 type arm of the immune response could be exploited as vaccine candidates against VL [15–21]. The induction of $CD4^+$ Th1 cells response for parasite antigens is crucial in controlling infection. Cytokines like IFN-γ are able to induce the production of nitric oxide and other compounds by infected phagocityc cells, thereby assisting to control of the parasites´ multiplication [21,22]. On the contrary, IL-4, IL-10, IL-13, and TGF-β represent important disease promoting cytokines, leading in turn to the suppression of the Th1 response and contributing to the disease [23,24]. Concomitantly to the role of $CD4^+$ T cells, the cytotoxic activity performed by $CD8^+$ T cells also contributes to protection against VL. These cells were linked to act against re-infection, but studies have also showed that $CD8^+$ T cells act also with an important role in controlling the primary infection, by increasing the Th1 response [20,25–27].

Protozoan parasites of the genus *Leishmania* have a dimorphic life cycle, consisting of extracellular promastigotes that multiply and develop within the alimentary tract of the sand fly vector, and intracellular amastigotes that multiply within the phagolysosomes of their host

macrophages [6,27–29]. The most studies on *Leishmania spp*. vaccines have focused on promastigote antigens. On the other hand, as described in detail previously [30], amastigote antigens have been far less tested as vaccine candidates against VL. However, the amastigotes seem to be the more appropriate target for the immune responses elicited by a vaccine, since after a few hours of initial infection and during the active disease, only this parasite stage is present in the host tissues. In addition, in contrast to promastigotes, the amastigote forms reside inside host cells and are targets for $CD8^+$ T cells, elements involved in the protective immunity against VL [31]. In this context, aiming to produce a vaccine composed by both amastigote and promastigote antigens; the present study evaluated three proteins: LiHyp1 (XP_001468941.1), LiHyp6 (XP_001467126.1) and the IgE-dependent histamine-releasing factor (HRF) (CAJ05086.1). The first two proteins were identified within the proteins extracted from amastigote-like cells by an immunoproteomic approach using sera from asymptomatic and symptomatic VL dogs [5]. The last one was characterized by the same procedure, but employing promastigote extracts [5].

The LiHyp1 protein (36.6 kDa) was recently shown to be protective against *L. infantum* [13]. In the present work, and in order to increase its prophylactic efficacy, this antigen was combined with other new characterized antigens for developing a new composition. The LiHyp6 gene is predicted to encode a protein with a theoretical molecular weight of 21.4 kDa, while the HRF gene is predicted to encode a protein with 19.4 kDa. Interestingly, mammals´ HRF protein, has been described as having a stimulatory activity on the immune system of the hosts by activating both T and B cells [32,33]. Therefore, the first purpose of this study is to analyze the antigenicity of the three recombinant proteins using canine patients. In addition, the immunogenicity and protective properties of the three proteins, administered individually or in combination, in the presence of an adjuvant able to induce cellular response; have been also studied.

Material and Methods

Ethics Statement

Experiments were performed in compliance with the National Guidelines of the Institutional Animal Care, and Committee on the Ethical Handling of Research Animals (CEUA) from the Federal University of Minas Gerais (UFMG) (Law number 11.794, 2008), with code number 043/2011. In addition, the owners of the domestic dogs (*Canis familiaris*) gave permission to collect blood samples from their animals.

Canine sera

Dog blood samples (10 mL) were collected by venipuncture of jugular vein in tubes without anticoagulant, and were kept at 37°C by 15 min, when they were centrifuged at 3,000 × *g* for 15 min, and sera samples were separated and kept at −80°C, until use. The sample size of domestic animals was 86 domestic animals (*Canis familiaris*) and consisted of males (n = 44) and females (n = 42) of different breeds and ages. CVL-positive animals were selected on the basis of two serological tests (IFAT [IFAT- LVC Bio-Manguinhos kit] and ELISA [EIE-LVC Bio-Manguinhos kit], both from Biomanguinhos, Fiocruz, Brazil), for *Leishmania spp*. Dogs with an IFAT titre < 1/40 or ELISA reactivity below the cut-off value indicated by the manufacturer were considered to be seronegative. Animals with an IFAT titre > 1/40 and an ELISA value over the cut-off were considered to be seropositive. Thus, symptomatic VL dogs (n = 15) were those positive by IFAT and ELISA, and when submitted to PCR, also presented positive results for *L. infantum* kDNA; and presenting three or more of the following clinical symptoms: weight loss, alopecia, adenopathy, onychogryposis, hepatomegaly, conjunctivitis; and

exfoliative dermatitis on the nose, tail, and ear tips. Asymptomatic VL dogs (n = 9) presented positive serological (IFAT and ELISA) and parasitological (PCR) results, but they do not present clinical signs or symptoms of leishmaniasis. Non-infected dogs were selected from an endemic (n = 15; Belo Horizonte, Minas Gerais, Brazil) or non-endemic (n = 15; Poços de Caldas, Minas Gerais, Brazil) area of leishmaniasis, but they presented negative serological (IFAT and ELISA) results, as well as present any clinical signs or symptoms of leishmaniasis. Non-infected animals immunized with the Leish-Tec® vaccine (n = 12), which were isolated in kennels to prevent their contact with transmitting vectors of leishmaniasis; as well as dogs experimentally infected with *Trypanosoma cruzi* (n = 12) or *Ehrlichia canis* (n = 8), were used in the ELISA assays.

Mice, parasite and antigen preparation

Female BALB/c mice (8 weeks age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, UFMG; and were maintained under specific pathogen-free conditions. Experiments were carried out using the *L. infantum* (MOM/BR/1970/BH46) strain. Parasites were grown at 24°C in Schneider's medium (Sigma, St. Louis, MO, USA), which was supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 100 U/mL penicillin, and 50 μg/mL streptomycin, at pH 7.4. The soluble *L. infantum* antigenic extract (SLA) was prepared from 10^9 stationary-phase promastigotes, like previously described [34]. Briefly, parasites were washed three times in 5 mL of cold sterile phosphate-buffered saline (PBS). After seven cycles of freezing (-196°C) and thawing (+37°C), the suspension was centrifuged at 8,000 x *g* for 20 min at 4°C; and supernatant containing SLA was collected in 500 μL aliquots, being stored at -80°C, until used. The protein concentration was estimated by the Bradford method [35].

Cloning, expression, and purification of recombinant proteins

LiHyp1 was cloned, expressed, and the recombinant protein was purified as previously described [13]. The primers used to amplify the LiHyp6 and HRF genes from *L. infantum* genomic DNA were: 5´-TTTGCTAGCATGAGCTTCTTTGACTTCTCA-3′ (*forward*) and 5´-TTTAAGCTTTCATTGCAGAACTTTGAGTACA-3´ (*reverse*) for LiHyp6, and 5´-GGATCCAT GAAGATCTTCAAGGATGTG-3´ (*forward*) and 5´-AAGCTTAGACGCGCTCGCCCTTCAG-3´ (*reverse*) for HRF proteins. Cut sites for *Bam*HI and *Hin*dIII (underlined) were included for cloning purposes in both cases. For PCR, genomic DNA from *L. infantum* was used. After amplification, DNA fragments were excised from gels, purified, and linked into pGEM-T easy vector systems (Promega, USA). Recombinant plasmids were used to transform *Escherichia coli* XL1-Blue (Phoneutria, Brazil) competent cells. Positive clones were tested by restriction analysis with *Eco*RI, and those presenting LiHyp6 or HRF genes were propagated, double-stranded sequenced, and used in the construction of the expression vector. DNA fragments obtained from *Nhe*I/*Eco*RI and *Bam*HI/*Hin*dIII digestion of pGEM-LiHyp6 or pGEM-HRF plasmids were ligated into the corresponding cut sites of the pET-28a-c and pQE30 plasmids, respectively (Qiagen, Hilden, Germany), and transformed into BL21AI and M15 *E. coli* strains for overexpression induced by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Promega, Montreal, Canada). After induction, cells were ruptured by five cycles of ultrasound (15 sec each, 90 MHz) in a binding buffer (0.02 M phosphate buffer, pH 8.0, 0.5 M NaCl, 0.005 M imidazole, 8 M urea, and 0.001 M β-mercaptoethanol). The presence of the recombinant proteins in the supernatant extracts after centrifugation (13,000 × *g*, 20 min at 4°C) was analyzed by one dimensional SDS-PAGE. Both proteins proved to be soluble and had been purified under native conditions, following manufacturer's instructions (Qiagen).

Briefly, rLiHyp6 and rHFR proteins were transferred by gravity flow onto Ni-nitrilotriacetic-acid-agarose (Ni-NTA) columns. The recombinant proteins were successively washed (1 column vol.) in binding buffer and in wash buffer (0.02 M phosphate buffer pH 8.0, 0.5 M NaCl, 0.005 M imidazole, and 0.001 M β-mercaptoethanol), and eluted in elution buffer (0.02 M phosphate buffer pH 8.0, 0.5 M NaCl, 0.5 M imidazole, and 0.001 M β-mercaptoethanol). After elution, proteins were dialyzed against 1x phosphate saline buffer (PBS). The rLiHyp6 and rHRF proteins were concentrated in eppendorf Vacufuge vacuum concentrate, and further purified on a Superdex^{TM} 200 gel-filtration column (GE Healthcare Life Sciences, USA). After purification, the recombinant proteins were passed through a polymyxin-agarose column (Sigma) to remove any residual endotoxins content. The purity of the recombinant proteins was checked by an one dimensional SDS-PAGE.

Validation of the purified proteins by Western-Blot assays

To verify the antigenicity of the purified proteins in the CVL; the individual rLiHyp1, rLiHyp6, and rHRF proteins (10 μg each) were submitted to a 12% SDS-PAGE and blotted onto a nitrocellulose membrane (0.2 μm pore size, Sigma, St. Louis, USA), when they were evaluated in reaction to sera samples of VL dogs, or sera from non-infected animals (negative control). The technical protocol was performed as previously described [13].

ELISA experiments for the serodiagnosis of CVL

Previous titration curves were performed to determine the most appropriate antigen concentration and antibody dilution to be used. For the serodiagnosis of CVL, the rLiHyp1, rLiHyp6, rHRF, and SLA-specific IgG antibodies levels were evaluated using a canine serological panel. For this, microtiter immunoassay plates (Falcon) were coated with the individual recombinant proteins (1.5, 1.5, or 0.5 μg per well, for rLiHyp1, rLiHyp6, and rHRF, respectively), with their mixture (0.5 μg per well, of each protein), or SLA *L. infantum* (1.0 μg per well); all diluted in 100 μL of coating buffer (50 mM carbonate buffer pH 9.6), for 18 h at 4°C. After washing the plates three times with PBS-T (PBS plus Tween 20 0.05%), their free binding sites were blocked using 200 μL of PBS-T containing 2% casein, for 1 h at 37°C. After washed three times with PBS-T, plates were incubated with 100 μL of individual canine sera (1:200, diluted in PBS-T), for 1 h at 37°C. Plates were subsequently washed four times in PBS-T, and incubated with anti-dog IgG horseradish-peroxidase conjugated antibody (1:10,000, diluted in PBS-T; catalog A6792, Sigma Aldrich, USA), for 1 h at 37°C. After washing the plates four times with PBS-T, the reaction was developed by incubation with 100 μL per well of a solution composed by 2 μL H_2O_2, 2 mg orto-phenylenediamine, and 10 mL citrate-phosphate buffer at pH 5.0 for 30 min in the dark. The reaction was stopped by adding 25 μL 2 N H_2SO_4, and the optical density was read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nm.

Vaccination regimens, challenge infection and determination of parasite burden

Mice (n = 8, per group) were vaccinated subcutaneously in their left hind footpad with 25 μg of each recombinant protein (rLiHyp1, rLiHyp6, or rHRF), or with their mixture (using 25 μg of each protein), all associated with 25 μg of saponin (*Quillaja saponaria* bark saponin, Sigma). Additional mice were immunized with saponin or received saline. Three doses of the vaccines were administered at two-week intervals into the animals. Four weeks after the third and last immunization, mice (n = 4 per group) were euthanized and sera samples and spleen were collected to analyze the immune response induced by vaccination. At the same time, the

remaining animals of each group were infected subcutaneously in the right hind footpad with 10^7 stationary-phase promastigotes of *L. infantum*. Ten weeks after infection, animals were euthanized and sera samples, spleen, liver, bone marrow (BM), and draining lymph nodes (dLN) were collected. Spleen and blood were used to evaluate the generated immune response. For the evaluation of the parasite burden, spleen, liver, BM and dLN were processed and used in a limiting-dilution technique, as previously described [36]. In this case, triplicates instead of duplicates were used in the parasitological analysis.

Cytokine production before and after *L. infantum* infection

Splenocyte cultures were performed four weeks after the third and last immunization and before infection, as well as in the 10^{th} week after challenge, as described [34]. Briefly, single-cell suspensions from spleen tissue were plated in duplicate in 24-well plates (Nunc), at 10^6 cells per mL. Cells were incubated in RPMI 1640 medium (negative control), which was supplemented with 10% FBS, 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4; or separately stimulated with rLiHyp1, rLiHyp6 or rHRF proteins (20 μg mL^{-1}, each); with their mixture (10 μg mL^{-1} of each protein), or with SLA *L. infantum* (25 μg mL^{-1}), for 48 h at 37°C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12, and GM-CSF levels were assessed in the supernatants by a sandwich ELISA provided in commercial kits (BD OptEIA TM set mouse IFN-γ, IL-4, IL-10, IL-12, and GM-CSF; all obtained from Pharmingen, San Diego, CA, USA); following manufacturer's instructions. To evaluate the involvement of IL-12, and $CD4^+$ and $CD8^+$ T cells, spleen cells of mice vaccinated with rLiHyp1/saponin, rLiHyp6/saponin, rHRF/saponin or polyproteins/saponin and infected were *in vitro* stimulated with SLA *L. infantum* (25 μg mL^{-1}), and incubated in the presence of 5 μg mL^{-1} of monoclonal antibodies (mAb) against mouse IL-12 (C17.8), CD4 (GK 1.5), or CD8 (53–6.7). Appropriate isotype-matched controls—rat IgG2a (R35-95) and rat IgG2b (95–1)–were employed in the assays. Antibodies (no azide/low endotoxin^{TM}) were purchased from BD (Pharmingen).

Humoral response and nitrite production

For the analysis of the humoral response induced after vaccination, the rLiHyp1, rLiHyp6, rHRF, and SLA-specific IgG1 and IgG2a isotypes levels were evaluated using the sera samples collected from the vaccinated animals, four weeks after the third and last immunization and before infection; as well as in the 10^{th} week after challenge, by an ELISA technique, as previously described [13]. The used concentration of the recombinant proteins were: 1.0, 1.0, and 0.5 μg per well for rLiHyp1, rLiHyp6, and rHRF proteins, respectively; 0.5 μg per well of each protein to their mixture, and 2.0 μg per well of SLA *L. infantum*. The sera samples were diluted at 1:200, and the anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich, USA) were employed both in a 1:5,000 dilution. The nitrite production in the cultures supernatant was assessed by the Griess reaction [37]. Data were expressed as μM per 10^6 cells.

Statistical analysis

The results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed using GraphPad Prism^{TM} (version 6.0 for Windows). The mean optical density (OD) value was calculated by subtracting the mean blank OD from OD mean for each sample by using specific values obtained in the ELISA assays. The lower limit of positivity (cut-off) for SLA *L. infantum*, rLiHyp1, rLiHyp6, rHRF, and their mixture was established for optimal sensitivity and specificity using the Receiver Operator Curve (ROC curve). The accuracy was evaluated according to the area under the curve (AUC) relative to the ROC curve, 95% confidence interval (95%

CI). The ROC curves were plotted with the values from serum samples from dogs presenting symptomatic and asymptomatic CVL as compared to those from the control groups (*T. cruzi*-infected, *E. canis*-infected, Leish-Tec-immunized, and non-infected from endemic and non-endemic area dogs), according to a sick/non-sick rating method, in which one inclusion criterion in each group was the positivity or negativity of PCR for *L. infantum* kDNA in blood samples. Statistical analysis of the data from vaccinated and/or infected mice was performed by one-way analysis of variance (ANOVA), using Tukey's post-test for multiple comparisons between the groups. The mean ± standard deviation (SD) of each experimental group is shown. Differences were considered significant when $P < 0.05$. The vaccination experiments were repeated once, and presented similar results. Data showed in this study are representative of one of them.

Results

Serodiagnosis of CVL using the recombinant proteins

Initially, to analyze the antigenicity of the recombinant proteins, a Western-blot assay was performed. As it is shown (Fig 1), the rLiHyp1, rLiHyp6, and rHRF proteins were recognized by the sera from CVL-infected animals (in the right, in Fig 1). However, when sera from non-infected dogs were used, no significant reactivity was observed with the evaluated three recombinant proteins (in the left, in Fig 1). In this context, antigens were evaluated individually or in combination in ELISA experiments to determine their sensitivity and specificity for the serodiagnosis of CVL (Fig 2). The SLA *L. infantum* was used as a control and a comparative antigen. In the results, all CVL sera recognized the SLA antigen, but a significant cross-reactivity was observed when sera samples from *T. cruzi*-infected animals were evaluated (Fig 2A). On the contrary, the rLiHyp1 (Fig 2B), rLiHyp6 (Fig 2C), and rHRF (Fig 2D) proteins were recognized by all asymptomatic and symptomatic CVL sera, but no cross-reactivity with sera from *T. cruzi*-infected animals was observed. When the three proteins were used as a mixture, it was able to better distinguish between the CVL sera from the other sera groups (Fig 2E). To determine the diagnostic performance of the rLiHyp1, rLiHyp6 and rHRF proteins, as well as their mixture; ROC curves were constructed. The proteins presented sensitivity and specificity values of 100.0% and 98.4%, respectively; whereas using SLA *L. infantum*, these values were of 83.3% and 98.47%, respectively. It was concluded that the mixture of the three proteins presented a better performance than SLA for the serodiagnosis of CVL (Table 1).

Immunogenicity of the recombinant proteins in BALB/c mice

The immunogenicity of rLiHyp1, rLiHyp6, and rHRF proteins administered with saponin individually or in a mixed formulation was evaluated in BALB/c mice (Fig 3). Cellular responses against the antigens were analyzed four weeks after receiving three vaccine doses, and just before infection. Following *in vitro* stimulation with recombinant proteins used in the immunizations, spleen cells from vaccinated mice with rLiHyp1, rLiHyp6, or rHRF plus saponin produced significantly higher levels of IFN-γ, IL-12, and GM-CSF than those secreted by spleen cells of the animals that received saline or saponin (stimulated with the protein mixture). Comparatively, the groups receiving the three mixed proteins presented higher levels of these cytokines when compared to the animals immunized with the individual proteins (Fig 3A). Also, a low production of IL-4 and IL-10 was observed in all experimental groups. The ratios between IFN-γ/IL-4 and IFN-γ/IL-10 levels (Fig 3B), as well as between IL-12/IL-4 and IL-12/IL-10 levels (Fig 3C) were calculated. The obtained results were showing that mice vaccinated with the recombinant proteins plus saponin mounted a protein-specific Th1-like response. Data of immunogenicity before challenge infection that were obtained in the 2nd experiment are also

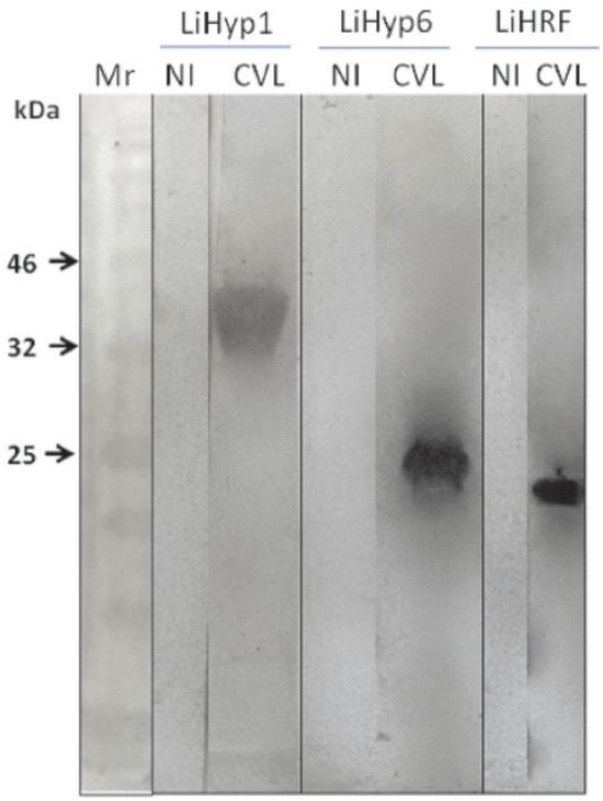


Fig 1. Immunoblotting experiments using the recombinant proteins and a canine serological panel. For immunoblotting experiments, the rLiHyp1 (36.6 kDa), rLiHyp6 (21.4 kDa), and rHRF (19.4 kDa) proteins (10 μg, each) were submitted to a 12% SDS-PAGE and blotted onto a nitrocellulose membrane, which were blocked with a PBS-T containing 5% BSA solution, and incubated with a pool of sera of asymptomatic and symptomatic VL dogs, or with a pool containing sera of non-infected dogs (1:200 and 1:100 diluted in PBS-T, respectively). Peroxidase conjugated anti-dog IgG (1:10,000) was used as a second antibody. The reactivities against the rLiHyp1, rLiHyp6 and rHRF proteins are shown. A low range protein ladder (Invitrogen™, Life Technologies, USA) was used (Mr). The individual reactions of the rLiHyp1, rLiHyp6, and rHRF proteins with the pools of sera from non-infected or *L. infantum*-infected dogs (NI and CVL, respectively) are shown. Immunoblottings were derived from three independent experiments, and one representative preparation is showed in this study.

doi:10.1371/journal.pone.0137683.g001

shown (Figure A in S1 File). In addition, the humoral response was also analyzed (Fig 4). Using SLA *L. infantum* as an antigen, very low responses were observed (Fig 4A), although mice vaccinated with rLiHyp1/saponin, rLiHyp6/saponin, or polyproteins/saponin had produced higher IgG2a levels than the rHRF/saponin, saponin and saline groups. When the recombinant proteins were used as antigens, all rLiHyp1/saponin, rLiHyp6/saponin, rHRF/saponin and polyproteins/saponin groups presented higher levels of protein-specific IgG2a isotype, in relation to the obtained IgG1 levels (Fig 4B). Using the OD values of each serum samples in all experimental groups, the ratio between the IgG2a and IgG1 levels was calculated, and either SLA (Fig 4C) or the recombinant proteins (Fig 4D) as antigen, a higher IgG2a production was encountered in relation to the IgG1 levels in the animals immunized with the recombinant

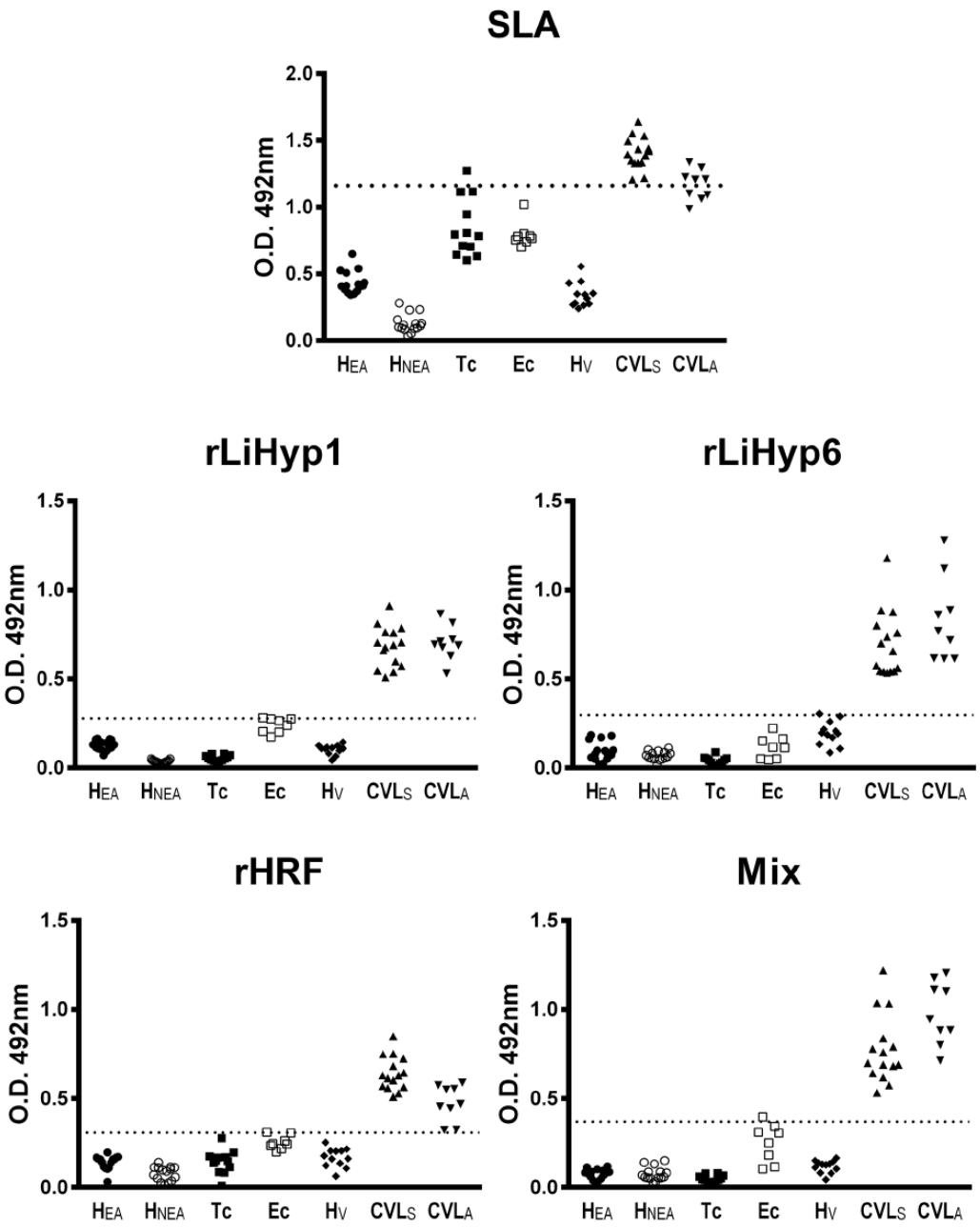


Fig 2. Evaluation of the ELISA reactivity using the recombinant proteins against different sera samples. ELISA experiments were performed using sera samples from *T. cruzi*-infected (n = 19), asymptomatic (n = 20) and symptomatic (n = 25) *L. infantum*-infected, and non-infected (n = 40) dogs. Reactions against SLA *L. infantum* (1.0 μg per well) (**A**), rLiHyp1 (1.5 μg per well) (**B**), rLiHyp6 (1.5 μg per well) (**C**), rHRF (0.5 μg per well) (**D**) and their mixture (0.5 μg per well of each protein) (**E**), were performed. The mean optical density (OD) value was calculated by subtracting the mean blank O.D. from O.D. mean for each sample, by using specific values obtained in the ELISA assays. The lower limit of positivity (cut-off) for rLiHyp1, rLiHyp6, rHRF, their mixture, and SLA *L.*

infantum was established for optimal sensitivity and specificity using the ROC curves. Abbreviations: H_{EA}: healthy from endemic area, H_{NEA}: healthy from non-endemic area, Tc: *Trypanosoma cruzi*, Ec: *Ehrlichia canis*, H_V: healthy and vaccinated, CVL_S: symptomatic canine visceral leishmaniasis, CVL_A: asymptomatic canine visceral leishmaniasis.

doi:10.1371/journal.pone.0137683.g002

proteins isolated or in combination, added with saponin. In this context, it could be speculated that the immunization with the recombinant proteins was able to induce a higher *in vitro* IFN-γ, IL-12 and GM-CSF production, as well as low levels of IL-4, IL-10 and parasite-specific IgG1 isotype antibodies in the vaccinated animals.

Protective efficacy against challenge infection

This study analyzed whether or not immunization with the recombinant proteins, administered isolately or in combination, all associated with saponin, could protect against *L. infantum*. The infection was followed up over a 10-week period, after which parasite burden in the spleen, liver, dLN, and BM was evaluated. In the results, significant reductions in the parasite load were observed in all evaluated organs of the vaccinated mice, when compared to those that received saline or saponin (Fig 5). In comparison to the saline group, mice vaccinated with rLiHyp1/saponin, rLiHyp6/saponin, rHRF/saponin, or with polyproteins/saponin presented reductions in the parasite load in the spleen (1.9-, 2.2-, 1.6-, and 5.2-log reductions; Fig 5A), liver (1.6-, 1.8-, 1.5-, and 4.0-log reductions; Fig 5B), dLN (2.0-, 2.1-, 1.7- and 4.5-log reductions; Fig 5C), and BM (1.7-, 1.9-, 1.6-, and 5.7-log reductions; Fig 5D), respectively. Comparing the results obtained in relation to the saponin group, it was possible to observe that mice vaccinated with rLiHyp1/saponin, rLiHyp6/saponin, rHRF/saponin, or with polyproteins/saponin presented reductions in the parasite load in the spleen (1.7-, 2.0-, 1.5-, and 4.8-log reductions; Fig 5A), liver (1.5-, 1.7-, 1.4-, and 3.8-log reductions; Fig 5B), dLN (1.8-, 1.9-, 1.6- and 4.2-log reductions; Fig 5C), and BM (1.3-, 1.4-, 1.2-, and 4.3-log reductions; Fig 5D), respectively. Evaluating comparatively the protective efficacy between the individual protein-based vaccines and polyproteins vaccine, it can be speculate that the polyproteins/saponin vaccine was able to induce a better degree of protection, due to the more significant reductions of parasite burden that were encountered in the evaluated organs in the animals of this group. Data of parasite burden obtained in the 2nd experiment are also shown (Figure B in S1 File).

Cellular response elicited after *Leishmania infantum* infection

The production of cytokines in the supernatants of spleen cell cultures stimulated with SLA or recombinant proteins was also evaluated, 10 weeks after infection (Fig 6). In the results, spleen cells from mice vaccinated with rLiHyp1/saponin, rLiHyp6/saponin, rHRF/saponin or

Table 1. Diagnostic performance of recombinant proteins and their mixture using a serological panel. Samples from asymptomatic (n = 9) or symptomatic (n = 15) *L. infantum*-infected dogs, sera from animals with no clinical signs of VL, and negative parasitological and serological results to *Leishmania spp*. antigens, and living in an endemic (n = 15) or non-endemic (n = 15) area of leishmaniasis; as well as sera from animals experimentally infected by *Trypanosoma cruzi* (n = 12) or *Ehrlichia canis* (n = 8), and sera from dogs vaccinated with Leish-Tec (n = 12); were tested in the ELISA assays. ROC curves were used to determine sensitivity, specificity and AUC (area under the curve). 95%CI: Confiance Interval. LR: Likehood Ratio. SLA: Soluble *Leishmania infantum* antigenic extract.

Antigen	AUC	95%CI	Se	95%CI	Sp	95%CI	LR
SLA	0.99	0.9–1.0	83.3	62.6–95.3	98.4	91.3–100.0	51.7
rLiHyp1	1.0	1.0–1.0	100.0	85.8–100.0	98.4	91.3–100.0	62.0
rLiHyp6	1.0	1.0–1.0	100.0	85.8–100.0	98.4	91.3–100.0	62.0
rHRF	1.0	1.0–1.0	100.0	85.8–100.0	98.4	91.3–100.0	62.0
Mix	1.0	1.0–1.0	100.0	85.8–100.0	98.4	91.3–100.0	62.0

doi:10.1371/journal.pone.0137683.t001

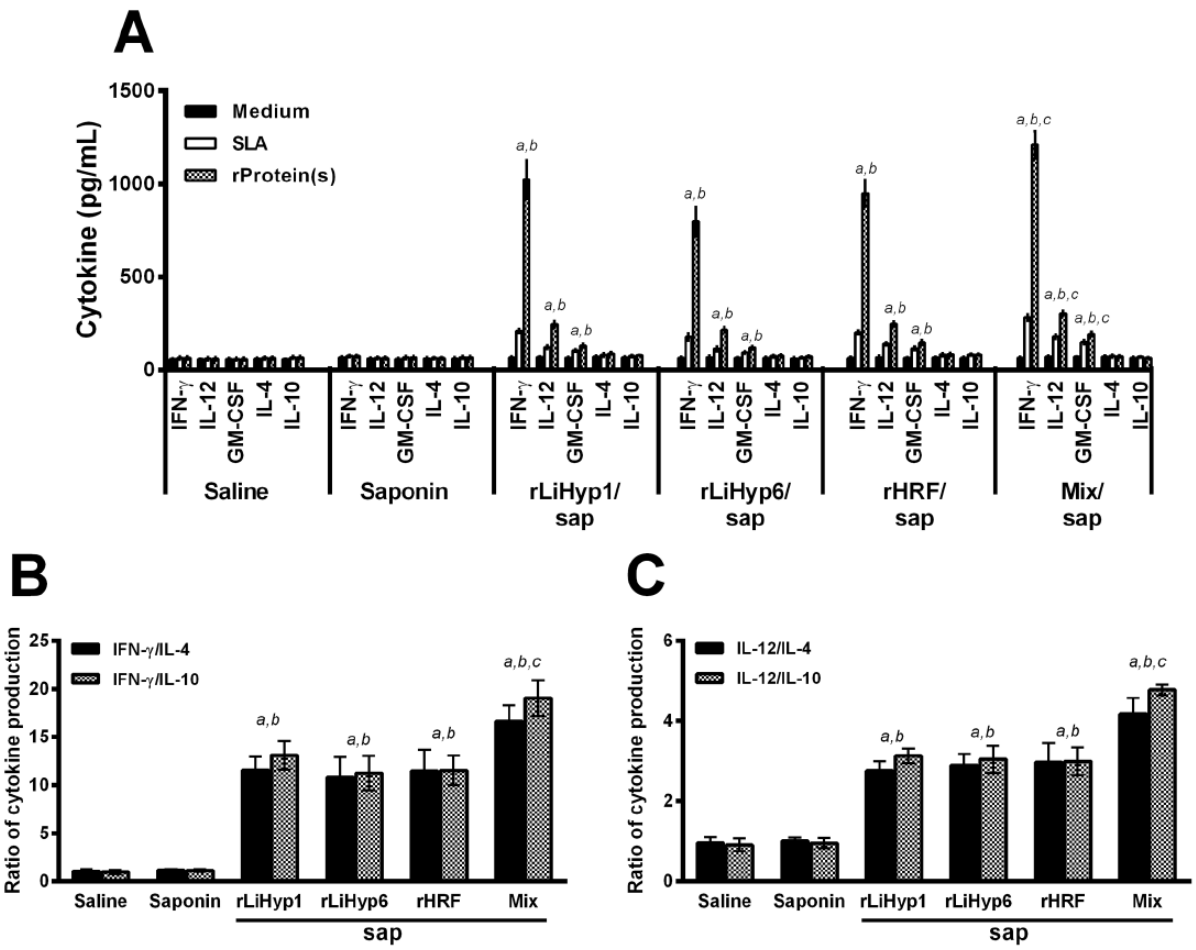


Fig 3. Cellular response induced in BALB/c mice by immunization using the recombinant proteins plus saponin. Mice (n = 8, per group) were vaccinated subcutaneously in their left hind footpad with 25 μg of each recombinant protein (rLiHyp1, rLiHyp6, or rHRF), or with their mixture (using 25 μg of each protein), all associated with 25 μg of saponin (*Quillaja saponaria* bark saponin, Sigma). Additional mice were immunized with saponin or received saline. Three doses were administered, at two-week intervals. Four weeks after the last immunization, mice (n = 4 per group) were euthanized and spleens were collected to evaluate the cellular response induced by vaccination. For this, single cell suspensions were incubated in RPMI 1640 medium (negative control), which was supplemented with 10% FBS, 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4; or separately stimulated with rLiHyp1 (rLiHyp1/saponin group), rLiHyp6 (rLiHyp6/saponin group), rHRF (rHRF/saponin group) proteins (20 μg mL^{-1}, each one); with their mixture (saline, saponin, and polyproteins/saponin groups; using 10 μg mL^{-1} of each protein), or SLA *L. infantum* (25 μg mL^{-1}), for 48 h at 37°C in 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4, and IL-10 levels were measured by ELISA in the culture supernatants (**A**). Bars represent the mean ± standard deviation (SD) of the groups. In addition, the ratios between IFN-γ/IL-4 and IFN-γ/IL-10 levels (**B**), and between IL-12/IL-4 and IL-12/IL-10 levels (**C**) were calculated and are shown. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0.001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0.001$). (*c*) indicates statistically significant difference in relation to the rLiHyp1/saponin, rLiHyp6/saponin, and rHRF/saponin groups ($P < 0.001$).

doi:10.1371/journal.pone.0137683.g003

polyproteins/saponin produced high levels of protein- or parasite-specific IFN-γ, IL-12, and GM-CSF, in comparison to those secreted by spleen cells of animals that received saline or saponin (Fig 6A). In contrast, the protein- or parasite-driven production of IL-4 and IL-10 showed that vaccination induced no significant production of these cytokines, whereas mice from the saline and saponin groups showed a high IL-4 and IL-10 production, using both

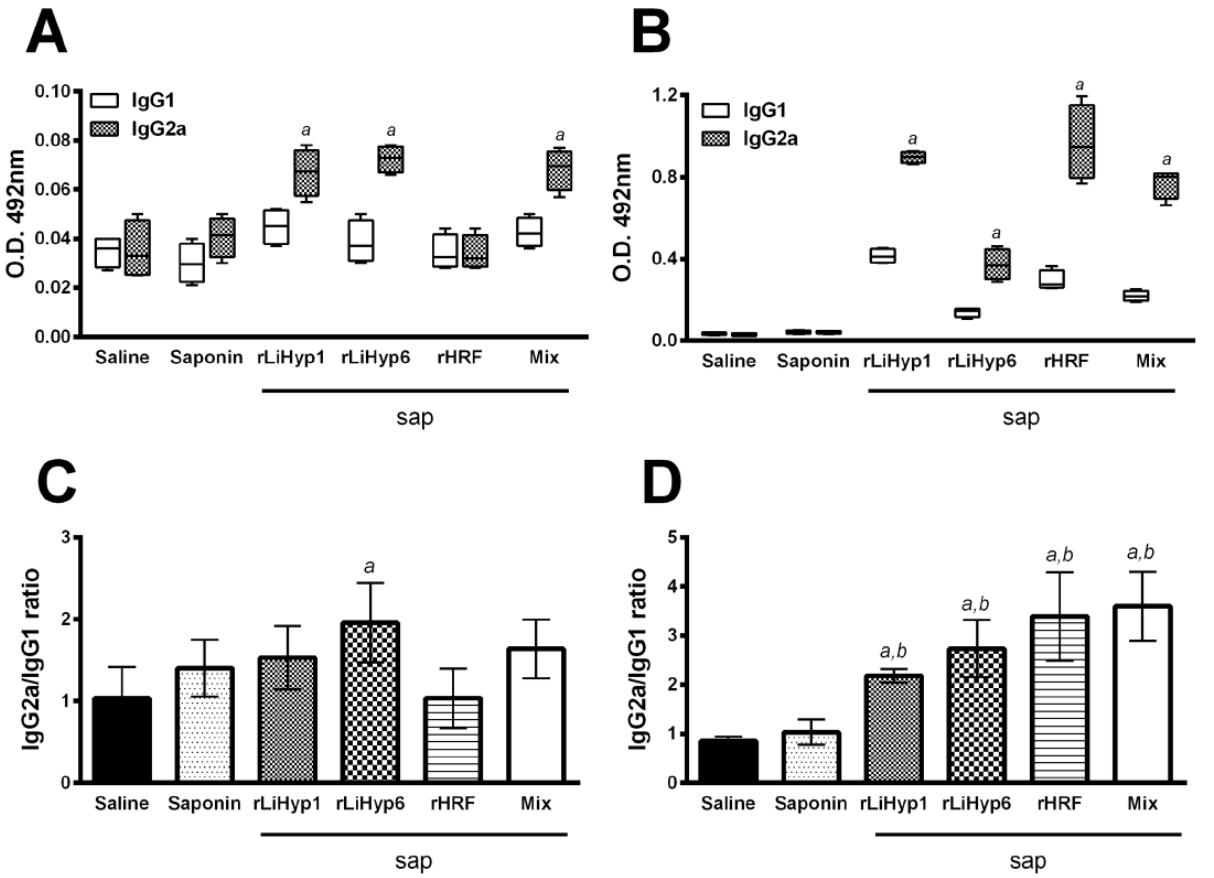


Fig 4. Humoral response generated in BALB/c mice by immunization with the recombinant proteins plus saponin. Mice (n = 8, per group) were vaccinated subcutaneously in their left hind footpad with 25 μg of each recombinant protein (rLiHyp1, rLiHyp6, or rHRF), or with their mixture (using 25 μg of each protein), all associated with 25 μg of saponin (*Quillaja saponaria* bark saponin, Sigma). Additional mice were immunized with saponin or received saline. Three doses were administered, at two-week intervals. Four weeks after the last immunization, mice (n = 4 per group) were euthanized and sera samples were collected to evaluate the humoral response induced by vaccination. The reactivity based on IgG1 and IgG2a isotypes represented by whisker (min to max) plots against SLA (**A**) and the respective recombinant proteins (**B**) are shown. In each group vaccinated with the recombinant protein, the respective antigen was used. In the saline and saponin groups, as well as in the polyproteins/saponin group, the protein mixture was used as antigen. Also, SLA was used as antigen in all groups. For the assays, 1.0, 1.0, and 0.5 μg per well of rLiHyp1, rLiHyp6, and rHRF proteins, respectively; 0.5 μg per well of each protein composing the antigenic mixture, and 2.0 μg per well of SLA *L. infantum*, were used in the ELISA assays. The sera samples were diluted at 1:200, and the anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies were employed in a 1:5,000 and 1:10,000 dilution, respectively. The ratios between the IgG2a/IgG1 levels against SLA (**C**) and the respective recombinant proteins (**D**) were calculated, and are also shown. Bars represent the mean ± standard deviation (SD) of the groups. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0.001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0.001$).

doi:10.1371/journal.pone.0137683.g004

recombinant proteins and SLA in the stimulation of their spleen cells. As observed before infection, the ratios between IFN-γ/IL-4 and IFN-γ/IL-10 (Fig 6B), as well as between IL-12/IL-4 and IL-12/IL-10 (Fig 6C), using SLA to stimulate the cultures were calculated, and results showed that mice vaccinated with rLiHyp1/saponin, rLiHyp6/saponin, rHRF/saponin or polyproteins/saponin developed a parasite-specific Th1 response, after challenge infection. The

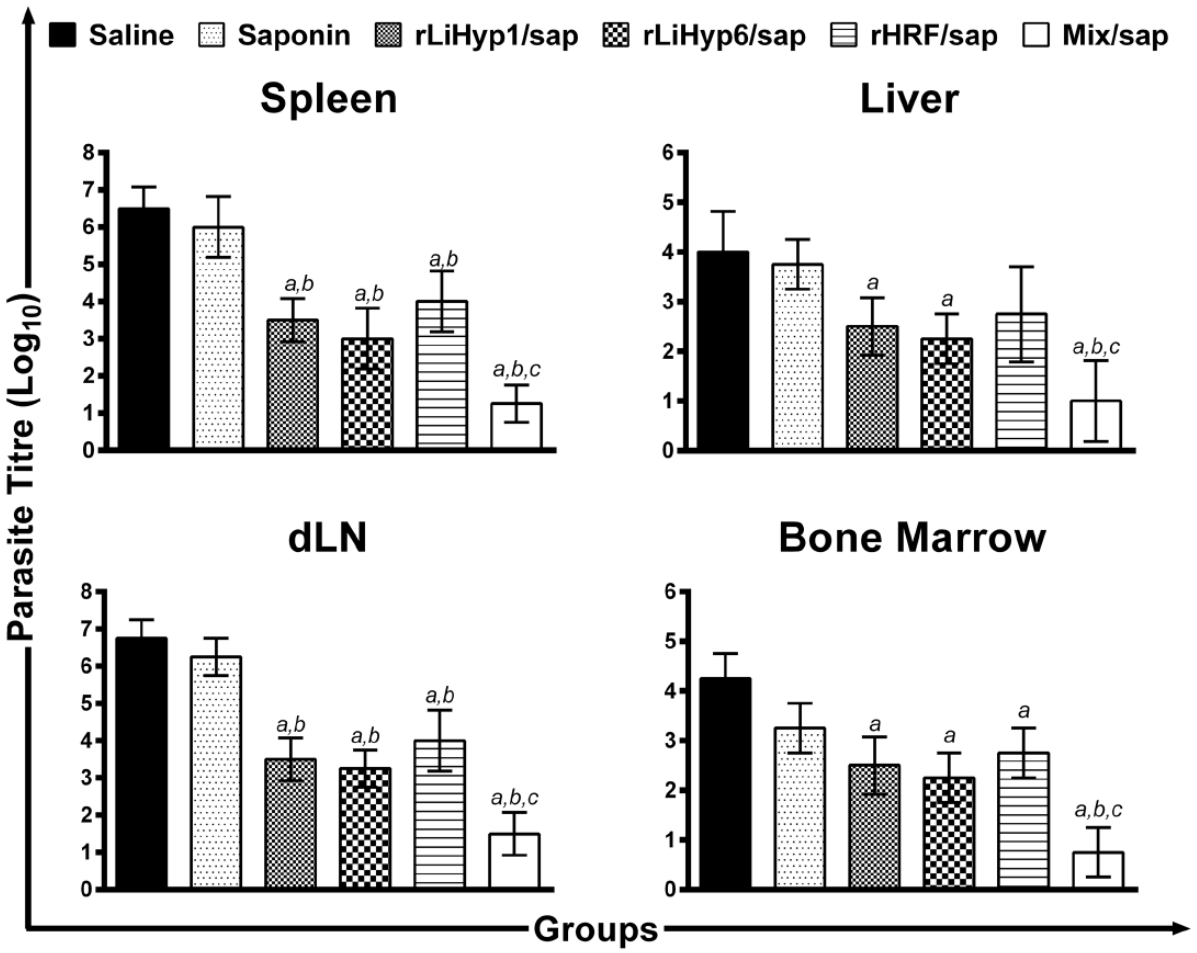


Fig 5. Protection of BALB/c mice against *Leishmania infantum* infection. Mice (n = 8, per group) were vaccinated subcutaneously in their left hind footpad with 25 μg of each recombinant protein (rLiHyp1, rLiHyp6, or rHRF), or with their mixture (using 25 μg of each protein), all associated with 25 μg of saponin (*Quillaja saponaria* bark saponin, Sigma). Additional mice were immunized with saponin or received saline. Three doses were administered, at two-week intervals. Four weeks after the last immunization, mice (n = 4 per group) were subcutaneously infected in the right hind footpad with 1 x 10^7 stationary-phase promastigotes of *L. infantum*. Ten weeks after challenge, the parasite burden in the spleen (**A**), liver (**B**), infected paws´draining lymph nodes (**C**), and bone marrow (**D**) were measured by a limiting-dilution technique. Bars represent the mean ± standard deviation (SD) of the groups. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0.001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0.001$). (*c*) indicates statistically significant difference in relation to the rLiHyp1/saponin, rLiHyp6/saponin, and rHRF/saponin groups ($P < 0.001$). Data shown in this study are representative of two independent experiments, which presented similar results.

doi:10.1371/journal.pone.0137683.g005

polyproteins/saponin group was able to mount a more pronounced Th1 response, which was corroborated with the results obtained before challenge; indicating that this stronger immune response could contribute to the higher reduction of the parasite burden found in the vaccinated and infected animals of this group.

The contribution of $CD4^+$ and $CD8^+$ T cells for the parasite-specific IFN-γ production from the spleen cells of immunized and infected mice was evaluated, 10 weeks after challenge (Fig 7). In the results, animals vaccinated with rLiHyp1/saponin (Fig 7A), rLiHyp6/saponin (Fig

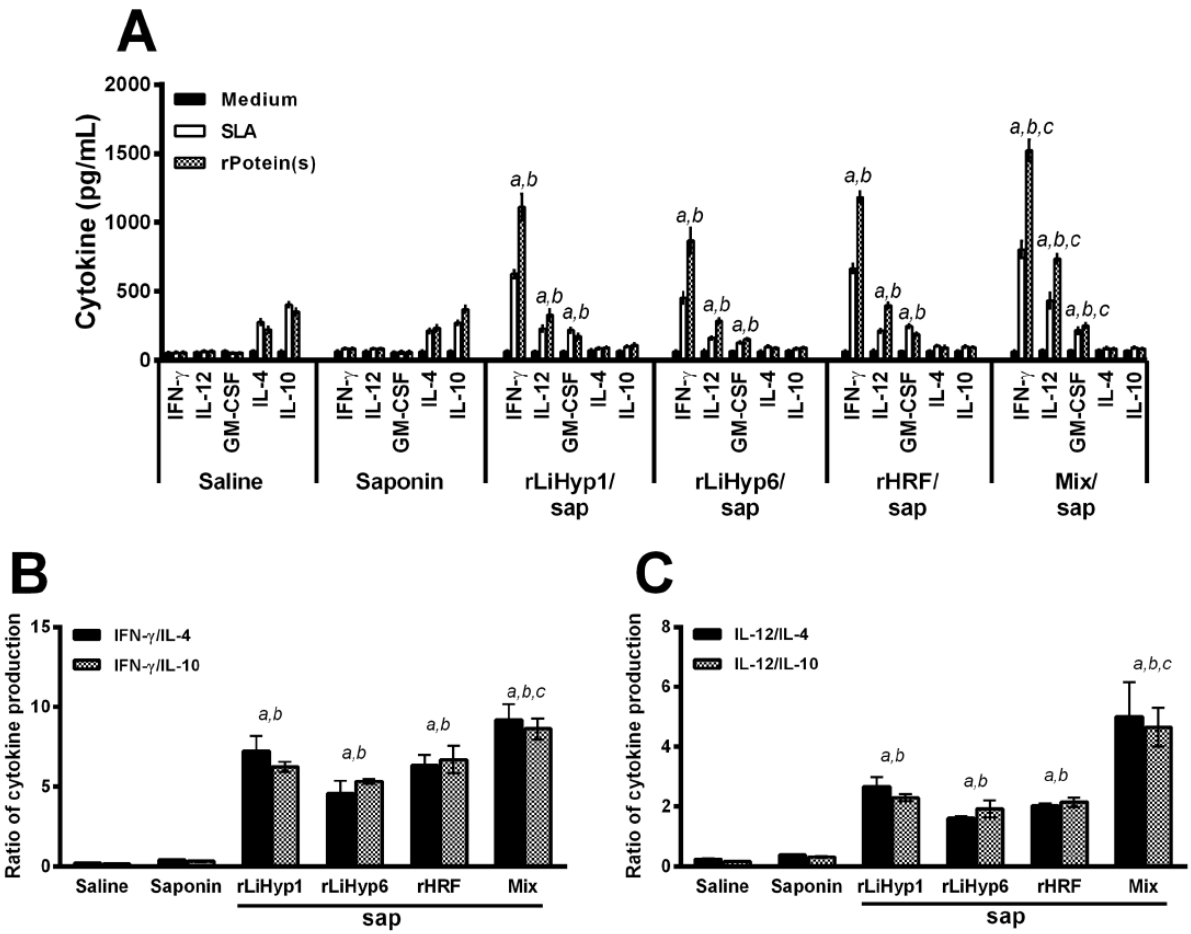


Fig 6. Analysis of the cellular response induced in the vaccinated animals, 10 weeks after *Leishmania infantum* infection. Mice (n = 8, per group) were vaccinated subcutaneously in their left hind footpad with 25 μg of each recombinant protein (rLiHyp1, rLiHyp6, or rHRF), or with their mixture (using 25 μg of each protein), all associated with 25 μg of saponin (*Quillaja saponaria* bark saponin, Sigma). Additional mice were immunized with saponin or received saline. Three doses were administered, at two-week intervals. Four weeks after the last immunization, mice (n = 4 per group) were subcutaneously infected using stationary-phase promastigotes of *L. infantum*, and 10 weeks after challenge, single cell suspensions were obtained from the spleens of the animals. Cells were incubated in RPMI 1640 medium (negative control), which was supplemented with 10% FBS, 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4; or separately stimulated with rLiHyp1 (rLiHyp1/saponin group), rLiHyp6 (rLiHyp6/saponin group), rHRF (rHRF/saponin group) proteins (20 μg mL^{-1}, each one); with their mixture (saline, saponin, and polyproteins/saponin groups; using 10 μg mL^{-1} of each protein), or SLA *L. infantum* (25 μg mL^{-1}), for 48 h at 37°C in 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4, and IL-10 levels were measured by ELISA in the culture supernatants (**A**). Bars represent the mean ± standard deviation (SD) of the groups. In addition, the ratios between IFN-γ/IL-4 and IFN-γ/IL-10 levels (**B**), as well as between IL-12/IL-4 and IL-12/IL-10 levels (**C**) were calculated, and are shown. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0.001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0.001$). (*c*) indicates statistically significant difference in relation to the rLiHyp1/saponin, rLiHyp6/saponin, and rHRF/saponin groups ($P < 0.001$).

doi:10.1371/journal.pone.0137683.g006

7B), rHRF/saponin (Fig 7C), or with polyproteins/saponin (Fig 7D) presented a significant reduction in the IFN-γ production, when anti-IL-12, anti-CD4 or anti-CD8 monoclonal antibodies were used in the cells cultures. The addition of anti-CD8 antibodies induced a lower IFN-γ production in the rLiHyp1/saponin and rLiHyp6/saponin groups (Fig 7A and 7B,

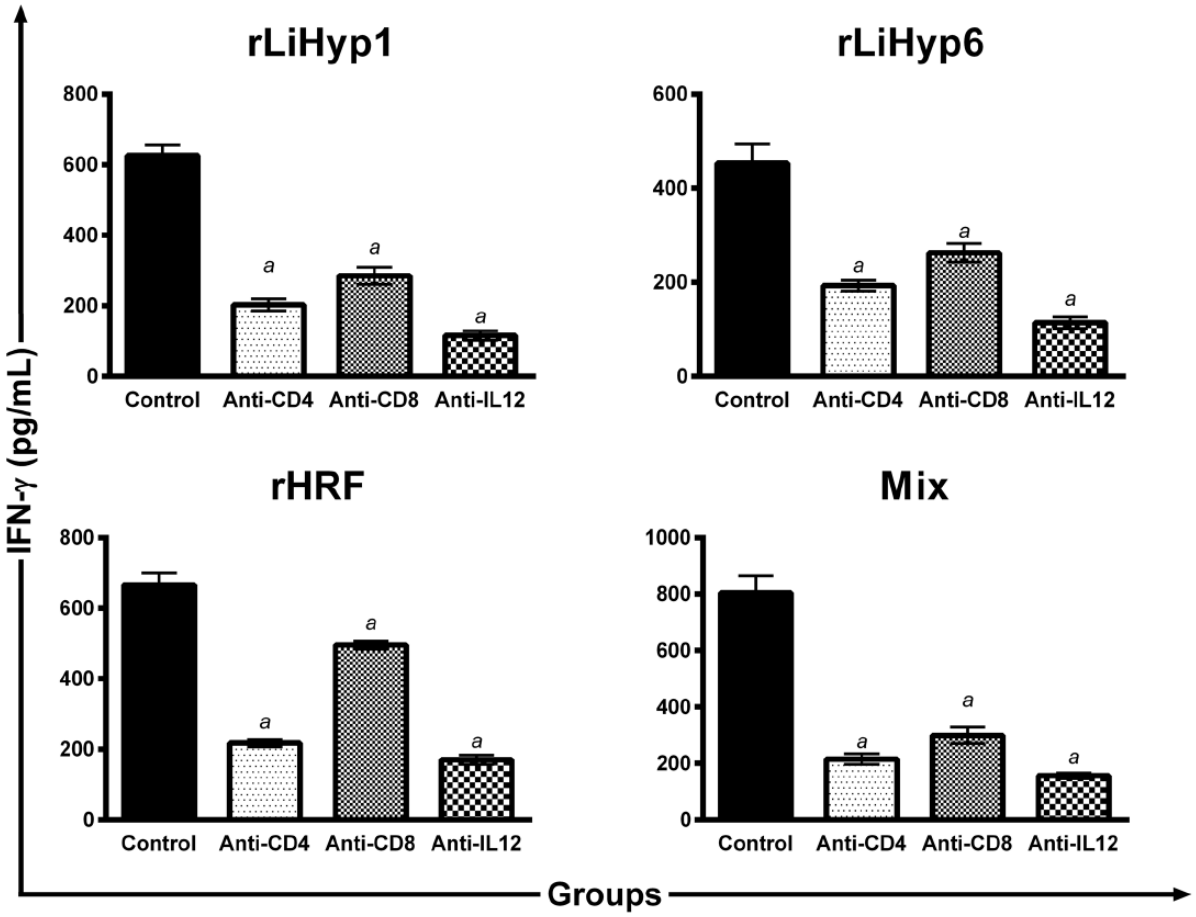


Fig 7. Analysis of the involvement of CD4$^+$ and CD8$^+$ T cells in IFN- production after *Leishmania infantum* infection. Mice (n = 8, per group) were vaccinated subcutaneously with rLiHyp1/saponin (**A**), rLiHyp6/saponin (**B**), rHRF/saponin (**C**) or polyproteins/saponin (**D**). Three doses were administered at two-week intervals. Four weeks after the last immunization, mice (n = 4 per group) were subcutaneously infected in the right hind footpad with 1 x 10^7 stationary-phase promastigotes of *L. infantum*. Ten weeks after challenge, single cell suspensions were obtained from the spleens of the mice and *in vitro* stimulated with SLA (25 μg mL^{-1}) for 48 h at 37°C in 5% CO_2, in the absence (positive control) or presence of 5 μg mL^{-1} of monoclonal antibodies (mAb) against mouse IL-12, CD4, or CD8. Statistically significant differences between the positive control cells and cultures incubated with anti-CD4, anti-CD8 or anti-IL-12 monoclonal antibodies were obtained and are shown. Bars represent the mean ± standard deviation (SD) of the groups. (*a*) indicates statistically significant difference in relation to the positive control ($P < 0.001$).

doi:10.1371/journal.pone.0137683.g007

respectively), in comparison to the rHRF/saponin group (Fig 7C), possibly due to the fact that these hypothetical proteins were found in the amastigote stage of the parasites. In the polyproteins/saponin group, the reduction of the IFN-γ production was achieved when both anti-CD4 and anti-CD8 monoclonal antibodies were used in the cells cultures (Fig 7D). Data of immunogenicity after challenge infection that were obtained in the 2nd experiment are also shown (Figures C, D and E in S1 File).

Humoral response and nitrite production induced after *Leishmania infantum* infection

Evaluating the antibody production in the vaccinated and infected animals (Fig 8), 10 weeks after infection, it was possible to verify that animals vaccinated with rLiHyp1/saponin, rLiHyp6/saponin, rHRF/saponin, or polyproteins/saponin presented higher levels of parasite- and protein-specific IgG2a antibodies in relation to the IgG1 levels (Fig 8A and 8B, respectively). The rHRF/saponin group presented the higher levels of both IgG1 and IgG2a levels, although the IgG2a production has been significantly higher than IgG1 levels. The ratio between the IgG2a and IgG1 levels using SLA (Fig 8C) or recombinant proteins (Fig 8D) as antigens was also calculated, and it was possible to observe that all vaccinated groups presented higher IgG2a/IgG1 values, when compared to the saline and saponin groups.

In an attempt to determine the influence of the immunizations of the different vaccines on the *L. infantum* specific killing effectors functions, the nitrite concentration (employed as an indicator of NO production) was analyzed in the culture supernatants after an *in vitro* stimulation using parasite antigens (Fig 9). In the results, the nitrite production was significantly higher in the mice vaccinated with rLiHyp1/saponin, rLiHyp6/saponin, rHRF/saponin, or polyproteins/saponin, when compared to the saline and saponin groups. The highest level on nitrite in supernatants was observed in cultures established form mice inoculated with the polyproteins/saponin vaccine.

Discussion

It has been showed that *Leishmania spp.* proteins that react with antibodies from VL dogs could be associated with antigenicity and protective responses, representing potential diagnostic markers and vaccine candidates against leishmaniasis [5,11]. As described in detail previously [30], amastigote antigens have been far less evaluated as vaccine candidates against VL. One could speculate that this could be due to the fact that promastigotes are more easily cultured *in vitro*, as opposed to amastigotes; thus hampering the identification of amastigote-specific antigens [13,30,38]. However, a vaccine that is able to elicit a protective immune response against amastigote proteins could present advantages for both prophylactic and therapeutic conditions. Also, in contrast to promastigote forms, the amastigotes reside within host cells and are targets for $CD8^+$ T cells, elements involved in the protection against *Leishmania spp.* infection. In the present study, the DNA encoding of two hypothetical proteins, which were recently identified expressed in the amastigote stage of *L. infantum*, were cloned and combined with a promastigote protein; with the purpose of composing a polyproteins vaccine to be evaluated against VL. Also, due to their recognition by antibodies of VL dogs, these proteins were evaluated for the serodiagnosis of CVL.

Vaccine studies concerning leishmaniasis call for the critical evaluation of the parasitological and immunological parameters in murine models [13,28]. To properly evaluate new antigens, it is important to optimize their dose and route of administration [39]. The present study aimed to evaluate a polyproteins vaccine composed by three different antigens, based on their potential to induce a more effective protective immunity against murine VL. In relation to the immunogenic composition, some authors have used smaller amounts of single proteins in a 1:1:1 ratio, in the attempt to evaluate the efficacy of their vaccine candidates [40,41]. Taking this into account, our group chose to compose the polyproteins vaccine with the same amount of protein in a fixed ratio of 1:1:1. Nonetheless, it was possible to verify that the polyproteins vaccine plus saponin was able to induce a more pronounced Th1 response in the vaccinated animals, which was based on *in vitro* protein- and parasite-specific production of IFN-γ, IL-12, and GM-CSF, combined with the presence of low levels of IL-4 and IL-10 in the spleen cells of

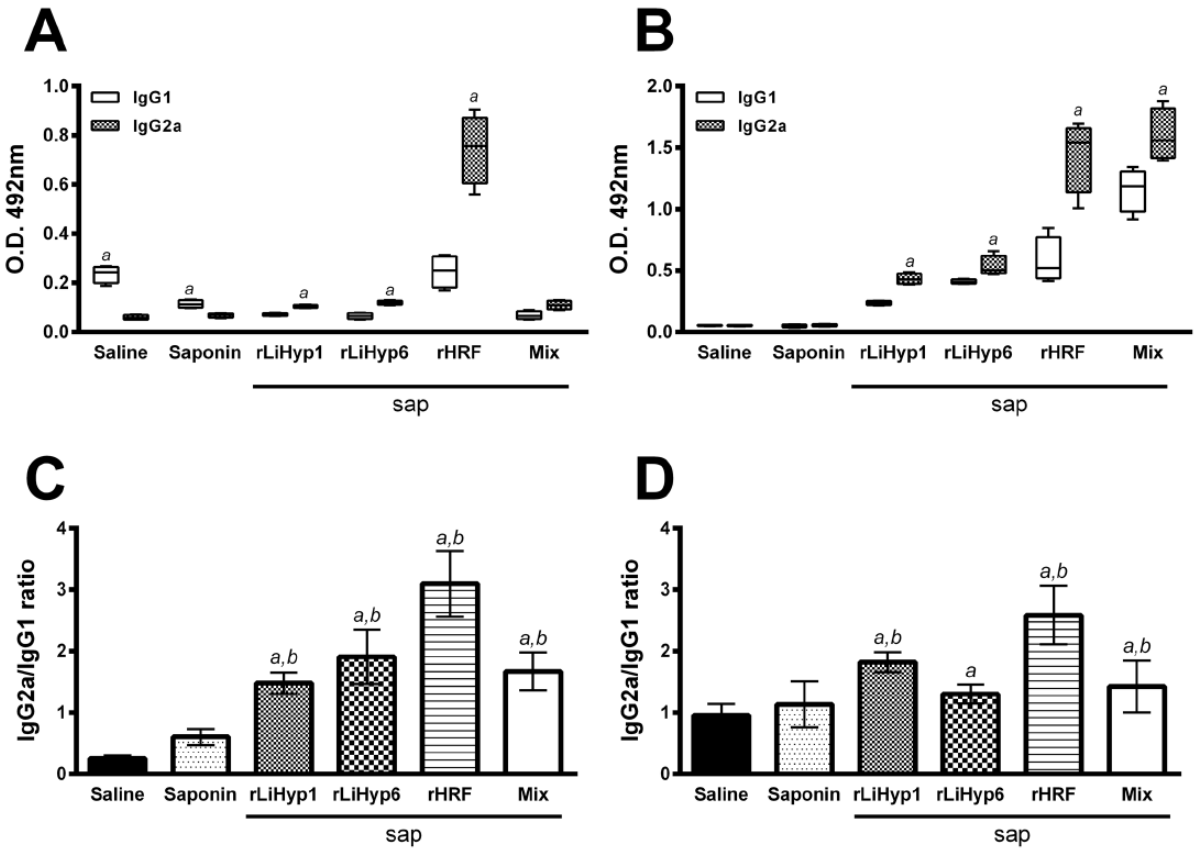


Fig 8. Humoral response induced in the vaccinated and infected BALB/c mice, 10 weeks after *Leishmania infantum* infection. Mice (n = 8, per group) were vaccinated subcutaneously with rLiHyp1/saponin, rLiHyp6/saponin, rHRF/saponin or polyproteins/saponin. Three doses were administered at two-week intervals. Four weeks after the last immunization, mice (n = 4 per group) were subcutaneously infected using stationary-phase promastigotes of *L. infantum*, and 10 weeks after challenge, sera samples were obtained from the animals to evaluate the humoral response. The reactivity based on IgG1 and IgG2a isotypes represented by whisker (min to max) plots against SLA (**A**) and the respective recombinant proteins (**B**) are shown. In each group vaccinated with the recombinant protein, the respective antigen was used. In the saline and saponin groups, as well as in the polyproteins/saponin group, the protein mixture was used as antigen. Also, SLA was used as antigen in all groups. For the assays, 1.0, 1.0, and 0.5 μg per well of rLiHyp1, rLiHyp6, and rHRF proteins, respectively; 0.5 μg per well of each protein composing the antigenic mixture, and 2.0 μg per well of SLA *L. infantum*, were used in the ELISA assays. The sera samples were diluted at 1:200, and the anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies were employed in a 1:5,000 and 1:10,000 dilution, respectively. The ratios between the IgG2a/IgG1 levels against SLA (**C**) and the respective recombinant proteins (**D**) were calculated, and are also shown. Bars represent the mean ± standard deviation (SD) of the groups. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0.001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0.001$).

doi:10.1371/journal.pone.0137683.g008

the immunized animals, as well as by low levels of protein- and parasite-specific IgG1 isotype antibodies. The results of immunogenicity proved to be better when the polyproteins vaccine, as compared to the employ of individual proteins, was used. In addition, after infection, mice immunized with the polyproteins vaccine plus saponin displayed more significant reductions in the parasite burden in all evaluated organs, which were correlated with a parasite-dependent IFN-γ production in the spleen of the animals, which is one of the main cytokines implicated in the acquired immunity against VL [16,42].

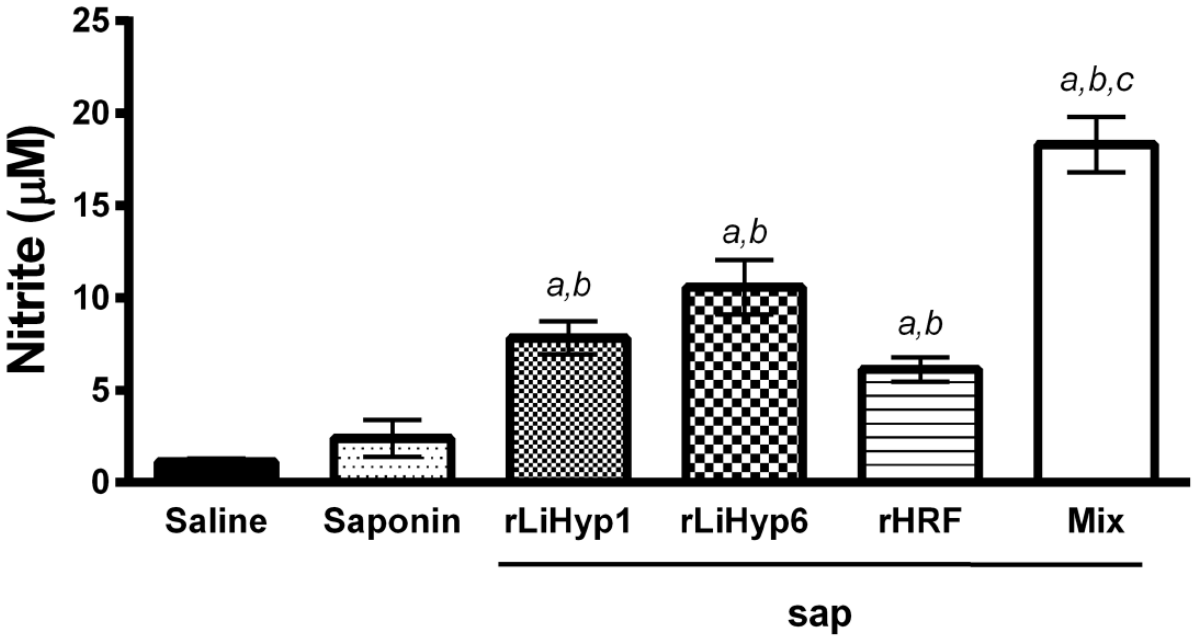


Fig 9. Nitric oxide production by vaccinated and infected animals, 10 weeks after challenge infection. Mice (n = 8, per group) were vaccinated subcutaneously with rLiHyp1/saponin, rLiHyp6/saponin, rHRF/saponin or polyproteins/saponin. Three doses were administered at two-week intervals. Four weeks after the last immunization, mice (n = 4 per group) were subcutaneously infected in the right hind footpad with 1 x 10^7 stationary-phase promastigotes of *L. infantum*. Ten weeks after challenge, single cell suspensions were obtained from the spleens of the animals and incubated in RPMI 1640 medium (negative control), which was supplemented with 10% FBS, 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4; or stimulated with SLA *L. infantum* (25 μg mL^{-1}), for 48 h at 37°C in 5% CO_2. Then, the nitrite production was evaluated in the culture supernatant using a nitric oxide assay kit, and the results are expressed in μM. Bars represent the mean ± standard deviation (SD) of the groups. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0.001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0.001$). (*c*) indicates statistically significant difference in relation to the rLiHyp1/saponin, rLiHyp6/saponin, and rHRF/saponin groups ($P < 0.001$).

doi:10.1371/journal.pone.0137683.g009

The evaluation of parasite burden was performed at week 10 after infection, since in the murine model employed; high numbers of parasites are present in different organs in this period of time [43]. In the absence of parasite determination at longer times after challenge, it cannot be discarded that vaccinated mice can present a delay in parasite spreading. Further investigation should be made to study parasite evolution in different organs, since mice infected with *L. infantum* have organ specific responses that destroy parasites in the liver, while they become chronic in the spleen. Like demonstrated here, the development and design of combining-protein vaccines has showed an improved protective efficacy against VL. In a recent study, a vaccine containing *L. tarentolae* expressing the *L. donovani* A2 protein along with cysteine proteinases (CPA and CPB) was evaluated in the protection of BALB/c mice against *L. infantum* infection. The authors showed that the combined vaccine induced a protective immunity, which was based on a Th1 response with high levels of IFN-γ prior and after challenge, as well as by low levels of IL-10 produced after infection. Moreover, the protection was correlated with a high nitric oxide production, and a low parasite burden in the vaccinated and infected animals was observed in this study [44]. In other work, the efficacy of a combined vaccine using the same system, live recombinant *L. tarentolae* expressing cysteine proteinases (CPA and CPB), but added with PpSP15, an immunogenic salivary protein from *Phlebotomus*

papatasi; was also protective against *L. infantum*, being this protection also based on the development of a Th1 immune response in the vaccinated and infected animals [45].

Evaluating the involvement of the T cells in the immune response induced after infection in the present study, $CD4^+$ T cells proved to be the major source of IFN-γ in the protected mice, since depletion of these cells in cultures of spleen cells stimulated with SLA *L. infantum* significantly reduced this response. In relation to the involvement of $CD8^+$ T cells, although the IFN-γ production had been also diminished in the vaccinated and infected animals, this production was higher in relation to that obtained when the anti-CD4 monoclonal antibody was used. Previous reports have shown that the activation of both $CD4^+$ and $CD8^+$ T cells subsets is important for the killing of parasites in mice vaccinated with *Leishmania spp*. recombinant proteins [13,20,46]; although the $CD4^+$ T cells response had been more important in the IFN-γ production, as well as in the induction of protection against challenge infection [13]. However, although the results of the present study have showed a discrimination in the IFN-γ production in the spleen cells cultures after using anti-CD4 and anti-CD8 monoclonal antibodies, this results could be considered a proof-of-concept of the involvement of the T cells, once these cells were not separate of others, such as macrophages in the *in vitro* cultures; being then this production considered as an indirect implication of the involvement of $CD4^+$ and $CD8^+$ T cells in the IFN-γ production.

Spleen cells derived from the vaccinated mice, when compared to the control groups, produced higher levels of protein- and parasite-specific GM-CSF, a cytokine linked to macrophage activation and resistance of murine models against infection caused by some *Leishmania spp*. species, such as *L. infantum* [13,17,20], *L. major* [47], and *L. donovani* [48]. GM-CSF plays also with an important role in activation and functional maturation of dendritic cells [49]. Moreover, it can recruits cells, such as monocytes and neutrophils, to the *Leishmania spp*. infection site, and contributing to the development of an effective immune response. It has been also used as an adjuvant in vaccine candidates for malaria [50], HIV [51], leishmaniasis [52], and others. These studies have shown that GM-CSF significantly increases the immunogenicity of the evaluated antigens, contributing to the protection against disease. The present study showed that the polyproteins vaccine plus saponin induced a low production of IL-4 and IL-10 before infection, which was maintained in low levels after challenge. On the other hand, animals from the control (saline and saponin) groups showed a significantly higher production of these cytokines after *L. infantum* infection. Indeed, the control of the parasite-mediated IL-10 response in mice may be important for protection against visceral disease, since IL-10 is considered to be the most important factor for disease progression caused by viscerotropic *Leishmania spp*. species, like described in both IL-10 deficient mice [17,53,54], and in mice treated with an anti-IL-10 receptor antibody [55].

As described in detail previously [13], in BALB/c mice, the IL-4-dependent production of IgG1 isotype antibodies is associated with disease progression caused by *L. infantum* [13,20,36], and *L. amazonensis* [34,56]. For instance, in studies of immunization of BALB/c mice with the recombinant A2 protein [16,34] or *Leishmania* ribosomal proteins [17] plus saponin, the animals were protected against infection, being this protection correlated with a decrease in parasite-specific IL-4 and IL-10 mediated response, as well as by low levels of parasite-specific IgG1 isotype antibodies. In this context, in the present study, mice that received saline or saponin and that were not protected against infection, presented high parasite-specific IgG1 antibody levels, when compared to their IgG2a levels. An important immunological control of intracellular parasitism is based on the production of oxygen derivative metabolites [57]. Therefore, the present study investigated the NO production by spleen cells of the vaccinated and infected animals, 10 weeks after infection. It was observed that the protected animals, which were immunized with the recombinant proteins isolate or in combination,

produced higher levels of NO, when compared to the control groups. According to these data, it could be speculated a possible activation of an anti-parasite effectors mechanisms mediated by NO, as previously described by others [17,58,59].

Serological tests are currently recommended for the laboratorial diagnosis of CVL. Some of them, such as IFAT and ELISA, are used as diagnostic markers; however, their low sensitivity to detect cases from animals with low levels of antileishmanial antibodies, as well as by their specificity hampered due to cross-reactivity with antibodies of animals presenting other pathologies, such as Chagas´ disease, babesiosis and erlichiose; are problems to be solved [60,61]. In Brazil, there are two commercial vaccines against CVL, Leishmune® [62], and Leish-Tec® [16]. However, although protective, these vaccines can induce seroconversion in the immunized animals, causing them to be classified as false-positive in the serological assays performed [63–65]. In the present study, it was possible to verify that all three evaluated recombinant proteins were specifically recognized by antibodies of symptomatic and asymptomatic VL dogs, and the results of sensitivity and specificity values were better in comparison to data obtained using an antigenic preparation of the parasites. In addition, the serological results obtained with the rLiHyp1 protein are in accordance with previous data [13]. Moreover, the ability of these antigens to distinguish the CVL sera from those from non-infected animals living in endemic and non-endemic areas of leishmaniasis, leads to the possibility of reducing the number of false-positive results usually encountered when these sera are evaluated in the serological assays performed [66]. However, the number and variety of sera used in the present study could be considered a limiting factor of the serological assays. In this context, data here presented should be taken as a proof-of-concept of the efficacy of these proposed antigens to be employed in the serodiagnosis of CVL, and may well serve as a reference for further assays. On the other hand, we believe that, after an adequate validation, the rLiHyp1, rLiHyp6 and rHRF proteins may be promptly applied for a sensitive and specific serodiagnosis of CVL.

In recent years, advances in the development of vaccines against VL have been based on molecularly defined antigens [13,16,19,27,29,67]. Although second generation vaccines are currently being tested in clinical trials, the screening of new candidates will help to further increase the prophylactic efficacy of effective candidates against this disease. It has been proposed that a combination of different parasite proteins leading to the development of polyproteins vaccines could help to produce a more robust and effective prophylactic vaccine, presenting more protective characteristics [1,43,44]. The WHO has postulated that multiproteic vaccines could present a more protective efficacy against *Leishmania spp*. infection [1]. In addition, to be effective as a vaccine candidate against leishmaniasis, its components should either be shared by different parasite species, or be based on antigens expressed in both parasite stages. The use of chimeric vaccines combining multi-proteins and/or poly-epitopes may well provide benefits in terms of simplicity and production costs, since only one vaccine would be produced, besides of it could be protective against different *Leishmania spp*. species [68].

Although offering advantages in terms of safety and production´ costs, protein-based vaccines are less immunogenic and must be supplemented with immune adjuvants, in order to boost their immunogenicity [69]. In this context, for the development of a successful vaccine, one could speculate that the association of an effective adjuvant will be desirable. Recently, the rLiHyp1 protein was evaluated as a vaccine candidate against *L. infantum* infection, being associated with two polysaccharide-rich fractions (F2 and F4 fractions), which were derived from *Agaricus blazei* mushroom. The authors showed that the immunization using the rLiHyp1 protein plus F2 or F4 fractions of *A. blazei* was able to induce protection against challenge infection, which was based on the development of a Th1 immune response in the vaccinated and infected animals [70]. In the same study, the rLiHyp1 protein was administered isolated, and the results showed that it was not protective without the association of an immune adjuvant.

Thus, the data of the present study could be taken as a proof-of-concept of the efficacy from a developed polyproteins vaccine that, when administered in association with an adjuvant, could be used in the protection against VL. Additional studies evaluating this polyproteins vaccine against other *Leishmania spp*. species, such as against species causing tegumentary leishmaniasis; could be performed. In relation to their diagnostic performance, the rLiHyp1, rLiHyp6 and rHRF proteins could be also evaluated in future studies as new diagnostic markers for the CVL.

Supporting Information

S1 File. Immunological and parasitological parameters evaluated in the vaccinated animals, before and after the *Leishmania infantum* challenge infection. Mice (n = 8, per group) were vaccinated subcutaneously in their left hind footpad with 25 g of each recombinant protein (rLiHyp1, rLiHyp6, or rHRF), or with their mixture (using 25 g of each protein), all associated with 25 g of saponin (*Quillaja saponaria* bark saponin, Sigma). Additional mice were immunized with saponin or received saline. Three doses were administered, at two-week intervals. Four weeks after the last immunization, mice (n = 4 per group) were subcutaneously infected in the right hind footpad with 1 x 10^7 stationary-phase promastigotes of *L. infantum*. Before and 10 weeks after challenge, the cytokine levels were evaluated. For this, cells were incubated in complete RPMI 1640 medium (negative control), or separately stimulated with rLiHyp1 (rLiHyp1/saponin group), rLiHyp6 (rLiHyp6/saponin group), rHRF (rHRF/saponin group) proteins (20 g mL^{-1}, each one); with their mixture (saline, saponin, and polyproteins/saponin groups; using 10 g mL^{-1} of each protein), or SLA *L. infantum* (25 μg mL^{-1}), for 48 h at 37°C in 5% CO_2. IFN-, IL-12, GM-CSF, IL-4, and IL-10 levels were measured by ELISA in the culture supernatants (**Figure A**, before and **Figure D**, after infection). Also 10 weeks after challenge, the parasite burden was determined in the spleen, liver, infected paws´ draining lymph nodes, and bone marrow of the animals (**Figure B**). Using the supernatants of the SLA *L. infantum*-stimulated spleen cells, the nitrite production was evaluated in this time (**Figure C**). (*a*) indicates statistically significant difference in relation to the saline group ($P < 0.001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0.001$). (*c*) indicates statistically significant difference in relation to the rLiHyp1/saponin, rLiHyp6/saponin, and rHRF/saponin groups ($P < 0.001$). To evaluate the involvement of $CD4^+$ and $CD8^+$ T cells in IFN- production after *L. infantum* infection, single cell suspensions that were stimulated with SLA (25 g mL^{-1}) were incubated in the absence (positive control) or presence of 5 g mL^{-1} of monoclonal antibodies (mAb) against mouse IL-12, CD4, or CD8 (**Figure E**). (*a*) Statistically significant differences between the positive control cells and cultures incubated with anti-CD4, anti-CD8 or anti-IL-12 monoclonal antibodies were obtained and are shown ($P < 0.001$). In all panels, bars represent the mean ± standard deviation (SD) of the groups.
(TIF)

Author Contributions

Conceived and designed the experiments: EAFC MACF VTM CAPT APF MS. Performed the experiments: VTM MCD LEC VGS JSO DPL EG. Analyzed the data: EAFC MS CAPT MACF VTM DFMS APF SMRT. Contributed reagents/materials/analysis tools: DFMS SMRT. Wrote the paper: EAFC MACF MS SMRT CAPT DFMS.

References

1. World Health Organization (2010) Control of the leishmaniases. World Health Organ Tech Rep Ser: 22–26.

2. Croft SL, Coombs GH (2003) Leishmaniasis: current chemotherapy and recent advances in the search for novel drugs. Trends Parasitol 19: 502–508. PMID: 14580961
3. Minodier P, Parola P (2007) Cutaneous leishmaniasis treatment. Travel Med Infect Dis 5: 150–158. doi: 10.1016/j.tmaid.2006.09.004 PMID: 17448941
4. Oliveira LF, Schubach AO, Martins MM, Passos SL, Oliveira RV, Marzochi MC, et al. (2011) Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. Acta Trop 118: 87–96. doi: 10.1016/j.actatropica.2011.02.007 PMID: 21420925
5. Coelho VTS, Oliveira JS, Valadares DG, Chávez-Fumagalli MA, Duarte MC, Lage PS, et al. (2012) Identification of proteins in promastigote and amastigote-like Leishmania using an immunoproteomic approach. PLoS Negl Trop Dis 6: 1–10. doi: 10.1371/journal.pntd.0001430
6. Gramiccia M, Gradoni L (2005) The current status of zoonotic leishmaniases and approaches to disease control. Int J Parasitol 35: 1169–1180. doi: 10.1016/j.ijpara.2005.07.001 PMID: 16162348
7. Miró G, Cardoso L, Pennisi MG, Oliva G, Baneth G (2008) Canine leishmaniosis—new concepts and insights on an expanding zoonosis: part two. Trends Parasitol 24: 371–377. doi: 10.1016/j.pt.2008.05.003 PMID: 18603476
8. Barbiéri CL (2006) Immunology of canine leishmaniasis. Parasite Immunol 28: 329–337. doi: 10.1111/j.1365-3024.2006.00840.x PMID: 16842269
9. Ciaramella P, Oliva G, Luna R, Ambrosio R, Cortese L, Cortese L, et al. (1997) A retrospective clinical study of canine leishmaniasis in 150 dogs naturally infected by Leishmania infantum. Vet Rec 141: 539–543. doi: 10.1136/vr.141.21.539 PMID: 9413121
10. Garcia-Alonso M, Nieto CG, Blanco A, Requena JM, Alonso C, Navarrete I (1996) Presence of antibodies in the aqueous humour and cerebrospinal fluid during Leishmania infections in dogs. Pathological features at the central nervous system. Parasite Immunol 18: 539–546. PMID: 9226692
11. Alves WA, Bevilacqua PD (2004) Reflexões sobre a qualidade do diagnóstico da leishmaniose visceral canina em inquéritos epidemiológicos: o caso da epidemia de Belo Horizonte, Minas Gerais, Brasil, 1993–1997. Cad Saude Publica 20: 259–265. doi: 10.1590/S0102-311X2004000100043
12. Miró G, Montoya A, Mateo M, Alonso A, García S, García A, et al. (2007) A leishmaniosis surveillance system among stray dogs in the region of Madrid: Ten years of serodiagnosis (1996–2006). Parasitol Res 101: 253–257. doi: 10.1007/s00436-007-0497-8 PMID: 17323100
13. Martins VT, Chávez-Fumagalli MA, Costa LE, Martins AMCC, Lage PS, Lage DP, et al. (2013) Antigenicity and protective efficacy of a Leishmania amastigote-specific protein, member of the super-oxygenase family, against visceral leishmaniasis. PLoS Negl Trop Dis 7: e2148. doi: 10.1371/journal.pntd.0002148 PMID: 23573301
14. Baharia RK, Tandon R, Sharma T, Suthar MK, Das S, Siddiqi MI, et al. (2015) Recombinant NAD-dependent SIR-2 protein of Leishmania donovani: immunobiochemical characterization as a potential vaccine against visceral leishmaniasis. PLoS Negl Trop Dis 9: e0003557. doi: 10.1371/journal.pntd.0003557 PMID: 25745863
15. Afonso LC, Scott P (1993) Immune responses associated with susceptibility of C57BL/10 mice to Leishmania amazonensis. Infect Immun 61: 2952–2959. PMID: 8514400
16. Blackwell JM (1996) Genetic susceptibility to leishmanial infections: studies in mice and man. Parasitology 112 Suppl: S67–S74. PMID: 8684837
17. Fernandes AP, Costa MMS, Coelho EAF, Michalick MSM, Freitas E, Melo MN, et al. (2008) Protective immunity against challenge with Leishmania (Leishmania) chagasi in beagle dogs vaccinated with recombinant A2 protein. Vaccine 26: 5888–5895. PMID: 18786587
18. Chávez-Fumagalli MA, Costa MAF, Oliveira DM, Ramírez L, Costa LE, Duarte MC, et al. (2010) Vaccination with the Leishmania infantum ribosomal proteins induces protection in BALB/c mice against Leishmania chagasi and Leishmania amazonensis challenge. Microbes Infect 12: 967–977. doi: 10.1016/j.micinf.2010.06.008 PMID: 20601076
19. Das A, Ali N (2012) Vaccine prospects of killed but metabolically active Leishmania against visceral leishmaniasis. Expert Rev Vaccines 11: 783–785. Available: http://www.ncbi.nlm.nih.gov/pubmed/22913255. doi: 10.1586/erv.12.50 PMID: 22913255
20. Ramírez L, Santos DM, Souza AP, Coelho EAF, Barral A, Alonso C, et al. (2013) Evaluation of immune responses and analysis of the effect of vaccination of the Leishmania major recombinant ribosomal proteins L3 or L5 in two different murine models of cutaneous leishmaniasis. Vaccine 31: 1312–1319. doi: 10.1016/j.vaccine.2012.12.071 PMID: 23313653
21. Costa LE, Goulart LR, Jesus-Pereira NC, Lima MI, Duarte MC, et al. (2014) Mimotope-based vaccines of Leishmania infantum antigens and their protective efficacy against visceral leishmaniasis. PLoS One 9: e110014. doi: 10.1371/journal.pone.0110014 PMID: 25333662

22. Green SJ, Mellouk S, Hoffman SL, Meltzer MS, Nacy CA (1990) Cellular mechanisms of nonspecific immunity to intracellular infection: cytokine-induced synthesis of toxic nitrogen oxides from L-arginine by macrophages and hepatocytes. Immunol Lett 25: 15–19. doi: 10.1016/0165-2478(90)90083-3 PMID: 2126524

23. Wilson ME, Jeronimo SMB, Pearson RD (2005) Immunopathogenesis of infection with the visceralizing Leishmania species. Microb Pathog 38: 147–160. doi: 10.1016/j.micpath.2004.11.002 PMID: 15797810

24. Stäger S, Alexander J, Carter KC, Brombacher F, Kaye PM (2003) Both interleukin-4 (IL-4) and IL-4 receptor α signaling contribute to the development of hepatic granulomas with optimal antileishmanial activity. Infect Immun 71: 4804–4807. doi: 10.1128/IAI.71.8.4804-4807.2003 PMID: 12874364

25. Joshi J, Kaur S (2014) Studies on the protective efficacy of second-generation vaccine along with standard antileishmanial drug in Leishmania donovani infected BALB/c mice. Parasitology 141: 554–562. Available: http://www.ncbi.nlm.nih.gov/pubmed/24618257. doi: 10.1017/S0031182013001959 PMID: 24618257

26. Kharazmi A, Kemp K, Ismail A, Gasim S, Gaafar A, Kurtzhals JA, et al. (1999) T-cell response in human leishmaniasis. Immunology Letters 65: 105–108. doi: 10.1016/S0165-2478(98)00132-1 PMID: 10065635

27. Stäger S, Smith DF, Kaye PM (2000) Immunization with a recombinant stage-regulated surface protein from Leishmania donovani induces protection against visceral leishmaniasis. J Immunol 165: 7064–7071. PMID: 11120835

28. Iborra S, Parody N, Abánades DR, Bonay P, Prates D, Novais FO, et al. (2008) Vaccination with the Leishmania major ribosomal proteins plus CpG oligodeoxynucleotides induces protection against experimental cutaneous leishmaniasis in mice. Microbes Infect 10: 1133–1141. doi: 10.1016/j.micinf.2008.06.002 PMID: 18603012

29. Agallou M, Smirlis D, Soteriadou KP, Karagouni E (2012) Vaccination with Leishmania histone H1-pulsed dendritic cells confers protection in murine visceral leishmaniasis. Vaccine 30: 5086–5093. doi: 10.1016/j.vaccine.2012.05.075 PMID: 22704924

30. Fernandes AP, Coelho EAF, Machado-Coelho GLL, Grimaldi G, Gazzinelli RT (2012) Making an anti-amastigote vaccine for visceral leishmaniasis: Rational, update and perspectives. Curr Opin Microbiol 15: 476–485. PMID: 22698479

31. Wenzel UA, Bank E, Florian C, Forster S, Zimara N, Steinacker J, et al. (2012) Leishmania major parasite stage-dependent host cell invasion and immune evasion. FASEB J 26: 29–39. doi: 10.1096/fj.11-184895 PMID: 21908716

32. Kang HS, Lee MJ, Song H, Han SH, Kim YM, Im JI, et al. (2001) Molecular identification of IgE-dependent histamine-releasing factor as a B cell growth factor. J Immunol 166: 6545–6554. PMID: 11359806

33. Kang J-A, Kim W-S, Park S-G (2014) Notch1 is an important mediator for enhancing of B-cell activation and antibody secretion by Notch ligand. Immunology 143: 550–559. doi: 10.1111/imm.12333 PMID: 24913005

34. Coelho EAF, Tavares CAP, Carvalho FAA, Chaves KF, Teixeira KN, Rodrigues RC, et al. (2003) Immune responses induced by the Leishmania (Leishmania) donovani A2 antigen, but not by the LACK antigen, are protective against experimental Leishmania (Leishmania) amazonensis infection. Infect Immun 71: 3988–3994. doi: 10.1128/IAI.71.7.3988-3994.2003 PMID: 12819086

35. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72: 248–254. doi: 10.1016/0003-2697(76)90527-3 PMID: 942051

36. Zanin FHC, Coelho EAF, Tavares CAP, Marques-da-Silva EA, Costa MMS, Rezende SA, et al. (2007) Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against Leishmania chagasi and Leishmania amazonensis experimental infections. Microbes Infect 9: 1070–1077. doi: 10.1016/j.micinf.2007.05.012 PMID: 17644455

37. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR (1982) Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal Biochem 126: 131–138. doi: 10.1016/0003-2697(82)90118-X PMID: 7181105

38. Kaur S, Kaur T, Garg N, Mukherjee S, Raina P, Athokpam V (2008) Effect of dose and route of inoculation on the generation of $CD4^+$ Th1/Th2 type of immune response in murine visceral leishmaniasis. Parasitol Res 103: 1413–1419. doi: 10.1007/s00436-008-1150-x PMID: 18751727

39. Galli V, Simionatto S, Marchioro SB, Klabunde GHF, Conceição FR, Dellagostin AO (2013) Recombinant secreted antigens from Mycoplasma hyopneumoniae delivered as a cocktail vaccine enhance the immune response of mice. Clin Vaccine Immunol 20: 1370–1376. doi: 10.1128/CVI.00140-13 PMID: 23803903

40. Tsenova L, Harbacheuski R, Moreira AL, Ellison E, Dalemans W, Alderson MR, et al. (2006) Evaluation of the Mtb72F polyprotein vaccine in a rabbit model of tuberculous meningitis. Infect Immun 74: 2392–2401. PMID: 16552069

41. Resende DM, Caetano BC, Dutra MS, Penido MLO, Abrantes CF, Verly RM, et al. (2008) Epitope mapping and protective immunity elicited by adenovirus expressing the Leishmania amastigote specific A2 antigen: correlation with IFN-γ and cytolytic activity by $CD8^+$ T cells. Vaccine 26: 4585–4593. PMID: 18588933

42. Rachamim N, Jaffe CL (1993) Pure protein from Leishmania donovani protects mice against both cutaneous and visceral leishmaniasis. J Immunol 150: 2322–2331. PMID: 8450215

43. Oliveira DM, Valadares DG, Duarte MC, Costa LE, Martins VT, Costa LE, et al. (2012) Evaluation of parasitological and immunological parameters of *Leishmania chagasi* infection in BALB/c mice using different doses and routes of inoculation of parasites. Parasitol Res 110: 1277–1285. doi: 10.1007/s00436-011-2628-5 PMID: 21915627

44. Saljoughian N, Taheri T, Zahedifard F, Taslimi Y, Doustdari F, Bolhassani A, et al. (2013) Development of novel prime-boost strategies based on a tri-gene fusion recombinant L. tarentolae vaccine against experimental murine visceral leishmaniasis. PLoS Negl Trop Dis 7: 1–15. doi: 10.1371/journal.pntd.0002174

45. Zahedifard F, Gholami E, Taheri T, Taslimi Y, Doustdari F, Seyed N, et al. (2014) Enhanced protective efficacy of nonpathogenic recombinant Leishmania tarentolae expressing cysteine proteinases combined with a sand fly salivary antigen. PLoS Negl Trop Dis 8. doi: 10.1371/journal.pntd.0002751

46. Bhowmick S, Ravindran R, Ali N (2008) Gp63 in stable cationic liposomes confers sustained vaccine immunity to susceptible BALB/c mice infected with Leishmania donovani. Infect Immun 76: 1003–1015. doi: 10.1128/IAI.00611-07 PMID: 18195029

47. Dumas C, Muyombwe A, Roy G, Matte C, Ouellette M, Olivier M, et al. (2003) Recombinant Leishmania major secreting biologically active granulocyte-macrophage colony-stimulating factor survives poorly in macrophages in vitro and delays disease development in mice. Infect Immun 71: 6499–6509. doi: 10.1128/IAI.71.11.6499-6509.2003 PMID: 14573672

48. Murray HW, Cervia JS, Hariprashad J, Taylor AP, Stoeckle MY, Hockman H (1995) Effect of granulocyte-macrophage colony-stimulating factor in experimental visceral leishmaniasis. J Clin Invest 95: 1183–1192. doi: 10.1172/JCI117767 PMID: 7883967

49. Bayih AG, Daifalla NS, Gedamu L (2014) DNA-protein immunization using Leishmania peroxidoxin-1 induces a strong $CD4^+$ T cell response and partially protects mice from cutaneous leishmaniasis: role of fusion murine granulocyte-macrophage colony-stimulating factor DNA adjuvant. PLoS Negl Trop Dis 8: e3391. doi: 10.1371/journal.pntd.0003391 PMID: 25500571

50. Weiss WR, Ishii KJ, Hedstrom RC, Sedegah M, Ichino M, Barnhart K, et al. (1998) A plasmid encoding murine granulocyte-macrophage colony-stimulating factor increases protection conferred by a malaria DNA vaccine. J Immunol 161: 2325–2332. PMID: 9725227

51. Lai L, Kwa S, Kozlowski PA, Montefiori DC, Ferrari G, Johnson WE, et al. (2011) Prevention of infection by a granulocyte-macrophage colony-stimulating factor co-expressing DNA/modified vaccinia Ankara simian immunodeficiency virus vaccine. J Infect Dis 204: 164–173. doi: 10.1093/infdis/jir199 PMID: 21628671

52. Follador I, Araujo C, Orge G, Cheng LH, Carvalho LP, Bacellar O, et al. (2002) Immune responses to an inactive vaccine against American cutaneous leishmaniasis together with granulocyte-macrophage colony-stimulating factor. Vaccine 20: 1365–1368. doi: 10.1016/S0264-410X(01)00469-8 PMID: 11818154

53. Murphy ML, Wille U, Villegas EN, Hunter CA, Farrell JP (2001) IL-10 mediates susceptibility to Leishmania donovani infection. Eur J Immunol 31: 2848–2856. doi: 10.1002/1521-4141(2001010)31:10<2848::AID-IMMU2848>3.0.CO;2-T PMID: 11592059

54. Awasthi A, Kumar MR, Saha B (2004) Immune response to Leishmania infecction. Indian J Med Res 119: 238–258. PMID: 15243162

55. Murray HW, Lu CM, Mauze S, Freeman S, Moreira AL, Kaplan G, et al. (2002) Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. Infect Immun 70: 6284–6293. doi: 10.1128/IAI.70.11.6284-6293.2002 PMID: 12379707

56. Pereira BAS, Alves CR (2008) Immunological characteristics of experimental murine infection with Leishmania (Leishmania) amazonensis. Vet Parasitol 158: 239–255. doi: 10.1016/j.vetpar.2008.09.015 PMID: 18922635

57. Balaraman S, Tewary P, Singh VK, Madhubala R (2004) Leishmania donovani induces interferon regulatory factor in murine macrophages: a host defense response. Biochem Biophys Res Commun 317: 639–647. doi: 10.1016/j.bbrc.2004.03.097 PMID: 15063806

58. Carrión J, Folgueira C, Soto M, Fresno M, Requena JM (2011) Leishmania infantum HSP70-II null mutant as candidate vaccine against leishmaniasis: a preliminary evaluation. Parasit Vectors 4: 150. Available: http://www.parasitesandvectors.com/content/4/1/150. doi: 10.1186/1756-3305-4-150 PMID: 21794145

59. Soudi S, Hosseini AZ, Hashemi SM (2011) Co-administration of rectal BCG and autoclaved Leishmania major induce protection in susceptible BALB/c mice. Parasite Immunol 33: 561–571. doi: 10.1111/j.1365-3024.2011.01318.x PMID: 21781137

60. Courtenay O, Quinnell RJ, Garcez LM, Shaw JJ, Dye C (2002) Infectiousness in a cohort of brazilian dogs: why culling fails to control visceral leishmaniasis in areas of high transmission. J Infect Dis 186: 1314–1320. doi: 10.1086/344312 PMID: 12402201

61. Reis AB, Teixeira-Carvalho A, Vale AM, Marques MJ, Giunchetti RC, Mayrink W, et al. (2006) Isotype patterns of immunoglobulins: hallmarks for clinical status and tissue parasite density in brazilian dogs naturally infected by Leishmania (Leishmania) chagasi. Vet Immunol Immunopathol 112: 102–116. doi: 10.1016/j.vetimm.2006.02.001 PMID: 16621021

62. Palatnik-de-Sousa CB, Barbosa AF, Oliveira SM, Nico D, Bernardo RR, Santos WR, et al. (2008) FML vaccine against canine visceral leishmaniasis: from second-generation to synthetic vaccine. Expert Rev Vaccines 7: 833–851. doi: 10.1586/14760584.7.6.833 PMID: 18665780

63. Strauss-Ayali D, Jaffe CL, Burshtain O, Gonen L, Baneth G (2004) Polymerase chain reaction using noninvasively obtained samples, for the detection of Leishmania infantum DNA in dogs. J Infect Dis 189: 1729–1733. doi: 10.1086/383281 PMID: 15116312

64. Mettler M, Grimm F, Capelli G, Camp H (2005) Evaluation of enzyme-linked immunosorbent assays, an immunofluorescent-antibody test, and two rapid tests and gel tests for serological diagnosis of symptomatic and asymptomatic Leishmania infections in dogs. J Clin Microbiol 43: 5515–5519. PMID: 16272479

65. Figueiredo FB, Madeira MF, Nascimento LD, Abrantes TR, Mouta-Confort E, Passos SR, et al. (2010) Canine visceral leishmaniasis: study of methods for the detection of IgG in serum and eluate samples. Rev Inst Med Trop Sao Paulo 52: 193–196. doi: 10.1590/S0036-46652010000400005 PMID: 21748226

66. Tavares CAP, Fernandes AP, Melo MN (2003) Molecular diagnosis of leishmaniasis. Expert Rev Mol Diagn 3: 657–667. doi: 10.1586/14737159.3.5.657 PMID: 14510185

67. Mutiso JM, Macharia JC, Kiio MN, Ichagichu JM, Rikoi H, Gicheru MM (2013) Development of Leishmania vaccines: predicting the future from past and present experience. J Biomed Res 27: 85–102. doi: 10.7555/JBR.27.20120064 PMID: 23554800

68. Beaumier CM, Gillespie PM, Hotez PJ, Bottazzi ME (2013) New vaccines for neglected parasitic diseases and dengue. Transl Res 162: 144–155. doi: 10.1016/j.trsl.2013.03.006 PMID: 23578479

69. Cerpa-Cruz S, Paredes-Casillas P, Landeros Navarro E, Bernard-Medina AG, Martínez-Bonilla G, Gutiérrez-Ureña S (2013) Adverse events following immunization with vaccines containing adjuvants. Immunol Res 56: 299–303. doi: 10.1007/s12026-013-8400-4 PMID: 23576057

70. Jesus-Pereira NC, Régis WCB, Costa LE, de Oliveira JS, Silva AG, Martins VT, et al. (2015) Evaluation of adjuvant activity of fractions derived from Agaricus blazei, when in association with the recombinant LiHyp1 protein, to protect against visceral leishmaniasis. Exp Parasitol 153: 180–190. doi: 10.1016/j.exppara.2015.03.027 PMID: 25845753

5.4. Artigo 2 - Conclusão

As proteínas recombinantes LiHyp1, LiHyp6 e HRF, separadas ou conjugadas, apresentaram uma elevada performance em detectar soros de cães com leishmaniose visceral, possibilitando a utilização como novos marcadores sensíveis e específicos para o sorodiagnóstico da LVC. Quando as proteínas foram adicionadas a saponina, ainda demonstraram induzir uma resposta imune Th1 com redução da carga parasitária em todos os camundongos BALB/c imunizados e desafiados com *L. infantum*. Entretanto, ao realizar uma comparação da eficácia vacinal dos grupos experimentais em estudo, animais imunizados com as poliproteínas apresentaram uma maior proteção em relação aos imunizados com as proteínas individuais, sendo que estes três grupos apresentaram resultados semelhantes entre eles.

5.5. Artigo 3 – Breve introdução e objetivo

O terceiro artigo a ser apresentado foi intitulado *"Avaliação da eficácia vacinal de uma quimera recombinante contendo epitopos de células T contra a leishmaniose visceral",* publicado pela revista internacional *Parasite Immunology.*

A Organização Mundial de Saúde tem preconizado o uso de vacinas polipeptídicas contra as leishmanioses, uma vez que esta estratégia possibilitaria o englobamento de diferentes regiões antigênicas e imunogências de várias proteínas do parasito em uma única vacina, aumentando as chances de uma resposta protetora estendida contra diversas espécies do parasito e reduzido custo de produção em relação a outras estratégias. O atual trabalho, focado nesta informação, realizou um estudo de epitopos de linfócitos T $CD4^+$ e $CD8^+$, humanos e murinos, presentes nas proteínas LiHyV, LiHyp1, LiHyp6 e HRF, as quais já demonstraram ser imunogênicas e protetoras em camundongos BALB/c desafiados com *L. infantum*. As regiões imunodominantes das quatro proteínas foram selecionadas e os genes codificadores foram interligados, com a finalidade de se construir uma quimera polipeptídica. Desta forma, o objetivo deste trabalho foi avaliar a imunogenicidade e eficácia vacinal da proteína quimérica sob a forma recombinante, associada à saponina, em relação aos grupos controle, e ainda, comparar a eficiência desta estratégia vacinal em relação ao uso das poliproteínas (LiHyp1+LiHyp6+HRF+Saponina) em camundongos BALB/c desafiados com *L. infantum*.

Received: 17 June 2016 | Accepted: 30 August 2016

DOI: 10.1111/pim.12359

ORIGINAL ARTICLE

WILEY Parasite Immunology

A recombinant chimeric protein composed of human and mice-specific CD4$^+$ and CD8$^+$ T-cell epitopes protects against visceral leishmaniasis

V. T. Martins[1] | M. C. Duarte[2,3] | D. P. Lage[3] | L. E. Costa[3] | A. M. R. S. Carvalho[2] | T. A. O. Mendes[1] | B. M. Roatt[2] | D. Menezes-Souza[2,3] | M. Soto[4] | E. A. F. Coelho[2,3]

[1]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

[2]Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

[3]Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

[4]Departamento de Biología Molecular, Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Universidad Autónoma de Madrid, Madrid, Spain

Correspondence
Eduardo Antonio Ferraz Coelho, Laboratório de Pesquisa do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.
Email: eduardoferrazcoelho@yahoo.com.br

Funding information
Instituto Nacional de Ciência e Tecnologia em Nano-biofarmacêutica; FAPEMIG, Grant/Award Number: CBB-APQ-00819-12 and CBB-APQ-01778-2014; CNPq, Grant/Award Number: APQ-482976/2012-8, APQ-488237/2013-0 and APQ-467640/2014-9

Summary

In this study, a recombinant chimeric protein (RCP), which was composed of specific CD4$^+$ and CD8$^+$ T-cell epitopes to murine and human haplotypes, was evaluated as an immunogen against *Leishmania infantum* infection in a murine model. BALB/c mice received saline were immunized with saponin or with RCP with or without an adjuvant. The results showed that RCP/saponin-vaccinated mice presented significantly higher levels of antileishmanial IFN-γ, IL-12 and GM-CSF before and after challenge, which were associated with the reduction of IL-4 and IL-10 mediated responses. These animals showed significant reductions in the parasite burden in all evaluated organs, when both limiting dilution and quantitative real-time PCR techniques were used. In addition, the protected animals presented higher levels of parasite-specific nitrite, as well as the presence of anti-*Leishmania* IgG2a isotype antibodies. In conclusion, the RCP/saponin vaccine could be considered as a prophylactic alternative to prevent against VL.

KEYWORDS

immune response, *Leishmania infantum*, recombinant chimeric protein, T-cell epitopes, vaccine, visceral leishmaniasis

1 | INTRODUCTION

Leishmaniasis is a parasitic disease complex caused by different *Leishmania* species, which affects primarily the poorest regions in 98 countries worldwide, where the main clinical manifestations comprise the visceral and tegumentary leishmaniasis.[1,2] Post-kala-azar dermal leishmaniasis is considered a clinical complication of visceral leishmaniasis (VL), in areas where *Leishmania donovani* species is endemic.[3] About two millions of new cases of leishmaniasis are registered annually, of which between 200 000 and 400 000 are associated with VL, leading to approximately 20 000-30 000 deaths per year.[4]

Although parasites are distributed worldwide, in some geographic regions, more than one *Leishmania* species is found as responsible to cause clinical manifestations in infected mammalian hosts. This is the

case of the Americas, where Brazil is responsible by 90% of the VL cases, and there are a high number of cases of tegumentary leishmaniasis (TL) being diagnosed.[2,5] The treatment of leishmaniasis is mainly based on the administration of the pentavalent antimonials; however, it is hampered due to the severe side effects observed in the patients, as well as by increase in the parasite resistance.[6-9] In this context, alternative approaches for disease control are desirable and, although an effective human vaccine did not exist, *Leishmania* species are related antigenically, raising the possibility for developing cross-protective vaccines.[10]

It is postulate that to be considered as an effective vaccine candidate against leishmaniasis, a formulation should present components either shared by different *Leishmania* species or composed of antigens expressed in both parasite stages.[11] Like previously reported,[12] the use of vaccines combining immunogenic proteins could provide benefits in terms of protection efficacy against distinct *Leishmania* species.[13] However, there are a few studies evaluating multi-antigenic candidates as vaccine to protect against leishmaniasis,[14-16] as studies have been developed evaluating single antigens or usually employed these immunogens to protect against one parasite species.[17-22]

The development of multiproteins or polypeptides-based vaccines requires the adequate choice of the biological targets to be used in the formulation. Recently, proteins were identified in protein extracts from amastigote and promastigote forms of *Leishmania infantum* by an immunoproteomic approach, using sera of VL dogs,[23] postulating their biological use on leishmaniasis. Some of these proteins were successfully evaluated as vaccine candidates against infection caused by this parasite species. LiHyp1 (XP_001468941.1),[19] which was characterized as a hypothetical amastigote-specific protein conserved between different *Leishmania* species, LiHyp6 (XP_001568689.1) and IgE-dependent histamine-releasing factor (HRF) (CAJ05086.1),[12] amastigote and promastigote-specific proteins of the parasites, respectively; and LiHyV (XP_888524.1),[16] a hypothetical protein also found conserved in *Leishmania*, were all showed to be individually protective against *L. infantum*, being able to mount a Th1 immune response in the vaccinated hosts, which was primed by high levels of IFN-γ and a antileishmanial nitrite production.[16]

The major advantages derived from the use of recombinant proteins have been based in terms of standardization and purity of production.[20] On the other hand, although whole proteins had been well studied,[16-19,21,22] the protective immunity is known to be triggered by both $CD4^+$ and $CD8^+$ T-cells epitopes.[24,25] Bioinformatics approaches have utilized a number of algorithms for predicting epitopes, HLA-binding, transporter of antigen processing affinity and proteasome cleavage, aiming to identify those with a higher probability to be immunogenic and, consequently, able to induce protection against pathogens.[16,26,27]

In this context, in this study, specific $CD4^+$ and $CD8^+$ T-cell epitopes derived from the LiHyp1, LiHyp6, LiHyV and HRF proteins were identified using two bioinformatics assays, and a recombinant chimeric protein (RCP) was constructed and evaluated as a vaccine candidate against *L. infantum*. The purpose of this study was to investigate the immunogenicity and protective efficacy of a new protein, based on *Leishmania*-specific T-cells epitopes derived of different immunogenic proteins and found conserved between different parasite species, aiming to develop a new biotechnological product that could be used in future as a protective vaccine against both tegumentary and visceral leishmaniasis.

2 | MATERIAL AND METHODS

2.1 | Animals

Female BALB/c mice (8 weeks age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil. The Committee on the Ethical Handling of Research Animals (CEUA) from the UFMG approved this study (code number 333/2015). To evaluate the lymphoproliferation, the study was also approved by Ethics Committee from UFMG (protocol number CAAE−323431 14.9.0000.5149).

2.2 | Parasites

Leishmania infantum (MOM/BR/1970/BH46) strain was used. Stationary promastigotes were grown at 24°C in complete Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), which was composed of Schneider's medium plus 20% heat-inactivated foetal bovine serum (FBS, Sigma-Aldrich), 20 mmol/L L-glutamine, 100 U/mL penicillin and 50 μg/mL streptomycin, at pH 7.4. The soluble antigen (SLA) extract of the parasites was prepared as previously described.[11]

2.3 | Cloning, expression and purification of the recombinant proteins

For the construction of the RCP, amino acid sequences of the LiHyp1, LiHyp6, LiHyV and HRF proteins were subjected to bioinformatics assays, aiming to select rich regions containing immunogenic epitopes and specific to murine and human T-cells haplotypes. For the prediction of $CD8^+$ T-cell epitopes, the NetCTLPan program (version 1.1) was used, where epitopes able to bind to A2, A3 and B7 alleles of human MHC class I with a peptide length of nine amino acids, weight on C-terminal cleavage site of 0.225, weight on TAP transport efficiency of 0.025 and threshold of epitope identification of 1.0 were identified. These alleles represent more than 90% of the human population of any ethnic group.[28,29] The program was also used to identify epitopes able to bind to H-2-Kd, H-2-Ld and H-2-Dd alleles from BALB/c mice, using the same parameter values described above. For the prediction of specific $CD4^+$ T-cell epitopes, the NetMHCII 2.2 server was used. It evaluates the binding affinity of peptides to 26 alleles from humans.[30] The epitopes able to interact with a binding affinity lower than 500 nmol/L and, at least, 30% of the alleles were selected. This server was also used to identify epitopes able to bind to the I-Ad and I-Ed alleles from BALB/c mice, using the same parameter values. The antigenic regions rich in specific T-cell epitopes from humans, but not specific to B-cell epitopes, were included in the chimeric

protein sequence (Fig. S1). Then, it was submitted for a selection using specific codons, aiming its expression in *Escherichia coli* with the web codon optimization tool (https://www.idtdna.com/CodonOpt) and optimized to reduce the presence of intramolecular interactions of messenger RNA, being calculated with the MFOLD Program.[31] The RCP was synthesized by GenScript® (USA) and expressed in an Artic Express strain (DE3, Agilent Technologies, Stevens Creek Blvd., Santa Clara, CA, 95051, USA) using 1 mmol/L of IPTG for 24 hours at 12°C. After, the protein was purified in a nickel affinity column. Like technically described to rLiHyp1,[29] rLiHyp6,[12] rLiHyV[16] and rHRF[12] proteins, the antigens were expressed and purified as recombinant proteins. After, all of them were passed on a polymyxin-agarose column (Sigma-Aldrich) to remove the residual endotoxin content (less than 10 ng of lipopolysaccharide per 1 mg of protein, measured by the Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000, BioWhittaker, Walkersville, USA, was detected).

2.4 | Validating the recombinant chimeric protein by a Western blot assay

To validate the antigenicity of the RCP, Western blot assays were performed. For this, sera obtained in previous studies evaluating the rLiHyp1,[29] rLiHyp6,[12] rLiHyV[16] and rHRF[12] proteins as vaccine candidates were used. In these previous studies, the recombinant proteins were individually administered associated with saponin in BALB/c mice, and serum samples were collected before challenge infection. All of these sera showed high levels of IgG antibodies against the recombinant proteins used in the immunization protocols, as well as to parasite extracts. As controls, sera from *L. infantum*-infected mice of those immunized with RCP/saponin or from naive (noninfected and nonvaccinated) mice were used. The RCP (10 µg) was submitted to a 12% SDS-PAGE and blotted onto a nitrocellulose membrane (0.2 µm pore size, Sigma-Aldrich). After, blots were incubated with the pools of sera listed above, and the technical protocol was developed as accordingly described.[12]

2.5 | Experimental design

2.5.1 | Vaccination, challenge infection and follow-up or the infection

Mice (n=16, per group) were vaccinated subcutaneously in their left hind footpad with 25 µg of RCP with or without 25 µg of saponin (*Quillaja saponaria* bark saponin, Sigma-Aldrich). Additional mice received saponin (25 µg) or saline (diluent). Three doses were administered at two-week intervals as accordingly described.[12] Thirty days after the third immunization, mice (n=8 per group) were euthanized and serum samples and spleen were collected to evaluate the immune response. At the same time, the remaining animals (n=8 per group) were subcutaneously infected in their right hind footpad with 1×10^7 stationary promastigotes of *L. infantum*. Animals were followed by 10 weeks, when they were euthanized and serum samples, spleen, liver, bone marrow (BM) and draining lymph nodes (dLN) were collected for the parasitological and immunological evaluations.

2.5.2 | Evaluation of the cellular response

Capture ELISA

Splenocyte cultures were performed 30 days after the last vaccine dose and before infection, as well as in the 10th week after challenge, as described.[19] Cells (5×10^6) were incubated in complete DMEM (negative control), which was composed of DMEM plus 20% FBS, 20 mmol/L L-glutamine, 200 U/mL penicillin and 100 µg/mL streptomycin, at pH 7.4, or separately stimulated with RCP, rLiHyp1, rLiHyp6, rLiHyV or rHRF proteins (10 µg/mL, each) or with *L. infantum* SLA (25 µg/mL), for 48 hours at 37°C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12 and GM-CSF levels were assessed in the culture supernatant by a capture ELISA (BD OptEIA TM set mouse kits, Pharmingen®, San Diego, CA, USA), following manufacturer's instructions. To evaluate the involvement of IL-12, and $CD4^+$ and $CD8^+$ T cells, spleen cells of RCP/saponin-vaccinated mice and later infected were in vitro stimulated with SLA (25 µg/mL) and incubated in the absence (control) or presence of 5 µg/mL of monoclonal antibodies (mAb) against mouse IL-12 (C17.8), CD4 (GK 1.5) or CD8 (53-6.7). Appropriate isotype-matched controls—rat IgG2a (R35-95) and rat IgG2b (95-1)—were employed in the assays. Antibodies (no azide/low endotoxin™) were purchased from BD (Pharmingen). The nitrite production was evaluated in the culture supernatants, using the Griess method.[32]

Flow cytometry

The spleen of the infected and vaccinated animals was removed, and in vitro cultures were performed as described.[33] The frequency of $CD4^+$ and $CD8^+$ T cells expressing intracytoplasmic cytokines (IFN-γ, TNF-α and IL-10) was determined following a conventional strategy analysis as previously described.[34] The results were expressed as indexes, which were determined by dividing the percentage of $CD4^+$ and $CD8^+$ cytokine-positive T cells in the stimulated culture, by this observed in the unstimulated control culture (stimulated/unstimulated culture ratio).

2.6 | PBMCs purification and CFSE labelling for proliferation assay

The peripheral blood mononuclear cells (PBMCs) were purified from blood samples of five healthy volunteers as accordingly described.[35] For proliferation assays, PBMCs (1×10^7 cells) were labelled using carboxy fluorescein diacetate succinimidyl ester (CFSE) and the in vitro cultures were performed as described,[36] with few modifications. Briefly, PBMCs were cultured in 48-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA), with each well containing 800 µL of complete RPMI medium. Then, these cells (1×10^6 per well) were added in triplicates to the wells together with 100 µL of RPMI medium (control culture) or stimulated with RCP or SLA (20 and 25 µg/mL, respectively). Incubation was carried out in a 5% CO_2 atmosphere at 37°C for 5 days, after which the PBMCs were removed and the lysing/fixation was performed, and cells were washed twice with FACS buffer and fixed in a FACS fixing solution (10 g/L paraformaldehyde, 10.2 g/L sodium cacodylate and 6.63 g/L sodium chloride, pH 7.2)

for storage at 4°C prior to flow cytometric acquisition and analysis. Flow cytometric measurements were performed on a FACScalibur® instrument (Becton Dickson, BD, USA), and the Cell-Quest™ software package (Franklin Lakes, NJ, USA) was used for data acquisition and analysis based on 30 000 events/sample.

2.7 | Humoral response

To analyse the antibody production, serum samples were collected from the vaccinated animals, 30 days after the last immunization and before infection, as well as in the 10th week after challenge. The IgG1 or IgG2a isotype levels were investigated against RCP, rLiHyp1, rLiHyp6, rLiHyV and rHRF proteins, as well as against SLA (1.0, 1.0, 1.0, 0.5, 0.5 and 2.0 μg per well, respectively), as described.[12] Serum samples were diluted at 1:200, and both anti-mouse IgG1 and IgG2a horseradish-peroxidase-conjugated antibodies (Sigma-Aldrich) were used in a 1:5000 dilution.

2.8 | Parasite burden

Spleen, liver, BM and dLN of the infected and vaccinated animals were collected and cultured to evaluate the parasite load, following a limiting-dilution protocol as described by[37] and modified by.[12] Results were expressed as the log of the titre (ie the dilution corresponding to the last positive well), adjusted per milligram of organ. In addition, the splenic parasite load was evaluated by a *q*PCR assay, as described.[34]

2.9 | Statistical analysis

The results were entered into Microsoft Excel (version 10.0) spreadsheets and analysed using GRAPHPAD PRISM$_{TM}$, Targetware Informatica Ltda, São Paulo, Brazil (version 6.0 for Windows). The statistical analysis was performed by one-way analysis of variance (ANOVA), followed by Bonferroni's post-test, which was used for multiple comparisons between the groups. Experiments were repeated once and results presented are representative of one of them. Bars in the graphs represent the mean plus standard deviation (SD) of the groups. Differences were considered significant when $P<.05$.

3 | RESULTS

3.1 | Constructing and validating the recombinant chimeric protein

In this study, two bioinformatics tools were used to predict *Leishmania*-specific T-cell epitopes from LiHyp1,[29] LiHyp6,[12] LiHyV[16] and HRF[12] proteins. To select MHC class I-specific epitopes, LiHyp1, LiHyp6, LiHyV and HRF proteins showed five, two, five and seven potential epitope sequences to human haplotypes, respectively, whereas the prediction to identify epitopes able to bind to H-2-Kd, H-2-Ld e H-2-Dd alleles from BALB/c mice showed that the LiHyp1, LiHyp6 and LiHyV proteins presented six, one and two potential epitope sequences, respectively (Table 1). To select the MHC class II-specific epitopes, the LiHyp6 and HRF proteins showed five and one potential epitope sequences to human haplotypes, respectively, whereas the prediction to BALB/c mice haplotypes identified six, five and ten potential epitope sequences in the LiHyp1, LiHyp6 and LiHyV proteins, respectively (Table 2). A schematic representation of the RCP is shown (Figure 1). Then, the protein was cloned, purified and its antigenicity was evaluated in immunoblotting assays (Figure 2). In the results, serum samples derived from mice that were vaccinated with the recombinant versions of the different antigens showed reactivity with the RCP, demonstrating the antigenicity of this new protein. In addition, any reactivity was detected when serum samples obtained from naive animals were used in the assays.

TABLE 1 In silico predicted human and mouse MHC class I—restricted epitopes of *Leishmania* proteins

MHC class I—human				
Protein	**No**	**Peptide sequence**	**Allele**	**Net CTL score**
LiHyp1	1	69-ILNDGRFQL-77	A2	1.008
	2	156-MVPDRSVYI-164	A2	0.748
	3	83-ASFMPLLER-91	A3	0.754
	4	77-LPPLPPASF-85	B7	0.801
	5	80-LPPASFMPL-88	B7	0.995
LiHyp6	6	37-SLATAFGLV-45	A2	0.853 92
	7	52-LLYRSTFRH-60	A3	0.694 86
HRF	8	87-YMAHIRSYM-95	A2	0.784 78
	9	111-FQTNAAAFV-119	A2	0.761 62
	10	88-MAHIRSYMK-96	A3	0.681 51
	11	112-QTNAAAFVK-120	A3	0.6143
	12	115-AAAFVKKVL-123	B7	0.4089
LiHyV	13	90-SMSMAITTV-98	A2	0.943 97
	14	69-VSGNGLTIK-77	A3	0.5355
	15	83-TPSSARLSM-91	B7	0.971 49
	16	97-TVAQSAITL-105	B7	0.509 25
	17	109-MPANSDIRI-117	B7	0.726 04
	18	116-RIVATTSSL-124	B7	0.780 72
	19	125-APAQSLFDF-133	B7	0.748 18
MHC class I—mouse				
Protein	**No**	**Peptide sequence**	**Allele**	**Net CTL score**
LiHyp1	1	77-LPPLPPASF-85	H-2-Ld	0.271 86
	2	79-PLPPASFMP-87	H-2-Ld	0.082 48
	3	165-MSGPARYVY-173	H-2-Dd	0.232 80
	4	59-DVYTRASDR-67	H-2-Kd	0.072 71
	5	83-ASFMPLLER-91	H-2-Kd	0.207 10
	6	156-MVPDRSVYI-164	H-2-Ld	0.231 40
LiHyp6	7	29-LTYAETVVS-37	H-2-Kd	–
LiHyV	8	82-STPSSARLS-90	H-2-Ld	0.113 16
	9	124-LAPAQSLFD-132	H-2-Ld	–

TABLE 2 In silico predicted human and mouse MHC class II–restricted epitopes of *Leishmania* proteins

MHC class II—human				
Protein	**No**	**Peptide sequence**	**Percentage of binding to alleles**	
LiHyp6	1	52-LLYRSTFRHAMLLRV-66	38.46	
	2	53-LYRSTFRHAMLLRVQ-67	38.46	
	3	54-YRSTFRHAMLLRVQR-68	38.46	
	4	55-RSTFRHAMLLRVQRE-69	42.31	
	5	56-STFRHAMLLRVQRET-70	38.46	
HRF	6	108-RKAFQTNAAAFVKKV-122	30.77	
MHC class II—mouse				
Protein (acession number)	**No**	**Peptide sequence**	**Allele**	**Net MHCII score**
LiHyp1	1	169-ARYVYFHMVLPVEAQ-183	H-2-IAd	394.4
	2	170-RYVYFHMVLPVEAQR-184	H-2-IAd	446.9
	3	171-YVYFHMVLPVEAQRF-185	H-2-IAd	161.2
	4	172-VYFHMVLPVEAQRFS-186	H-2-IAd	160.5
	5	173-YFHMVLPVEAQRFSL-187	H-2-IAd	169.2
	6	174-FHMVLPVEAQRFSLV-188	H-2-IAd	255.3
LiHyp6	7	55-RSTFRHAMLLRVQRE-69	H-2-IAd	204.8
	8	56-STFRHAMLLRVQRET-70	H-2-IAd	182.5
	9	57-TFRHAMLLRVQRETR-71	H-2-IAd	221.1
	10	58-FRHAMLLRVQRETRF-72	H-2-IAd	252
	11	59-RHAMLLRVQRETRFD-73	H-2-IAd	484.1
LiHyV	12	83-TPSSARLSMSMAITT-97	H-2-IAd	411.1
	13	84-PSSARLSMSMAITTV-98	H-2-IAd	347.9
	14	85-SSARLSMSMAITTVA-99	H-2-IAd	193.3
	15	86-SARLSMSMAITTVAQ-100	H-2-IAd	145.8
	16	87-ARLSMSMAITTVAQS-101	H-2-IAd	113.9
	17	88-RLSMSMAITTVAQSA-102	H-2-IAd	93.5
	18	89-LSMSMAITTVAQSAI-103	H-2-IAd	165.8
	19	90-SMSMAITTVAQSAIT-104	H-2-IAd	231.5
	20	97-TVAQSAITLSGVMPA-111	H-2-IAd	382.5
	21	98-VAQSAITLSGVMPAN-112	H-2-IAd	391

3.2 | Immune response generated by vaccination from BALB/c mice, before and after *L. infantum* infection

The cellular response was evaluated in the vaccinated animals, before and after challenge infection. Evaluating the immunogenicity of the vaccine candidate, animals immunized with RCP/saponin produced higher levels of protein and parasite-specific IFN-γ, IL-12 and GM-CSF, when compared to the values found in the control (saline and saponin) groups, using both RCP and SLA as stimuli (Figure 3A), as well as when the rLiHyp1, rLiHyp6, rLiHyV or rHRF proteins were used as stimuli (Figure 3B). RCP-immunized animals did not show difference in the cytokine levels in comparison with the control groups (data not shown). In all evaluated groups, IL-4 and IL-10 levels were low and similar between them. After challenge, this same immune profile was observed in the animals that were immunized with RCP/saponin, where higher levels of IFN-γ, IL-12 and GM-CSF were found, being also associated with lower levels of IL-4 and IL-10, using both RCP and SLA as stimuli (Figure 3C). Also, a high production of these cytokines was found when rLiHyp1, rLiHyp6, rLiHyV or rHRF proteins were used as stimuli (Figure 3D). On the other hand, animals from the control groups showed higher levels of anti-*Leishmania* IL-4 and IL-10 levels.

To evaluate the T-cell profile as responsible to produce IFN-γ in the RCP/saponin-vaccinated group, anti-CD4 and anti-CD8 monoclonal antibodies were added to the cultures of the spleen cells, which were stimulated with *L. infantum* SLA, and the levels of this cytokine were also evaluated by a capture ELISA. In the results, splenocytes of the infected and vaccinated animals showed significant reductions

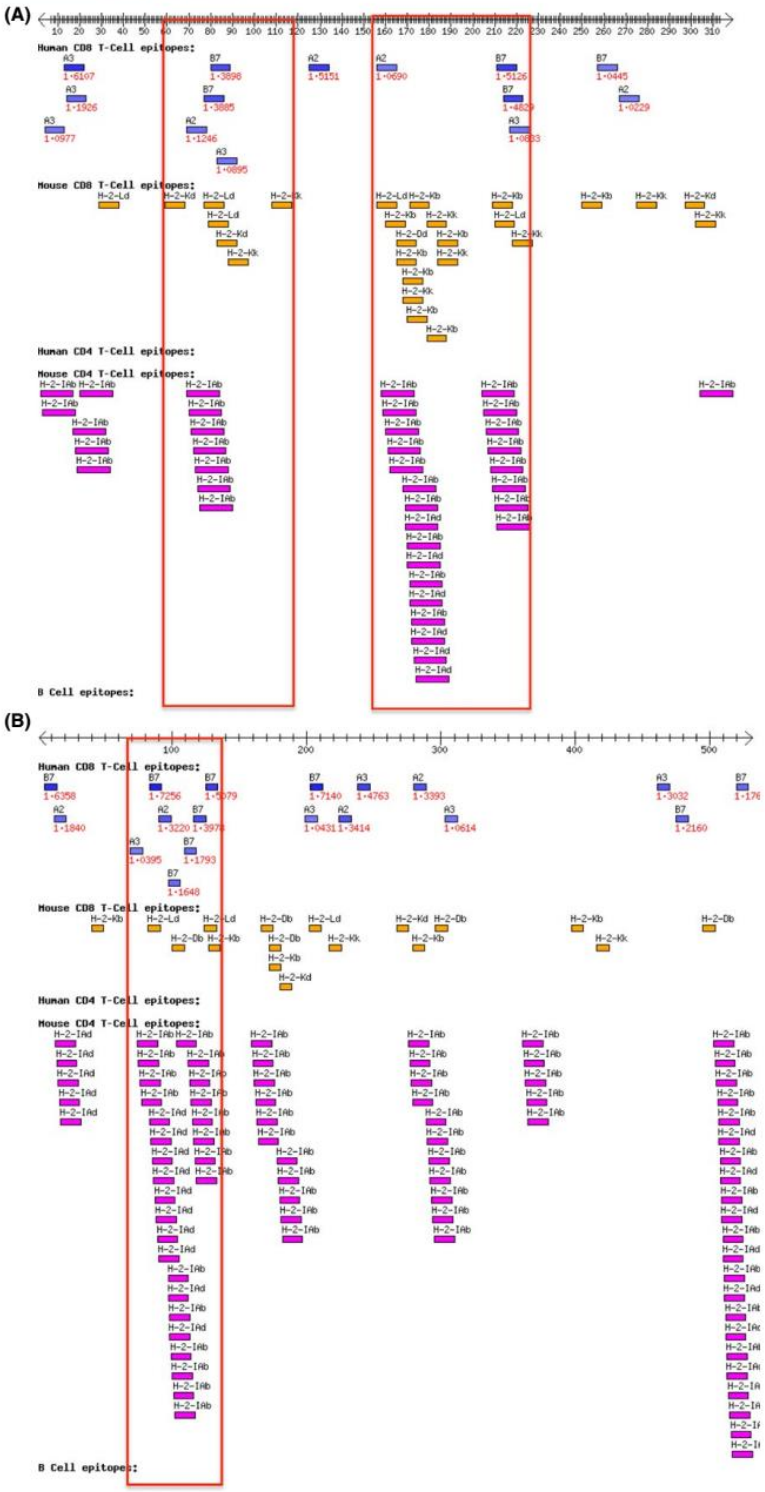


FIGURE 1 Schematic representation of the chimeric protein sequence. The high potential epitope density prediction and regions selection of the target proteins used in the construction of the recombinant chimeric protein sequence is shown. (A) Epitopes that were predicted in (A) LiHyp1 and (B) LiHyV. (B) Epitopes that were predicted in (C) LiHyp6 and (D) histamine-releasing factor. Arrowhead-ticked lines represent the protein sequence. Selected regions are shown inside red lines

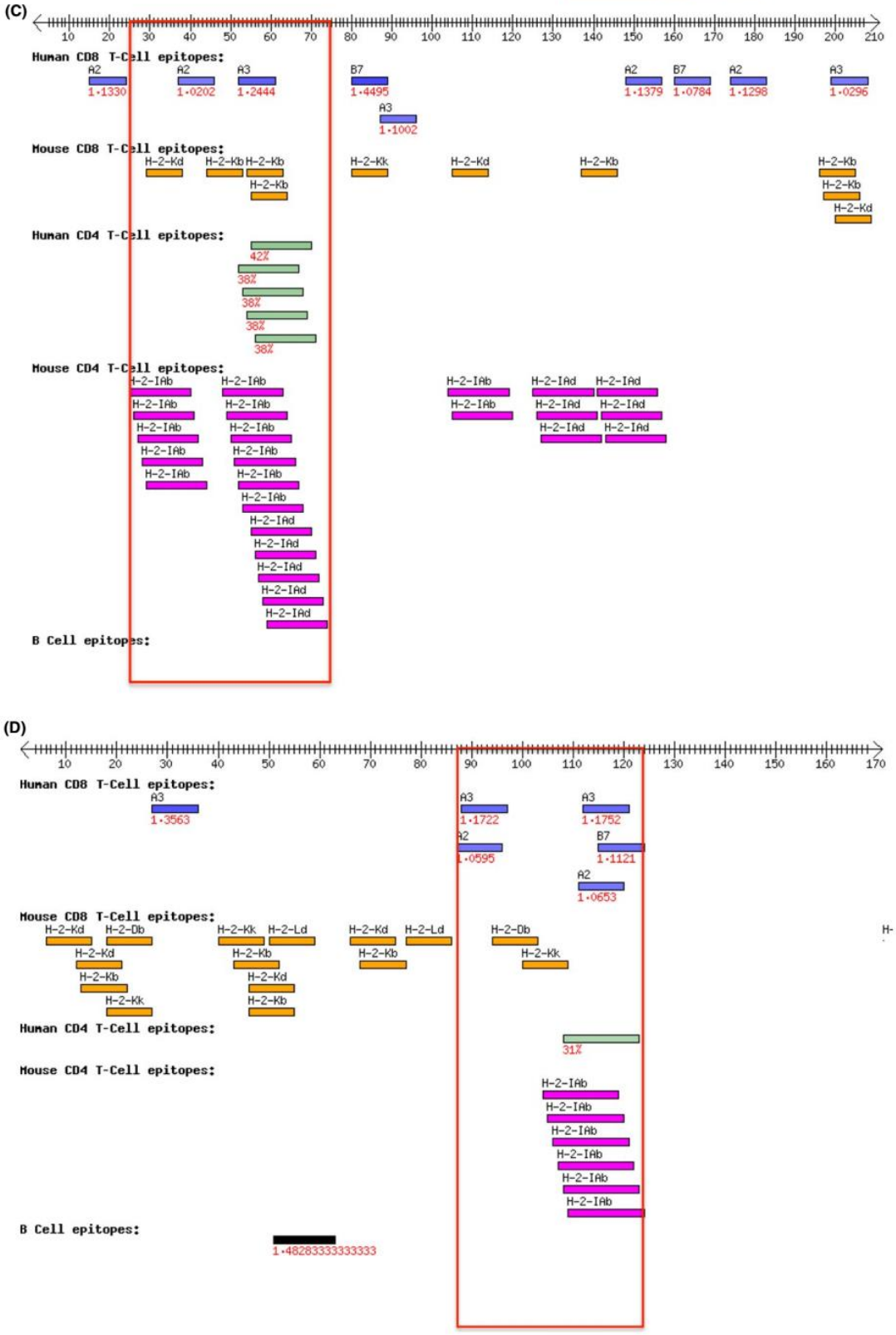


FIGURE 1 Continued

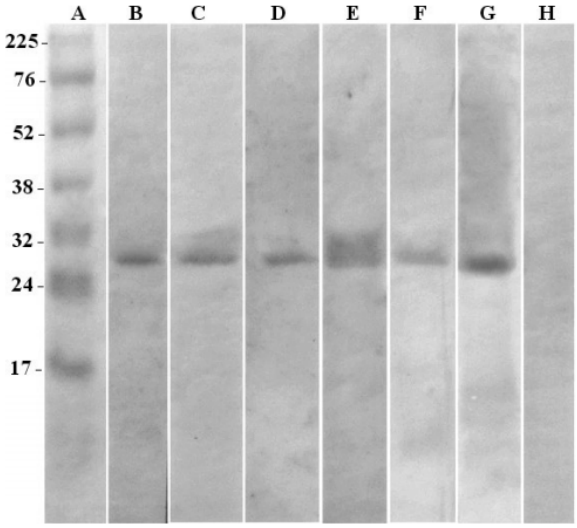


FIGURE 2 Antigenicity of the recombinant chimeric protein (RCP). Immunoblotting assays were performed to evaluate the antigenicity of this new protein. For this, the recombinant protein (10 µg, ~31.6 kDa) was submitted to a 12% SDS-PAGE and blotted onto nitrocellulose membrane. A low range protein ladder (Invitrogen™, Waltham, MA, USA) was used (lane A). Membranes were incubated with pools of sera from mice immunized with rLiHyp1/saponin (lane B), rLiHyV/saponin (lane C), rLiHyp6/saponin (lane D) or rHRF/saponin (lane E). As controls, sera of animals vaccinated with RCP/saponin (lane F), from those infected with *Leishmania infantum* promastigotes (lane G) or from naive mice (lane H) were used. Immunoblottings were derived from three independent experiments, and one preparation is shown

in the IFN-γ production when anti-CD4 or anti-CD8 antibodies were added into the cultures, in comparison with the noninhibited cell cultures (control); however, a more significant reduction in the IFN-γ production was found when the anti-CD4 antibody was used into the in vitro cultures (Figure 4). The T-cell profile was also evaluated by a flow cytometry assay and; in the results, it was possible to verify that both $CD4^+$ and $CD8^+$ T cells were also able to produce IFN-γ, when compared to the unstimulated cultures (Figure 5). Also, these cells showed low IL-10 producers, when compared to the control cells, demonstrating their Th1 immune profile. The flow cytometry analyses that were performed are also shown (Fig. S2).

When the lymphoproliferation from PBMCs obtained from healthy subjects using the RCP as a stimulus was evaluated, it was observed that this cellular population was able to proliferate in higher levels when *L. infantum* SLA was used as a stimulus (2.21%±1.21%), when compared to the stimulus using RCP (0.95%±0.28%). However, the chimeric protein showed a higher efficacy in stimulates PBMCs, when results were compared to those obtained using unstimulated cells (0.50%±0.37%). This fact can demonstrate the possible efficacy of RCP, after performing standardized studies from dose-response curves; it can be used as an immunogen in humans.

The nitrite production was also evaluated in the spleen cells of the infected and vaccinated animals. In the results, splenocytes of the RCP/saponin group showed levels of 14.4±1.5 µmol/L and 18.4±1.8 µmol/L, respectively, when RCP or SLA was used as stimuli. On the other hand, animals from the saline and saponin groups showed nitrite levels of 1.3±0.1 and 1.5±0.2 µmol/L, respectively, when the RCP was used as a stimulus, and of 1.0±0.1 and 1.8±0.2 µmol/L, respectively, when the SLA was used as a stimulus. Evaluating the antibody production in the infected and/or vaccinated animals, RCP/saponin group showed higher levels of protein and parasite-specific IgG2a isotype antibodies, when compared to the IgG1 levels, which were evaluated before (Figure 6A) and after (Figure 6B) challenge infection. On the other hand, mice from the saline and saponin groups produced higher levels of *Leishmania*-specific IgG1 isotype antibodies, when compared to the IgG2a levels.

3.3 | Protective efficacy from recombinant chimeric protein plus saponin in BALB/c mice against *L. infantum*

This study evaluated whether the immunization using the RCP, administered alone or in association with saponin, could protect BALB/c mice against *L. infantum* infection. After a 10-week follow-up period, infected animals were euthanized and their spleen, liver, dLN and BM were collected to evaluate the parasite load. In the results, significant reductions of the number of parasites were obtained in all evaluated organs of the RCP/saponin-vaccinated mice, when compared to those that received saline or saponin, when both a limiting-dilution technique (Figure 7A) and a qPCR assay (Figure 7B) were developed. In addition, the immunization of RCP without adjuvant was not protective against challenge (data not shown).

4 | DISCUSSION

Studies evaluating antileishmanial vaccine candidates have advanced in recent decades, due to the understanding of T-cell-mediated immunological mechanisms associated with the control of infection.[10,38] Most of the candidates have been evaluated as single recombinant molecules, being administered associated with immune adjuvants,[39] whereas a mixture composed of different immunogens based on the use of multiproteins or polypeptides-based vaccines has been less studied, although a formulation composed of immunogenic parts of distinct *Leishmania* proteins could present advantages, such as comprising a higher number of $CD4^+$ and/or $CD8^+$ T-cells epitopes of several antigens conserved between different *Leishmania* species, and could be protective against most of them.[29,40]

The success of candidate antigens to protect against *Leishmania* is dependent on the activation of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells to induce protection against infection.[41,42] $CD4^+$ T cells are activated in terms of recognition of peptide-MHC class II complexes in the surface of antigen processing cells (APCs). Activation of IFN-γ-producing $CD4^+$ T cells plays a main role in primary immune response against leishmaniasis. Specifically, IFN-γ mediates macrophages activation against parasite in a H_2O_2-dependent manner,[43,44] which is

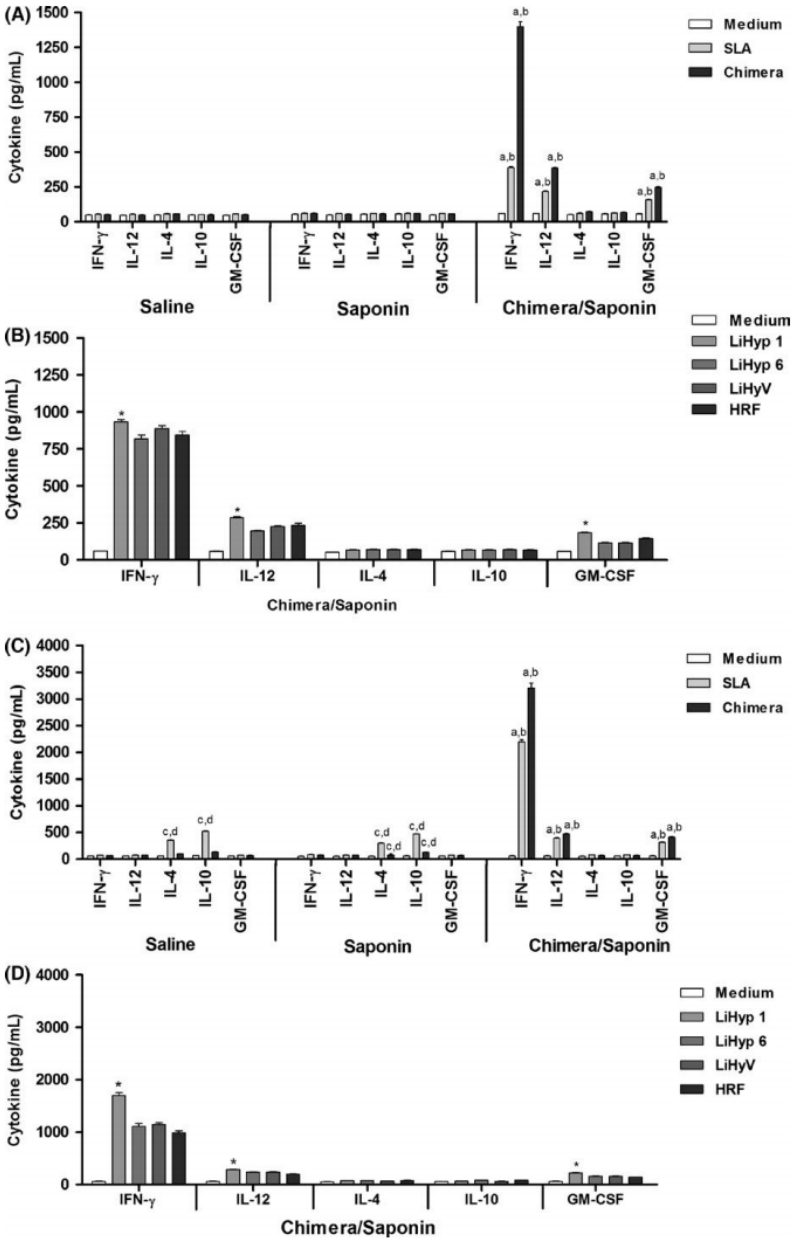


FIGURE 3 Cellular response induced before and after infection. Mice (n=16, per group) received saline or were immunized with saponin or recombinant chimeric protein (RCP) plus saponin. Thirty days after, animals (n=8) were euthanized and their spleen were collected and cultured in complete DMEM (negative control), or separately stimulated with the recombinant proteins (20 μg/mL, each) or *Leishmania infantum* SLA (25 μg/mL), for 48 h at 37°C in 5% CO_2. The other animals (n=8) were infected with *L. infantum* promastigotes and, 10 wks after, their spleen were collected and also used in the immunological evaluations. For this, IFN-γ, IL-12p70, GM-CSF, IL-4 and IL-10 levels were measured by a capture ELISA after both RCP and SLA stimuli, before (A) and after (C) challenge; as well as after the rLiHyp1, rLiHyp6, rLiHyV or rHRF stimuli, before (B) and after (D) infection. Bars represent the mean plus standard deviation (SD) of the groups. The letters *a*, *b* and *c* indicate statistically significant differences in relation to the saline, saponin and recombinant chimera plus saponin groups, respectively ($P<.05$). (*) indicates significant difference in relation to the rLiHyp6, rLiHyV and rHRF stimuli

based on NO production for killing them.[45] $CD8^+$ T cells are linked to a long-term immunological memory, which could be boosted by natural infections or by administration of new doses of the immunogens, although they participate of the primary response against parasites.[10]

Derived from this line of investigation, detection of the major antigenic determinants within the whole proteins and the identification of epitopes able to stimulate the development of a protective immune response against leishmaniasis are also the focus of current research.[29,46] It is noted that peptides are usually more stable, easier to produce and cheaper than recombinant proteins,[40] although their isolated use as peptide-based vaccine usually present low protective efficacy due to the immunological restriction to one protein of origin.[22] In the light of our knowledge, the protective characteristics of these vaccine candidates could be improved by increasing the number of doses of the vaccine, the amount of peptide used in each dose and/or even using carrier molecules.[47-49] In addition, the employ of vaccines combining $CD4^+$ and $CD8^+$ T-cell epitopes could optimize the protective efficacy of these candidates, when compared to their individual use.[20,50,51]

In this context, the present study aimed to develop a new chimeric protein based on *Leishmania*-specific $CD4^+$ and $CD8^+$ T-cell epitopes, which were identified in the amino acid sequences from LiHyp1,

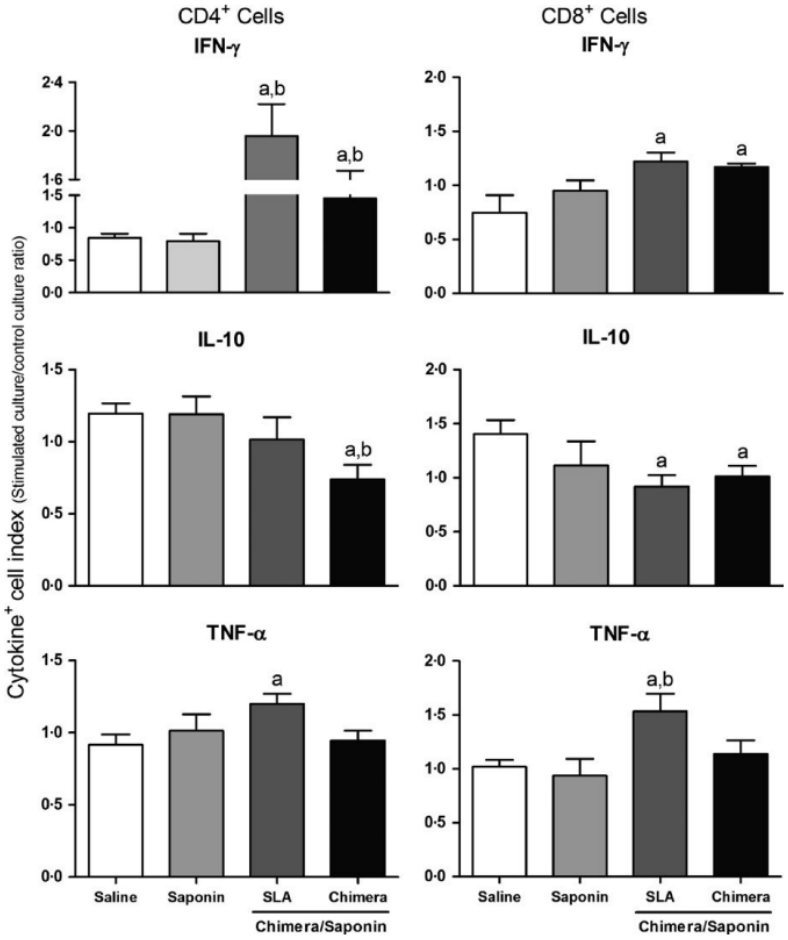


FIGURE 4 $CD4^+$ and $CD8^+$ T-cell involvement in the IFN-γ production. Mice (n=8 per group) were vaccinated and challenged with *Leishmania infantum* promastigotes. Ten weeks after, their spleen cells were collected, cultured in complete DMEM and stimulated with recombinant chimeric protein (20 μg/mL) or *L. infantum* SLA (25 μg/mL), for 48 h at 37°C in 5% CO_2; in the absence (control) or presence of 5 μg/mL of monoclonal antibodies (mAb) against mouse IL-12, $CD4^+$ or $CD8^+$, when the IFN-γ production was measured by a capture ELISA. Bars represent the mean plus standard deviation (SD) of the experimental groups. The letters *a* and *b* indicate significant differences in relation to the saline and saponin groups, respectively ($P<.05$)

LiHyp6, LiHyV and HRF proteins.[12,16,19] This new protein was composed of 56 epitopes, being 31 of them with high specificity to bind to mouse haplotypes (22 and 9 $CD4^+$ and $CD8^+$ T-cells epitopes, respectively) and 25 epitopes showing high specificity to bind to human haplotypes (6 and 19 $CD4^+$ and $CD8^+$ T epitopes, respectively). The choice to select epitopes able to bind to mice and human haplotypes was mainly based on the necessity to develop a new antigen able to stimulate the immune response in different mammalian hosts, since until the present moment, there are no available bioinformatics tools and that could be considered safe to perform predictions for $CD4^+$ and $CD8^+$ T-cells epitopes in dogs, considered also relevant mammalian hosts to *Leishmania* parasites.[52] In this context, and also due to the fact that most vaccine candidates against leishmaniasis are tested in BALB/c mice,[11,12,14,15] these two mammalian hosts were chosen to identify target epitopes and develop the RCP to be evaluated in the present study.

Here, the immunization using RCP/saponin induced a polarized Th1 immune response before infection, which was based on high levels of both protein and parasite-specific IFN-γ, combined with low levels of IL-4 and IL-10 in the spleen cells of the mice, as well as by higher levels of antileishmanial IgG2a isotype antibodies. After infection, the immune profile was maintained in the vaccinated animals, besides high levels of nitrite encountered, demonstrating a possible activation by this mechanism to eliminate internalized parasites, as described in other studies.[12,22,53] In addition, IL-12 and GM-CSF were also found in high levels in the protected animals. These cytokines are related to macrophages activation and resistance of the infected hosts against different *Leishmania* species, such as *L. infantum*,[19,53,54] *L. major*,[55] *L. amazonensis*[11,56] and *L. donovani*.[49] The use of the RCP without adjuvant was not protective against challenge infection, lighting up the necessity of the association of immune adjuvants with vaccine candidates against leishmaniasis, as described also by others.[11,19,57-59]

To investigate the T-cell subtypes profile involved in the immune response in the RCP/saponin group, the participation of the $CD4^+$ and $CD8^+$ T cells in the IFN-γ production was evaluated by adding monoclonal antibodies into the cultures. This strategy has permitted to evaluate the in vitro contribution of both T-cells subtypes in the IFN-γ production in experimental models, which are based on the block of antigen presentation.[12,19,22] The results showed that $CD4^+$ T cells were the main responsible to produce IFN-γ in the protected mice, as the deactivation of these cells using anti-CD4 monoclonal antibody significantly abrogated the parasite-dependent IFN-γ production.

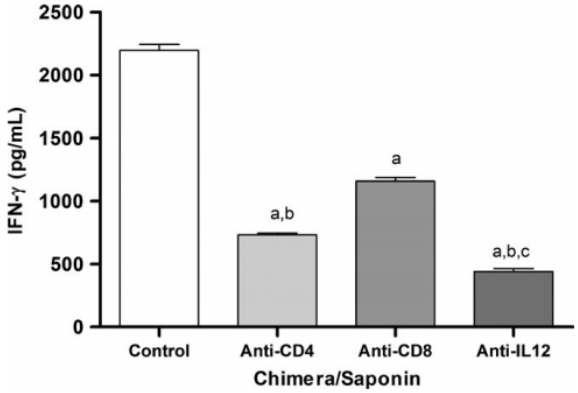


FIGURE 5 Intracytoplasmic cytokine indexes in the spleen cells of the infected and vaccinated animals. Cytokine indexes were determined by ratio between the values of cytokine-producing T cells obtained after stimulation with *Leishmania infantum* soluble antigen (SLA) or recombinant chimeric protein (RCP) in the control or stimulated cultures (SLA/CC or chimera/CC ratio). For this, mice (n=16 per group) were inoculated with saline (white rectangle), saponin (light grey rectangle) or with RCP/saponin (dark grey and black rectangles after the SLA or RCP stimuli, respectively) and challenged with *L. infantum*. Ten weeks after, their spleen cells (n=8) were collected and also stimulated. Results are showed as cytokine indexes (ratio between stimulated cultures/control cultures) in A, B and C for $CD4^+$ T cells, and in D, E and F, for $CD8^+$ cells. Bars represent the mean plus standard deviation (SD) of the groups. The letters *a* and *b* indicate significant differences in relation to the saline and saponin groups, respectively ($P<.05$)

However, other experiments such as those evaluating the depletion of T cells will be performed to validate the contribution of both T cells in the IFN-γ production in our experimental model.

When a flow cytometry assay was used to evaluate the intracellular cytokines production, the RCP/saponin-immunized animals and later challenged showed a higher ratio of IFN-γ^+-producing T cells, which was corroborated with lower levels of IL-10^+ T cells in the spleen cells of these animals, demonstrating that when the immune profile was evaluated by both a capture ELISA and flow cytometry, results converged to show a Th1 immune response that was developed in these protected animals.

In a previous study, Martins et al.[12] performed a study in BALB/c mice that were immunized with an equal combination of the rLiHyp1, rLiHyp6 and rHRF proteins plus saponin. Vaccinated animals were challenged with *L. infantum* and, 10 weeks after infection, they showed significant reductions in the parasite burden in all evaluated organs, when compared to mice that received saline or saponin, as controls. In the present study, although similar results of protection were obtained using the RCP/saponin combination to immunize animals, one formulation was produced, whereas in the previous study, three recombinant proteins were individually cloned, expressed and purified, reflecting possibly a higher cost of production in relation to our chimeric protein.

Besides recombinant proteins, DNA plasmid had been also studied as vaccine delivery against leishmaniasis.[56,59,60] These candidates are able to induce a Th1 immune response, resulting in a strong cytotoxic T-cell immunity. In addition, stability, long-term protection, ease of administration and cost-effectiveness are issues associated with this form of vaccine delivery. Das et al.[61] developed a DNA vaccine expressing proteins from different *Leishmania* species and found it to be immunogenic in subjects living in different endemic regions of leishmaniasis. In this context, our RCP, although had showed a satisfactory protection against VL, could be also evaluated as a DNA vaccine to protect against *Leishmania* infection. This fact could be also considered

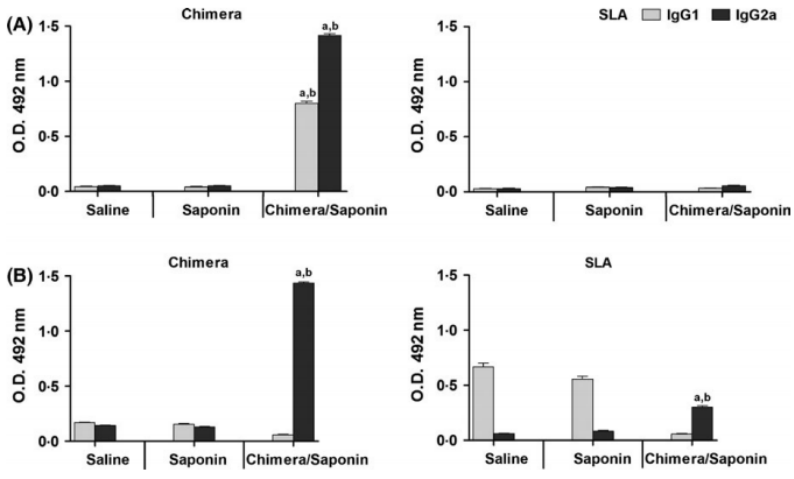


FIGURE 6 Humoral response generated in the infected and/or immunized animals. Mice (n=16 per group) were vaccinated and, 30 d after the last dose, their sera (n=8) were collected. In the other (n=8 per group), a challenge infection was performed using *Leishmania infantum* promastigotes, and 10 wks after, their sera were also collected. The IgG1 and IgG2a isotypes production against recombinant chimeric protein or *L. infantum* soluble antigen was evaluated, both before (A) and after (B) infection. Bars represent the mean plus standard deviation of the groups. The letters *a* and *b* indicate significant differences in relation to the saline and saponin groups, respectively ($P<.05$)

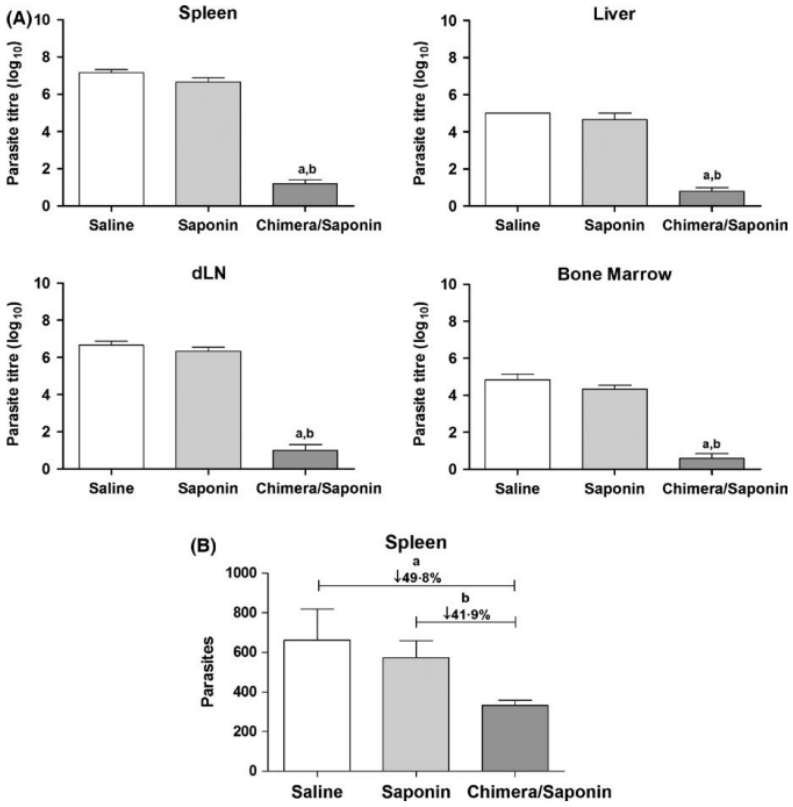


FIGURE 7 Evaluation of the parasite load in the infected and vaccinated animals. Mice (n=8, per group) were vaccinated and challenged with *Leishmania infantum* promastigotes. Ten weeks after, the parasite burden in the spleen, liver, infected paws' draining lymph nodes (dLN) and bone marrow was measured by a limiting-dilution (A) or *q*PCR (in spleen, B). Bars represent the mean plus standard deviation of the groups. The letters *a* and *b* indicate statistically significant differences in relation to the saline and saponin groups ($P<.05$)

since when PBMCs derived from healthy individuals were stimulated with RCP, a lymphoproliferation was observed in these cells, when compared to the unstimulated controls. Although *L. infantum* SLA had been able to induce a higher cellular proliferation, this preparation is composed of different *Leishmania* proteins, most of them considered as immunogenic. In this context, the results showed here can be considered to future studies evaluating RCP as a human vaccine to protect against VL.

As a limiting factor of the present study, the evaluation of vaccine efficacy was performed only one month after the last immunization, since our[16,19,22,53,58] and other groups[62,63] have evaluated vaccine candidates to protect against murine VL in this same period of time. In the absence of the evaluation of efficacy of the RCP/saponin composition at longer times before challenge, it cannot be discarded that vaccinated mice could maintain or eventually present a decrease in the prophylactic effect of RCP/saponin against challenge infection. In this context, further investigations are certainly necessary to be developed, aiming to evaluate different periods of time in the immunization schedules to define the longer time to our vaccine candidate guarantee protection against *L. infantum* infection. In conclusion, the present study's data could be taken as a proof-of-concept of the efficacy from a new recombinant chimeric protein plus saponin to induce protection against VL. Additional studies evaluating this protein against other *Leishmania* species causing either tegumentary or visceral leishmaniasis, as well as in other mammalian hosts, will be performed.

ACKNOWLEDGEMENTS

This work was supported by grants from Instituto Nacional de Ciência e Tecnologia em Nano-biofarmacêutica (INCT-NanoBiofar), FAPEMIG (CBB-APQ-00819-12 and CBB-APQ-01778-2014) and CNPq (APQ-482976/2012-8, APQ-488237/2013-0, and APQ-467640/2014-9). EAFC is a grant recipient of CNPq.

DECLARATION OF INTEREST

The authors hereby declare that there is no conflict of interest.

REFERENCES

1. World Health Organization. Control of the leishmaniases. World Health Organ Tech Rep Ser. 2010:22-26.
2. Alvar J, Vélez ID, Bern C, et al. Leishmaniasis worldwide and global estimates of its incidence. *PLoS One*. 2012;7:e35671.
3. Grimaldi G Jr, Tesh RB. Leishmaniasis of the New World: current concepts and implications for future research. *Clin Microbiol Rev*. 1993;6:230-250.
4. Singh S, Sharma U, Mishra J. Post-kala-azar dermal leishmaniasis: recent developments. *Int J Dermatol*. 2011;50:1099-1108.
5. Reithinger R, Dujardin J, Louzir H, Pirmez C, Alexander B, Brooker S. Cutaneous leishmaniasis. *Lancet Infect Dis*. 2007;7:581-596.
6. Croft SL, Coombs GH. Leishmaniasis: current chemotherapy and recent advances in the search for novel drugs. *Trends Parasitol*. 2003;19:502-508.

7. Minodier P, Parola P. Cutaneous leishmaniasis treatment. *Trav Med Infect Dis*. 2007;5:150-158.
8. Carvalho L, Luque-Ortega JR, Manzano JI, Castanys S, Rivas L, Gamarro F. Tafenoquine, an antiplasmodial 8-aminoquinoline, targets *Leishmania* respiratory complex III and induces apoptosis. *Antimicrob Agents Chemother*. 2010;54:5344-5351.
9. Chávez-Fumagalli MA, Ribeiro TG, Castilho RO, et al. New delivery systems for amphotericin B applied to the improvement of leishmaniasis treatment. *Rev Soc Bras Med Trop*. 2015;48:235-242.
10. Reed SG, Coler RN, Mondal D, Kamhawi S, Valenzuela JG. *Leishmania* vaccine development: exploiting the host-vector-parasite interface. *Expert Rev Vaccines*. 2016;15:81-90.
11. Coelho EAF, Tavares CAP, Carvalho FAA, et al. Immune responses induced by the *Leishmania* (*Leishmania*) *donovani* A2 antigen, but not by the LACK antigen, are protective against experimental *Leishmania* (*Leishmania*) *amazonensis* infection. *Infect Immun*. 2003;71:3988-3994.
12. Martins VT, Chávez-Fumagalli MA, Lage DP, et al. Antigenicity, immunogenicity and protective efficacy of three proteins expressed in the promastigote and amastigote stages of *Leishmania infantum* against visceral leishmaniasis. *PLoS One*. 2015;10:e0141496.
13. Beaumier CM, Gillespie PM, Hotez PJ, Bottazzi ME. New vaccines for neglected parasitic diseases and dengue. *Transl Res*. 2013;162:144-155.
14. Saljoughian N, Taheri T, Zahedifard F, et al. Development of novel prime-boost strategies based on a tri-gene fusion recombinant *L. tarentolae* vaccine against experimental murine visceral leishmaniasis. *PLoS Negl Trop Dis*. 2013;7:1-15.
15. Zahedifard F, Gholami E, Taheri T, et al. Enhanced protective efficacy of nonpathogenic recombinant *Leishmania tarentolae* expressing cysteine proteinases combined with a sand fly salivary antigen. *PLoS Negl Trop Dis*. 2014;8:e2751.
16. Martins VT, Duarte MC, Chávez-Fumagalli MA, et al. A *Leishmania*-specific hypothetical protein expressed in both promastigote and amastigote stages of *Leishmania infantum* employed for the serodiagnosis of, and as a vaccine candidate against, visceral leishmaniasis. *Parasit Vectors*. 2015;8:363.
17. Rachamim N, Jaffe CL. Pure protein from *Leishmania donovani* protects mice against both cutaneous and visceral leishmaniasis. *J Immunol*. 1993;150:2322-2331.
18. Agallou M, Smirlis D, Soteriadou KP, Karagouni E. Vaccination with *Leishmania* histone H1-pulsed dendritic cells confers protection in murine visceral leishmaniasis. *Vaccine*. 2012;30:5086-5093.
19. Martins VT, Chávez-Fumagalli MA, Costa LE, et al. Antigenicity and protective efficacy of a *Leishmania* amastigote-specific protein, member of the super-oxygenase family, against visceral leishmaniasis. *PLoS Negl Trop Dis*. 2013;7:e2148.
20. Joshi J, Kaur S. Studies on the protective efficacy of second-generation vaccine along with standard antileishmanial drug in *Leishmania donovani* infected BALB/c mice. *Parasitology*. 2014;141:554-562.
21. Baharia RK, Tandon R, Sharma T, et al. Recombinant NAD-dependent SIR-2 protein of *Leishmania donovani*: immunobiochemical characterization as a potential vaccine against visceral leishmaniasis. *PLoS Negl Trop Dis*. 2015;9:e0003557.
22. Lage DP, Martins VT, Duarte MC, et al. Prophylactic properties of a *Leishmania*-specific hypothetical protein in a murine model of visceral leishmaniasis. *Parasite Immunol*. 2015;37:646-656.
23. Coelho VTS, Oliveira JS, Valadares DG, et al. Identification of proteins in promastigote and amastigote-like *Leishmania* using an immunoproteomic approach. *PLoS Negl Trop Dis*. 2012;6:1-10.
24. Nico D, Gomes DC, Alves-Silva MV, et al. Cross-protective immunity to *Leishmania amazonensis* is mediated by $CD4^+$ and $CD8^+$ epitopes of *Leishmania donovani* nucleoside hydrolase terminal domains. *Front Immunol*. 2014;5:189.
25. Santos SC, Brodskyn CI. The role of $CD4^+$ and $CD8^+$ T cells in human cutaneous leishmaniasis. *Front Public Health*. 2014;2:165.
26. Nielsen M, Lundegaard C, Lund O, Kesmir C. The role of the proteasome in generating cytotoxic T-cell epitopes: insights obtained from improved predictions of proteasomal cleavage. *Immunogenetics*. 2005;57:33-41.
27. Tenzer S, Peters B, Bulik S, et al. Modeling the MHC class I pathway by combining predictions of proteasomal cleavage, TAP transport and MHC class I binding. *Cell Mol Life Sci*. 2005;62:1025-1037.
28. Stranzl T, Larsen MV, Lundegaard C, Nielsen M. NetCTLpan: pan-specific MHC class I pathway epitope predictions. *Immunogenetics*. 2010;62:357-368.
29. Bakker AH, Hoppes R, Linnemann C, et al. Conditional MHC class I ligands and peptide exchange technology for the human MHC gene products HLA-A1, -A3, -A11, and -B7. *Proc Natl Acad Sci USA*. 2008;105:3825-3830.
30. Nielsen M, Lund O. NN-Align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction. *BMC Bioinformatics*. 2009;10:296.
31. Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*. 2003;31:3406-3415.
32. Panaro MA, Acquafredda A, Lisi S, et al. Inducible nitric oxide synthase and nitric oxide production in *Leishmania infantum*-infected human macrophages stimulated with interferon-gamma and bacterial lipopolysaccharide. *Int J Clin Lab Res*. 1999;29:122-127.
33. Vieira PM, Francisco AF, Machado EM, et al. Different infective forms trigger distinct immune response in experimental Chagas disease. *PLoS One*. 2012;7:e32912.
34. Duarte MC, Lage DP, Martins VT, et al. A vaccine combining two *Leishmania braziliensis* proteins offers heterologous protection against *Leishmania infantum* infection. *Mol Immunol*. 2016;76:70-79.
35. Khamesipour A, Nateghi RM, Tasbihi M, et al. Phenotyping of circulating $CD8^+$ T cell subsets in human cutaneous leishmaniasis. *Microbes Infect*. 2012;14:702-711.
36. Roatt BM, Aguiar-Soares RD, Vitoriano-Souza J, et al. Performance of LBSap vaccine after intradermal challenge with *L. infantum* and saliva of *Lu. longipalpis*: immunogenicity and parasitological evaluation. *PLoS One*. 2012;7:e49780.
37. Titus RG, Marchand M, Boon T, Louis JA. A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. *Parasite Immunol*. 1985;7:545-555.
38. Palatnik-de-Sousa CB. Vaccines for leishmaniasis in the fore coming 25 years. *Vaccine*. 2008;26:1709-1724.
39. Fernandes AP, Coelho EAF, Machado-Coelho GLL, Grimaldi G, Gazzinelli RT. Making an anti-amastigote vaccine for visceral leishmaniasis: rational, update and perspectives. *Curr Opin Microbiol*. 2012;15:476-485.
40. Chávez-Fumagalli MA, Martins VT, Testasicca MC, et al. Sensitive and specific serodiagnosis of *Leishmania infantum* infection in dogs by using peptides selected from hypothetical proteins identified by an immunoproteomic approach. *Clin Vaccine Immunol*. 2013;20:835-841.
41. Okwor I, Uzonna J. Persistent parasites and immunologic memory in cutaneous leishmaniasis: implications for vaccine designs and vaccination strategies. *Immunol Res*. 2008;41:123-136.
42. Nateghi-Rostami M, Keshavarz H, Edalat R, et al. CD8+ T cells as a source of IFN-γ production in human cutaneous leishmaniasis. *PLoS Negl Trop Dis*. 2010;4:e845.
43. Murray HW, Byrne GI, Rothermel CD, Cartelli DM. Lymphokine enhances oxygen-independent activity against intracellular pathogens. *J Exp Med*. 1983;158:234-239.
44. Murray HW, Cartelli DM. Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes. Evidence for oxygen-dependent and -independent leishmanicidal activity. *J Clin Invest*. 1983;72:32-44.
45. Cunningham AC. Parasitic adaptive mechanisms in infection by *Leishmania*. *Exp Mol Pathol*. 2002;72:132-141.
46. Costa MM, Andrade HM, Bartholomeu DC, et al. Analysis of *Leishmania chagasi* by 2-D difference gel electrophoresis (2-D DIGE)

and immunoproteomic: identification of novel candidate antigens for diagnostic tests and vaccine. *J Proteome Res*. 2011;10:2172-2178.
47. Frankenburg S, Axelrod O, Kutner S, et al. Effective immunization of mice against cutaneous leishmaniasis using an intrinsically adjuvanted synthetic lipopeptide vaccine. *Vaccine*. 1996;14:923-929.
48. Topuzogullari M, Cakir KR, Dincer IS, et al. Conjugation, characterization and toxicity of lipophosphoglycan-polyacrylic acid conjugate for vaccination against leishmaniasis. *J Biomed Sci*. 2013;20:35.
49. Murray HW, Cervia JS, Hariprashad J, Taylor AP, Stoeckle MY, Hockman H. Effect of granulocyte-macrophage colony-stimulating factor in experimental visceral leishmaniasis. *J Clin Invest*. 1995;95:1183-1192.
50. Kharazmi A, Kemp K, Ismail A, et al. T-cell response in human leishmaniasis. *Immunol Lett*. 1999;65:105-108.
51. Seyed N, Taheri T, Vauchy C, et al. Immunogenicity evaluation of a rationally designed polytope construct encoding HLA-A*0201 restricted epitopes derived from *Leishmania major* related proteins in HLA-A2/DR1 transgenic mice: steps toward polytope vaccine. *PLoS One*. 2014;9:e108848.
52. Baneth G, Koutinas AF, Solano-Gallego L, Bourdeau P, Ferrer L. Canine leishmaniosis – new concepts and insights on an expanding zoonosis: part one. *Trends Parasitol*. 2008;24:324-330.
53. Lage DP, Martins VT, Duarte MC, et al. Cross-protective efficacy of *Leishmania infantum* LiHyD protein against tegumentary leishmaniasis caused by *Leishmania major* and *Leishmania braziliensis* species. *Acta Trop*. 2016;158:220-230.
54. Chávez-Fumagalli MA, Costa MAF, Oliveira DM, et al. Vaccination with the *Leishmania infantum* ribosomal proteins induces protection in BALB/c mice against *Leishmania chagasi* and *Leishmania amazonensis* challenge. *Microbes Infect*. 2010;12:967-977.
55. Dumas C, Muyombwe A, Roy G, et al. Recombinant Leishmania major secreting biologically active granulocyte-macrophage colony-stimulating factor survives poorly in macrophages *in vitro* and delays disease development in mice. *Infect Immun*. 2003;71:6499-6509.
56. Zanin FHC, Coelho EAF, Tavares CAP, et al. Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against *Leishmania chagasi* and *Leishmania amazonensis* experimental infections. *Microbes Infect*. 2007;9:1070-1077.
57. Ramírez L, Santos DM, Souza AP, et al. Evaluation of immune responses and analysis of the effect of vaccination of the *Leishmania major* recombinant ribosomal proteins L3 or L5 in two different murine models of cutaneous leishmaniasis. *Vaccine*. 2013;31:1312-1319.
58. Costa LE, Goulart LR, Jesus-Pereira NC, et al. Mimotope-based vaccines of *Leishmania infantum* antigens and their protective efficacy against visceral leishmaniasis. *PLoS One*. 2014;9:e110014.
59. Coelho EA, Tavares CA, Lima KM, et al. Mycobacterium hsp65 DNA entrapped into TDM-loaded PLGA microspheres induces protection in mice against *Leishmania* (*Leishmania*) *major* infection. *Parasitol Res*. 2006;98:568-575.
60. Zarrati S, Mahdavi M, Tabatabaie F. Immune responses in DNA vaccine formulated with PMMA following immunization and after challenge with *Leishmania major*. *J Parasit Dis*. 2016;40:427-435.
61. Das S, Freier A, Boussoffara T, et al. Modular multiantigen T cell epitope-enriched DNA vaccine against human leishmaniasis. *Sci Transl Med*. 2014;6:234-256.
62. Iborra S, Soto M, Carrión J, Alonso C, Requena JM. Vaccination with a plasmid DNA cocktail encoding the nucleosomal histones of *Leishmania* confers protection against murine cutaneous leishmaniosis. *Vaccine*. 2004;22:3865-3876.
63. Duthie MS, Favila M, Hofmeyer KA, et al. Strategic evaluation of vaccine candidate antigens for the prevention of visceral leishmaniasis. *Vaccine*. 2016;34:2779-2786.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

5.6. Artigo 3 – Conclusão

A proteína recombinante quimérica, composta por epitopos de células T selecionados a partir das proteínas LiHyV, LiHyp1, LiHyp6 e HRF, foi capaz de induzir uma elevada produção de IFN-γ e uma reduzida produção de IL-4 e IL-10, apresentando desta forma um perfil de resposta imune Th1. A imunização de camundongos BALB/c com a proteína quimérica ainda foi capaz de reduzir a carga parasitária e de combater a infecção experimental por *L. infantum*. Esta estratégia vacinal, quando equiparada às outras avaliadas previamente, como, por exemplo, às poliproteínas, demonstrou possuir uma maior eficácia e um menor custo de produção, se fazendo de grande interesse para o uso em uma vacina.

5.7. Artigo 4 – Breve introdução e objetivo

O quarto e último artigo a ser apresentado foi intitulado de *"Uma proteína recombinante fusionada apresentando epitopos específicos ao MHC de classe I e II de humanos e camundongos protege contra a infecção por Leishmania amazonensis"*, e foi publicado na revista internacional *Cellular Immunology*.

Sabe-se que um antígeno vacinal desejável para as leishmanioses deve ser conservado em diversas espécies do parasito, induzir uma resposta imune Th1, ser seguro e apresentar um custo acessível à população. Foi percebido que o uso de uma quimera recombinante composta de peptídeos conservados em diferentes espécies de *Leishmania* spp, apresentou-se segura e com custo assecível, além de induzir uma resposta imune Th1 e proteção em camundongos BALB/c infectados por *L. infantum*. Desta forma, este trabalho teve por objetivo avaliar a imunogenicidade e a eficácia vacinal desta mesma quimera polipeptídica recombinante em camundongos BALB/c desafiados com *L. amazonensis*, uma das espécies responsáveis pela doença tegumentar em mamíferos.

Cellular Immunology 313 (2017) 32–42

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Research paper

A recombinant fusion protein displaying murine and human MHC class I- and II-specific epitopes protects against *Leishmania amazonensis* infection

Vívian T. Martins [a,b], Daniela P. Lage [b], Mariana C. Duarte [b,c], Ana Maria R.S. Carvalho [c], Lourena E. Costa [b], Tiago A.O. Mendes [a], Danniele L. Vale [c], Daniel Menezes-Souza [b,c], Bruno M. Roatt [c], Carlos A.P. Tavares [a], Manuel Soto [d], Eduardo A.F. Coelho [b,c,*]

[a] Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901 Belo Horizonte, Minas Gerais, Brazil
[b] Programa de Pós-Graduação em Ciências Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 30130-100 Belo Horizonte, Minas Gerais, Brazil
[c] Departamento de Patologia Clínica, Coltec, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901 Belo Horizonte, Minas Gerais, Brazil
[d] Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Departamento de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain

ARTICLE INFO

Article history:
Received 5 August 2016
Revised 24 October 2016
Accepted 25 December 2016
Available online 28 December 2016

Keywords:
Tegumentary leishmaniasis
Polypeptide vaccine
Th1 immune response
CD4+ T cells
CD8+ T cells
Leishmania amazonensis

ABSTRACT

Tegumentary leishmaniasis (TL) constitutes a major public health problem with significant morbidity worldwide. Synthetic peptide-based vaccines are attractive candidates to protect against leishmaniasis, since T cell-specific epitopes can be delivery to antigen-presenting cells, leading to the generation of a Th1 cell-mediated immunity. In this context, the present study aims to evaluate the immunogenicity and protective efficacy of a vaccine composed of major histocompatibility complex class I and II-restricted epitopes derived from four *Leishmania infantum* proteins to protect mice against *Leishmania amazonensis* infection. This recombinant fusion protein was administered in BALB/c mice alone or with saponin. As controls, animals received saline or saponin. In the results, the administration of the recombinant protein plus saponin induced a specific IFN-γ, IL-12 and GM-CSF production, as well as high IgG2a isotype antibody levels, which protected mice against a challenge using *L. amazonensis* promastigotes. Lower parasite burden was found in the infected footpads, liver, spleen and draining lymph node of vaccinated mice, when compared to those from the control groups. In addition, protection was associated with a lower IL-4 and IL-10 response, which was accompanied by the antileishmanial nitrite production by spleen cells of the animals. Interestingly, the recombinant protein administered alone induced a partial protection against challenge. In conclusion, this study shows a new vaccine candidate based on T cell-specific epitopes that was able to induce protection against *L. amazonensis* infection.

1. Introduction

Leishmaniasis is a disease caused by infection with the protozoan *Leishmania*, which is transmitted by the bite of an infected phlebotomine sand fly. It is a tropical disease threatening 350 million people in endemic regions of the world [63,2]. The drugs available to treat against disease are inadequate, since limitations such as side effects, parasite resistance and/or high cost have been observed. The main clinical manifestations of the disease are tegumentary and visceral leishmaniasis. Tegumentary leishmaniasis (TL) is the most frequent clinical form of the disease and is commonly found in Europe, Africa and Asia, being caused by *Leishmania major* species; whereas in the Americas, it is often caused by *L. mexicana*, *L. braziliensis* and *L. amazonensis* [26,18].

The experimental infection of BALB/c mice using *L. major* is one of the well-described models to evaluate the immune response associated with the T cell differentiation. Usually, this lineage develops a Th1 immune response and resistance to infection, when animals are infected with a low number of parasites [16]. However, when an infection using a high inoculum is performed in this

* Corresponding author at: Laboratório do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Av. Dr. Alfredo Balena, 30130-100, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.
E-mail address: eduardoferrazcoelho@yahoo.com.br (E.A.F. Coelho).

http://dx.doi.org/10.1016/j.cellimm.2016.12.008
0008-8749/

lineage, animals develop a Th2-type immune response and susceptibility to infection [8]. The resistance against disease is based on the development of antileishmanial Th1 immunity, which is characterized by the production of IFN-γ, IL-12 and other pro-inflammatory cytokines [23,47]. On the other hand, the production of high levels of IL-4, IL-10 and other anti-inflammatory cytokines usually allows to the development of the infection in these animals [46,59].

Leishmania amazonensis is one of the major etiologic agents of leishmaniasis, being associated with a broad spectrum of diseases in humans ranging from cutaneous to the potentially fatal visceral disease [6,56]. This species presents a wide geographical distribution in the Americas, which overlaps with transmission areas of other parasite species [25]. BALB/c mice are susceptible to the *L. amazonensis* infection, and they develop a Th2 response against infection, which is demonstrated by secretion of high levels of parasite-specific IL-4 and IL-10, accompanied by the antileishmanial IgG1 isotype antibodies production [11,50,52].

The understanding of the aspects related to the protective immunity is a requirement for the development of vaccines to protect against diseases, including the selection of both immunogens and adjuvants [27,54]. Since the development of Th1 immune response is important to protection against leishmaniasis [11,37,38], molecules able to stimulates this response could be considered as possible candidates to protect against *Leishmania* infection [53,35,36]. In addition, unlike other protozoa, parasites do not undergo significant antigenic variation, having a single host cell, the macrophage, although they can also interact with other cells, such as neutrophils and dendritic cells. They also present a single morphological form inside of these cells, the amastigote, which is associated with pathology in the infected mammalian hosts [54].

The use of immunogens able to protect against different *Leishmania* species has been encouraged by the World Health Organization, since these products could protect against both tegumentary and visceral leishmaniasis [63]. In this way, in a recent immunoproteomic study performed, VL dogs sera were employed to identify antigenic proteins in *L. infantum* amastigote and promastigote extracts [12]. As a consequence, four proteins were shown to be individually protective in mice against *L. infantum* infection: LiHyp1 (XP_001468941.1) [37], LiHyp6 (XP_001568689.1), IgE-dependent histamine-releasing factor (CAJ05086.1) [38] and LiHyV (XP_888524.1) [39].

In the present study, $CD4^+$ and $CD8^+$ T cell-specific epitopes derived for these four proteins were identified according to their binding affinity to humans and mice haplotypes, aiming to construct a new fusion protein to be tested as a vaccine candidate able to protect against *Leishmania* infection. A chimeric protein containing these T cell-specific epitopes was obtained as a recombinant fusion protein after cloning and expressing a gene chemically synthesized. Then, the resulting protein was purified and evaluated in BALB/c mice as a vaccine to cross-protect against *L. amazonensis* infection. Besides the construction of this new protein, immunological and parasitological parameters were investigated in the vaccinated and infected animals, aiming to evaluate the efficacy of this new candidate to protect against TL.

2. Materials and methods

2.1. Ethics

This study was approved by the Committee on the Ethical Handling of Research Animals (CEUA) from the Federal University of Minas Gerais (UFMG), with the code number 0333/2015.

2.2. Mice and parasites

BALB/c mice (female, 8 weeks age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, UFMG. Animals were maintained under specific pathogen-free conditions. Stationary-phase promastigotes of *L. amazonensis* (IFLA/BR/1967/PH-8) were grown at 24 °C in complete Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), which was composed by Schneider's medium plus 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 20 mM L-glutamine, 100 U/mL penicillin, and 50 μg/mL streptomycin, at pH 7.4. The soluble antigenic extract of the parasites (SLA) was prepared from 1×10^9 stationary-phase promastigotes, as described [11].

2.3. Construction and purification of the recombinant proteins

For the construction of the recombinant fusion protein, the amino acid sequences of the LiHyp1, LiHyp6, LiHyV and HRF proteins were subjected to bioinformatics assays, aiming to select rich regions containing immunogenic epitopes and specific to bind to murine and human T cell haplotypes. When the prediction to $CD8^+$ T cell epitopes was performed, the NetCTLPan program (version 1.1) was used, where epitopes able to bind to A2, A3 and B7 alleles of human MHC class I with a peptide length of 9 amino acids, weight on C-terminal cleavage site of 0.225, weight on TAP transport efficiency of 0.025 and threshold of epitope identification of 1.0, were selected. These alleles represent more than 90% of the human population of any ethnic group [4,58]. The program was also used to identify epitopes able to bind to H-2-Kd, H-2-Ld and H-2-Dd alleles from BALB/c mice, by using the same parameter values as described above. For the prediction of $CD4^+$ T cell-specific epitopes, the NetMHCII 2.2 server was used. It evaluates the binding affinity of peptides to 26 alleles from humans [44]. The epitopes able to interact with a binding affinity lower than 500 nM and, at least, 30% of the alleles, were selected. This server was also used to identify epitopes able to bind to the I-Ad and I-Ed alleles from BALB/c mice, by using the same parameter values. The antigenic regions rich in T cell-specific epitopes from humans, but not specific to B-cell epitopes, were included in the protein sequence. Then, it was submitted for the selection by using specific codons, aiming its expression in *Escherichia coli* with the web codon optimization tool (https://www.idtdna.com/CodonOpt), and optimized to reduce the presence of intramolecular interactions of messenger RNA, being calculated with the MFOLD Program [62]. The recombinant plasmid sequence containing the fusion protein was synthesized by GenScript® (USA) and transformed into Artic Express strain (DE3, Agilent Technologies, USA), where the over-expression of the recombinant protein was performed by adding 1.0 mM IPTG (isopropyl-β-D-thiogalactopyranoside, Promega, Montreal, Canada), for 24 h at 12 °C. For protein purification, cells were lysed by a homogenizer after five passages through the apparatus. The product was centrifuged at 13,000×*g* for 20 min at 4 °C. The recombinant fusion protein, containing a tag of 6× residues of histidine fused at its *N*-terminal region, was purified under non-denaturing conditions, using a 5 mL His-Trap column (GE Healthcare Life Science), attached to an FPLC (GE Healthcare Life Science) system. The, it was dialyzed using saline as a buffer. The other recombinant proteins used in this study were cloned and purified according described to LiHyp1 [37], LiHyp6 [38], HRF [38] and LiHyV [39]. All recombinant proteins were passed on a polymyxin-agarose column (Sigma-Aldrich) in order to remove the residual endotoxin content (less than 10 ng of lipopolysaccharide per 1 mg of protein, measured by the Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000, BioWhittaker, USA).

2.4. Immunization and challenge infection

BALB/c mice (n = 12, per group) were subcutaneously vaccinated in their left hind footpad with 25 μg of recombinant fusion protein, alone or associated with 25 μg of saponin (*Quillaja saponaria* bark saponin, Sigma-Aldrich). Additional animals received saponin (25 μg) or saline. Three doses were administered at two-week intervals. Thirty days after the last immunization, mice (n = 6 per group) were euthanized when spleen and serum samples were collected to evaluation of the immune response. At the same time, the remaining animals (n = 6, per group) were subcutaneously challenged in their right hind footpad with 1×10^6 stationary-phase promastigotes of *L. amazonensis*. The footpad swelling was monitored weekly, during 10 weeks using a metric caliper. After, animals were euthanized and serum samples, tissue and organs were collected to evaluate some immunological and parasitological parameters associated to the vaccination and challenge infection. Experiments were repeated and presented similar results.

2.5. Limiting dilution assay

The infected footpad, spleen, liver and draining lymph node (dLN) of the mice were collected 10 weeks after *L. amazonensis* infection. A limiting-dilution protocol was developed to evaluate the parasite burden, as described in Titus et al. [60], with modifications performed by Martins et al. [37]. Results were expressed as the log of the titer (*i.e.*, the dilution corresponding to the last positive well), adjusted per milligram of tissue or organ.

2.6. qPCR assay

The parasite load in the infected footpads was also evaluated by a *q*PCR assay. For this, lesion fragments DNA was extracted using a phenol-chloroform method, as described [20]. The resulting material was resuspended in 100 μL milli-Q water and amplified using the oligonucleotides: *forward* (5′-CCTATTTTACACCAACCCCCAGT-3′) and *reverse* (5′-GGGTAGGGGCGTTCTGCGAAA-3′). Standard curves were obtained from DNA extracted from 1×10^8 parasites under the same conditions used to perform the samples extraction. PCR was performed in StepOne™ Instrument (48 wells-plate, Life Technologies USA) using 2× SYBR® Green PCR Master Mix (5 μL; Applied Biosystems USA), with 2 mM of each primer (1 μL) and 4 μL of DNA (5 ng/μL). The samples were incubated at 95 °C for 10 min, and then submitted to 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and during each time, fluorescence data were collected. The parasite load was calculated by the interpolation with the standard curve included in the same run. Assays were performed in duplicate. Data are expressed as the parasite number per total DNA [21].

2.7. Cytokine and nitrite production

Spleen cells were collected from the animals 30 days after the last vaccine dose and before infection (n = 6), as well as in the 10th week after challenge (n = 6), as described in Martins et al. [37]. Cell cultures were performed in duplicate (5×10^6 cells/mL) in 24-well plates (Nunc, Nunclon), incubated in complete DMEM (background control), which was composed of DMEM plus 20% FBS, 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, pH 7.4. For specific stimulation, cells were separately cultured in the presence of rLiHyp1, rLiHyp6, rLiHyV, rHRF or the fusion protein (10 μg/mL, in all cases). Also, *L. amazonensis* SLA (25 μg/mL) was employed for stimulation. All cultures were performed for 48 h at 37 °C, 5% CO_2. Concanavalin A (ConA, 5 μg/mL) was used as a positive control. IFN-γ, IL-4, IL-10, IL-12p70 and GM-CSF were measured in the supernatants by a capture ELISA (BD OptEIA TM set mouse, Pharmingen®, San Diego, CA, USA), following manufacturer's instructions. To evaluate the involvement of the $CD4^+$ and $CD8^+$ T cells in the IFN-γ production, spleen cells cultures were established from mice vaccinated with the fusion protein plus saponin and later infected with *L. amazonensis*. Splenocytes were stimulated with the recombinant fusion protein (10 μg/mL) or *L. amazonensis* SLA (25 μg/mL), in the absence (control) or presence of 5 μg/mL of monoclonal antibodies (mAb) against mouse CD4 (GK 1.5) or CD8 (53-6.7). Isotype-matched controls – rat IgG2a (R35-95) and rat IgG2b (95-1) – were employed in the assays. Antibodies (no azide/low endotoxin™) were purchased from BD (Pharmingen®). The nitrite production was also evaluated in the stimulated *in vitro* cultures by using the Griess method, as described in Lage et al. [35].

2.8. Humoral response

The humoral response was evaluated in the infected and/or immunized animals. For this, serum samples were collected 30 days after the last immunization and before infection, as well as in the 10th week after challenge. Reactions were developed against the rLiHyp1, rLiHyp6, rLiHyV and rHRF proteins, as well as against the recombinant fusion protein or *L. amazonensis* SLA. The IgG1 and IgG2a isotypes antibodies levels were determined by an ELISA assay, as described in [38]. Briefly, rLiHyp1, rLiHyp6, rLiHyV, rHRF, fusion protein and SLA (1.0, 1.0, 1.0, 0.5, 1.0 and 1.0 μg per well, respectively) were employed to coat plates. The serum samples were 1:100 diluted, and the anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich) were both used in a 1:10,000 dilution. Reactions were developed through incubation with ortho-phenylenediamine (2 mg), H_2O_2 30 vol (2 μL) in 10 mL of a citrate-phosphate buffer, pH 5.0. After incubation in the dark for 30 min, reactions were stopped by adding H_2SO_4 2 N (25 μL). The optical density was read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Concord, Canada), at 492 nm.

2.9. Immunoblotting assays

Immunoblotting experiments were performed to evaluate the antigenicity of recombinant fusion protein, by using serum samples of mice that were immunized with the fusion protein plus saponin, as well as with serum samples of *L. amazonensis*-infected mice (positive control) and samples from non-immunized and non-infected animals (negative control), as described in Martins et al. [38].

2.10. Statistical analysis

The results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed using GraphPad Prism™ (version 6.0 for Windows). The one-way analysis of variance (ANOVA) and the Student *t* test were used for comparisons between the groups. Vaccination experiments were repeated and presented similar results. Differences were considered significant when $P < 0.05$.

3. Results

3.1. Construction of the recombinant fusion protein

In the present study, bioinformatics tools were used to predict $CD4^+$ and $CD8^+$ T cell-specific epitopes to humans and mice haplotypes. Firstly, the NetCTLPan program was used to select MHC class I-specific epitopes. The LiHyp1, LiHyp6, LiHyV and HRF proteins

presented five, two, five and seven potential epitope sequences, respectively, able to bind with a high specificity to the A2, A3 and B7 human haplotypes (Table 1). When the mice haplotypes were evaluated, LiHyp1, LiHyp6 and LiHyV proteins presented six, one and two potential epitope sequences, respectively. Secondly, the NetMHCII 2.2 server was used to select MHC class II-specific epitopes. The LiHyp6 and HRF proteins showed, respectively, five and one potential epitope sequences to human haplotypes, whereas to the prediction of murine haplotypes, six, five and ten potential epitope sequences were identified in the LiHyp1, LiHyp6 and LiHyV proteins, respectively (Table 2).

3.2. Protection against L. amazonensis infection by immunization with the recombinant fusion protein plus saponin

The protective efficacy of the immunization using the recombinant fusion protein plus saponin was evaluated in BALB/c mice against *L. amazonensis* infection. Animals were vaccinated and later infected with a high inoculum of the parasites. When the parasite burden was evaluated, the mice group immunized with the fusion protein plus saponin presented significant reductions in the parasite load in the infected footpads, spleen, liver and dLN, when compared to the saline (6.6-, 4.8-, 2.9- and 5.2-log, respectively) and saponin (6.0-, 3.4-, 2.4-, and 4.9-log, respectively) groups (Fig. 1A). Comparing the administration of the recombinant protein alone or with adjuvant, animals immunized with the protein plus saponin presented higher reductions when the parasite burden was evaluated in the infected footpads, spleen, liver and dLN, in the order of 4.4-, 2.6-, 2.0- and 4.0-log, respectively. However, animals immunized only with the fusion protein showed lower parasitism in the infected footpads, spleen, liver and dLN, when compared to the saline (3.0-, 2.3-, 1.5- and 3.2-log, respectively) and saponin (2.4-, 2.0-, 1.0-, and 2.8-log, respectively) groups. These results were corroborated by a *q*PCR assay, since the immunization using the recombinant protein plus saponin showed reductions in the order of 93.1% and 87.5% in the parasite number in the infected footpads, when compared to the saline and saponin groups, respectively. Similarly to the results found in the parasite evaluation, mice immunized with the fusion protein plus saponin showed significant reductions in the lesion average size, when compared to the other groups (Fig. 1B). Interestingly, mice immunized only with the fusion protein showed reduction in the footpad swelling, when compared with the results found to the saline and saponin groups, demonstrating an efficacy of this vaccine candidate against *Leishmania* infection, when administered without the association of an immune adjuvant.

3.3. Immune response developed in the recombinant fusion protein plus saponin-immunized mice, before and after infection

The cellular response was evaluated in the vaccinated animals, before and after challenge infection. Before challenge, spleen cells from mice immunized with the recombinant fusion protein plus saponin produced significantly higher anti-fusion protein and anti-parasite IFN-γ, IL-12, and GM-CSF levels, when compared with the results found in the other groups (Fig. 2). On the other hand, IL-4 and IL-10 levels were low and similar in all groups. When animals were immunized only with the fusion protein, higher levels of IFN-γ, IL-12, and GM-CSF were also found in relation to the saline and saponin groups. After challenge, the Th1 immunity was maintained in the fusion protein/saponin-vaccinated group, when both the protein and *L. amazonensis* SLA were used as stimuli of the spleen cells (Fig. 3). However, animals from the saline and saponin groups showed higher anti-protein and anti-parasite IL-4 and IL-10 levels, when compared to the results obtained in the recombinant fusion protein/saponin-immunized mice group.

Table 1
In silico predicted murine and human MHC class I-specific epitopes for *Leishmania* proteins.

Protein	Nº	Peptide sequence	Allele	Net CTL Score
MHC class I – human				
LiHyp1	1	69-ILNDGRFQL-77	A2	1,008
	2	156-MVPDRSVYI-164	A2	0,748
	3	83-ASFMPLLER-91	A3	0,754
	4	77-LPPLPPASF-85	B7	0,801
	5	80-LPPASFMPL-88	B7	0,995
LiHyp6	6	37-SLATAFGLV-45	A2	0,85392
	7	52-LLYRSTFRH-60	A3	0,69486
HRF	8	87-YMAHIRSYM-95	A2	0,78478
	9	111-FQTNAAAFV-119	A2	0,76162
	10	88-MAHIRSYMK-96	A3	0,68151
	11	112-QTNAAAFVK-120	A3	0,6143
	12	115-AAAFVKKVL-123	B7	0,4089
LiHyV	13	90-SMSMAITTV-98	A2	0,94397
	14	69-VSGNGLTIK-77	A3	0,5355
	15	83-TPSSARLSM-91	B7	0,97149
	16	97-TVAQSAITL-105	B7	0,50925
	17	109-MPANSDIRI-117	B7	0,72604
	18	116-RIVATTSSL-124	B7	0,78072
	19	125-APAQSLFDF-133	B7	0,74818
MHC class I – mouse				
LiHyp1	1	77-LPPLPPASF-85	H-2-Ld	0.27186
	2	79-PLPPASFMP-87	H-2-Ld	0.08248
	3	165-MSGPARYVY-173	H-2-Dd	0.23280
	4	59-DVYTRASDR-67	H-2-Kd	0.07271
	5	83-ASFMPLLER-91	H-2-Kd	0.20710
	6	156-MVPDRSVYI-164	H-2-Ld	0.23140
LiHyp6	7	29-LTYAETVVS-37	H-2-K_d	–
LiHyV	8	82-STPSSARLS-90	H-2-Ld	0.11316
	9	124-LAPAQSLFD-132	H-2-Ld	–

Table 2
In silico predicted murine and human MHC class II-specific epitopes for *Leishmania* proteins.

Protein	N°	Peptide sequence	Percentage of binding to alleles	
MHC class II – human				
LiHyp6	1	52-LLYRSTFRHAMLLRV-66	38,46	
	2	53-LYRSTFRHAMLLRVQ-67	38,46	
	3	54-YRSTFRHAMLLRVQR-68	38,46	
	4	55-RSTFRHAMLLRVQRE-69	42,31	
	5	56-STFRHAMLLRVQRET-70	38,46	
HRF	6	108-RKAFQTNAAAFVKKV-122	30,77	
MHC class II – mouse				
LiHyp1	1	169-ARYVYFHMVLPVEAQ-183	H-2-IAd	394,4
	2	170-RYVYFHMVLPVEAQR-184	H-2-IAd	446,9
	3	171-YVYFHMVLPVEAQRF-185	H-2-IAd	161,2
	4	172-VYFHMVLPVEAQRFS-186	H-2-IAd	160,5
	5	173-YFHMVLPVEAQRFSL-187	H-2-IAd	169,2
	6	174-FHMVLPVEAQRFSLV-188	H-2-IAd	255,3
LiHyp6	7	55-RSTFRHAMLLRVQRE-69	H-2-IAd	204,8
	8	56-STFRHAMLLRVQRET-70	H-2-IAd	182,5
	9	57-TFRHAMLLRVQRETR-71	H-2-IAd	221,1
	10	58-FRHAMLLRVQRETRF-72	H-2-IAd	252
	11	59-RHAMLLRVQRETRFD-73	H-2-IAd	484,1
LiHyV	12	83-TPSSARLSMSMAITT-97	H-2-IAd	411,1
	13	84-PSSARLSMSMAITTV-98	H-2-IAd	347,9
	14	85-SSARLSMSMAITTVA-99	H-2-IAd	193,3
	15	86-SARLSMSMAITTVAQ-100	H-2-IAd	145,8
	16	87-ARLSMSMAITTVAQS-101	H-2-IAd	113,9
	17	88-RLSMSMAITTVAQSA-102	H-2-IAd	93,5
	18	89-LSMSMAITTVAQSAI-103	H-2-IAd	165,8
	19	90-SMSMAITTVAQSAIT-104	H-2-IAd	231,5
	20	97-TVAQSAITLSGVMPA-111	H-2-IAd	382,5
	21	98-VAQSAITLSGVMPAN-112	H-2-IAd	391

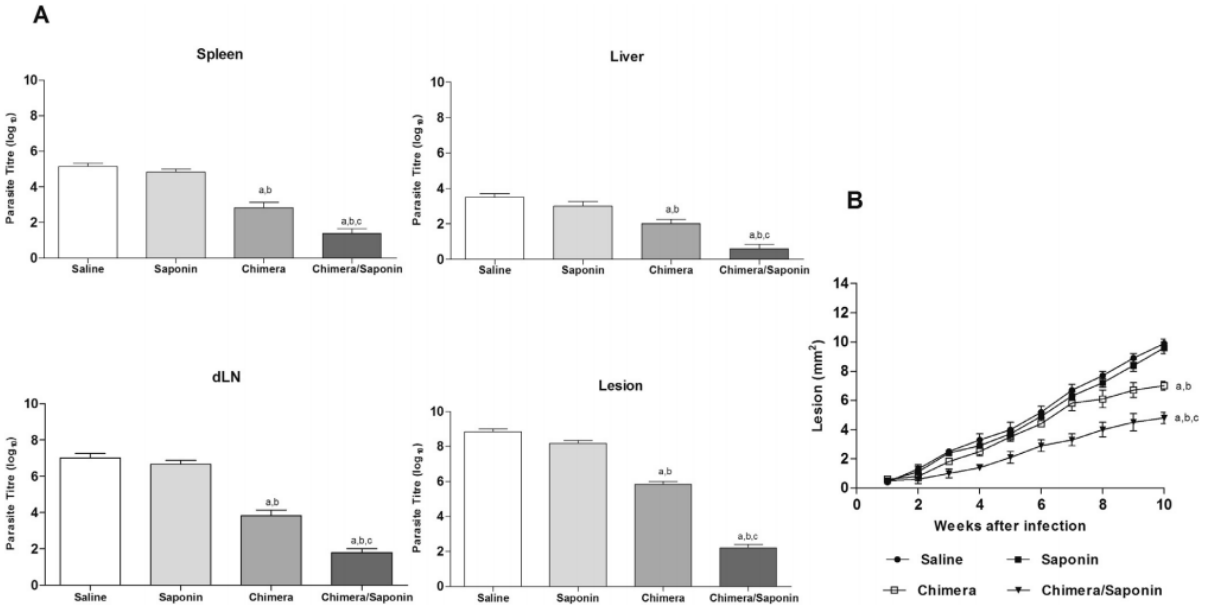


Fig. 1. Evaluation of the parasite load. BALB/c mice (n = 12, per group) were subcutaneously vaccinated with 25 μg of the recombinant fusion protein added or not with saponin (25 μg). Additional mice received saline or saponin. Three doses were administered at two-week intervals. Thirty days after the last immunization, animals were subcutaneously infected with 1×10^6 stationary promastigotes of *L. amazonensis*. Ten weeks after challenge, the parasite load in the infected footpads (lesion), spleen, liver, and draining lymph node (dLN) was evaluated by a limiting dilution technique (A). Bars represent the mean plus standard deviation of the groups. In addition, the lesion development was monitored during 10 weeks in the infected animals (B). The mean ± standard deviation of the footpad swelling is given as the difference of thickness between the infected and the uninfected contralateral footpad. The letters *a*, *b* and *c* indicate statistically significant differences in relation to the saline, saponin and recombinant fusion protein groups, respectively ($P < 0.05$).

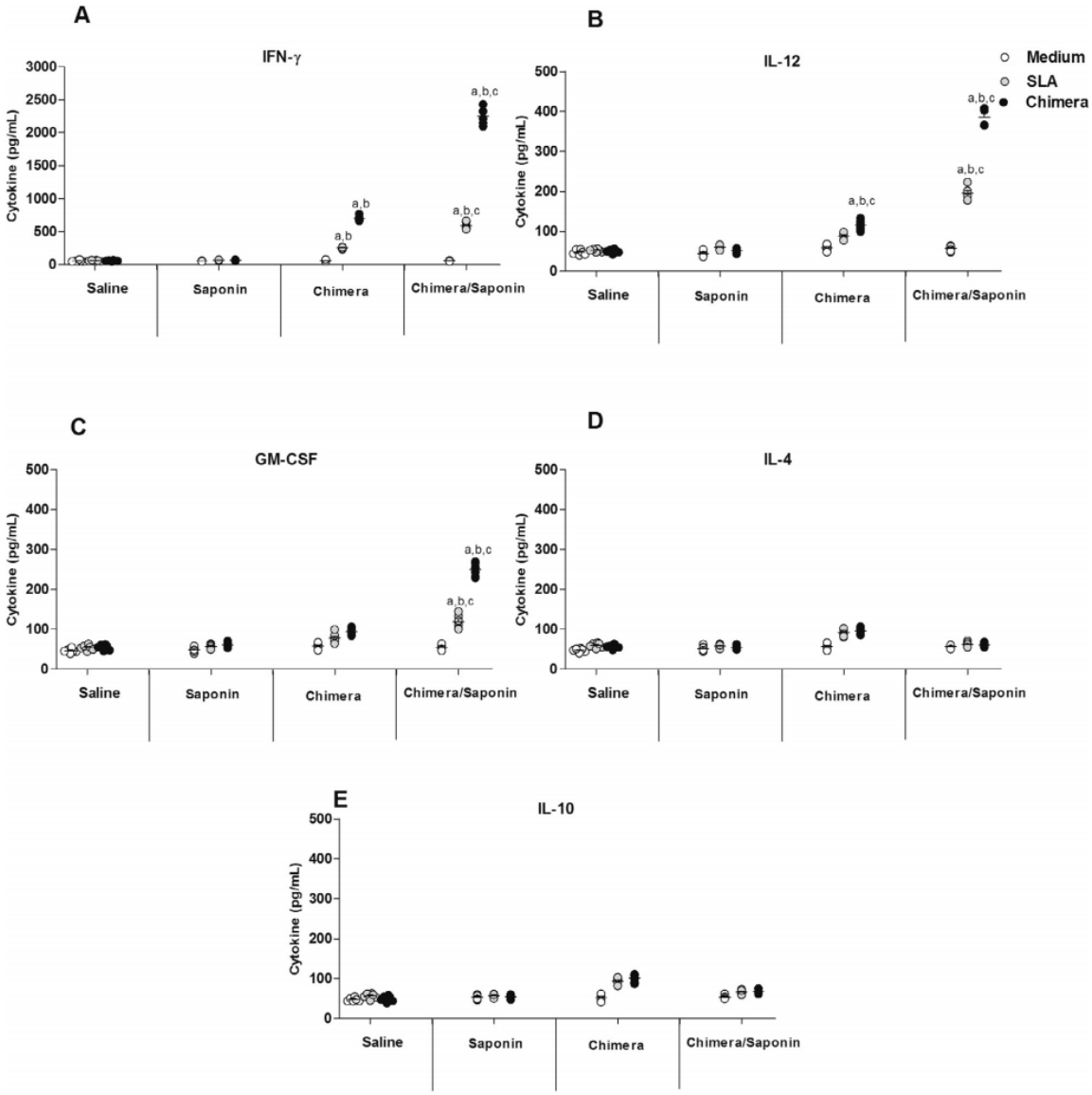


Fig. 2. Anti-recombinant fusion protein and anti-parasite cytokine production before infection. BALB/c mice (n = 12, per group) were subcutaneously vaccinated and, 30 days after the last immunization, they were euthanized (n = 6, per group) and their spleens were collected to evaluation of the cellular response induced by vaccination. For this, single cell suspensions were incubated in complete DMEM (medium), or separately stimulated with the recombinant fusion protein or *L. amazonensis* SLA (20 and 25 μg/mL, respectively), for 48 h at 37 °C in 5% CO_2. IFN-γ (A), IL-12p70 (B), GM-CSF (C), IL-4 (D) and IL-10 (E) levels were measured by a capture ELISA. The results are shown as individual scatter plots of the different groups. The letters *a*, *b* and *c* indicate statistically significant difference in relation to the saline, saponin and recombinant protein groups, respectively ($P < 0.05$).

When stimuli using the rLiHyp1, rLiHyp6, rLiHyV and rHRF proteins were performed, it was observed that both before (Fig. 4A) and after (Fig. 4B) infection, all proteins were able to simulate the spleen cells of the infected and/or recombinant fusion protein/saponin-immunized mice, where high levels of IFN-γ, IL-12, and GM-CSF were encountered. The involvement of the $CD4^+$ and $CD8^+$ T cells in the IFN-γ production was evaluated after infection in the spleen cells of this protected group. In the results, both T cell subtypes were responsible for producing this cytokine, since IFN-γ levels were significantly inhibited when both anti-CD4 and anti-CD8 monoclonal antibodies were added to the *in vitro* cultures. In this context, when splenocytes of these mice were stimulated with the fusion protein or SLA, IFN-γ values were of 3341.5 ± 179.5 and 1479.8 ± 100.8 pg/mL, respectively. When the anti-CD4 monoclonal antibody was added to the cultures, IFN-γ levels were of 885.4 ± 78.8 and 566.0 ± 75.6 pg/mL, when the fusion protein and SLA were used as stimuli, respectively. In addition, using the anti-CD8 monoclonal antibody in the cultures, IFN-γ values were of 1044.6 ± 94.5 and 688.9 ± 86.6 pg/mL, respectively, when the fusion protein and SLA were used as stimuli, respectively.

The nitrite production was also investigated as an indicator of cellular activation via NO production. In the results, it was shown that infected and recombinant fusion protein/saponin-immunized mice produced significantly higher recombinant anti-protein and

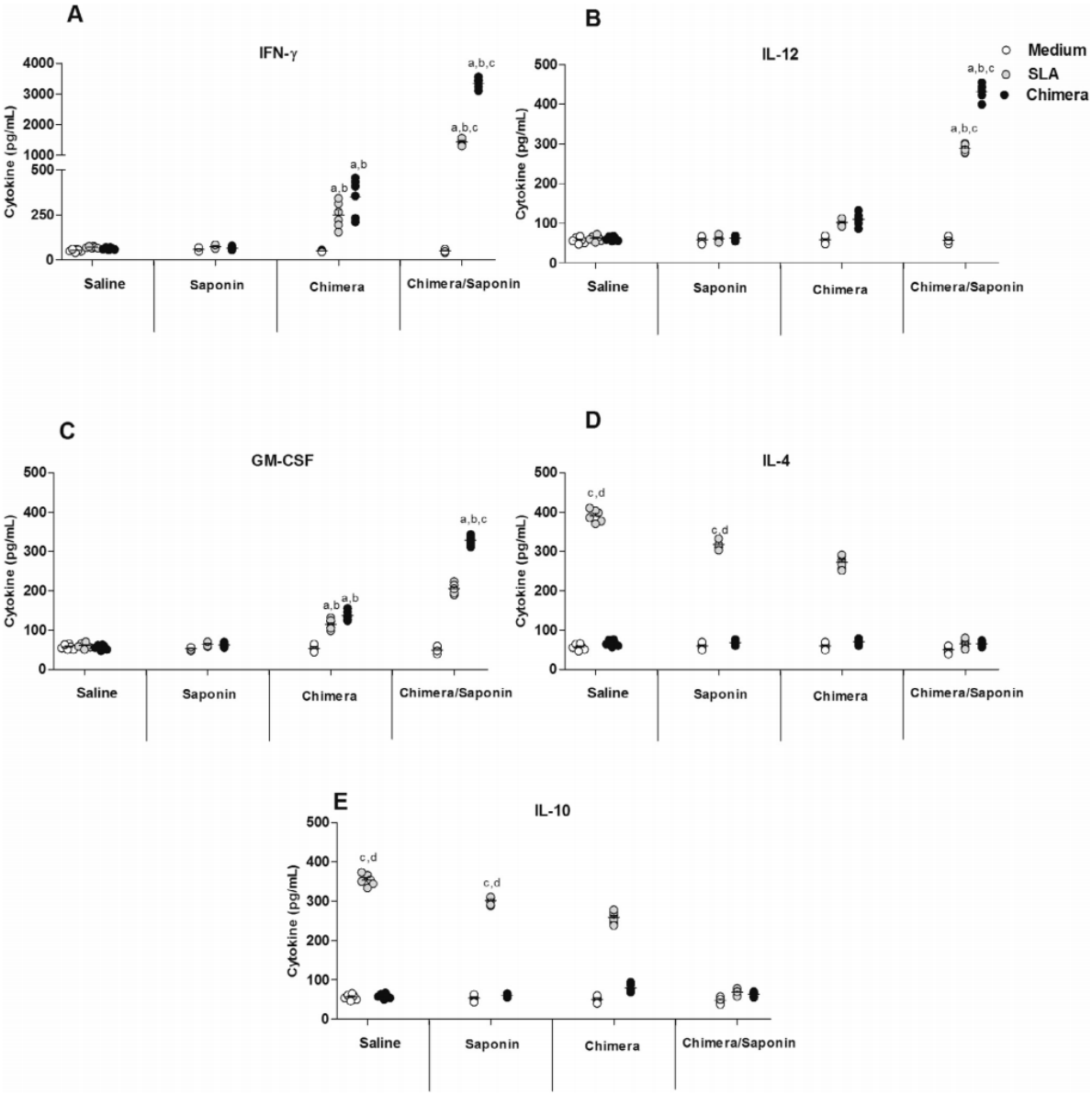


Fig. 3. Anti-recombinant fusion protein and anti-parasite cytokine production after *L. amazonensis* challenge. BALB/c mice (n = 12, per group) were vaccinated, subcutaneously infected and, ten weeks after challenge, single cell suspensions were obtained of their spleens (n = 6, per group) and incubated in complete DMEM (medium), or separately stimulated with the fusion protein or SLA (20 and 25 μg/mL, respectively), for 48 h at 37 °C in 5% CO_2. IFN-γ (A), IL-12p70 (B), GM-CSF (C), IL-4 (D) and IL-10 (E) levels were also measured by a capture ELISA. The results are shown as individual scatter plots of the different groups. The letters *a*, *b*, *c* and *d* indicate statistically significant difference in relation to the saline, saponin, fusion protein and fusion protein/saponin groups, respectively ($P < 0.05$).

anti-parasite nitrite levels, when compared to the other groups. However, mice immunized with the recombinant fusion protein without adjuvant showed higher levels of this molecule, when compared to the results obtained in the saline and saponin groups (Fig. 5). Additionally, the humoral response was investigated. In the results, serum samples from recombinant fusion protein/saponin-immunized animals showed a protein and parasite-specific IgG2a response, whereas animals receiving the recombinant protein alone presented a mixed IgG1/IgG2a response. After *L. amazonensis* infection, mice from the saline, saponin and fusion protein groups showed a higher production of anti-*Leishmania* IgG1 isotype antibodies, whereas an anti-parasite IgG2a response was found in the fusion protein/saponin-vaccinated mice (Fig. 6).

4. Discussion

A vaccine against leishmaniasis is highly desirable, due to the current challenges related for the treatment against disease. Studies evaluating candidate antigens have advanced in recent decades, and there is a growing interest in develop vaccines employing well-defined antigens [48,19,55,54], by using recombinant

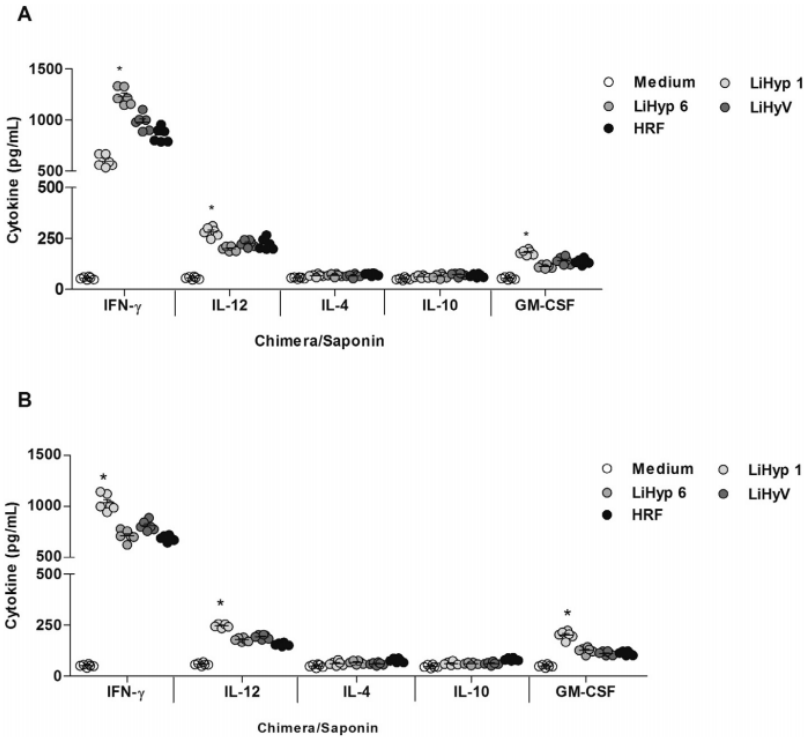


Fig. 4. Immune response induced by the four recombinant proteins before and after challenge infection. BALB/c mice (n = 12, per group) were subcutaneously vaccinated and, 30 days after the last immunization, they were euthanized and their spleens (n = 6, per group) were collected. The remaining animals (n = 6, per group) were subcutaneously infected with *L. amazonensis* promastigotes and, ten weeks after challenge, single cell suspensions were also obtained of their spleens. In both cases, cells were incubated in complete DMEM (medium), or separately stimulated with rLiHyp1, rLiHyp6, rLiHyV or rHRF proteins (20 μg/mL, in all cases) or SLA (25 μg/mL), for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-12p70, IL-4, IL-10 and GM-CSF levels were measured by a capture ELISA before (A) and after (B) infection. The results are shown as individual scatter plots of the different groups. (*) indicates statistically significant difference in relation to the other results obtained intra-groups ($P < 0.05$).

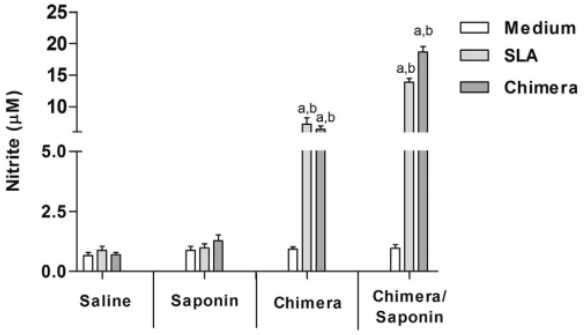


Fig. 5. Nitrite production after *Leishmania* infection. Spleen cells from mice that received saline or were immunized with recombinant fusion protein added or not with saponin (n = 12, per group) and that were later challenged, were stimulated with the recombinant fusion protein (20 μg/mL) or SLA (25 μg/mL), for 48 h at 37 °C in 5% CO_2. Then, the nitrite production was analyzed by the Griess method. Bars represent the mean plus standard deviation of the groups. The letters *a*, *b* and *c* indicate statistically significant differences in relation to the saline, saponin and recombinant protein groups, respectively ($P < 0.05$).

proteins, synthetic peptides, among others [11,37,52,35,36]. However, combinations between different immunogens, based on the use of multi-protein and/or polypeptide vaccines, have been less studied, although they offer the possibility to associate distinct immunogens in a same vaccine composition [38].

In an immunoproteomic study recently performed, *L. infantum* antigenic proteins were identified in amastigotes and promastigotes total extracts of the parasites using VL dogs sera [12]. As a consequence of this study, some of these proteins were evaluated as immunogens and well-successfully employed in the protection against *Leishmania* infection. LiHyp1, an amastigote-specific hypothetical protein, was shown to be protective against *L. infantum* [37], as well as the LiHyp6 and HRF proteins, which were also protective against this parasite species [38]. LiHyV, an antigen expressed in both promastigote and amastigote stages of the parasites, was also able to induce protection against *L. infantum* [39]. Since the development of cross-protective vaccines composed of different parasite proteins and/or their T cell-specific epitopes are encouraged [63], in the present study, a new recombinant protein was developed and based on the selection of $CD4^+$ and $CD8^+$ T cell-specific epitopes derived from these four immunogens. The objective is to create a new product to be tested as a vaccine candidate to protect against a *Leishmania* species able to cause TL in the mammalian hosts [26].

It is well-established that T cells recognize epitopes linked to appropriate MHC molecules, which are expressed in the surface of the phagocytic cells [1]. These epitopes are peptides recognized by effector T cell receptors (TCR), when they are presented in the binding groove of HLA molecules on the surface of antigen presenting cells (APCs). T cell epitopes able to bind to class I HLA molecules have an optimal size of 9 amino acids, showing a range of 8–11 residues. Those able to bind to class II HLA molecules have an optimal size between 12 and 25 amino acid residues. The set

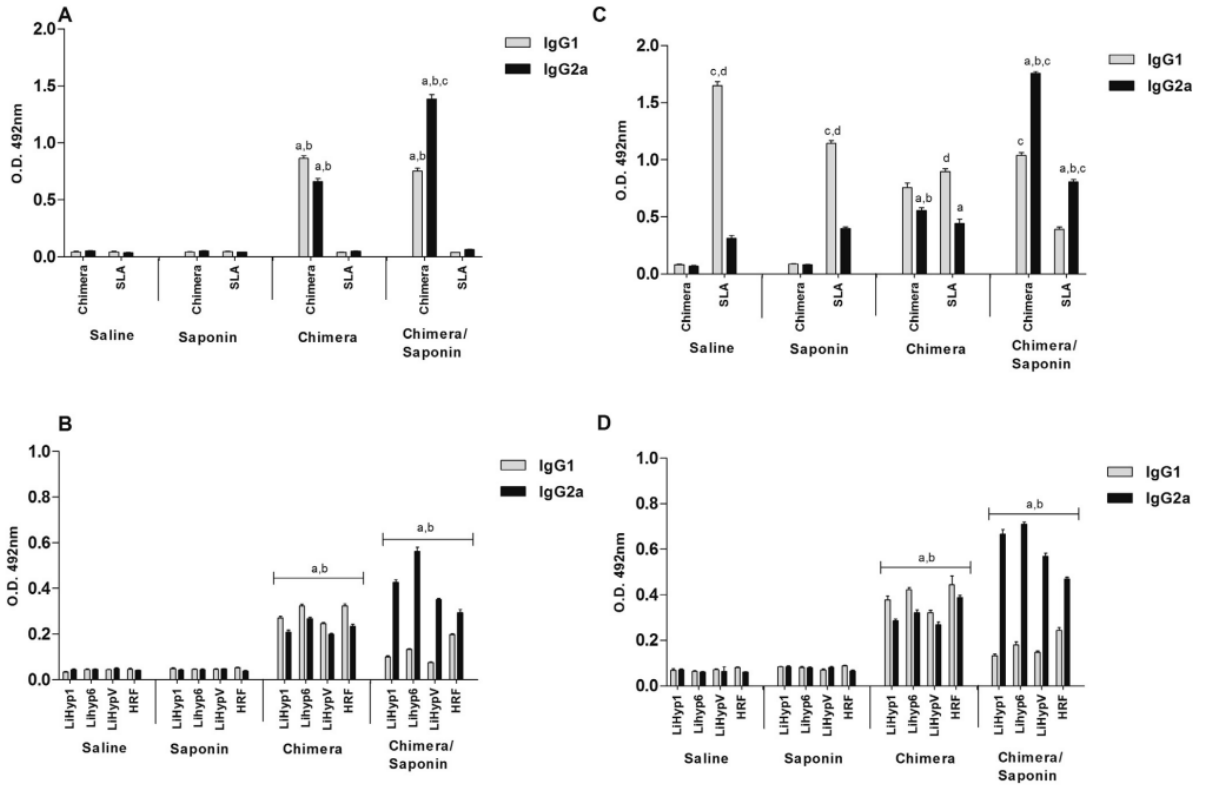


Fig. 6. Humoral response before and after infection. Mice (n = 12, per group) were vaccinated and, 30 days after the last immunization, serum samples were collected. Also, serum samples from animals that were immunized and later infected with *L. amazonensis* promastigotes were collected, 10 weeks after infection. Sera were used to perform the serological assays, when the IgG1 and IgG2a antibodies levels were determined. The reactivities against the recombinant fusion protein or SLA (A and C, before and after infection, respectively) and against the four recombinant proteins (B and D, before and after infection, respectively) are shown. Bars represent the mean plus standard deviation of the groups. The letters *a*, *b* and *c* indicate statistically significant differences in relation to the saline, saponin and recombinant protein groups, respectively ($P < 0.05$).

of HLA molecules which are expressed by a host determines which peptide fragments will be presented as epitopes [29,57,15,17].

The prediction and analysis of HLA-binding sequences within antigenic proteins are central to develop modern vaccines. The use of T cell epitopes prediction is to accurately identify peptide sequences within any protein, which could induce a protective T cell response when presented to the immune system in the context of HLA molecules on the cell surface [30]. In the present study, the NetCTLPan program was used to predict T cell epitopes able to specifically bind to MHC class I molecules, whereas the NetMHCII 2.2 server was used to predict MHC class II molecules-specific epitopes. $CD4^+$ and $CD8^+$ T cell epitopes derived from LiHyp1, LiHyp6, HRF and LiHyV proteins were selected due to their specificity and a new fusion protein was prepared. This recombinant protein comprised 56 epitopes, being 31 of them showing high specificity to bind to murine haplotypes (22 and 9 $CD4^+$ and $CD8^+$ T cell-specific epitopes, respectively) and 25 epitopes presenting high specificity to bind to human haplotypes (6 and 19 $CD4^+$ and $CD8^+$ T cell-specific epitopes, respectively). The choice to select epitopes able to bind to mice and human haplotypes was based on the necessity to develop a new immunogen able to stimulate the immune response in different mammalian hosts. The search of dogs T epitopes was not performed, since until the present moment there is no available bioinformatics tools that could be considerable safe to perform these predictions for this mammalian host, also considered relevant on leishmaniasis [5].

When the vaccine efficacy was evaluated using parasitological parameters, mice immunized with the recombinant fusion protein plus saponin displayed significant reductions in their infected footpad swellings, as well as in the parasite burden in the infection site (infected footpad), liver, spleen and dLN of the animals. On the other hand, mice from the control (saline and saponin) groups developed a severe infection and animals were euthanized ten weeks after infection due to their clinical signs. When animals were immunized with the recombinant protein in the absence of an adjuvant, a partial protection was observed. Reduction in the clinical symptoms as well as in the parasite burden were observed when compared to control groups, although this vaccine was less effective in comparison to the administration of the protein associated with saponin. The decrease in parasite burden found in the infected and vaccinated mice reinforces the prophylactic properties of this tested vaccine, mainly due to its capacity to control parasite replication inside the *L. amazonensis*-infected mammalian hosts.

The efficacy of antigens to protect against leishmaniasis is based on the activation of IFN-γ-producing $CD4^+$ and $CD8^+$ T cells [45,43,24]. $CD4^+$ T cells play a central role in primary immunity against *Leishmania* infection. IFN-γ mediates macrophages activation in a H_2O_2-dependent manner [41,42], and based on NO production for killing the parasites [14]. In addition, $CD8^+$ T cells are also related with the primary and secondary immunity, the last one that can be boosted by natural infections and/or by

administration of new vaccine doses [54]. The macrophage-activating IFN-γ and TNF-α, corroborating with the enhanced antileishmanial activity via NO of these phagocytic cells, usually are able to control *Leishmania* infection [61,9]. Therefore, an effective antileishmanial vaccine should induce a strong and long-lasting Th1 immunity, in order to either prevent the establishment of an initial infection or to control the infection progression and the development of the disease.

In our study, recombinant fusion protein/saponin-vaccinated mice mounted an anti-protein and anti-parasite Th1 immune response before challenge, which was based on high levels of IFN-γ, IL-12 and GM-CSF, being associated with an antileishmanial IgG2a isotype antibodies production. After infection, the immune profile was maintained in these animals, and higher levels of nitrite were also found, suggesting a possible activation of the parasitized phagocytic cells by an IFN-γ-dependent mechanism. IFN-γ has showed to play a central role in resistance to *Leishmania* infection in mammalian hosts against different *Leishmania* species, such as *L. amazonensis* (Chávez-Fumagalli et al., 2010), *L. major* (Stober et al., 2007; Soto et al., 2015) and *L. infantum* (Bhowmick et al., 2007; Rosa et al., 2007; Iborra et al., 2008, [37,38,35].

The involvement of $CD4^+$ and $CD8^+$ T cell subtypes in the IFN-γ production was evaluated in the recombinant fusion protein/saponin-vaccinated mice. In the results, it was observed that both T cell subtypes proved to have an important role in the production of this cytokine. Although our experiments were based on inhibition of IFN-γ production by treatment using specific antibodies against $CD4^+$ and $CD8^+$ molecules in spleen cells of this experimental group, we believe that an improved scientific quality of the results could be obtained if the percentage of $CD4^+$ and $CD8^+$ T cells neutralization was determined. In our study, this cell proportion was not evaluated, although in other works this experimental strategy has been also employed [37,35,38,39,36]; however, we consider this fact one important limitation of our study, since the percentage of $CD4^+$ and $CD8^+$ T cells contributing to the vaccine efficacy mediated by IFN-γ production is considered an important hallmark of success for any therapy or vaccine candidate and it was not evaluated in the present work.

Another key cytokine that has been shown to regulate *Leishmania* infection outcome is IL-10. The disruption of the IL-10 gene confers resistance while the over-expression of this gene in resistant strains renders them susceptible to infection [32]. IL-10 production by natural $CD4^+$ $CD25^+$ regulatory T cells plays an important role in disease pathology, since this cytokine appears to mediate its immunomodulatory action by blocking the IFN-γ-activating effects [7,40,51]. Corroborating with these findings, our results showed that animals that were susceptible to *L. amazonensis* infection presented high levels of IL-10, whereas recombinant protein/saponin-vaccinated mice produced very low levels of this cytokine.

Vaccines based on defined antigens, including recombinant proteins, while offering significant advantages over traditional vaccines in terms of safety and cost of production, generally have limited immunogenicity and require the addition of immune adjuvants, aiming to induce a protective and long-lasting immunity [28,11,49,34,3,10,13]. Adjuvants are able to increase the immunity to vaccine candidates by a variety of mechanisms of action. Saponins are natural glycosides derived from steroid or triterpene, which exhibit distinct biological activities. Notably, they can activate the mammalian immune system. Their unique capacity to stimulate both the Th1 immune response and cytotoxic T cells makes saponins ideal for use in vaccine compositions, aiming to develop a stronger immune response to protect against intracellular pathogens, such as *Leishmania*. However, these products are considered toxic for use in humans and can cause granulomatous reactions and hemolysis, due to their affinity to cholesterol in erythrocyte membranes, besides local pain after injection [33,22,31].

Although in our study the fusion protein had presented better protection in mice against *L. amazonensis* infection when it was associated with saponin, the use of the recombinant protein alone also induced some protection degree in the infected animals, since a significant reduction in the footpad swelling, as well as in the parasite load, associated with high levels of IFN-γ, were obtained in animals immunized with this antigen, when compared with the results found in the saline and saponin groups. These results are encouraging to test the recombinant fusion protein in other vaccination schedules, aiming to improve its protective efficacy against *L. amazonensis* infection. The vaccine efficacy was short-term evaluated, and any study was made at long-term, aiming to analyze the long-lasting protection of this vaccine candidate. In this context, the present study could be considered a proof-of-concept of the efficacy of our antigen to be used as protective against *L. amazonensis* infection. Certainly, additional studies are also necessary to evaluate the protective efficacy of our antigen, when it is administered without the association of immune adjuvants, aiming to offers protection against infection.

Acknowledgments

This work was supported by grants from Instituto Nacional de Ciência e Tecnologia em Nano-biofarmacêutica (INCT-NanoBiofar), FAPEMIG (CBB-APQ-00819-12 and CBB-APQ-01778-2014), and CNPq (APQ-482976/2012-8, APQ-488237/2013-0, and APQ-467640/2014-9). EAFC is a grant recipient of CNPq.

References

[1] M. Agallou, E. Athanasiou, O. Koutsoni, E. Dotsika, E. Karagouni, Experimental validation of multi-epitope peptides including promising MHC class I- and II-restricted epitopes of four known *Leishmania infantum* proteins, Front. Immunol. 5 (2014) 268.
[2] J. Alvar, I.D. Vélez, C. Bern, M. Herrero, P. Desjeux, J. Cano, W.H.O. Leishmaniasis Control Team, Leishmaniasis worldwide and global estimates of its incidence, PLoS One 7 (2012) e35671.
[3] I.F. Amorim, E. Freitas, C.F. Alves, W.L. Tafuri, M.N. Melo, M.S. Michalick, A.P. Costa-Val, Humoral immunological profile and parasitological statuses of Leishmune vaccinated and visceral leishmaniasis infected dogs from an endemic area, Vet. Parasitol. 173 (2010) 55–63.
[4] A.H. Bakker, R. Hoppes, C. Linnemann, M. Toebes, B. Rodenko, C.R. Berkers, S.R. Hadrup, W.J. Van Esch, M.H. Heemskerk, H. Ovaa, T.N. Schumacher, Conditional MHC class I ligands and peptide exchange technology for the human MHC gene products HLA-A1, -A3, -A11, and -B7, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 3825–3830.
[5] G. Baneth, A.F. Koutinas, L. Solano-Gallego, P. Bourdeau, L. Ferrer, Canine leishmaniosis: new concepts and insights on an expanding zoonosis: part one, Trends Parasitol. 24 (2008) 324–330.
[6] A. Barral, D. Pedral-Sampaio, G. Grimaldi, H. Momen, D. McMahon-Pratt, A. Ribeiro-de-Jesus, R. Almeida, R. Badaro, M. Barral-Netto, E.M. Carvalho, Leishmaniasis in Bahia, Brazil: evidence that *Leishmania amazonensis* produces a wide spectrum of clinical disease, Am. J. Trop. Med. Hyg. 44 (1991) 536–546.
[7] Y. Belkaid, C.A. Piccirillo, S. Mendez, E.M. Shevach, D.L. Sacks, $CD4^+CD25^+$ regulatory T cells control Leishmania major persistence and immunity, Nature 420 (2002) 502–507.
[8] P.A. Bretscher, G. Wei, J.N. Menon, H. Bielefeldt-Ohmann, Establishment of stable, cell-mediated immunity that makes "susceptible" mice resistant to *Leishmania major*, Science 257 (1992) 539–542.
[9] E. Carrillo, J. Moreno, Cytokine profiles in canine visceral leishmaniasis, Vet. Immunol. Immunopathol. 128 (2009) 67–70.
[10] D. Carter, S.G. Reed, Role of adjuvants in modeling the immune response, Curr. Opin. HIV AIDS 5 (2010) 409–413.
[11] E.A.F. Coelho, C.A.P. Tavares, F.A.A. Carvalho, K.F. Chaves, K.N. Teixeira, R.C. Rodrigues, H. Charest, G. Matlashewski, R.T. Gazzinelli, A.P. Fernandes, Immune responses induced by the *Leishmania (Leishmania) donovani* A2 antigen, but not by the LACK antigen, are protective against experimental *Leishmania (Leishmania) amazonensis* infection, Infect. Immun. 71 (2003) 3988–3994.
[12] V.T.S. Coelho, J.S. Oliveira, D.G. Valadares, M.A. Chávez-Fumagalli, M.C. Duarte, P.S. Lage, M. Soto, M.M. Santoro, C.A. Tavares, A.P. Fernandes, E.A. Coelho, Identification of proteins in promastigote and amastigote-like *Leishmania* using an immunoproteomic approach, PLoS Negl. Trop. Dis. 6 (2012) e1430.

[13] L.E. Costa, L.R. Goulart, N.C. Pereira, M.I. Lima, M.C. Duarte, V.T. Martins, P.S. Lage, D. Menezes-Souza, T.G. Ribeiro, M.N. Melo, A.P. Fernandes, M. Soto, C.A. Tavares, M.A. Chávez-Fumagalli, E.A. Coelho, Mimotope-based vaccines of *Leishmania infantum* antigens and their protective efficacy against visceral leishmaniasis, PLoS One 9 (2014) e110014.
[14] A.C. Cunninghan, Parasitic adaptive mechanisms in infection by *Leishmania*, Exp. Mol. Pathol. 72 (2002) 132–141.
[15] M.N. Davies, D.R. Flower, Static energy analysis of MHC class I and class II peptide-binding affinity, Methods Mol. Biol. 409 (2007) 309–320.
[16] T.M. Doherty, R.L. Coffman, *Leishmania major*: effect of infectious dose on T cell subset development in BALB/c mice, Exp. Parasitol. 84 (1996) 124–135.
[17] M.E. Elfaki, E.A. Khalil, A.S. Groot, A.M. Musa, A. Gutierrez, B.M. Younis, K.A. Salih, A.M. El-Hassan, Immunogenicity and immune modulatory effects of in silico predicted *L. donovani* candidate peptide vaccines, Hum. Vaccine Immunother. 8 (2012) 1769–1774.
[18] T.C. Felizardo, L.S. Toma, N.B. Borges, G.M. Lima, I.A. Abrahamsohn, *Leishmania (Leishmania) amazonensis* infection and dissemination in mice inoculated with stationary-phase or with purified metacyclic promastigotes, Parasitology 134 (2007) 1699–1707.
[19] A.P. Fernandes, E.A.F. Coelho, G.L.L. Machado-Coelho, G. Grimaldi, R.T. Gazzinelli, Making an anti-amastigote vaccine for visceral leishmaniasis: rational, update and perspectives, Curr. Opin. Microbiol. 15 (2012) 476–485.
[20] A.S. Ferreira, R.S. Leite, L.T. Ituassu, G.G. Almeida, D.M. Souza, R.T. Fujiwara, A.S. Andrade, M.N. Melo, Canine skin and conjunctival swab samples for the detection and quantification of *Leishmania infantum* DNA in an endemic urban area in Brazil, PLoS Negl. Trop. Dis. 6 (2012) e1596.
[21] J.A. Fiuza, S. Gannavaram, H.C. Santiago, A. Selvapandiyan, D.M. Souza, L.S. Passos, L.Z. Mendonça, D.S. Lemos-Giunchetti, N.D. Ricci, D.C. Bartholomeu, R. C. Giunchetti, L.L. Bueno, R. Correa-Oliveira, H.L. Nakhasi, R.T. Fujiwara, Vaccination using live attenuated *Leishmania donovani* centrin deleted parasites induces protection in dogs against *Leishmania infantum*, Vaccine 33 (2015) 280–288.
[22] K.L. Goldenthal, J.A. Cavagnaro, C.R. Alving, F.R. Vogel, National cooperative vaccine development working group. Safety evaluation of vaccine adjuvants, AIDS Res. Hum. Retroviruses. 9 (1993) S45–S49.
[23] J.A. Gomes, L.M. Bahia-Oliveira, M.O. Rocha, O.A. Martins-Filho, G. Gazzinelli, R. Correa-Oliveira, Evidence that development of severe cardiomyopathy in human Chagas' disease is due to a Th1-specific immune response, Infect. Immun. 71 (2003) 1185–1193.
[24] L. Gradoni, Canine *Leishmania* vaccines: still a long way to go, Vet. Parasitol. 208 (2015) 94–100.
[25] G. Grimaldi Jr., R.B. Tesh, D. McMahon-Pratt, A review of the geographic distribution and epidemiology of leishmaniasis in the New World, Am. J. Trop. Med. Hyg. 41 (1989) 687–725.
[26] G. Grimaldi Jr., R.B. Tesh, Leishmaniasis of the New World: current concepts and implications for future research, Clin. Microbiol. Rev. 6 (1993) 230–250.
[27] E.T. Guimarães, L.A. Santos, R. Ribeiro-dos-Santos, M.M. Teixeira, W.L. Santos, M.B. Soares, Role of interleukin-4 and prostaglandin E2 in *Leishmania amazonensis* infection of BALB/c mice, Microbes Infect. 8 (2006) 1219–1226.
[28] S. Gurunathan, D.L. Sacks, D.R. Brown, S.L. Reiner, H. Charest, N. Glaichenhaus, R.A. Seder, Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with *Leishmania major*, J. Exp. Med. 186 (1997) 1137–1147.
[29] J. Hammer, E. Bono, F. Gallazzi, C. Belunis, Z. Nagy, F. Sinigaglia, Precise prediction of major histocompatibility complex class II-peptide interaction based on peptide side chain scanning, J. Exp. Med. 180 (1994) 2353–2358.
[30] J. Hammer, New methods to predict MHC-binding sequences within protein antigens, Curr. Opin. Immunol. 7 (1995) 263–269.
[31] N.C. Jesus-Pereira, W.C. Régis, L.E. Costa, J.S. Oliveira, A.G. Silva, V.T. Martins, M. C. Duarte, J.R. Souza, P.S. Lage, M.S. Schneider, M.N. Melo, M. Soto, S.A. Soares, C.A. Tavares, M.A. Chávez-Fumagalli, E.A. Coelho, Evaluation of adjuvant activity of fractions derived from *Agaricus blazei*, when in association with the recombinant LiHyp1 protein, to protect against visceral leishmaniasis, Exp. Parasitol. 153 (2015) 180–190.
[32] M.M. Kane, D.M. Mosser, The role of IL-10 in promoting disease progression in leishmaniasis, J. Immunol. 166 (2001) 1141–1147.
[33] C.R. Kensil, U. Patel, M. Lennick, D. Marciani, Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria Molina* cortex, J. Immunol. 146 (1991) 431–437.
[34] N. Khoshgoo, F. Zahedifard, H. Azizi, Y. Taslimi, M.J. Alonso, S. Rafati, Cysteine proteinase type III is protective against *Leishmania infantum* infection in BALB/c mice and highly antigenic in visceral leishmaniasis individuals, Vaccine 26 (2008) 5822–5829.
[35] D.P. Lage, V.T. Martins, M.C. Duarte, E. Garde, M.A. Chávez-Fumagalli, D. Menezes-Souza, B.M. Roatt, C.A. Tavares, M. Soto, E.A. Coelho, Prophylactic properties of a *Leishmania*-specific hypothetical protein in a murine model of visceral leishmaniasis, Parasite Immunol. 37 (2015) 646–656.
[36] D.P. Lage, V.T. Martins, M.C. Duarte, L.E. Costa, G.S. Tavares, F.F. Ramos, M.A. Chávez-Fumagalli, D. Menezes-Souza, B.M. Roatt, C.A. Tavares, E.A. Coelho, Cross-protective efficacy of *Leishmania infantum* LiHyD protein against tegumentary leishmaniasis caused by *Leishmania major* and *Leishmania braziliensis* species, Acta Trop. 158 (2016) 220–230.
[37] V.T. Martins, M.A. Chávez-Fumagalli, L.E. Costa, A.M.C.C. Martins, P.S. Lage, D.P. Lage, M.C. Duarte, D.G. Valadares, R.D. Magalhães, T.G. Ribeiro, R.A. Nagem, W. D. DaRocha, W.C. Régis, M. Soto, E.A. Coelho, A.P. Fernandes, C.A. Tavares, Antigenicity and protective efficacy of a *Leishmania* amastigote-specific protein, member of the super-oxygenase family, against visceral leishmaniasis, PLoS Negl. Trop. Dis. 7 (2013) e2148.
[38] V.T. Martins, M.A. Chávez-Fumagalli, D.P. Lage, M.C. Duarte, E. Garde, L.E. Costa, V.G. Silva, J.S. Oliveira, D.F. Magalhães-Soares, S.M. Teixeira, A.P. Fernandes, M. Soto, C.A. Tavares, E.A. Coelho, Antigenicity, immunogenicity and protective efficacy of three proteins expressed in the promastigote and amastigote stages of *Leishmania infantum* against visceral leishmaniasis, PLoS One 10 (2015) e0141496.
[39] V.T. Martins, M.C. Duarte, M.A. Chávez-Fumagalli, D. Menezes-Souza, C.S. Coelho, D.F. Magalhães-Soares, A.P. Fernandes, M. Soto, C.A. Tavares, E.A. Coelho, A *Leishmania*-specific hypothetical protein expressed in both promastigote and amastigote stages of *Leishmania infantum* employed for the serodiagnosis of, and as a vaccine candidate against, visceral leishmaniasis, Parasite Vectors 8 (2015) 363.
[40] S. Mendez, S.K. Reckling, C.A. Piccirillo, D. Sacks, Y. Belkaid, Role for CD4(+) CD25(+) regulatory T cells in reactivation of persistent leishmaniasis and control of concomitant immunity, J. Exp. Med. 200 (2004) 201–210.
[41] H.W. Murray, G.I. Byrne, C.D. Rothermel, D.M. Cartelli, Lymphokine enhances oxygen-independent activity against intracellular pathogens, J. Exp. Med. 158 (1983) 234–239.
[42] H.W. Murray, D.M. Cartelli, Killing of intracellular *Leishmania donovani* by human mononuclear phagocytes. Evidence for oxygen-dependent and -independent leishmanicidal activity, J. Clin. Invest. 72 (1983) 32–44.
[43] M. Nateghi-Rostami, H. Keshavarz, R. Edalat, A. Sarrafnejad, T. Shahrestani, F. Mahboudi, A. Khamesipour, $CD8^+$ T cells as a source of IFN-γ production in human cutaneous leishmaniasis, PLoS Negl. Trop. Dis. 4 (2010) e845.
[44] M. Nielsen, O. Lundi, NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction, BMC Bioinformatics 10 (2009) 296.
[45] I. Okwor, J. Uzonna, Persistent parasites and immunologic memory in cutaneous leishmaniasis: implications for vaccine designs and vaccination strategies, Immunol. Res. 41 (2008) 123–136.
[46] M. Olivier, D.J. Gregory, G. Forget, Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view, Clin. Microbiol. Rev. 18 (2005) 293–305.
[47] U.M. Padigel, J. Alexander, J.P. Farrell, The role of interleukin-10 in susceptibility of BALB/c mice to infection with *Leishmania mexicana* and *Leishmania amazonensis*, J. Immunol. 171 (2003) 3705–3710.
[48] C.B. Palatnik-de-Sousa, Vaccines for leishmaniasis in the fore coming 25 years, Vaccine 26 (2008) 1709–1724.
[49] L.E. Parra, G.P. Borja-Cabrera, F.N. Santos, L.O. Souza, C.B. Palatnik-de-Sousa, I. Menz, Safety trial using the Leishmune vaccine against canine visceral leishmaniasis in Brazil, Vaccine 25 (2007) 2180–2186.
[50] B.A. Pereira, C.R. Alves, Immunological characteristics of experimental murine infection with *Leishmania (Leishmania) amazonensis*, Vet. Parasitol. 158 (2008) 239–255.
[51] V.S. Raman, M.S. Duthie, C.B. Fox, G. Matlashewski, S.G. Reed, Adjuvants for *Leishmania* vaccines: from models to clinical application, Front. Immunol. 3 (2012) 144.
[52] L. Ramírez, D.M. Santos, A.P. Souza, E.A.F. Coelho, A. Barral, C. Alonso, M.R. Escutia, P. Bonay, C.I. Oliveira, M. Soto, Evaluation of immune responses and analysis of the effect of vaccination of the *Leishmania major* recombinant ribosomal proteins L3 or L5 in two different murine models of cutaneous leishmaniasis, Vaccine 31 (2013) 1312–1319.
[53] L. Ramirez, L.C. Villén, M.C. Duarte, M.A. Chávez-Fumagalli, D.G. Valadares, D. M. Santos, C.I. Oliveira, M.R. Escutia, C. Alonso, P. Bonay, C.A. Tavares, E.A. Coelho, M. Soto, Cross-protective effect of a combined L5 plus L3 *Leishmania major* ribosomal protein based vaccine combined with a Th1 adjuvant in murine cutaneous and visceral leishmaniasis, Parasites Vectors 2 (2014) 3–10.
[54] S.G. Reed, R.N. Coler, D. Mondal, S. Kamhawi, J.G. Valenzuela, *Leishmania* vaccine development: exploiting the host-vector-parasite interface, Expert Rev. Vaccines 15 (2015) 81–90.
[55] B. Singh, S. Sundar, Leishmaniasis: vaccine candidates and perspectives, Vaccine 30 (2012) 3834–3842.
[56] L. Soong, C.A. Henard, P.C. Melby, Immunopathogenesis of non-healing American cutaneous leishmaniasis and progressive visceral leishmaniasis, Semin. Immunopathol. 34 (2012) 735–751.
[57] L.J. Stern, D.C. Wiley, Antigenic peptide binding by class I and class II histocompatibility proteins, Behring Inst. Mitt. 1 (1994) 1–10.
[58] T. Stranzl, M.V. Larsen, C. Lundegaard, M. Nielsen, NetCTLPan: pan-specific MHC class I pathway epitope predictions, Immunogenetics 62 (2010) 357–368.
[59] F. Tacchini-Cottier, T. Weinkopff, P. Launois, Does T Helper differentiation correlate with resistance or susceptibility to infection with *L. major*? Some insights from the murine model, Front. Immunol. 3 (2012) 32.
[60] R.G. Titus, M. Marchand, T. Boon, J.A. Louis, A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice, Parasite Immunol. 7 (1985) 545–555.
[61] R. Zafra, J.R. Jaber, R.A. Pérez-Ecija, A. Barragán, A. Martínez-Moreno, J. Pérez, High iNOS expression in macrophages in canine leishmaniasis is associated with low intracellular parasite burden, Vet. Immunol. Immunopathol. 123 (2008) 353–359.
[62] M. Zuker, Mfold web server for nucleic acid folding and hybridization prediction, Nucleic Acids Res. 31 (2003) 3406–3415.
[63] World Health Organization, Control of the leishmaniases, World Health Organ. Tech. Rep. Ser. (2010) 22–26.

5.8. Artigo 4 – Conclusão

A proteína recombinante quimérica, quando associada à saponina, foi capaz de induzir, em camundongos BALB/c, uma imunogenicidade baseada principalmente na produção de citocinas pró-inflamatórias, sendo que este perfil de resposta imune foi responsável por uma significativa redução da carga parasitária quando estes animais foram infectados experimentalmente por *L. amazonensis*. Estudos adicionais devem ser realizados com diferentes hospedeiros mamíferos, com a finalidade de validar a eficácia desta nova formulação vacinal contra as leishmanioses.

6. CONSIDERAÇÕES FINAIS E CONCLUSÃO

As leishmanioses são doenças que afetam milhões de pessoas anualmente e que podem se apresentar com manifestações clínicas leves, nos casos mais brandos, podendo causar até mesmo o óbito, nos casos mais severos (WHO, 2010). Como as medidas de controle atuais são pouco eficientes, os testes diagnósticos são de eficácia variável e o tratamento da doença ainda é insatisfatório, o desenvolvimento de uma vacina profilática é altamente desejável como uma medida efetiva de prevenção contra as leishmanioses (Grimaldi & Tesh, 1993; Gonzalo *et al*., 2002; Sukumaran *et al*., 2003; Ramiro *et al*., 2003; Desjeux, 2004; Palatnik-de-Sousa, 2008).

Objetivando a busca por novas proteínas de *Leishmania* que apresentem propriedades antigênicas e imunogênicas, recentemente foram identificadas diversas proteínas em extratos antigênicos de formas promastigotas e/ou amastigotas-like de *L. infantum*, as quais foram reconhecidas por anticorpos presentes em amostras de soros de cães com leishmaniose (Coelho *et al*., 2012). O fato dos anticorpos terem reconhecido algumas proteínas indica que as mesmas são expressas pelos parasitos durante a doença e que, dessa forma, podem ser consideradas antigênicas e/ou imunogênicas frente ao sistema imune adaptativo do hospedeiro mamífero, sendo consideradas de interesse para o desenvolvimento de vacinas ou testes diagnósticos (Coelho *et al*., 2012). Através destas análises, quatro proteínas conservadas em *Leishmania* sp foram selecionadas e nomeadas de LiHyV, LiHyp1, LiHyp6 e HRF, com a finalidade de serem testadas frente à capacidade antigênica e imunogênica contra as leishmanioses.

Estudos de predição de epitopos de células T $CD4^+$ e $CD8^+$ de camundongos e humanos foram realizados através de ferramentas de bioinformática, com a finalidade de se obter maior conhecimento a respeito de uma possível imunogenicidade destas proteínas. As formas recombinantes das proteínas foram associadas à saponina como adjuvante e testadas, tanto isoladas quanto conjugadas, como vacinas contra as leishmanioses em camundongos BALB/c. Dois epitopos de células T $CD8^+$, identificados na proteína LiHyV e uma quimera polipeptídica recombinante, composta por epitopos de células T das quatro proteínas selecionadas, também foram avaliados, quando associadas à saponina, quanto a capacidade de induzir proteção neste mesmo modelo experimental.

As proteínas recombinantes testadas foram capazes de induzir um perfil de resposta predominante Th1, antes e após a infecção desafio, o que foi responsável pela proteção contra a doença nestes animais. Esta resposta foi coordenada por uma produção elevada de IFN-ɣ, IL-12 e GM-CSF, combinados a baixos níveis de IL-4 e IL-10. As células T $CD4^+$ provaram produzir elevadas concentrações de IFN-ɣ nestes animais, embora tenha sido possível perceber que linfócitos citotóxicos também participam ativamente desta produção. A avaliação da carga parasitária em diferentes órgãos é um importante marcador da eficácia de uma vacina contra as leishmanioses, dado o fato de que a resposta imune específica de cada órgão pode ser observada individualmente (Garg & Dube, 2006). Neste trabalho, foi possível perceber que os camundongos imunizados com as proteínas recombinantes, quando comparados aos grupos controle, apresentaram reduções significativas no número de parasitos em todos os órgãos avaliados, demonstrando que estes antígenos foram eficientes contra as leishmanioses. Em relação à resposta humoral, sabe-se que os isotipos IgG1 e IgG2a apresentam uma correlação com a susceptibilidade e resistência, respectivamente, em modelos murinos experimentais para as leishmanioses, sendo que a produção de IgG2a encontra-se associada a uma resposta imune celular e ao controle da replicação dos parasitos (Passero *et al.*, 2012). Estas informações corroboram e reforçam os resultados encontrados em nosso trabalho, uma vez que as proteínas recombinantes foram capazes de induzir uma maior produção de IgG2a nos animais imunizados.

Desta forma, os dados encontrados neste trabalho indicam que as imunizações de camundongos BALB/c foram capazes de induzir uma resposta imune protetora após o desafio com *L. infantum* ou *L. amazonensis.* Entretanto, a eficácia dos diferentes antígenos e métodos utilizados foi variável, uma vez que as proteínas recombinantes isoladas apresentaram uma menor eficácia em relação às conjugadas; e que estas, por sua vez, apresentaram uma eficiência reduzida em relação ao uso da proteína quimérica. Os peptídeos sintéticos, entretanto, não foram capazes de induzir uma proteção nos animais imunizados. Estes dados nos mostram que as proteínas recombinantes, utilizadas em diferentes estratégias, apresentaram-se como possíveis candidatas a uma vacina efetiva contra as leishmanioses.

Em relação ao potencial antigênico, as proteínas foram analisadas através da técnica de ELISA sendo testadas frente a um amplo painel de soros de cães. Os resultados demonstraram que as proteínas recombinantes LiHyV, LiHyp1, LiHyp6 e HRF, foram reconhecidas com elevada sensibilidade e especificidade, tanto utilizadas individualmente, quanto utilizadas

conjugadas. Sendo assim, os resultados encontrados demonstraram um elevado potencial para estudos adicionais e para o emprego destas proteínas no diagnóstico sorológico da LVC.

7. PERSPECTIVAS

⇒ Avaliar o potencial imunogênico e o grau de proteção induzido pela proteína quimérica recombinate em outros mamíferos contra diferentes espécies de *Leishmania* sp.

⇒ Avaliar a proteína quimérica polipeptídica como candidata ao sorodiagnóstico da leishmaniose visceral canina através da técnica ELISA.

8. REFERÊNCIAS

AGUILAR-BE, I.; DA SILVA ZARDO, R.; PARAGUAI DE SOUZA, E.; BORJACABRERA, G.P.; ROSADO-VALLADO, M.; MUT-MARTIN, M.; GARCÍA-MISS MDEL, R.; PALATNIK DE SOUSA, C.B.; DUMONTEIL, E. Cross-protective efficacy of a prophylactic *Leishmania donovani* DNA vaccine against visceral and cutaneous murine leishmaniasis. *Infect. Immun*. v. 73, n. 2, p. 812–819. 2005.

ALVAR, J.; VÉLEZ, I.D.; BERN, C.; HERRERO, M.; DESJEUX, P.; CANO, J.; JANNIN, J.; BOER, M.D.; THE WHO LEISHMANIASIS CONTROL TEAM. Leishmaniasis Worldwide and Global Estimates of Its Incidence. *PLoS One*. v. 7, n. 5, p. e35671. 2012.

ARAÚJO, M.S.; ANDRADE, R.A.D.; VIANNA, L.R. Despite Leishvaccine and Leishmune trigger distinct immune profiles, their ability to activate phagocytes and CD8+ T-cells support their high-quality immunogenic potential against canine visceral leishmaniasis," *Vaccine*, v. 26, n. 18, p. 2211–2224. 2008.

BADARÓ, R.; JONES, T.C.; CARVALHO, E.M.; SAMPAIO, D.; REED, S.G.; BARRAL, A.; TEIXEIRA, R.; JOHNSON, W.D.JR. New perspectives on a subclinical form of visceral leishmaniasis. *J Infect Dis*. v. 154, n. 6, p. 1003-11. 1986.

BALASEGARAM, M.; RITMEIJER, K.; LIMA, M.A.; BURZA, S.; ORTIZ GENOVESE, G.; MILANI, B.; GASPANI, S.; POTET, J.; CHAPPUIS, F. Liposomal amphotericin B as a treatment for human leishmaniasis. *Expert Opin Emerg Drugs*. 2012.

BANETH, G.; KOUTINAS, A.F.; SOLANO-GALLEGO, L.; BOURDEAU, P.; FERRER, L. Canine leishmaniasis - new concepts and insights on an expanding zoonosis: part one. *Trends Parasitol*. v. 24, n. 7, p. 324–330. 2008.

BHARDWAJ, S.; VASISHTA, R.K.; ARORA, S.K. Vaccination with a novel recombinant *Leishmania* antigen plus MPL provides partial protection against *L. donovani* challenge in experimental model of visceral leishmaniasis. *Exp Parasitol*. v. 121, n. 1, p. 29-37. 2009.

BHOWMICK, S.; ALI, N. Identification of novel *Leishmania donovani* antigens that help define correlates of vaccine-mediated protection in visceral leishmaniasis. *PLoS One*. v. 4, n. 6, p. e5820. 2009.

BLACKWELL, J.M. Genetic susceptibility to leishmanial infections: studies in mice and man. *Parasitology*. v. 112, p. 67-74. 1996.

BOHME, M.W; EVANS, D.A; MILES, M.A.; HOLBOROW, E.J. Occurrence of autoantibodies to intermediate filament proteins in human visceral leishmaniasis and their induction by experimental polyclonal B-cell activation. *Immunology*. v. 59, p. 583–588. 1986.

BORJA-CABRERA, G.P.; CRUZ MENDES, A.; PARAGUAI DE SOUZA, E.; OKADA, L.Y.H.; TRIVELLATO, F.A.A.; KAWASAKI, J.K.A.; COSTA, A.C.; REIS, A.B.; GENARO, O.; PALATNIK, M.; PALATNIK DE SOUSA, C.B. Effective immunotherapy against canine visceral leishmaniasis with the FML-vaccine. *Vaccine.* v. 22, n. 17-18, p. 2234–2243. 2004.

BORJA-CABRERA, G.P.; SANTOS, F.N.; BAUER, F.S.; PARRA, L.E.; MENZ, I.; MORGADO, A.A.; SOARES, I.S.; BATISTA, L.M.; PALATNIK DE SOUSA, C.B. Immunogenicity assay of the Leishmune vaccine against canine visceral leishmaniasis in Brazil. *Vaccine.* v. 26, n. 39, p. 4991–4997. 2008.

BORJA-CABRERA, G.P.; SANTOS, F.N.; SANTOS, F.B.; TRIVELLATO, F.A.; KAWASAKI, J.K.; COSTA, A.C.; CASTRO,T.; NOGUEIRA, F.S.; MOREIRA,

M.A.; LUVIZOTTO, M.C.; PALATNIK, M.; PALATNIK DE SOUSA, C.B. Immunotherapy with the saponin enriched-Leishmune vaccine versus immunochemotherapy in dogs with natural canine visceral leishmaniasis. *Vaccine*. v. 28, n. 3, p. 597–603. 2010.

BRADFORD, M.M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. v. 72, p. 248-254. 1976.

BRASIL, MINISTÉRIO DA SAÚDE. SECRETARIA DE VIGILÂNCIA EM SAÚDE. DEPARTAMENTO DE VIGILÂNCIA EPIDEMIOLÓGICA. Manual de Vigilância e Controle da Leishmaniose Visceral. Brasília/DF. Ministério da Saúde, 120 p., 2006.

CARRIÓN, J.; NIETO, A.; IBORRA, S.; INIESTA, V.; SOTO, M.; FOLGUEIRA, C.; ABANADES, D.R.; REQUENA, J.M.; ALONSO, C. Immunohistological features of visceral leishmaniasis in BALB/c mice. *Parasite Immunol*. v. 28, p. 173-183. 2006.

CARVALHO, P.B.; ARRIBAS, M.A.G.; FERREIRA, E.I. Leishmaniasis. What do we know about its chemotherapy? *Braz. J. Pharmac. Sci.* v. 36, p. 69-96. 2000.

CHAPPUIS, F.; SUNDAR, S.; HAILU, A.; GHALIB, H.; RIJAL, S.; PEELING, R.W;ALVAR, J.; BOELAERT, M. Visceral leishmaniasis: what are the needs for diagnosis, treatment and control? *Nature Reviews Microbiology*. v. 5. 2007.

CHÁVEZ-FUMAGALLI, M.A.; COSTA, M.A.; OLIVEIRA, D.M.; RAMÍREZ, L.; COSTA, L.E.; DUARTE, M.C.; MARTINS, V.T.; OLIVEIRA, J.S.; OLORTEGI, C.C.; BONAY, P.; ALONSO, C.; TAVARES, C.A.; SOTO, M.; COELHO, E.A. Vaccination with the *Leishmania infantum* ribosomal proteins induces protection in BALB/c mice against *Leishmania chagasi* and *Leishmania amazonensis* challenge. *Microbes Infect*. v. 12, n. 12-13, p. 967-77. 2010.

CLAASSEN, E.; BOERSMA, W.J.A. Characteristics and practical use of new-generation adjuvants as an acceptable alternative to Freund´s complet adjuvant. *Res. Immunol*. v. 143, n. 5, p. 475-477. 1992.

COELHO, E.A.F.; TAVARES, C.A.; CARVALHO, F.A.; CHAVES, K.F.; TEIXEIRA, K.N.; RODRIGUES, R.C.; CHAREST, H.; MATLASHEWSKI, G.; GAZZINELLI, R.T.; FERNANDES, A.P. Immune responses induced by the *Leishmania (Leishmania) donovani* A2 antigen, but not by the LACK antigen, are protective against experimental *Leishmania (Leishmania) amazonensis* infection. *Infect.& Immun*. v. 71, n. 7, p. 3988-94, 2003.

COELHO, E.A.; RAMÍREZ, L.; COSTA, M.A.; COELHO, V.T.; MARTINS, V.T.; CHÁVEZ-FUMAGALLI, M.A.; OLIVEIRA, D.M.; TAVARES, C.A.; BONAY, P.; NIETO, C.G.; ABÁNADES, D.R.; ALONSO, C.; SOTO, M. Specific serodiagnosis of canine visceral leishmaniasis using *Leishmania* species ribosomal protein extracts. *Clin Vaccine Immunol*. v. 16, n. 12, p. 1774-80. 2009.

COELHO, V.T.; OLIVEIRA, J.S.; VALADARES, D.G.; CHÁVEZ-FUMAGALLI, M.A.; DUARTE, M.C.; LAGE, P.S.; SOTO, M.; SANTORO, M.M.; TAVARES, C.A.; FERNANDES, A.P.; COELHO, E.A. Identification of proteins in promastigote and amastigote-like *Leishmania* using an immunoproteomic approach. *PLoS Negl Trop Dis.* v. 6, n. 1, p. e1430. 2012.

COLER, R.N.; GOTO, Y.; BOGATZKI, L.; RAMAN, V.; REED, S.G. Leish-111f, a recombinant polyprotein vaccine that protects against visceral leishmaniasis by elicitation of CD4+ T cells. *Infect Immun*. v. 75, n. 9, p. 4648-54. 2007.

COSTA, L.E.; GOULART, L.R.; PEREIRA, N.C.J.; LIMA, M.I.; DUARTE, M.C.; MARTINS, V.T.; LAGE, P.S.; MENEZES-SOUZA, D.; RIBEIRO, T.G.; MELO, M.N.; FERNANDES, A.P.; SOTO, M.; TAVARES, C.A.; CHÁVEZ-FUMAGALLI,

M.A.; COELHO, E.A. Mimotope-based vaccines of *Leishmania infantum* antigens and their protective efficacy against visceral leishmaniasis. *PLoS One*. v. 9, n. 10. 2014.

DA COSTA-VAL, A.P.; CAVALCANTI, R.R.; FIGUEIREDO GONTIJO, N.; MICHALICK, M.S.; ALEXANDER, B.; WILLIAM, S.P.; MELO, M.N. Canine visceral leishmaniasis: relationships between clinical status, humoral immune response, haematology and *Lutzomyia (Lutzomyia) longipalpis* infectivity. *Vet. J.* v. 174, n. 3, p. 636–643. 2007.

DANESH-BAHREINI, M.A.; SHOKRI, J.; SAMIEI, A.; KAMALI-SARVESTANI, E.; BARZEGAR-JALALI, M.; MOHAMMADI-SAMANI, S. Nanovaccine for leishmaniasis: preparation of chitosan nanoparticles containing *Leishmania* superoxide dismutase and evaluation of its immunogenicity in BALB/c mice. v. 6, p. 835-42. 2011.

DAS, A.; ALI, N. Vaccine development against *Leishmania donovani*. *Frontiers in Immunology*. v. 3, n. 99, p. 1-19. 2012.

De JESUS PEREIRA, N.C.; RÉGIS, W.C.; COSTA, L.E.; De OLIVEIRA, J.S.; Da SILVA, A.G.; MARTINS, V.T.; DUARTE, M.C.; De SOUZA, J.R.; LAGE, P.S.; SCHNEIDER, M.S.; MELO, M.N.; SOTO, M.; SOARES, S.A.; TAVARES, C.A.; CHÁVEZ-FUMAGALLI, M.A.; COELHO, E.A. Evaluation of adjuvante activity of fractions derived from *Agaricus blazei*, when in association with the recombinant LiHyp1 protein, to protect against visceral leishmaniasis. *Exp Parasitol*. v. 153, p. 180-190. 2015.

De LUCA, P.M.; MACEDO, A.B.B. Cutaneous leishmaniasis vaccination: A matter of quality. *Front Immunol.* v. 7, n. 151, p. 1-8. 2016.

DESJEUX, P. Leishmaniasis: current situation and new perspectives. *Comp. Immunol. Microbiol. Infect. Dis*. v. 27, p. 305-18. 2004.

ETTINGER, S.J.; FELDMAN, E.C. Tratado de Medicina Interna Veterinária - Doenças do Cão e do Gato. Rio de Janeiro: Editora Guanabara Koogan S.A, 2004.

FALEIRO, R.B.; KUMAR, R.; HAFNER, L.M., ENGWERDA, C.R. Immune Regulation during Chronic Visceral Leishmaniasis. *PLoS Negl Trop Dis*. v. 8, p. 1-11. 2014.

FARIA, A.R.; ANDRADE, H.M. Diagnóstico da Leishmaniose Visceral Canina: grandes

avanços tecnológicos e baixa aplicação prática. *Rev Pan-Amaz Saude*.v. 3, n. 2, p. 47-57. 2012.

FERNANDES, A.P.; COSTA, M.M.; COELHO, E.A.; MICHALICK, M.S.; DE FREITAS, E.; MELO, M.N.; TAFURI, W.L.; RESENDE, M.; HERMONT, V.; ABRANTES, C.D.E.F.; GAZZINELLI, R.T. Protective immunity against challenge with *Leishmania (Leishmania) chagasi* in beagle dogs vaccinated with recombinant A2 protein. *Vaccine.* v. 26, n. 46, p. 5888-95. 2008.

FERNANDES, A.P.; COELHO, E.A.; MACHADO-COELHO, G.L.; GRIMALDI, G.JR.; GAZZINELLI, R.T. Making an anti-amastigote vaccine for visceral leishmaniasis: rational, update and perspectives. *Curr Opin Microbiol*. v. 15, n. 4, p. 476-85. 2012.

FERREIRA, A.S.; LEITE, R.S.; ITUASSU, L.T.; ALMEIDA, G.G.; SOUZA, D.M.; FUJIWARA, R.T.; ET, A.L. Canine skin and conjunctival swab samples for the detection and quantification of *Leishmania infantum* DNA in an endemic urban area in Brazil. *Plos Negl Trop Dis.* v. 6, n. 4, p. 1-9. 2012.

FERREIRA, E.C.; de LANA, M.; CARNEIRO, M.; REIS, A.B.; PAES, D.V.; da SILVA, E.S.; SCHALLIG, H.; GONTIJO, C.M. Comparison of serological assays for the diagnosis of canine visceral leishmaniasis in animals presenting different clinical manifestations. *Vet. Parasitol*. v. 146, n. 3-4, p. 235–241. 2007.

FIGUEIREDO, E.M.; COSTA E SILVA, J.; BRAZIL, R.P. Experimental treatment with sodium stibogluconate of hamsters infected with *Leishmania (Leishmania) chagasi* and *Leishmania (Leishmania) amazonensis. Rev Soc Bras Med Trop*. v. 32, n. 2, p. 191-3. 1999.

FIROUZMANDA, H.; BADIEEB, A.; KHAMESIPOURD, A.; SHARGH, V.H.; ALAVIZADEH, S.H.; ABBASI, A; JAAFARIC, M.R. Induction of protection against leishmaniasis in susceptible BALB/c mice using simple DOTAP cationic nanoliposomes containing soluble *Leishmania* antigen (SLA). *Acta Trop*. v. 128, n. 3, p. 528-35. 2013.

FIUZA, J.A.; GANNAVARAM, S.; SANTIAGO, H.C.; SELVAPANDIYAN, A.; SOUZA, D.M.; PASSOS, L.S.; ET, A.L. Vaccination using live attenuated *Leishmania donovani* centrin deleted parasites induces protection in dogs against *Leishmania infantum*. *Vaccine*. v. 33, p. 280-8. 2015.

FOROUGHI-PARVAR, F.; HATAM, G. Vaccines for canine leishmaniasis. *Adv Prev Med*. v. 2014, n. 569193, p. 1-9. 2014.

GAMA, M.E.; COSTA, J.M.; GOMES, C.M.; CORBETT, C.E. Subclinical form of the American visceral leishmaniasis. *Mem Inst Oswaldo Cruz*. v. 99, n. 8, p. 889-93. 2004.

GARG, R.; DUBE, A. Animal models for vaccine studies for visceral leishmaniasis. *Indian J Med Res.* v. 123, n. 3, p. 439–454. 2006.

GOMES, Y.M.; CAVALCANTI, M.P.; LIRA, R.A.; ABATH, F.G.C.; ALVES, L.C. Diagnosis of canine visceral leishmaniasis: Biotechnological advances. *The veterinary Journal*. v. 175, n. 1, p. 45–52. 2008.

GONZALO, R.M.; DEL REAL, G.; RODRIGUEZ, J.R.; RODRIGUEZ, D.; HELJASVAARA, R.; LUCAS, P.; LARRAGA, V.; ESTEBAN, M. A heterologous prime-boost regime using DNA and recombinant vaccinia virus expressing the Leishmania infantum P36/LACK antigen protects BALB/c mice from cutaneous leishmaniasis. *Vaccine*. v. 20, n. 7-8, p. 1226-31. 2002.

GONTIJO, B.; CARVALHO, M.L.R. Leishmaniose tegumentar americana. *Revista da Sociedade Brasileira de Medicina Tropical.* v. 36, n. 1, p. 71-80. 2003.

GONTINJO, C.M.F.; MELO, M.N. Leishmaniose Visceral no Brasil: quadro atual, desafios e perspectivas. *Revista Brasileira de Epidemiologia*. v. 7, n. 3, p. 338-349. 2004.

GOTO, Y, HOWARD RF, BHATIA A, TRIGO J, NAKATANI M, NETTO EM, REED SG. Distinct antigen recognition pattern during zoonotic visceral leishmaniasis in humans and dogs. *Vet. Parasitol*. v. 160, n. 3-4, p. 215–220. 2009.

GOTO, Y.; BHATIA, A.; RAMAN, V.S.; LIANG, H.; MOHAMATH, R.; PICONE, A.F.; VIDAL, S.E.; VEDVICK, T.S.; HOWARD, R.F.; REED, S.G. KSAC, the first defined polyprotein vaccine candidate for visceral leishmaniasis. *Clin Vaccine Immunol.* v. 18, n. 7, p. 1118-24. 2011.

GRAMICCIA, M.; GRADONI, L. The current status of zoonotic leishmaniases and approaches to disease control. *Int J Parasitol*. v. 35, n.11-12, p. 1169-80. 2005.

GREEN, S.J.; CRAWFORD, R.M.; HOCKMEYER, J.T.; MELTZER, M.S.; NACY, C.A. *Leishmania major* amastigotes initiate the L-arginine-dependent killing mechanism in IFN-gamma-stimulated macrophages by induction of tumor necrosis factor-alpha. *J Immunol*. v. 145, n. 12, p. 4290-7. 1990.

GRIMALDI, G.; TESH, R.B. Leishmaniasis of the New World: current concepts and implications for future research. Clin. Microbiol. Rev. v. 6, p. 230–50. 1993.

GRIMALDI, G.Jr.; TEVA, A.; FERREIRA, A.L.; SANTOS, C.B.; PINTO, I.D.; AZEVEDO, C.T.; FALQUETO, A. Evaluation of a novel chromatographic immunoassay based on Dual-Path Platform technology (DPP® CVL rapid test) for the serodiagnosis of canine visceral leishmaniasis. *Trans R Soc Trop Med Hyg*. v. 106, n. 1, p. 54-9. 2012.

GROGL, M.; MARTIN, R.K.; ODUOLA, A.M.J.; MILHOUS, W.K.; KYLE, D.E. Characteristics of multidrug resistance in *Plasmodium* and *Leishmania*: detection of P-glycoprotein-like components. *Am. J. Trop. Med. Hyg*. v. 45, p. 98-111. 1991.

GUHA, R.; DAS, S.; GHOSH, J.; NASKAR, K.; MANDALA, A.; SUNDAR, S.; DUJARDIN, J.C.; ROY, S. Heterologous priming–boosting with DNA and vaccinia virus expressing kinetoplastid membrane protein-11 induces potent cellular immune response and confers protection against infection with antimony resistant and sensitive strains of *Leishmania (Leishmania) donovani.* v. 31, n. 15, p. 1905-15. 2013.

HANDMAN, E. Leishmaniasis: current status of vaccine development. *Clin Microbiol Rev*. v. 14, n. 2, p. 229-43. 2001.

HUGENTOBLER, F.; DI ROBERTO, R.B.; GILLARD, J.; COUSINEAU, B. Oral immunization using live *Lactococcus lactis* co-expressing LACK and IL-12 protects BALB/c mice against *Leishmania major* infection. *Vaccine*. v. 30, n. 39, p. 5726-32. 2012.

JOSHI, S.; RAWAT, K.; YADAV, N.K.; KUMAR, V.; SIDDIQI, M.I.; DUBE, A. Visceral leishmaniasis: advancements in vaccine development via classical and molecular approaches. Frontiers in Immunology. v. 5, n. 380, p. 1-18. 2014.

KANE, M.M.; MOSSER, D.M. *Leishmania* parasites and their ploys to disrupt macrophage activation. *Curr Opin Hematol*. v. 7, n. 1, p. 26-31. 2000.

ROSTAMI, N.M.; KESHAVARZ, H.; EDALAT, R.; SARRAFNEJAD, A.; SHAHRESTANI, T.; FKHAMESIPOUR, M.A. $CD8^+$ T cells as a source of IFN-γ production in human cutaneous leishmaniasis. *Plos Negl Trop Dis*. v. 4, n. 10, p. 1-9. 2010.

KAR, K. Serodiagnosis of leishmaniasis. *Crit. Rev. Microbiol*. v. 21, n. 2, p. 123–152. 1995.

KEDZIERSKI, L.; EVANS, K.J. E. Immune responses during cutaneous and visceral leishmaniasis. *Parasitology*. p. 1-19. 2014.

KUBAR, J.; FRAGAKI, K. Recombinant DNA-derived *Leishmania* proteins: from the laboratory to the field. *Lancet Infect. Dis.* v. 5, n. 2, p. 107–114. 2005.

KUMAR, R.; NYLÉN, S. Immunobiology of visceral leishmaniasis. *Frontiers in Immunology*. v. 3, n. 251, p. 1 – 10. 2012.

KUMAR, R.; ENGWERDA, C. Vaccines to prevent leishmaniasis. *Clin Transl Immunology*. v. 3, n. 3, p. e13. 2014.

LAEMMLI, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, v. 227, n. 5259, p. 680-685. 1970.

LANOTTE G.; RIOUX J.A.; PERIERES J.; VOLLHARDT Y. Ecology of the leishmaniasis in the south of France. Developmental stages and clinical characterization of canine leishmaniasis in relation to epidemiology. *Ann. Parasitol. Hum. Comp.* v. 54, p. 277-295. 1979.

LEMESRE, J.L.; HOLZMULLER, P.; GONÇALVES R.B.; BOURDOISEAU, G.; HUGNET, C.; CAVALEYRA, M.; PAPIEROK, G. Longlasting protection against canine visceral leishmaniasis using the LiESAp-MDP vaccine in endemic areas of France: doubleblind randomised efficacy field trial. *Vaccine*, v. 25, n. 21, p. 4223–4234. 2007.

LIU, G.; ANDERSON, C.; SCALTRETO, H.; BARBON, J.; KENSIL, C.R. QS-21 structure/function studies: effect of acylation on adjuvant activity. Vaccine. v. 20, n. 21-22, p. 2808-15. 2002.

MARTINS, V.T., CHAVEZ-FUMAGALLI, M.A.; COSTA, L.E. MARTINS, A.M.C.C; LAGE, P.S.; LAGE, D.P.; DUARTE, M.C.; VALADARES, D.G.; MAGALHÃES, R.D.M.; RIBEIRO, T.G.; NAGEM, R.A.P.; DAROCHA, W.D.; RÉGIS, W.C.B.; SOTO, M.; COELHO, E.A.F.; FERNANDES, A.P.; TAVARES, C.A.P. Antigenicity and protective efficacy of a Leishmania amastigote specific protein,member of the super-oxygenase family, against visceral leishmaniasis. *PLoS Negl Trop Dis*. v. 7, n. 3. 2013.

MARTINS, V.T.; LAGE, D.P.; DUARTE, M.C.; COSTA, L.E.; CHÁVEZ-FUMAGALLI, M.A.; ROATT, B.M.; MENEZES-SOUZA, D.; TAVARES, C.A.; COELHO, E.A. Cross-protective efficacy from a immunogen firstly identified in *Leishmania infantum* against tegumentary leishmaniasis. *Parasite Immunol.* v. 38, n. 2, p.108-17. 2016'.

MARTINS, V.T.; LAGE, D.P.; DUARTE, M.C.; COSTA, L.E.; GARDE, E.; RODRIGUES, M.R.; CHÁVEZ-FUMAGALLI, M.A.; MENEZES-SOUZA, D.; ROATT, B.M.; TAVARES, C.A.; SOTO, M.; COELHO, E.A. A new *Leishmania*-specific hypothetical protein, LiHyT, used as a vaccine antigen against visceral leishmaniasis. *Acta Trop.* v. 154, p.73-81. 2016".

MARZOCHI, M.C.; COUTINHO, S.G.; DE SOUZA, W.J.; DE TOLEDO, L.M.; GRIMALDI JUNIOR, G.; MOMEN, H.; PACHECO, R.D.A.S.; SABROZA, P.C.; DE SOUZA, M.A.; RANGEL JUNIOR, F.B. Canine visceral leishmaniasis in Rio de Janeiro, Brazil. Clinical, parasitological, therapeutical and epidemiological findings (1977-1983). *Mem Inst Oswaldo Cruz*. v. 80, p. 349-57. 1985.

MARZOCHI, M.C.A.; MARZOCHI, K.B.F. Tegumentary and visceral leishmaniasis in Brazil – Emerging anthropozoonosis and possibilities for their control. *Caderno de Saúde Pública*. v. 10, n. 2, p. 359-375. 1994.

MATOS, I.; MIZENINA, O.; LUBKIN, A.; STEINMAN, R.M.; IDOYAGA, J. Targeting *Leishmania major* antigens to dendritic cells in vivo induces protective immunity. *PLoS One*. v. 8, n. 6, e67453. 2013.

MAZUMDER, T.; ANAM, K.; ALI, N. A mixed Th1/Th2 response elicited by a liposomal formulation of *Leishmania* vaccine instructs Th1 response sand resistance to *Leishmania donovani* in susceptible BALB/c mice. *Vaccine*. v. 22, n. 9-10, p. 1162–1171. 2004.

MEDEIROS, I.M.; NASCIMENTO, E.L.T.; HINRICHSEN, S.L. Leishmanioses (Visceral e Tegumentar). In: DIP-Doenças Infecciosas e Parasitárias. Rio de Janeiro: Guanabara Koogan. 2005.

MICHALSKY, E.M.; ROCHA, M.F.; da ROCHA LIMA, A.C.; FRANCA-SILVA, J.C.; PIRES, M.Q.; OLIVEIRA, F.S.; PACHECO, R.S.; dos SANTOS, S.L.; BARATA, R.A.; ROMANHA, A.J.; FORTES-DIAS, C.L.; DIAS, E.S. Infectivity of seropositive dogs, showing different clinical forms of leishmaniasis, to *Lutzomyia longipalpis* phlebotomine sand flies. *Vet. Parasitol*. v. 147, n. 1-2, p. 67–76. 2007.

MINODIER, P.; PAROLA, P. Cutaneous leishmaniasis treatment. *Travel Med Infect Dis.* v. 5, n. 3, p. 150-8. 2007.

MIRO, G.; CARDOSO, L.; PENNISI, M.G.; OLIVA, G.; BANETH, G. Canine leishmaniosis—new concepts and insights on an expanding zoonosis: part two. *Trends Parasitol*. v. 24, n. 8, p. 371–377. 2008.

MOLANO, I.; ALONSO, M.G.; MIRÓN, C.; REDONDO, E.; REQUENA, J.M.; SOTO, M.; NIETO, C.G.; ALONSO, C. A *Leishmania infantum* multi-component antigenic protein mixed with live BCG confers protection to dogs experimentally infected with *L. infantum. Vet Immunol Immunopathol.* v. 92, n. 1-2, p. 1-13. 2003.

MOLINA, R.; AMELA, C.; NIETO, J.; SAN-ANDRES, M.; GONZALEZ, F.; CASTILLO, J.A.; LUCIENTES, J.; ALVAR, J. Infectivity of dogs naturally infected with *Leishmania infantum* to colonized *Phlebotomus perniciosus*. *Trans. R. Soc. Trop. Med. Hyg*. v. 88, n. 4, p. 491–493. 1994.

MONDAL, S.; BHATTACHARYA, P.; ALI, N. Current diagnosis and treatment of visceral leishmaniasis. *Expert Rev Anti Infect Ther*. n. 8, p. 919-44. 2010.

MORRIS, R.V.; SHOEMAKER, C.B.; DAVID, J.R.; LANZARO, G.C.; TITUS, R.G. Sandfly maxadilan exacerbates infection with *Leishmania major* and vaccinating against it protects against *L. major* infection. *J. Immunol.* v. 167, n. 9, p. 5226–5230. 2001.

MURRAY, H.W.; SQUIRES, K.E.; MIRALLES, C.D.; STOECKLE, M.Y.; GRANGER, A.M.; GRANELLI-PIPERNO, A.; BOGDAN, C. Acquired resistance and granuloma formation in experimental visceral leishmaniasis. Differential T cell and lymphokine roles in initial versus established immunity. *J. Immunol*. v. 148, p. 1858–1863. 1992.

NIELSEN, M.; LUNDEGAARD, C.; LUND, O. Prediction of MHC class II binding affinity using SMM-align, a novel stabilization matrix alignment method. *BMC Bioinformatics.* v. 8, p. 1-12. 2007.

NIELSEN, M.; LUND, O. NN-align. An artificial neural network-based alignment algorithm for MHC class II peptide binding prediction. *BMC Bioinformatics*. v. 18, n. 10, p. 1-10. 2009.

NIETO, A.; DOMÍNGUEZ-BERNAL, G.; ORDEN, J.A.; DE LA FUENTE, R.; MADRID-ELENA, N.; CARRIÓN, J. Mechanisms of resistance and susceptibility to experimental visceral leishmaniosis: BALB/c mouse versus syrian hamster model. *Vet.Res*. v. 42, n. 39, p. 1-13. 2011.

OLIVEIRA, D.M.; COSTA, M.A.F.; CHAVEZ-FUMAGALLI, M.A.; VALADARES, D.G.; DUARTE, M.C.; COSTA, L.E.; MARTINS, V.T.; GOMES, R.F.; MELO, M.N.; SOTO, M.; TAVARES, C.A.P.; COELHO, E.A.F. Evaluation of parasitological and immunological parameters of *Leishmania chagasi* infection in BALB/c mice using different doses and routes of inoculation of parasites. Parasitol Res. v. 110, p.1277-1285. 2012.

OLIVEIRA, C.D.; ASSUNÇÃO, R.M.; REIS, I.A.; PROIETTI, F.A. Spatial distribution of human and canine visceral leishmaniasis in Belo Horizonte, Minas Gerais State, Brasil, 1994-1997. Cad Saúde Pública. v. 17, n. 5, p. 1231-9. 2001.

OLIVEIRA, T.M.; FURUTA, P.I.; CARVALHO, D.; MACHADO, R.Z. A study of cross-reactivity in serum samples from dogs positive for *Leishmania* sp., *Babesia canis* and *Ehrlichia canis* in enzyme-linked immunosorbent assay and indirect fluorescent antibody test. *Rev Bras Parasitol Vet*. v. 17, n. 1, p. 7-11. 2008.

PALATNIK DE SOUSA, C.B.; MORENO, M.B.; PARAGUAI DE SOUSA, E.; BOROJEVIC, R. The FML vaccine (fucose-mannose ligand) protects hamsters from experimental kala-azar. *Braz.J.Assoc.Adv.Sci. Cienc.Cult.* v. 46, n. 4, p. 290–296.1994.

PALATNIK DE SOUSA, C.B.; SANTOS, W.R.; CASAS, C.P.; PARAGUAI DE SOUZA, E.; TINOCO, L.W.; DA SILVA, B.P.; PALATNIK, M.; PARENTE, J.P. Protective vaccination against murine visceral leishmaniasis using aldehyde-containing *Quillaja saponaria* sapogenins. *Vaccine*. v. 22, n. 19, p. 2470-9, 2004.

PALATINIK DE SOUZA, C.B. Vaccines for leishmaniasis in the fore coming 25 years. *Vaccine.* v. 26, n. 14, p. 1709-24. 2008.

PALATINIK DE SOUZA, C.B. Vaccines for canine leishmaniasis. *Frontiers in immunology.* v. 3, n. 69, p. 1-15. 2012.

PARAGUAI DE SOUZA, E.; BERNARDO, R.R.; PALATNIK, M.; PALATNIK DE SOUSA, C.B. Vaccination of Balb/c mice against experimental visceral leishmaniasis with the GP36 glycoprotein antigen of *Leishmania donovani*. *Vaccine.* v. 19, n. 23-24, p. 3104-15. 2001.

PASSERO, L.F.D.; CARVALHO, A.K.; BORDON, M.L.A.C.; BONFIM-MELO, A.; CARVALHO, K.; KALLÁS, E.G.; SANTOS, B.B.A.; TOYAMA, M.H.; PAES-LEME, A.; CORBETT, C.E.P.; LAURENTI, M.D. Proteins of *Leishmania (Viannia) shawi* confer protection associated with Th1 immune response and memory generation. *Parasit Vectors*. v. 5, n. 64, p. 1-10. 2012.

PESSOA, S.B.; MARTINS, A.V. Parasitologia médica. 11ª ed. Rio de Janeiro: Guanabara Koogan. 1988.

PIARROUX R, AZAIEZ R, LOSSI AM, REYNIER P, MUSCATELLI F, GAMBARELLI F, FONTES M, DUMON H, QUILICI M. Isolation and characterization of a repetitive DNA sequence from *Leishmania infantum*: development of a visceral leishmaniasis polymerase chain reaction. *Am J Trop Med Hyg*. v. 49, n. 3, p. 364-9, 1993.

PORROZZI, R.; SANTOS da COSTA, M.V.; TEVA, A.; FALQUETO, A.; FERREIRA, A.L.; dos SANTOS, C.D.; FERNANDES, A.P.; GAZZINELLI, R.T.; CAMPOS-NETO, A.; GRIMALDI, G.Jr. Comparative evaluation of enzyme-linked immunosorbent assays based on crude and recombinant leishmanial antigens for serodiagnosis of symptomatic and asymptomatic *Leishmania infantum* visceral infections in dogs. *Clin. Vaccine Immunol.* v. 14, n. 5, p. 544–548. 2007.

RAJPUT, Z.I.; HU, S.H.; XIAO, C.W.; ARIJO, A.G. Adjuvant effects of saponins on animal immune responses. *J Zhejiang Univ Sci B.* v. 8, n. 3, p. 153-61. 2007.

RAMIRO, M.J.; ZÁRATE, J.J.; HANKE, T.; RODRIGUEZ, D.; RODRIGUEZ, J.R.; ESTEBAN, M.; LUCIENTES, J.; CASTILLO, J.A.; LARRAGA, V. Protection in dogs against visceral leishmaniasis caused by *Leishmania infantum* is achieved by immunization with a heterologous prime-boost regime using DNA and vaccinia recombinant vectors expressing LACK. *Vaccine*. v. 21, n. 20, p. 2474-84. 2003.

RAVINDRAN, R.; ALI, N. Progress in vaccine research and possible effector mechanisms in visceral leishmaniasis. *Curr Mol Med*. v. 4, n. 6, p. 697-709. 2004.

REIS, A.B., GIUNCHETTI, R.C., CARRILLO, E., MARTINS-FILHO, O.A., and MORENO, J. Immunity to *Leishmania* and the rational search for vaccines against canine leishmaniasis. *Trends Parasitol.* v. 26, p. 341–349. 2010.

REITHINGER R, DUJARDIN JC, LOUZIR H, PIRMEZ C, ALEXANDER B, BROOKER S. Cutaneous leishmaniasis. *Lancet Infect Dis*. v. 7, n. 9, p. 581-96. 2007.

RESENDE, L.A.; ROATT, B.M.; AGUIAR-SOARES, R.D.; VIANA, K.F.; MENDONÇA, L.Z.; LANNA, M.F.; SILVEIRA-LEMOS, D.; CORRÊA-OLIVEIRA, R.; MARTINS-FILHO, O.A.; FUJIWARA, R.T.; CARNEIRO, C.M.; REIS, A.B.; GIUNCHETTI, R.C. Cytokine and nitric oxide patterns in dogs immunized with LBSap vaccine, before and after experimental challenge with *Leishmania chagasi* plus saliva of *Lutzomyia longipalpis*. *Veterinary Parasitology*. v. 198, n. 3-4, p. 371–381. 2013.

ROATT, B.M.; AGUIAR-SOARES, R.D.; VITORIANO-SOUZA, J.; COURA-VITAL, W.; BRAGA, S.L.; CORRÊA-OLIVEIRA, R.; MARTINS-FILHO, O.A.; TEIXEIRA-CARVALHO, A.; de LANA, M.; FIGUEIREDO GONTIJO, N.; MARQUES, M.J.; GIUNCHETTI, R.C.; REIS, A.B. Performance of LBSap vaccine after intradermal challenge with *L. infantum* and saliva of *Lu. longipalpis*: immunogenicity and parasitological evaluation. *PLoS One*. v. 7, n. 11, e49780. 2012.

ROMANO, A.; DORIA, N.A.; MENDES, J.; SACKS, D.L.; PETERS, N.C. Cutaneous infection with *Leishmania major* mediates heterologous protection against visceral infection with *Leishmania infantum*. *J Immunol*.v. 195, n. 8, p. 3816-27. 2015.

RUECKERT, C.; GUZMÁN, C.A. Vaccines: from empirical development to rational design. *PLoS Pathog*. v. 8, n. 11, p. e1003001. 2012.

SACKS, D.; KAMHAWI, S. Molecular aspects of parasite-vector and vector-host interactions in Leishmaniasis. *Annu. Rev. Microbiol*. v. 55, p. 453-483. 2001.

SAMANT, M.; GUPTA, R.; KUMARI, S.; MISRA, P.; KHARE, P.; KUSHAWAHA, P.K.; SAHASRABUDDHE, A.A.; DUBE, A. Immunization with the DNA-encoding N-terminal domain of proteophosphoglycan of *Leishmania donovani* generates Th1-type immunoprotective response against experimental visceral leishmaniasis. *J. Immunol*. v. 183, n. 1, p. 470-9. 2009.

SAMBROOK, J.; FRITSCH, E.F.; MANIATIS, T. Molecular cloning: Laboratory manual, 2nd ed. New York: Cold Spring Harbor Press. 1989.

SANTOS, F.N.; BORJA-CABRERA, G.P.; MIYASHIRO, L.M.; GRECHI, J.; REIS, A.B.; MOREIRA, M. A.; MARTINS FILHO, O.A.; LUVIZOTTO, M.C.; MENZ, I.; PESSOA, L.M.; GONCALVES, P.R.; PALATNIK, M.; PALATNIK-DE-SOUSA, C.B. Immunotherapy against experimental canine visceral leishmaniasis with the saponin enriched-Leishmune vaccine. *Vaccine.* v. 25, n. 33, p. 6176–6190. 2007.

SANTOS, W.R.; DE LIMA, V.M.F.; PARAGUAI DE SOUZA, E.; BERNARDO, R.R.; PALATNIK, M.; PALATNIK DE SOUSA, C.B. Saponins, IL12 and BCG adjuvante in the FML-vaccine formulation against murine visceral leishmaniasis. *Vaccine.* v. 21, n.1-2, p. 30–43. 2002.

SANTOS, W.R.; AGUIAR, I.A.; PARAGUAI DE SOUZA, E.; DELIMA, V.F.M.; PALATNIK, M.; PALA TNIK-DE-SOUSA, C.B. Immunotherapy against murine experimental visceral leishmaniasis with the FML-vaccine. *Vaccine.* v. 21, n. 32, p. 4668–4676. 2003.

SELVAPANDIYAN, A.; DEY, R.; GANNAVARAM, S.; SOLANKI, S.; SALOTRA, P.; NAKHASI, H.L. Generation of growth arrested Leishmania amastigote: a tool to develop live attenuated vaccine candidates against visceral leishmaniasis. *Vaccine*. v. 32, n. 31, p. 3895-901. 2014.

SKEIKY, Y.A.; COLER, R.N.; BRANNON, M.; STROMBERG, E.; GREESON, K.; CRANE, R.T.; WEBB, J.R.; CAMPOS-NETO, A.; REED, S.G. Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. *Vaccine*. v. 20, n. 27-28, p. 3292-303. 2002.

SRIVASTAVA, P.; DAYAMA, A.; MEHROTRA, S.; SUNDAR, S. Diagnosis of visceral leishmaniasis.*Trans R Soc Trop Med Hyg.* v. 105, n. 1, p. 1-6. 2011.

STAGER, S.; SMITH, D.F.; KAYE, P.M. Immunization with a recombinant stage-regulated surface protein from *Leishmania donovani* induces protection against visceral leishmaniasis. *J. Immunol*. v. 165, n. 12, p. 7064–7071. 2000.

STAGER, S.; ALEXANDER, J.; KIRBY, A.C.; BOTTO, M.; ROOIJEN, N.V.; SMITH, D.F.; BROMBACHER, F.; KAYE, P.M. Natural antibodies and complement are endogenous adjuvants for vaccine-induced CD8+ T-cell responses. *Nat Med*. v. 9,n. 10, p. 1287-92. 2003.

STEBUT, E.V. Leishmaniasis. *J Dtsch Dermatol Ges.* p. 191-201. 2014.

SUKUMARAN, B.; TEWARY, P.; SAXENA, S.; MADHUBALA, R. Vaccination with DNA encoding ORFF antigen confers protective immunity in mice infected with Leishmania donovani. *Vaccine*. v. 21, n. 11-12, p. 1292-9. 2003

SUNDAR, S.; RAI, M. Laboratory diagnosis of visceral Leishmaniasis. *Clin Diagn Lab Immunol*. v. 9, n. 5, p. 951-958. 2002.

THAKUR, A.; KAUR, H.; KAUR, S. Evaluation of the immunoprophylactic potencial of killed vaccine candidate in combination with different adjuvants against murine visceral leishmaniasis. *Parasitol Int.* v. 64, n. 1, p. 70-8. 2015.

TAVARES, C.A.; FERNANDES, A.P.; MELO, M.N. Molecular diagnosis of leishmaniasis. *Expert Rev Mol Diagn*. v. 3, n. 5, p. 657-67. 2003.

TAYLOR, C.E. Cytokines as adjuvants for vaccines: antigen-specific responses differ from polyclonal responses. *Infect Immun*. v. 63, n. 9, p. 3241–3244. 1995.

TESH, R.B. Control of zoonotic visceral leishmaniasis: is it time to change strategies?. *Am J Trop Med Hyg.* v. 52, n. 3, p. 287-92. 1995.

TOWBIN, H.; STAEHELIN, T.; BORDON, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci USA*. v. 76, n. 9, p. 4350-4354. 1979.

TROCARELLI, M.Z.; CAMARGO, J.B.; MACHADO, J.G.; LUCHEIS, S.B.; LANGONI, H. *Leishmania* spp. and/or *Trypanosoma cruzi* diagnosis in dogs from endemic and

nonendemic areas for canine visceral leishmaniasis. *Vet Parasitol*. v. 164, n. 2-4, p. 118-23. 2009.

VIEIRA, P.M.; FRANCISCO, A.F.; MACHADO, E.M.; NOGUEIRA, N.C.; FONSECA, K.S.; REIS, A.B.; TEIXEIRA, A.C.; MARTINS-FILHO, O.A.; TAFURI, W.L.; CARNEIRO, C.M. Different infective forms trigger distinct immune response in experimental Chagas disease. *Plos One*. v. 7, n. 3, p. 1-10. 2012.

VIOL, M.A.; LIMA, V.M.; AQUINO, M.C.; GALLO, G.; ALVES, I.P.; GENEROSO, D.; PERRI, S.H.; LUCHEIS, S.B.; LANGONI, H.; NUNES, C.M.; BRESCIANI, K.D. Detection of cross infections by *Leishmania spp*. and *Trypanosoma spp*. in dogs using indirect immunoenzyme assay, indirect fluorescent antibody test and polymerase chain reaction. *Parasitol Res*. v. 111, n. 4, p. 1607-13. 2012.

VITORIANO-SOUZA, J.; MOREIRA, N.D.; TEIXEIRA-CARVALHO, A.; CARNEIRO, C.M.; SIQUEIRA, F.A.; VIEIRA, P.M.; GIUNCHETTI, R.C.; MOURA, S.A.; FUJIWARA, R.T.; MELO, M.N.; REIS, A.B. Cell recruitment and cytokines in skin mice sensitized with the vaccine adjuvants: saponin, incomplete Freund's adjuvant, and monophosphoryl lipid A. *PLoS One*. v. 7, n. 7, p. e40745. 2012.

VOGUEL, F.R. Adjuvants in perspective. *Dev. Biol. Stand*. v. 92, p. 241-248. 1998.

WEIGLE K.A.; VALDERRAMA L.; ARIAS A.L.; SANTRICH C.; SARAVIA N.G. Leishmanin skin test standardization and evaluation of safety, dose, storage, longevity of reaction and sensitization. *Am J Trop Med Hyg*. v. 44, n. 3, p. 260-71. 1991.

WENZEL, U.A.; BANK, E.; FLORIAN, C.; FORSTER, S.; ZIMARA, N.; STEINACKER, J.; KLINGER, M.; REILING, N.; RITTER, U.; VAN ZANDBERGEN, G. *Leishmania major* parasite stage-dependent host cell invasion and immune evasion. *Faseb J*. v. 26. n. 1, p. 29–39. 2012.

WILSON, M.E.; YOUNG, B.M.; ANDERSEN, K.P.; WEINSTOCK, J.V.; METWALI, A.; ALI, K.M.; DONELSON, J.E. A recombinant *Leishmania chagasi* antigen that stimulates cellular immune responses in infected mice. *Infect Immun.* v. 63, n. 5, p. 2062-9. 1995.

WILSON, M.E.; JERONIMO, S.M.; PEARSON, R.D. Immunopathogenesis of infection with the visceralizing *Leishmania* species. *Microb. Pathog*. v. 38, p. 147-60. 2005.

WORLD HEALTH ORGANIZATION. Control of the leishmaniasis: report of a meeting of the WHO Expert Committee on the Control of Leishmaniases, Geneva, 22-26 March 2010. *WHO Technical Report Series*. (949). Disponível em: <http://whqlibdoc.who.int/trs/WHO_TRS_949_eng.pdf.>. Acesso em: 24 de março. 2015.

WORLD HEALTH ORGANIZATION. Leishmaniasis. Fact sheet. n. 375. 2015.

ZANIN, F.H.; COELHO, E.A.; TAVARES, C.A.; MARQUES-DA-SILVA, E.A.; SILVA COSTA, M.M.; REZENDE, S.A.; GAZZINELLI, R.T.; FERNANDES, A.P. Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against *Leishmania chagasi* and *Leishmania amazonensis* experimental infections. Microbes Infect v. 9, n. 9, p. 1070–1077. 2007.

ZUKER, M. Mfold web server for nucleic acid folding and hybridization prediction. *Nucleic Acids Res*. v. 31, n. 13, p. 3406-15. 2003.

9. ANEXOS

9.1. Certificado de aprovação do projeto junto ao Comitê de Ética em Experimentação Animal (CEUA) da UFMG.

9.2. Patentes desenvolvidas durante o doutorado

9.2.1. Patente de inovação intitulada “Proteína quimérica, composição vacinal contra leishmanioses e usos”.

9.2.2. Patente de inovação intitulada “Composição vacinal contra as leishmanioses tegumentar e visceral, e uso”.

9.3. Prêmio: 1º Lugar Prêmio Jovem Pesquisador da Sociedade Brasileira de Medicina Tropical, 2015.

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM EXPERIMENTAÇÃO ANIMAL
- C E T E A -

CERTIFICADO

Certificamos que o **Protocolo nº 43/2011,** relativo ao projeto intitulado "***Ferramenta imunoproteômica utilizando Leishmania Chagasi para a identificação de novos antigenos vacinais contra a Leishmaniose Visceral***", que tem como responsável(is) **Eduardo Antonio Ferraz Coelho** , está(ão) de acordo com os Princípios Éticos da Experimentação Animal, adotados pelo ***Comitê de Ética em Experimentação Animal*** **(CETEA/UFMG),** tendo sido aprovado na reunião de **6/ 07/2011.**

Este certificado expira-se em **6/ 07/ 2016.**

CERTIFICATE

We hereby certify that the **Protocol nº 43/2011,** related to the project entitled "***An immunoproteome approach using Leishmania Chagasi in order to identifying new vaccine antigens to protect against Visceral Leishmaniasis***", under the supervisiors of **Eduardo Antonio Ferraz Coelho,** is in agreement with the Ethical Principles in Animal Experimentation, adopted by the ***Ethics Committee in Animal Experimentation*** **(CETEA/UFMG),** and was approved in **July 6, 2011.**

This certificate expires in **July 6, 2016.**

Belo Horizonte, 14 de Julho de 2011.

Profª. Jacqueline Isaura Alvarez-Leite
Coordenadora do CETEA/UFMG

Universidade Federal de Minas Gerais
Avenida Antônio Carlos, 6627 – Campus Pampulha
Unidade Administrativa II – 2º Andar, Sala 2005
31270-901 - Belo Horizonte. MG - Brasil

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

21/03/2016 870160009908 10:01

00.000.2.2.16.0134329.3

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2016 006121 0

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: Universidade Federal de Minas Gerais

Tipo de Pessoa: Pessoa Jurídica

CPF/CNPJ: 17217985000104

Nacionalidade: Brasileira

Qualificação Jurídica: Instituição de Ensino e Pesquisa

Endereço: Av. Antônio Carlos, 6627 - Unidade Administrativa II - 2º andar- sala 2008

Cidade: Belo Horizonte

Estado: MG

CEP: 31270-901

País: Brasil

Telefone: (31) 34094774

Fax:

Email: patentes@ctit.ufmg.br

Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): "PROTEÍNA QUIMÉRICA, COMPOSIÇÃO VACINAL CONTRA LEISHMANIOSES E USOS"

Resumo: A presente invenção refere-se a uma composição vacinal baseada em uma quimera polipeptídica recombinante composta por epitopos específicos de linfócitos T CD4+ e CD8+ de humano e de camundongo derivados de quatro proteínas (LiHyp1, LiHyp6, LiHyV e HRF) de Leishmania, que foi capaz de induzir proteção contra a leishmaniose visceral e tegumentar e seu uso.

Figura a publicar: 1

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

22/03/2016 870160010093 09:53

00.000.2.2.16.0134320.0

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2016 006219 5

Dados do Depositante (71)

Depositante 1 de 1

Nome ou Razão Social: Universidade Federal de Minas Gerais

Tipo de Pessoa: Pessoa Jurídica

CPF/CNPJ: 17217985000104

Nacionalidade: Brasileira

Qualificação Jurídica: Instituição de Ensino e Pesquisa

Endereço: Av. Antônio Carlos, 6627 - Unidade Administrativa II - 2º andar- sala 2011

Cidade: Belo Horizonte

Estado: MG

CEP: 31270-901

País: Brasil

Telefone: (31) 34094774

Fax:

Email: patentes@ctit.ufmg.br

Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): “COMPOSIÇÃO VACINAL CONTRA AS LEISHMANIOSES TEGUMENTAR E VISCERAL, E USO”

Resumo: A presente invenção trata de uma composição vacinal composta por duas proteínas imunogênicas, definidas pelas SEQ ID No 1 e 2, e seu uso no tratamento e/ou prevenção das leishmanioses tegumentar e visceral no cão e no homem.

CERTIFICADO

A Sociedade Brasileira de Medicina Tropical (SBMT) tem a honra de parabenizar Vivian Tamietti Martins

1º lugar no Prêmio Jovem Pesquisador 2015. Reconhecemos a importância de seu trabalho para a ciência, bem como a necessidade de estimular a continuação de sua pesquisa na área da Medicina Tropical.

10. Apêndices

10.1. Publicação do artigo científico intitulado *"A Leishmania-specific hypothetical protein expressed in both promastigote and amastigote stages of Leishmania infantum employed for the serodiagnosis and as a vaccine candidate against visceral leishmaniasis"* pelo periódico internacional *Parasites & Vectors.*

10.2. Publicação do artigo científico intitulado *"Antigenicity, immunogenicity and protective efficacy of three proteins expressed in the promastigote and amastigote stages of Leishmania infantum against visceral leishmaniasis"* pelo periódico internacional *PLoS One.*

10.3. Publicação do artigo científico intitulado "*A recombinant chimeric protein composed of human and mice-specifc* $CD4^+$ *and* $CD8^+$ *T-cell epitopes protects against visceral leishmaniasis*" ao periódico *Parasite Immunology*.

10.4. Publicação do artigo científico intitulado *"A recombinant fusion protein displaying murine and human MHC class I- and II-specific epitopes protects against Leishmania amazonensis infection"* ao periódico Cellular Immunology.

10.5. Publicação do artigo científico intitulado *"A new Leishmania-specific hypothetical protein, LiHyT, used as a vaccine antigen against visceral leishmaniasis"* pelo periódico internacional *Acta Tropica*.

10.6. Publicação do artigo científico intitulado *"Cross-protective efficacy from a immunogen firstly identified in Leishmania infantum against tegumentary leishmaniasis"* pelo periódico internacional *Parasite Immunology*.

Martins et al. Parasites & Vectors (2015) 8:363
DOI 10.1186/s13071-015-0964-5

Parasites & Vectors

RESEARCH Open Access

A *Leishmania*-specific hypothetical protein expressed in both promastigote and amastigote stages of *Leishmania infantum* employed for the serodiagnosis of, and as a vaccine candidate against, visceral leishmaniasis

Vivian T. Martins[1], Mariana C. Duarte[2,3], Miguel A. Chávez-Fumagalli[2], Daniel Menezes-Souza[3,4], Cecília S. P. Coelho[5], Danielle F. de Magalhães-Soares[6], Ana Paula Fernandes[7], Manuel Soto[8], Carlos A. P. Tavares[1] and Eduardo A. F. Coelho[2,3,9*]

Abstract

Background: LiHyV is an antigenic hypothetical protein present in both promastigote and amastigote stages of *Leishmania infantum*, which was recently identified by an immunoproteomic approach. A recombinant version of this protein (rLiHyV) was evaluated as a diagnostic marker for canine VL (CVL). In addition, the prophylactic efficacy of the rLiHyV protein, and two of its $CD8^+$ T cell epitopes, has been analyzed in a murine model of visceral leishmaniasis (VL).

Methods: Initially, the rLiHyV protein was evaluated by an ELISA technique for the serodiagnosis of CVL. Secondly, vaccines composed of the recombinant protein and both chemically synthesized peptides, combined with saponin as an adjuvant; were administered subcutaneously into BALB/c mice. The cellular and humoral responses generated by vaccination were evaluated. In addition, the parasite burden and immune response were studied 10 weeks after *L. infantum* infection.

Results: The rLiHyV protein was recognized by antibodies of VL dogs. No cross-reactivity was obtained with sera from dogs vaccinated with a Brazilian commercial vaccine, with sera from animals infected with *Trypanosoma cruzi*, *Babesia canis* and *Ehrlichia canis*, or those from non-infected animals living in an endemic area for leishmaniasis. After challenge with *L. infantum*, spleen cells of BALB/c mice vaccinated with rLiHyV/saponin stimulated with parasite antigens showed a higher production of IFN-γ, IL-12 and GM-CSF, than the same cells obtained from mice vaccinated with the individual peptides, or mice from control (inoculated with saline or saponin) groups. This Th1-type cellular response observed in rLiHyV/saponin vaccinated mice was accompanied by the induction of parasite-specific IgG2a isotype antibodies. Animals immunized with rLiHyV/saponin showed significant reductions in the parasite burden in the liver, spleen, bone marrow and in the lymph nodes draining the paws relative to control mice.

Conclusions: The present study showed for the first time that the *L. infantum* LiHyV protein could be considered as a vaccine candidate against *L. infantum* infection, as well as a diagnostic marker for CVL.

Keywords: *Leishmania spp*, Hypothetical proteins, BALB/c mice, Vaccine, Serodiagnosis, Canine visceral leishmaniasis

* Correspondence: eduardoferrazcoelho@yahoo.com.br
[2]Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
[3]Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil
Full list of author information is available at the end of the article

PLOS | ONE

CORRECTION

Correction: Antigenicity, Immunogenicity and Protective Efficacy of Three Proteins Expressed in the Promastigote and Amastigote Stages of *Leishmania infantum* against Visceral Leishmaniasis

Vivian Tamietti Martins, Miguel Angel Chávez-Fumagalli, Daniela Pagliara Lage, Mariana Costa Duarte, Esther Garde, Lourena Emanuele Costa, Viviane Grazielle da Silva, Jamil Silvano Oliveira, Danielle Ferreira de Magalhães-Soares, Santuza Maria Ribeiro Teixeira, Ana Paula Fernandes, Manuel Soto, Carlos Alberto Pereira Tavares, Eduardo Antonio Ferraz Coelho

The seventh author's name is spelled incorrectly. The correct name is: Viviane Grazielle da Silva.

Reference

1. Martins VT, Chávez-Fumagalli MA, Lage DP, Duarte MC, Garde E, Costa LE, et al. (2015) Antigenicity, Immunogenicity and Protective Efficacy of Three Proteins Expressed in the Promastigote and Amastigote Stages of *Leishmania infantum* against Visceral Leishmaniasis. PLoS ONE 10(9): e0137683. doi:10.1371/journal.pone.0137683 PMID: 26367128

Citation: Martins VT, Chávez-Fumagalli MA, Lage DP, Duarte MC, Garde E, Costa LE, et al. (2015) Correction: Antigenicity, Immunogenicity and Protective Efficacy of Three Proteins Expressed in the Promastigote and Amastigote Stages of *Leishmania infantum* against Visceral Leishmaniasis. PLoS ONE 10(10): e0141496. doi:10.1371/journal.pone.0141496

Published: October 20, 2015

Received: 17 June 2016 | Accepted: 30 August 2016

DOI: 10.1111/pim.12359

ORIGINAL ARTICLE

WILEY Parasite Immunology

A recombinant chimeric protein composed of human and mice-specific CD4$^+$ and CD8$^+$ T-cell epitopes protects against visceral leishmaniasis

V. T. Martins[1] | M. C. Duarte[2,3] | D. P. Lage[3] | L. E. Costa[3] | A. M. R. S. Carvalho[2] | T. A. O. Mendes[1] | B. M. Roatt[2] | D. Menezes-Souza[2,3] | M. Soto[4] | E. A. F. Coelho[2,3]

[1]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

[2]Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

[3]Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

[4]Departamento de Biología Molecular, Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Universidad Autónoma de Madrid, Madrid, Spain

Correspondence
Eduardo Antonio Ferraz Coelho, Laboratório de Pesquisa do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.
Email: eduardoferrazcoelho@yahoo.com.br

Funding information
Instituto Nacional de Ciência e Tecnologia em Nano-biofarmacêutica; FAPEMIG, Grant/Award Number: CBB-APQ-00819-12 and CBB-APQ-01778-2014; CNPq, Grant/Award Number: APQ-482976/2012-8, APQ-488237/2013-0 and APQ-467640/2014-9

Summary

In this study, a recombinant chimeric protein (RCP), which was composed of specific CD4$^+$ and CD8$^+$ T-cell epitopes to murine and human haplotypes, was evaluated as an immunogen against *Leishmania infantum* infection in a murine model. BALB/c mice received saline were immunized with saponin or with RCP with or without an adjuvant. The results showed that RCP/saponin-vaccinated mice presented significantly higher levels of antileishmanial IFN-γ, IL-12 and GM-CSF before and after challenge, which were associated with the reduction of IL-4 and IL-10 mediated responses. These animals showed significant reductions in the parasite burden in all evaluated organs, when both limiting dilution and quantitative real-time PCR techniques were used. In addition, the protected animals presented higher levels of parasite-specific nitrite, as well as the presence of anti-*Leishmania* IgG2a isotype antibodies. In conclusion, the RCP/saponin vaccine could be considered as a prophylactic alternative to prevent against VL.

KEYWORDS
immune response, *Leishmania infantum*, recombinant chimeric protein, T-cell epitopes, vaccine, visceral leishmaniasis

1 | INTRODUCTION

Leishmaniasis is a parasitic disease complex caused by different *Leishmania* species, which affects primarily the poorest regions in 98 countries worldwide, where the main clinical manifestations comprise the visceral and tegumentary leishmaniasis.[1,2] Post-kala-azar dermal leishmaniasis is considered a clinical complication of visceral leishmaniasis (VL), in areas where *Leishmania donovani* species is endemic.[3] About two millions of new cases of leishmaniasis are registered annually, of which between 200 000 and 400 000 are associated with VL, leading to approximately 20 000-30 000 deaths per year.[4]

Although parasites are distributed worldwide, in some geographic regions, more than one *Leishmania* species is found as responsible to cause clinical manifestations in infected mammalian hosts. This is the

Cellular Immunology 313 (2017) 32–42

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Research paper

A recombinant fusion protein displaying murine and human MHC class I- and II-specific epitopes protects against *Leishmania amazonensis* infection

Vívian T. Martins [a,b], Daniela P. Lage [b], Mariana C. Duarte [b,c], Ana Maria R.S. Carvalho [c], Lourena E. Costa [b], Tiago A.O. Mendes [a], Danniele L. Vale [c], Daniel Menezes-Souza [b,c], Bruno M. Roatt [c], Carlos A.P. Tavares [a], Manuel Soto [d], Eduardo A.F. Coelho [b,c,*]

[a] Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901 Belo Horizonte, Minas Gerais, Brazil
[b] Programa de Pós-Graduação em Ciências Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 30130-100 Belo Horizonte, Minas Gerais, Brazil
[c] Departamento de Patologia Clínica, Coltec, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901 Belo Horizonte, Minas Gerais, Brazil
[d] Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Departamento de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain

ARTICLE INFO

Article history:
Received 5 August 2016
Revised 24 October 2016
Accepted 25 December 2016
Available online 28 December 2016

Keywords:
Tegumentary leishmaniasis
Polypeptide vaccine
Th1 immune response
$CD4^+$ T cells
$CD8^+$ T cells
Leishmania amazonensis

ABSTRACT

Tegumentary leishmaniasis (TL) constitutes a major public health problem with significant morbidity worldwide. Synthetic peptide-based vaccines are attractive candidates to protect against leishmaniasis, since T cell-specific epitopes can be delivery to antigen-presenting cells, leading to the generation of a Th1 cell-mediated immunity. In this context, the present study aims to evaluate the immunogenicity and protective efficacy of a vaccine composed of major histocompatibility complex class I and II-restricted epitopes derived from four *Leishmania infantum* proteins to protect mice against *Leishmania amazonensis* infection. This recombinant fusion protein was administered in BALB/c mice alone or with saponin. As controls, animals received saline or saponin. In the results, the administration of the recombinant protein plus saponin induced a specific IFN-γ, IL-12 and GM-CSF production, as well as high IgG2a isotype antibody levels, which protected mice against a challenge using *L. amazonensis* promastigotes. Lower parasite burden was found in the infected footpads, liver, spleen and draining lymph node of vaccinated mice, when compared to those from the control groups. In addition, protection was associated with a lower IL-4 and IL-10 response, which was accompanied by the antileishmanial nitrite production by spleen cells of the animals. Interestingly, the recombinant protein administered alone induced a partial protection against challenge. In conclusion, this study shows a new vaccine candidate based on T cell-specific epitopes that was able to induce protection against *L. amazonensis* infection.

1. Introduction

Leishmaniasis is a disease caused by infection with the protozoan *Leishmania*, which is transmitted by the bite of an infected phlebotomine sand fly. It is a tropical disease threatening 350 million people in endemic regions of the world [63,2]. The drugs available to treat against disease are inadequate, since limitations such as side effects, parasite resistance and/or high cost have been observed. The main clinical manifestations of the disease are tegumentary and visceral leishmaniasis. Tegumentary leishmaniasis (TL) is the most frequent clinical form of the disease and is commonly found in Europe, Africa and Asia, being caused by *Leishmania major* species; whereas in the Americas, it is often caused by *L. mexicana*, *L. braziliensis* and *L. amazonensis* [26,18].

The experimental infection of BALB/c mice using *L. major* is one of the well-described models to evaluate the immune response associated with the T cell differentiation. Usually, this lineage develops a Th1 immune response and resistance to infection, when animals are infected with a low number of parasites [16]. However, when an infection using a high inoculum is performed in this

* Corresponding author at: Laboratório do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Av. Dr. Alfredo Balena, 30130-100, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil.
E-mail address: eduardoferrazcoelho@yahoo.com.br (E.A.F. Coelho).

http://dx.doi.org/10.1016/j.cellimm.2016.12.008
0008-8749/

Acta Tropica 154 (2016) 73–81

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

A new *Leishmania*-specific hypothetical protein, LiHyT, used as a vaccine antigen against visceral leishmaniasis

Vívian Tamietti Martins[a], Daniela Pagliara Lage[b], Mariana Costa Duarte[c], Lourena Emanuele Costa[b], Esther Garde[d], Marcella Rezende Rodrigues[c], Miguel Angel Chávez-Fumagalli[b], Daniel Menezes-Souza[c], Bruno Mendes Roatt[c], Carlos Alberto Pereira Tavares[a], Manuel Soto[d], Eduardo Antonio Ferraz Coelho[b,*]

[a] *Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil*
[b] *Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil*
[c] *Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil*
[d] *Centro de Biología Molecular Severo Ochoa, CSIC-UAM, Departamento de Biología Molecular, Universidad Autónoma de Madrid, Madrid, Spain*

ARTICLE INFO

Article history:
Received 19 August 2015
Received in revised form
10 November 2015
Accepted 12 November 2015

Keywords:
Hypothetical proteins
Leishmania infantum
Vaccine
Immunoproteomic approach
Immune adjuvants
Th1 immune response

ABSTRACT

The present study aimed to evaluate a new *Leishmania*-specific hypothetical protein, LiHyT, as a vaccine candidate against VL. The immunogenicity of the recombinant protein (rLiHyT) plus saponin was evaluated in BALB/c mice. In the results, it is shown that rLiHyT plus saponin vaccinated mice produced high levels of IFN-γ, IL-12, and GM-CSF after *in vitro* stimulation of spleen cells using both rLiHyT and *Leishmania infantum* SLA. The protective efficacy was evaluated after subcutaneous challenge with stationary promastigotes of *L. infantum*. Immunized and infected mice, when compared to the controls, showed significant reductions in the number of parasites in the liver, spleen, bone marrow, and in the paws' draining lymph nodes. Protection was associated with an IL-12-dependent production of IFN-γ, mainly by $CD4^+$ T cells, with a minor contribution of $CD8^+$ T cells. In these mice, a decrease in the parasite-mediated IL-4 and IL-10 responses, as well as a predominance of LiHyT- and parasite-specific IgG2a isotype antibodies, were also observed. The present study showed that a new *Leishmania*-specific protein, when combined with a Th1-type adjuvant, presents potential to be used as a vaccine against VL.

1. Introduction

Leishmaniasis presents a broad spectrum of clinical manifestations and is caused by different species of protozoa belonging to the genus *Leishmania*. The disease exhibits a high morbidity and mortality in the world, with approximately 350 million people in 98 countries at risk of contracting the infection (WHO, 2010). Approximately 0.7–1.2 million cases of tegumentary leishmaniasis (TL), and 0.2–0.4 million cases of visceral leishmaniasis (VL) are registered annually. Visceral leishmaniasis represents an important disease worldwide, leading to nearly 50,000 deaths each year (Alvar et al., 2012). Because of its remarkable impact on global public health, VL is considered one of the six major tropical diseases, and it has gained greater importance in HIV-infected individuals as an opportunistic infection in areas where both infections are endemic (Lindoso et al., 2014). Therefore, the development of new strategies, such as a vaccine to prevent the disease, should be considered urgent.

In VL, the development of a Th1 cell-mediated immune response is considered relevant to prevention and/or cure of disease. Therefore, proteins able to stimulate the development of a Th1 response could be considered as potential vaccine candidates against VL (Fernandes et al., 2008; Chávez-Fumagalli et al., 2010; Ramírez et al., 2013). In addition, the induction of a $CD4^+$ Th1 cells response against parasite proteins is crucial in controlling infection. Cytokines such as IFN-γ are able to induce the production of nitric oxide (NO) and other compounds by infected phagocytic cells, thereby assisting to control parasitesí multiplication (Green et al., 1990; Costa et al., 2014). On the other hand, IL-4, IL-10, IL-13, and TGF-β represent important disease promoting cytokines, leading

* Corresponding author at: Laboratório de Biotecnologia Aplicada ao Estudo das Leishmanioses, Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Avenida Prof. Alfredo Balena, 190, 30.130-100, Belo Horizonte, Minas Gerais, Brazil.
E-mail address: eduardoferrazcoelho@yahoo.com.br (E.A.F. Coelho).

http://dx.doi.org/10.1016/j.actatropica.2015.11.006
0001-706X/

Parasite Immunology

Parasite Immunology, 2016, **38,** 108–117 DOI: 10.1111/pim.12304

Cross-protective efficacy from a immunogen firstly identified in *Leishmania infantum* against tegumentary leishmaniasis

V. T. MARTINS,[1] D. P. LAGE,[2] M. C. DUARTE,[3] L. E. COSTA,[2] M. A. CHÁVEZ-FUMAGALLI,[2] B. M. ROATT,[3] D. MENEZES-SOUZA,[3] C. A. P. TAVARES[1] & E. A. F. COELHO[2]

[1]Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, [2]Faculdade de Medicina, Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil, [3]Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil

SUMMARY

Experimental vaccine candidates have been evaluated to prevent leishmaniasis, but no commercial vaccine has been proved to be effective against more than one parasite species. LiHyT is a Leishmania*-specific protein that was firstly identified as protective against* Leishmania infantum*. In this study, LiHyT was evaluated as a vaccine to against two* Leishmania *species causing tegumentary leishmaniasis (TL):* Leishmania major *and* Leishmania braziliensis*. BALB/c mice were immunized with rLiHyT plus saponin and lately challenged with promastigotes of the two parasite species. The immune response generated was evaluated before and 10 weeks after infection, as well as the parasite burden at this time after infection. The vaccination induced a Th1 response, which was characterized by the production of IFN-γ, IL-12 and GM-CSF, as well as by high levels of IgG2a antibodies, after* in vitro *stimulation using both the protein and parasite extracts. After challenge, vaccinated mice showed significant reductions in their infected footpads, as well as in the parasite burden in the tissue and organs evaluated, when compared to the control groups. The anti-*Leishmania *Th1 response was maintained after infection, being the IFN-γ production based mainly on $CD4^+$ T cells. We described one conserved* Leishmania*-specific protein that could compose a pan-*Leishmania *vaccine.*

Correspondence: Eduardo Antonio Ferraz Coelho, Faculdade de Medicina, Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Universidade Federal de Minas Gerais (e-mail: eduardoferrazcoelho@yahoo.com.br).
Disclosure: None.
Received: 17 November 2015
Accepted for publication: 6 January 2016

Keywords *BALB/c mice, hypothetical proteins,* Leishmania braziliensis*,* Leishmania major*, Th1 immune response, vaccine*

INTRODUCTION

Leishmaniasis is a vector-transmitted disease, and at least 20 species of *Leishmania* parasites can cause it in mammalian hosts (1). Infection may either be asymptomatic or give rise to different clinical forms of tegumentary or visceral disease, depending on the *Leishmania* species and on the genetic background from the hosts (2). Leishmaniasis affects 12 million people in the world and there is an estimated incidence of 1·5–2·0 million of new cases annually (3). Cutaneous leishmaniasis (CL) is the most frequent clinical form of tegumentary leishmaniasis (TL), and it is commonly caused by *Leishmania major*, *L. tropica* and *L. aethiopica* species in Europe, Africa and Asia. In the Americas, the disease is often caused by *L. braziliensis*, *L. mexicana* and *L. amazonensis* species (4,5).

Chemotherapy to treat leishmaniasis is toxic and/or expensive (6,7). Spontaneous or drug induced recovery is usually accompanied by a cellular immunity, which can protect against re-infection by parasite, providing a rational basis for developing vaccines against leishmaniasis; however, there are no available vaccines for use in human (8). Murine models have contributed to clarify the role of T cells in the resistance to *Leishmania* infection (9–11). When infected with a low number of *L. major* promastigotes, BALB/c mice usually develop a Th1 response and resist to a subsequent challenge (12,13), whereas the infection with a high number of the parasites results in the development of a Th2 response and in the susceptibility to infection (14–16). Therefore, one could speculate that depending on the parasite species, the inoculum size and the strain of mice studied, a variable response regarding