Universidade Federal de Minas Gerais
Faculdade de Medicina
Programa de Pós-Graduação em Ciências da Saúde:
Infectologia e Medicina Tropical

# DESEMPENHO DIAGNÓSTICO E AVALIAÇÃO DA EFICÁCIA PROTETORA DE PROTEÍNAS RECOMBINANTES DE *Leishmania spp*. E DE UMA QUIMERA POLIPEPTÍDICA CONTRA AS LEISHMANIOSES TEGUMENTAR E VISCERAL

Belo Horizonte
2018

Daniel Silva Dias

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Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical da Faculdade de Medicina da UFMG, como requisito parcial para a obtenção do título de Doutor junto ao referido Programa.

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**Área de Concentração: Infectologia e Medicina Tropical**

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*Dedico:*

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*"O mecanismo do descobrimento não é lógico ou intelectual. É uma iluminação súbita, quase um êxtase. Em seguida, a inteligência analisa e a experiência confirma a intuição. Além disso, há uma conexão com a imaginação".*

*Albert Einstein*

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## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| **DAT** | Teste de Aglutinação Direta |
| **DPP®** | Dual-Path Platform |
| **DTH** | Hipersensibilidade do tipo tardio |
| **ELISA** | Ensaio imunoenzimático |
| **FAST** | Teste de avaliação rápida da aglutinação |
| **GM-CSF** | Fator estimulador de colônias de macrófagos e granulócitos |
| **ICT** | Teste imunocromatográfico |
| **IDRM** | Teste de intradermoreação de Montenegro |
| **IFAT** | Ensaio de imunofluorescência indireta |
| **IFI** | Imunofluorescência Indireta |
| **IFN-ɣ** | Interferon-gama |
| **IgG** | Imunoglobulina G |
| **IL** | Interleucina |
| **iNOS** | Enzima óxido nítrico sintase induzível |
| **LC** | Leishmaniose cutânea |
| **LCD** | Leishmaniose cutâneo-difusa |
| **LiHyS** | Proteína hipotética de *L. infantum* |
| **LM** | Leishmaniose mucosa |
| **LT** | Leishmaniose tegumentar |
| **LV** | Leishmaniose visceral |
| **LVC** | Leishmaniose visceral canina |
| **LVH** | Leishmaniose visceral humana |
| **MHC** | Complexo principal de histocompatibilidade |
| **NO -** | Óxido nítrico |
| **PBMC** | Células mononucleares de sangue periférico |
| **PCLV** | Programa de Controle da Leishmaniose Visceral |
| **PCR** | Reação em Cadeia da Polimerase |
| **PHB** | Prohibitina |
| **RIFI** | Reação de Imunofluorescência Indireta |
| **RT-PCR** | PCR em tempo real |
| **Sap** | Saponina |

| | |
|---|---|
| **SGT** | Proteína contendo repetições de tetratricopeptídeo ricos em glutamina |
| **SMF** | Sistema mononuclear fagocitário |
| **TGF-β** | Fator de transformação de crescimento beta |
| **Th** | T helper |
| **TNF-α** | Fator de necrose tumoral alfa |
| **WHO** | Organização Mundial de Saúde |

# *CONSIDERAÇÕES INICIAIS*

# CONSIDERAÇÕES INICIAIS

A escolha do tema desta tese ocorreu devido à elevada incidência das leishmanioses no mundo que, apesar de ser considerada como um problema de incidência global; investimentos de recursos voltados para a prevenção, diagnóstico e tratamento desse complexo de doenças ainda são precários.

A ineficácia das medidas de controle contra doença e a observação da aquisição de imunidade duradoura contra a reinfecção após a cura da infecção por espécies de *Leishmania* indicam a possibilidade do desenvolvimento de uma vacina protetora contra a doença. Também, um diagnóstico precoce que apresente elevadas sensibilidade e especificidade poderia possibilitar um tratamento mais rápido e eficaz, sendo benéfico tanto para os pacientes quanto para os sistemas de Saúde dos países onde a doença ocorre. Outro fator também relevante na escolha do tema baseia-se no fato de ter conhecido o meu atual orientador Dr. Prof. Eduardo Coelho, que se dedica desde o ano de 2000 a estudos de tratamento, diagnóstico e vacina, graças a uma parceria durante o mestrado para avaliar o potencial de diagnóstico de uma proteína quimérica recombinante no diagnóstico da leishmaniose visceral canina usando amostras de seu painel sorológico.

A linha de pesquisa deste estudo é em imunologia das doenças infecciosas e o objetivo geral da tese foi avaliar o desempenho diagnóstico de três proteínas recombinantes do parasito, usadas sob a forma isolada, bem como sua eficácia vacinal e de uma quimera polipeptídica composta por seus principais epitopos de células T em camundongos BALB/c contra a infecção experimental por *L. infantum*, principal espécie causadora de leishmaniose visceral nas Américas. O trabalho possibilitou a produção de cinco artigos científicos publicados em revistas de relevante impacto internacional, que serão apresentados neste documento, e no depósito de uma patente nacional.

A apresentação deste documento foi realizada de acordo com a Resolução nº 02/2013, de 18 de setembro de 2013; que regulamenta o formato dos trabalhos finais e de qualificação, estabelecendo condições para a marcação das defesas de teses e dissertações do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical da Faculdade de Medicina da UFMG.

## RESUMO

A proteção contra a reinfecção por espécies de *Leishmania spp.* indica a possibilidade do desenvolvimento de uma vacina profilática contra as leishmanioses. O avanço das ferramentas biotecnológicas vem se mostrando como uma boa perspectiva para o desenvolvimento de novos produtos. Neste trabalho, três proteínas: proteína contendo repetições de tetratricopeptídeo ricos em glutamina (SGT), uma proteína hipotética (LiHyS) e prohibitina (PHB), que foram identificadas em um estudo anterior pela técnica de imunoproteômica, na sua forma recombinante, foram utilizadas como marcador sorológico para as leishmanioses. A imunogenicidade das proteínas foi também avaliada nas suas formas recombinantes isoladas ou em associação, além de uma quimera proteica composta pelos principais epitopos de células T das moléculas. Todos associados com saponina como adjuvante e administradas em camundongos BALB/c. A eficácia vacinal foi avaliada contra a infecção desafio com *L. infantum.* Os resultados mostraram que os animais vacinados com as proteínas apresentaram uma produção elevada de IFN-ɣ, IL-12 e GM-CSF, aliados a uma baixa produção de IL-4 e IL-10, quando os comparados aos grupos controle. Os animais também apresentaram redução significativa na carga parasitária nos órgãos analisados. A proteção foi relacionada com uma elevada produção de IFN-ɣ, que ocorreu principalmente por meio de linfócitos T $CD4^+$. De forma interessante, a quimera mostrou maior eficácia de proteção e imunogenicidade quando comparada ao uso das proteínas de forma isolada ou em associação. Quanto aos testes sorológicos, as proteínas recombinantes apresentaram também elevados valores de sensibilidade e especificidade para o diagnóstico da leishmaniose visceral, quando comparado ao uso do antígeno rA2 e de uma preparação antigênica de *Leishmania* usados como controles. Dessa forma, o presente trabalho apresenta novas proteínas e uma quimera recombinante que podem ser utilizadas como candidatos à vacina contra a leishmaniose visceral, bem como marcadores sorológicos para a detecção da doença nos hospedeiros mamíferos infectados.



**Palavras-chave**: leishmanioses; proteínas recombinantes; quimera proteica; vacina; diagnóstico; imunoproteômica.

## ABSTRACT

Protection against reinfection by *Leishmania spp*. species indicates the possibility of developing a prophylactic vaccine against leishmaniasis. The advancement of biotechnology tools has been showing to be a good prospect for the development of new products. In the present work, three proteins: a protein containing glutamine-rich tetratricopeptide repetitions (SGT), a hypothetical protein (LiHyS) and prohibitin (PHB), which were identified in a previous study using an immunoproteomic technique, in their recombinant form, were used as serological markers for leishmaniasis. The immunogenicity of the proteins was also evaluated in their recombinant forms isolated or in association, as well as a protein chimera composed of the major T-cell epitopes of the molecules. All associated with saponin as adjuvant and administered in BALB/c mice. Vaccine efficacy was evaluated against challenge infection with *L. infantum*. The results showed that the animals vaccinated with the proteins presented a high production of IFN-γ, IL-12 and GM-CSF, allied to a low production of IL-4 and IL-10, when compared to the control groups. Also, the animals showed a significant reduction in the parasite load in all organs analyzed. The protection was related to a high production of IFN-γ, which occurred mainly through $CD4^+$ T cells. Interestingly, the chimera presented a greater protection effectiveness and immunogenicity when compared to the use of proteins alone or in combination. As for the serological tests, the recombinant proteins also presented higher sensitivity and specificity values for the diagnosis of visceral leishmaniasis when compared to controls using the rA2 antigen and an antigen preparation of *Leishmania*. Thus, the present work presents novel proteins and a recombinant chimera that can be used as vaccine candidates against visceral leishmaniasis, as well as serological markers for detection of the disease in infected mammalian hosts.



**Keywords:** Leishmaniasis; recombinant proteins; protein chimera; vaccine; diagnosis; immunoproteomics.

# *INTRODUÇÃO*

## 1. INTRODUÇÃO

As leishmanioses são doenças causadas por parasitos protozoários do gênero *Leishmania* e que afetam milhões de pessoas em todo o mundo, com alta taxa de morbidade e letalidade. No Continente Americano, o Brasil é o país mais afetado, registrando aproximadamente 90% dos casos de leishmaniose visceral e 40% dos casos de leishmaniose tegumentar (LT), fato que torna a doença um problema de saúde pública e que requer uma atenção especial pelas autoridades competentes (ALVAR *et al., 2012).*

As medidas profiláticas e de controle para as leishmanioses visam, principalmente, a interrupção do ciclo biológico do parasito; entretanto, o número variado de espécies de *Leishmania spp.*, o caráter zoonótico da doença e a manutenção do ciclo silvestre da doença; dificultam a adoção de medidas efetivas de controle (TESH, 1995; WHO, 2010). Até o momento, nenhum dos testes de diagnóstico disponível é totalmente eficaz, apresentando valores variáveis de sensibilidade e/ou especificidade (GOMES *et al.*, 2014). O diagnóstico sorológico da infecção por *Leishmania* na sua forma subclínica (assintomática) é complicado devido aos baixos títulos dos anticorpos de determinados antígenos e às reações-cruzadas com outras doenças (DAVIES *et al*., 2003). Além disso, as drogas disponíveis para o tratamento apresentam alta toxidade, diversos efeitos colaterais, custo elevado e um aumento da resistência dos parasitos às mesmas vêm sendo observado (GONTIJO, MELO, 2004; MINODIER; PAROLA, 2007; MONDAL *et al.*, 2010).

Diante disso, o desenvolvimento ou o aperfeiçoamento de estratégias que possam atuar na prevenção das leishmanioses, como a busca por vacinas eficazes capazes de induzir imunidade protetora contra a infecção pelo parasito *Leishmania spp.*, tem-se tornado prioridade e vem sendo considerado como uma medida possível para o controle da doença (COSTA *et al.*, 2011). Porém, o desenvolvimento de vacinas contra a doença apresenta-se como uma tarefa complexa, embora possa ser considerada uma solução real (GRIMALDI; TESH, 1993; RAMIRO *et al.*, 2003; SUKUMARAN *et al.*, 2003; DESJEUX, 2004).

A Organização Mundial de Saúde preconiza que, para uma vacina ou teste de diagnóstico ser efetivo contra as leishmanioses, os mesmos devem compartilhar de uma combinação de diferentes proteínas e/ou peptídeos imunogênicos, e contemplar

diferentes regiões antigênicas e imunogênicas de proteínas dos parasitos. A utilização de novas ferramentas biotecnológicas, como a imunoproteômica, tem levado à identificação de proteínas com potencial para o emprego na prevenção e/ou diagnóstico de distintas doenças. Esta técnica foi recentemente empregada por nosso grupo de pesquisa para identificar proteínas antigênicas do parasito *Leishmania infantum* na leishmaniose visceral canina. Dentre elas, as moléculas: proteína contendo repetições de tetratricopeptídeo ricos em glutamina (SGT), uma proteína hipotética (LiHyS) e prohibitina (PHB), foram reconhecidas por imunoglobulinas de cães com a doença assintomática e clínica (sintomática), e no presente trabalho foram avaliadas como candidatos vacinais em camundongos BALB/c contra a infecção por *L. infantum*. Uma quimera contendo epitopos específicos de linfócitos T de tais proteínas foi construída e também utilizada nos experimentos de vacinação. Quanto à eficácia diagnóstica, ensaios de ELISA foram realizados usando as moléculas recombinantes em amplo painel sorológico.

# *REVISÃO DA LITERATURA*

# 2. REVISÃO DA LITERATURA

## 2.1 EPIDEMIOLOGIA DAS LEISHMANIOSES

As leishmanioses são doenças causadas por parasitas protozoários do gênero *Leishmania,* que acorrem em cinco continentes e são endêmicas em 98 países, possuindo elevada taxa de morbidade e mortalidade (ALVAR *et al*., 2012). Estima-se que haja uma incidência anual de aproximadamente 0,2 a 0,4 milhões de casos de leishmaniose visceral (LV) e de 0,7 a 1,2 milhões de leishmaniose tegumentar (LT) (ALVAR *et al.*, 2012; WHO, 2014). Este complexo de doenças é prevalente em áreas tropicais, subtropicais e Sul da Europa, com mais de 20 espécies de parasitos que causam a doença no homem.(WHO, 2014).

A doença acomete, principalmente, pessoas de classe econômica baixa. Está associada à desnutrição, ao deslocamento da população, condições precárias de habitação, ao sistema imunológico debilitado e à falta de recursos. A diferença no avanço da doença está relacionada com a espécie do parasita infectante, bem como a suscetibilidade do hospedeiro, podendo variar entre distintas manifestações cutâneas até a forma visceral da doença (ASHFORD, 2000). Nos seres humanos, as formas clínicas da doença são divididas em LV e LT. A última, por sua vez, pode se apresentar nas formas cutânea, mucosa e cutâneo-difusa (WHO, 2014).

Nas Américas, as formas cutânea e mucosa acometem pessoas em 20 países, sendo endêmica em 18 deles. Já os casos humanos de LV estão presentes em 12 países. No período entre 2001 e 2015 foram reportados 843.931 novos casos de LT, sendo 70% no Brasil, Colômbia e Peru e 52.176 novos casos de LV, sendo 96% no Brasil (OPAS/OMS, 2017). A figura 1 mostra a distribuição da doença no mundo em 2014.

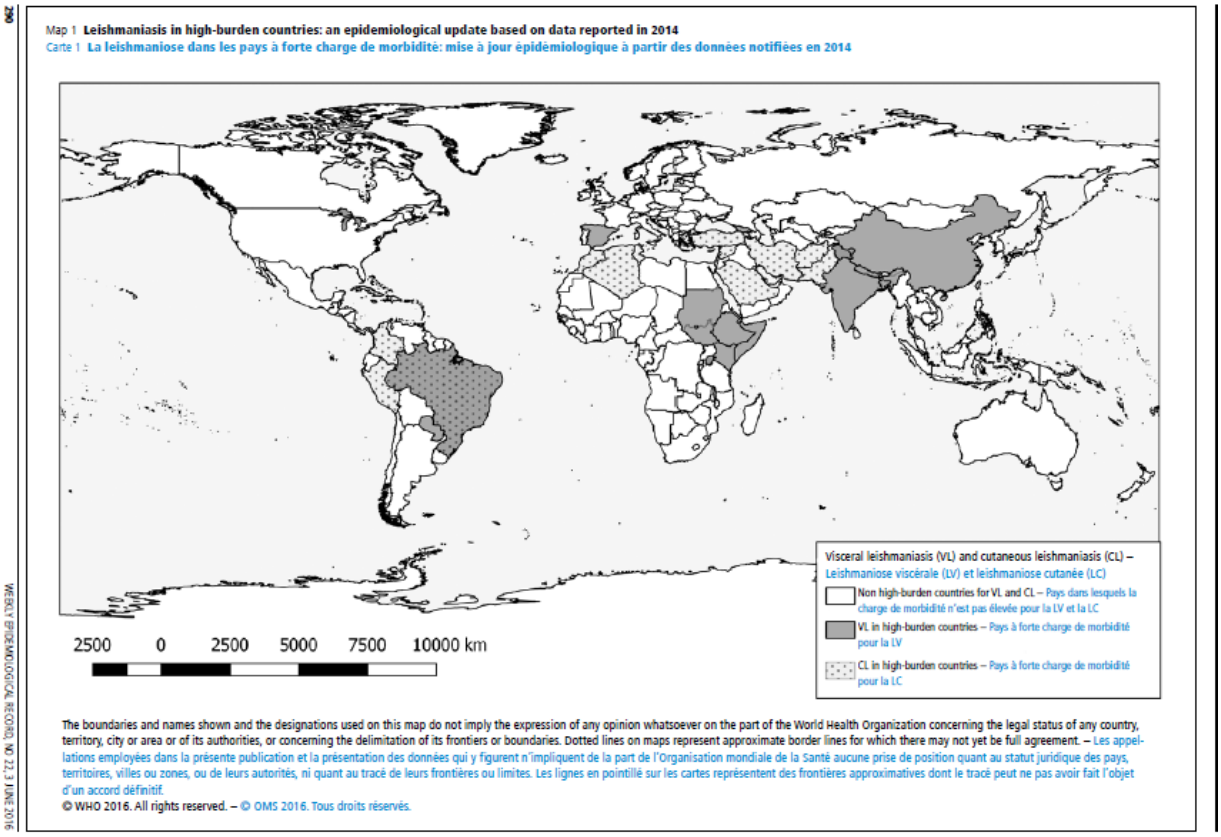


**Figura 1**: Epidemiologia das leishmanioses em 2014. Fonte: WHO 2014

As leishmanioses seguem sendo um problema de saúde pública devido à sua magnitude, complexidade clínica e biológica e epidemiológica. Apesar dos esforços já realizados para enfrentar a doença, novas estratégias e ações específicas ainda são necessárias para que os casos sejam mais precocemente diagnosticados e tratados de forma rápida e oportuna, evitando complicações e mortes (WHO 2014).

## 2.2 A ETIOLOGIA E CICLO BIOLÓGICO DO PARASITA *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 responsável pela transmissão é o inseto da Ordem Diptera, Família Psychodidae, Sub-Família Phlebotominae, pertencente aos gêneros *Phlebotomus* em países do Velho Mundo e *Lutzomyia* nas Américas (GRIMALDI; TESH, 1993). Dentre os hospedeiros primários incluem humanos, cães e gatos domésticos, gambás, raposas e ratos (QUINNELL;

COURTENAY, 2009). Os cães infectados são considerados como o principal reservatório urbano, responsáveis pela transmissão do parasito aos homens – o que torna a LVC não apenas um problema veterinário, mas também de saúde pública (BANETH *et al*., 2008; TEIXEIRA-NETO *et al*., 2010).

A transmissão do parasito ao inseto ocorre quando o mesmo realiza uma hematofagia em um hospedeiro vertebrado infectado. O vetor ingere juntamente com o sangue as formas amastigotas, que em seu intestino tornam-se promastigotas procíclicas e migram para o trato digestório médio e anterior, onde se multiplicam e diferenciam-se em promastigotas metacíclicas, colonizando as glândulas salivares do vetor. Quando o vetor flebotomíneo fêmea infectado realiza um repasto sanguíneo, as formas promastigotas metacíclicas são regurgitadas na pele do homem. Prontamente, as células do sistema fagocítico-mononuclear, neutrófilos, células dendríticas e especialmente os macrófagos migram para o local e fagocitam os parasitos (KAYE; SCOTT, 2011; RIBEIRO-GOMES, SACKS, 2012; HANDLER *et al*., 2015). No interior dos macrófagos ocorre a diferenciação das formas promastigotas em amastigotas e também sua replicação por divisão binária, podendo resultar no rompimento das células infectadas e consequentemente a liberação dos parasitos, iniciando uma infecção no mamífero. A proliferação também pode ocorrer em outras células de diferentes tecidos, como nos linfonodos e no fígado (BARSKY *et al*., 1978; REITHINGER *et al*., 2007). A Figura 2 resume o ciclo biológico do agente infeccioso das leishmanioses.

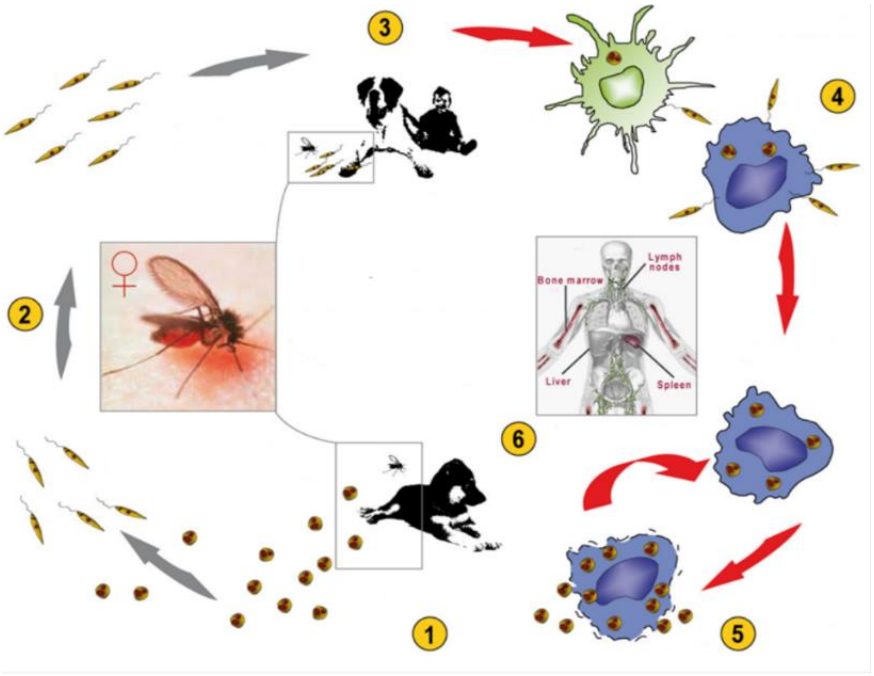


**Figura 2:** Ciclo biológico do parasito *Leishmania*: 1- Macrófagos infectados sendo ingeridos pelo vetor durante seu repasto sanguíneo. 2- No intestino do vetor, as amastigotas se diferenciam em promastigotas procíclicas e, posteriormente, em promastigotas metacíclicas, que migram para a porção anterior do intestino do mesmo. 3- Introdução de formas promastigotas metacíclicas na derme do hospedeiro mamífero. 4-Fagocitose das formas promastigotas metacíclicas por macrófagos do hospedeiro mamífero. 5- Rompimento do macrófago infectado liberando formas amastigotas, que poderão ser fagocitadas por novas células. 6- Principais órgãos acometidos pelo parasito causador da forma visceral. Adaptado de (NIETO *et al*., 2011)

## 2.3 MANIFESTAÇÕES CLÍNICAS DAS LEISHMANIOSES

A patogenia das leishmanioses é determinada pela espécie infectante e fatores relacionados ao hospedeiro, tais como o estado geral de saúde, genética e sistema imune (LOCKSLEY *et al.*, 1999; TRIPATHI; SINGH; NAIK, 2007). Deste modo, o hospedeiro pode resistir à infecção, desenvolver a forma assintomática ou ainda manifestar a doença na sua forma tegumentar ou visceral (KANE *et al.*, 2001). A doença é classificada de acordo com as manifestações clínicas em LT e LV (ASHFORD, 2000; DESJEUX, 2004).

A LT apresenta as formas clínicas: leishmaniose cutânea (LC), leishmanioses cutâneo-difusa (LCD) e leishmanioses mucosa (LM). Tais patologias podem causar desde uma lesão cutânea única, no local da picada do vetor e que pode apresentar cura espontânea, até lesões debilitantes, disseminadas por pela epiderme e mucosa do

paciente e que não apresentam cura espontânea, necessitando de tratamento quimioterápico (GRIMALDI; TESH, 1993).

A LC é a mais encontrada, ocorrendo em cerca de 90% dos pacientes. A doença se manifesta em partes mais expostas do corpo, como face, braços e pernas. Inicialmente apresenta-se como um nódulo no local da picada que evolui formando uma úlcera granulosa contornada por uma borda elevada. Em alguns casos, nódulos satélites podem ocorrer nas proximidades da lesão inicial. Quando curados, deixam cicatrizes permanentes, as quais podem levar a uma condição de morbidade (STEBUT, 2014).

A LCD constitui manifestação rara e grave, não apresentando cura espontânea. 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. Devido às frequentes recidivas, é considerada com 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).

A LM manifestam-se como lesões de caráter infiltrante, que podem ocasionar destruição parcial ou total da mucosa da cavidade do nariz, boca, faringe, laringe e, excepcionalmente, traqueia e árvore respiratória superior (WEIGLE, SARAVIA, 1996). Segundo a classificação proposta por MARZOCHI, MARZOCHI (1994) e considerando como parâmetros o tempo decorrido entre as lesões cutâneas e mucosas, o local de inoculação e a localização das lesões, a forma mucosa pode ser classificada em:

• Forma primária – situação em que a lesão de mucosa é causada pela picada do vetor diretamente na mucosa, restrita às mucosas labial e genital.

• Forma indeterminada – acometimento mucoso, sem identificação da porta de entrada, supondo-se que as lesões mucosas sejam originadas de infecção, sem manifestação cutânea clínica prévia.

• Forma tardia – caracterizada pelo aparecimento da lesão mucosa após alguns anos do surgimento da lesão cutânea, fato que costuma ocorrer nos dois primeiros anos. Mas, há relatos de sua ocorrência décadas após a erupção da lesão na pele.

A LV é caracterizada por um amplo espectro clínico, que pode variar em manifestações clínicas assintomáticas e sintomáticas que, por sua vez são classificadas como oligossintomáticas, moderadas e graves (CHAPPUIS *et al.*, 2007). A intensidade das manifestações clínicas varia de acordo com cada hospedeiro, podendo ser

assintomática por longos anos, dificultando o diagnóstico clínico (BADARÓ *et al*., 1986; GAMA *et al.,* 2004).

Na LV, a infecção parasitária acomete órgãos como o baço, fígado, órgãos linfoides 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, evidências sugestivas da presença dos parasitos, como hepatoesplenomegalia e inchaço dos gânglios linfáticos, que quando não tratadas podem levar o paciente ao óbito (CHAPPUIS *et al*., 2007).

A LV canina (LVC) apresenta manifestações clínicas diversas, que dependem de vários fatores, tais como: a imunidade inata do cão, a espécie infectiva do parasita e a resposta imune gerada pelo animal. O período de incubação da doença pode variar de poucos meses a alguns anos. Entretanto, cães infectados podem permanecer assintomáticos por longos períodos de tempo, ainda que infectivos durante tal época (LANOTTE *et al*., 1979; KEENAN *et al.*, 1984). Os cães sintomáticos podem apresentar: linfoadenomegalia, enfraquecimento crônico, alopécia, úlceras, dermatite esfoliativa, onicogrifose, anemia, hepatoesplenomegalia, disfunção renal severa, hipergamaglobulinemia e colites (CIARAMELLA *et al*., 1997; TAFURI *et al*., 2001).

As manifestações clínicas de acordo com cada espécie de parasito responsável em causar a infecção, e também os locais de ocorrência das doenças podem ser visualizados na tabela 1.

**Tabela 1:** Espécies de Leishmania. Adaptada de (WHO EXPERT COMMITTEE ON THE CONTROL OF THE LEISHMANIASES; WORLD HEALTH ORGANIZATION, 2010).

| Manifestação Clínica | Espécie |
|---|---|
| **Velho Mundo** | **Subgênero *Leishmania*** |
| Leishmaniose Visceral | *Leishmania donovani e Leishmania infantum* |
| Leishmaniose Cutânea | *Leishmania major, Leishmania tropica e Leishmania aethiopica* |
| Leishmaniose Cutâneo-Difusa | *Leishmania aethiopica* |
| **Novo Mundo** | **Subgênero *Leishmania*** |
| Leishmaniose Visceral | *Leishmania infantum* |
| Leishmaniose Cutânea | *Leishmania infantum, Leishmania mexicana, Leishmania pifanol e Leishmania amazonensis* |
| Leishmaniose Cutâneo-Difusa | *Leishmania mexicana e L. amazonensis* |
| **Novo Mundo** | **Subgênero *Viannia*** |
| Leishmaniose Cutânea | *Leishmania brasiliensis, Leishmania guyanensis, Leishmania panamensis e Leishmania peruviana* |
| Leishmaniose Mucosa | *Leishmania brasiliensis e Leishmania panamensis* |

## 2.4 DIAGNÓTICO DAS LEISHMANIOSES

O diagnóstico clínico das leishmanioses é um desafio, uma vez que a doença abrange um grande espectro de características clínicas (CHAPPUIS *et al*., 2007). Os sinais são frequentemente confundidos com os de outras doenças, tais como lepra e câncer para LC e malária, esquistossomose, tripanossomíase, tuberculose e desnutrição para a LV (SINGH, 2006). Desse modo, o diagnóstico deve ser realizado com base na associação entre parâmetros clínicos e exames laboratoriais confirmatórios (TESH, 1995).

O diagnóstico parasitológico é o mais conclusivo quando comparado aos demais testes, uma vez que a identificação do parasito realizada por meio de análises microscópicas em amostras supostamente contaminadas não deixa dúvidas sobre a infecção. Porém, apresenta limitações quando a infecção apresenta baixa carga parasitária, a leitura da lâmina exige de tempo e treinamento adequado e, além disso, é um método invasivo em relação à coleta das amostras (TAVARES *et al.*, 2003; REITHINGER *et al*., 2007).

O primeiro teste de diagnóstico utilizado na detecção da doença foi o teste de intradermoreação de Montenegro (IDRM), que se baseia na resposta de células T de

memória que tiveram contato prévio com o parasito, as suas desvantagens são as baixas especificidade e sensibilidade (WEIGLE *et al*., 1991; DE PAIVA-CAVALCANTI *et al*., 2015).

Testes sorológicos, tais como o ensaio imunoenzimático (ELISA), ensaio de imunofluorescência indireta (IFAT), teste de aglutinação direta (DAT), Western-blot e o teste imunocromatográfico (ICT) se baseiam na detecção de anticorpos e/ou antígenos específicos dos parasitos em amostras de soro ou plasma, também são utilizados no diagnóstico, porém apresentam variações na sensibilidade e/ou especificidade (MARZOCHI, MARZOCHI, 1994; TAVARES *et al*., 2003; DE PAIVA-CAVALCANTI *et al.*, 2015).

Uma das técnicas muito utilizadas no meio científico para a detecção das do material genético do parasito é a Reação em Cadeia da Polimerase (PCR) e a PCR-*Real Time*, porém são consideradas caras para uma aplicação em grandes inquéritos epidemiológicos e, além disso, apresentam limitações no processo de execução e qualidade do material coletado (PAIVA-CAVALCANTI *et al.*, 2015; SRIVASTAVA *et al.*, 2011; SUNDAR, RAI, PINHEIRO, 2002).

Normalmente, o diagnóstico laboratorial é realizado através de testes sorológicos e testes moleculares. A sensibilidade da pesquisa parasitológica é baixa quando comparada com a do IFAT, que é inferior à dos testes de ELISA e DAT, que geralmente apresentam uma alta sensibilidade e especificidade variável (ROMERO; BOELAERT, 2010).

Quando se trata da detecção na LVC, o maior problema enfrentado são os cães assintomáticos que atuam como reservatórios e potenciais transmissores da doença, o diagnóstico preciso neste caso é de extrema importância para a realização de estudos epidemiológicos e controle efetivo da (MOLINA *et al.*, 1994; DA COSTA-VAL *et al.*, 2007; MICHALSKY *et al.*, 2007). Porém, até o momento, nenhum dos testes de diagnóstico disponível apresenta-se totalmente eficazes, ocorrendo diferenças na sensibilidade e especificidade, tornando necessário um conhecimento epidemiológico e clínico da doença para concluir o diagnóstico (GOMES et al., 2014).

A fim de contornar esses problemas, proteínas recombinantes do parasito vêm sendo testadas como antígenos, com o objetivo de desenvolver um teste diagnostico mais sensível e específico para LVC, capaz de distinguir cães saudáveis vacinados de cães infectados (KUBAR, FRAGAKI, 2005). Além disso, objetiva-se diminuir a ocorrência de resultados falso-positivos devido à reatividade cruzada com anticorpos de

outras patologias, tais como: Doença de Chagas, ehrlichiose, babesiose, toxoplasmose, dentre outras (KAR, 1995; FERREIRA *et al.*, 2007; PORROZZI et al., 2007).

No Brasil, em 2011, o Ministério da Saúde através da Nota Técnica Conjunta 01/2011 estabeleceu como protocolo de diagnóstico da LVC o DPP® (Dual-Path Platform, Biomanguinhos, Fiocruz, Rio de Janeiro, Brasil) como teste de triagem e o ELISA (EIE-LVC kit, Biomanguinhos, Fiocruz, Rio de Janeiro, Brasil) como método confirmatório (FARIA, ANDRADE, 2012; COURA-VITAL *et al*., 2014; LAURENTI *et al.*, 2014). O DPP® trata-se de um teste imunocromatográfico composto pelos antígenos recombinantes rK39 e rK26, que apresenta elevada sensibilidade e especificidade na detecção de cães sintomáticos. Contudo, em relação à detecção de animais assintomáticos, apresenta uma variação em sua eficácia e baixa sensibilidade (GRIMALDI *et al*., 2012). O teste confirmatório de ELISA, por sua vez, apresenta uma elevada sensibilidade em identificar cães doentes, porém sua especificidade é baixa, o que pode ocasionar resultados falso-positivo (FARIA, ANDRADE, 2012.

Por fim, para considerar que um teste diagnóstico seja satisfatório, ele deve apresentar uma elevada sensibilidade e especificidade, além de fácil padronização, execução e custo acessível (SRIVASTAVA *et al*., 2011).

## 2.5 TRATAMENTO E MEDIDAS PROFILÁTICAS CONTRA AS LEISHMANIOSES

As leishmanioses foram descritas e reconhecidas pela ciência médica há mais de um século, ainda que existam poucas alternativas atuais para o controle e tratamento da doença (KLING, KÖRNER, 2013). O tratamento clínico das leishmanioses é realizado para evitar a mortalidade causada pela LV e reduzir a morbidade provocada pelas lesões desfigurantes nas formas mais graves de LT (FRANKE *et al*., 1990).

Os antimoniais pentavalentes são os fármacos de primeira linha na terapia medicamentosa contra as leishmanioses (GOTO, LINDOSO, 2010). Essa medicação está disponível em duas formulações: o antimoniato de N-metil glucamina, produzido sob o nome comercial de Glucantime® (Rhône Poulenc Rorer, França) e o estibogluconato de sódio, produzido sob o nome comercial de Pentostan® (Welcome Foundation, Inglaterra) (FRANKE *et al*., 1990; HERWALDT, 1999), sua administração é pela via intramuscular ou endovenosa e apresenta efeitos colaterais graves (TUON *et*

*al*., 2008). Infelizmente, um aumento na resistência dos parasitos a essas drogas tem sido documentado em várias regiões do mundo (GOTO, LINDOSO, 2010).

O desoxicolato de anfotericina B (Fungizone®) é um antifúngico com excelente atividade *in vitro* na eliminação de *Leishmania spp*. É usada extensivamente no caso de falhas no tratamento com os antimoniais, apesar de sua elevada toxicidade e do fato de necessitar de administração parenteral (AMATO *et al*., 2000; SERENO; HOLZMULLER; LEMESRE, 2000). Quatro formulações deste medicamento estão disponíveis: anfotericina B livre, anfotericina B lipossomal, anfotericina dispersão colesterol e anfotericina complexo lipídico. Todas as formulações compartilham eficácia semelhante, no entanto, foram observadas diferenças em relação aos seus efeitos colaterais, sendo os mais graves associados com o uso da AmpB livre (GOTO & LINDOSO, 2010).

A pentamidina (Lomidina®) tem sido usada no tratamento da LC e LM em algumas regiões do Mundo. É considerado um fármaco de segunda linha, mas comumente recomendado quando os antimoniais não podem ser administrados, embora também apresente efeitos adversos significantes e necessite de administração parenteral (MARZOCHI *et al*., 2010; NAGLE *et al*., 2014). Tal produto apresentou interesse renovado por seu possível uso como profilaxia secundária em pacientes co-infectados pelo HIV (DORLO, KAGER, 2008; PATEL, LOCKWOOD, 2009).

A paramomicina é um antibiótico aminoglicosídico, também chamado de aminosidina, é o único medicamento que apresenta atividade antileishmanial contra todas as formas de leishmaniose, sua via de administração é parenteral (CROFT *et al.*, 2006; DAVIDSON *et al.*, 2009; NAGLE *et al.*, 2014).

A miltefosina é uma alquilfosfocolina que tem atividade contra células cancerígenas e várias espécies de parasitos, assim como bactérias e fungos. Foi originalmente desenvolvida como um fármaco anticancerígeno, tendo sido aprovado na Índia como o primeiro tratamento oral para as leishmanioses (NAGLE *et al.*, 2014; PEYRON *et al*., 2005). Desde 2002, a miltefosina é o único agente oral utilizado no tratamento as formas de leishmaniose em diversos países no mundo (DORLO *et al.*, 2012).

Novos medicamentos eficazes e seguros, preferencialmente orais, são necessários. Os fármacos utilizados apresentam problemas bem conhecidos de toxicidade, eficácia, via de administração ou duração do tratamento e consequentemente

baixa adesão dos pacientes. Além disso, eles não eliminam completamente os parasitos dos indivíduos infectados (DESJEUX, 2004; EGGER *et al.*, 2010).

Devido às dificuldades inerentes ao tratamento e também pelo aumento do número de casos de recidiva da doença, algumas medidas preventivas podem ser adotadas, estas visam geralmente à interrupção do ciclo biológico dos parasitos, entretanto, seu caráter zoonótico, o número variado de espécies de *Leishmania spp.* e a manutenção do ciclo silvestre; dificultam a adoção de medidas de controle eficazes (TESH, 1995). Medidas aparentemente simples, como manter os cães em canis fechados durante períodos de atividade intensa do vetor, eliminar os microambientes que favorecem o desenvolvimento do vetor na residência, e utilizar nos cães coleiras impregnadas com inseticida (ALEXANDER, MAROLI, 2003; BANETH *et al., 2008).*Entretanto, a implementação dessas medidas não depende somente da consciência do proprietário do cão, mas, principalmente de questões socioeconômicas, os animais pertencentes a famílias com renda inferior a dois salários mínimo apresentam duas vezes mais chances de serem infectados quando comparados com os cães de famílias com maior renda (COURA-VITAL *et al.*, 2011).

O Programa de Controle da Leishmaniose Visceral (PCLV) tem encontrado várias dificuldades, incluindo: altas taxas de infecção; gestão ineficaz no controle de vetores; grande intervalo entre o diagnóstico e a eliminação de cães infectados, precisão insuficiente dos kits de diagnóstico para a detecção da doença, permitindo assim que os cães assintomáticos persistam como reservatórios (COURTENAY *et al.,* 2002; MAIA-ELKHOURY; SENA, 2008; COURA-VITAL *et al.*, 2011).

A eutanásia de cães soropositivos constitui-se em uma medida profilática adotada por órgãos governamentais, porém, devido ao fato de serem animais de estimação, e por tal procedimento não impactar significativamente na redução do número de casos da doença no homem; tal medida é considerada eticamente questionável e ineficaz (TESH, 1995; GONTIJO, MELO, 2004; NERY *et al*., 2017). A eliminação de reservatórios silvestres também não se encaixa como uma medida executável e ecologicamente correta, além da possibilidade de adaptação do parasito a outros reservatórios existentes naquele ambiente (GRAMICCIA; GRADONI, 2005; GRIMALDI; TESH, 1993) O tratamento dos cães é permitido apenas em alguns países, pois sua eficácia ainda se encontra em avaliação pelos pesquisadores, sendo necessários novos estudos de animais tratados (TRAVI *et al.*, 2018).

Devido às dificuldades enfrentadas no diagnóstico, no tratamento e na prevenção, o desenvolvimento de uma vacina eficaz se faz necessário e tem sido objeto de investigação de diversos grupos de pesquisa. Dentre os diferentes tipos de vacinas em desenvolvimento, aquelas que contêm o parasito vivo, mas atenuado ou avirulento, e as multiproteicas contendo antígenos de várias espécies de *Leishmania ssp*. se apresentam como as opções mais promissoras (ALVAR *et al.*, 2013; MUTISO *et al.*, 2013).

## 2.6 IMUNOLOGIA DAS LEISHMANIOSES

Para que um modelo animal possa ser utilizado em pesquisas, ele deve ser semelhante aos humanos, preferencialmente, quanto a etiologia, fisiopatologia, sintomatologia e resposta aos agentes terapêuticos ou profiláticos (BEHFOROUZ, WENGER, MATHISON; 1986). Uma boa opção de modelo animal é o modelo murino, pois apresenta fácil condição de manejo quando comparado com cães, hamsters, macacos, dentre outros animais. Isso facilita os estudos dos mecanismos imunológicos em diversas patologias (NATALE, MELLO, MANZONI-DE-ALMEIDA; 2016).

Um grande avanço no estudo das leishmanioses foi a elucidação do modelo de suscetibilidade, uma vez que camundongos BALB/c apresentam resposta do tipo T helper 1 (Th1) e T helper 2 (Th2) bem definidos quando da infecção com a espécie *L. major*, abrindo caminhos para a descrição dos papeis dos componentes celulares e humorais da resposta imunológica ao parasito *Leishmania* (MOSMANN *et al.*, 1986; LOCKSLEY *et al.*, 1987). Camundongos BALB/c são também utilizados como modelo de infecção para o estudo de candidatos vacinais contra várias espécies de *Leishmania spp.*, tais como *L. infantum* e *L. amazonensis* (SACKS, NOBEN-TRAUTH, 2002; WILSON; JERONIMO; PEARSON, 2005). Também, auxiliam na elucidação da relação parasito-hospedeiro; bem como na resposta imune às diversas espécies de *Leishmania*, favorecendo o entendimento das funções das células que compõem esse sistema complexo (NATALE, MELLO, MANZONI-DE-ALMEIDA; 2016).

A resistência da doença em camundongos C57BL/6 infectados por *L. major*, é devido à presença de linfócitos T $CD4^+$ com fenótipo Th1 produtores de níveis elevados de IFN-γ. Essa polarização ocorre nos linfonodos drenantes do sítio inflamatório devido a altos níveis de IL-12 produzidos pelas células dendríticas no local da infecção.

Linfócitos Th1 produzem níveis elevados de IFN-γ, citocina que atua diretamente sobre os macrófagos (MOSSER, EDWARDS, 2008). Uma população de macrófagos com atividade microbicida é ativada pela combinação dos sinais de IFN-γ e TNF. O macrófago infectado e ativado produz, então, ânions superóxido e radicais de oxigênio e nitrogênio. Através do metabolismo do aminoácido L-arginina pela enzima óxido nítrico sintase-indutível (iNOS), há produção de óxido nítrico (NO), que mata o parasito intracelular. A expressão de iNOS é induzida por TNF-α (TRACEY *et al.*, 2008).

Em camundongos BALB/C infectados por *L. major, a* susceptibilidade a doença acontece devido a resposta do tipo Th2, determinada pelo perfil de linfócitos $TCD4^{+}$, com altos níveis de IL-4 e baixos níveis de IFN-γ. Quando os macrófagos são ativados por IL-4, paralelamente a ativação de fatores de transcrição é promovida, aumentando então a expressão da enzima Aginase I, a qual converte L- arginina em ornitina e que não afeta a proliferação do parasito, diferentemente de quando os macrófagos são ativados por IFN-γ e TNF, onde produto da ação da enzima óxido nítrico sintase indutível (iNOS) lisam o parasito (KREIDER *et al.*, 2007; LOKE *et al.*, 2007). Outra citocina importante na proliferação dos parasitos e que independe do tipo de respostas Th1 ou Th2 é a IL-10. Quando macrófagos fagocitam parasitos opsonizados por IgG do hospedeiro, esses são induzidos a produzirem maiores quantidades de IL-10, inibe a produção de óxido nítrico (NO) por macrófagos infectados (KANE *et al.*, 2001; NOBEN-TRAUTH *et al.*, 2003).

### 2.6.1 Resposta Imune na Leishmaniose Visceral

O controle da forma visceral da doença está associado ao padrão de resposta do tipo Th1, com produção das citocinas IFN-γ ou TNF que culmina na ativação dos macrófagos e matam o parasito das espécies *L. infantum* e *L. donovani* (PEARSON, STEIGBIGEL, 1981; MURRAY, RUBIN, ROTHERMEL, 1983). Mesmo quando as manifestações clínicas não são apresentadas pelo hospedeiro humano, é produzido uma intensa resposta DTH (hipersensibilidade do tipo tardio) e alta produção de IL-2, IL-12 e IFN-γ por células mononucleares de sangue periférico (CARVALHO *et al.*, 1985; SACKS, PERKINS, 1985). Em pacientes sintomáticos, há ausência de produção de IL-2

e de IFN-γ e elevados níveis séricos de IL-10 (DE CAMARGO *et al.*, 2014; GOMES *et al.*, 2014).

A susceptibilidade e o desenvolvimento de LV em humanos são caracterizados pelo padrão de resposta Th2, com produção de citocinas IL-4 e IL-13, dentre outras, responsáveis por inibir células efetoras do sistema imune que levam à ativação clássica de macrófagos (BABALOO, KAYE, ESLAMI, 2001; THAKUR, MITRA, NARAYAN, 2003). A função da IL-17 ainda não é muito bem esclarecida na LV, entretanto, estudos correlacionam a presença de linfócitos Th17 secretando tal citocina em sinergismo com IFN-γ (NASCIMENTO *et al.*, 2015) e IL-22 (PITTA et al., 2009), exercendo papel protetor. Por outro lado, de acordo com o trabalho realizado por (ANDERSON *et al.*, 2007)**,** há maior gravidade da doença pela presença das células T reguladoras, que exerceriam efeito supressor sobre linfócitos Th1 por meio de mecanismos reguladores como a secreção de IL-10 e TGF-β.

### 2.6.2 Resposta Imune na Leishmaniose Tegumentar

Diferenças no desenvolvimento da doença causada por *L. major* ou *L. amazonensis* sugerem que diferentes aspectos da resposta imune podem estar envolvidos nas infecções por tais espécies, de forma que o papel protetor ou indutor de suscetibilidade não se aplica a todos os hospedeiros e a todas as espécies de parasitos (SOONG *et al.*, 1997; CAMPBELL *et al.*, 2003).

A suscetibilidade à infecção por *L. amazonensis* em modelos murinos não está diretamente ligada a uma polarização de resposta do tipo Th2, mas a uma baixa produção de citocinas como IFN-γ, IL-2, IL-12, dentre outras, o que mantêm a sobrevivência do parasito e uma elevada carga parasitária (JI, SUN, SOONG, 2003)**.** Estudos relataram que a infecção por essa espécie de parasito é capaz de dificultar a migração de células dendríticas do sítio de infecção para os linfonodos drenantes (HERMIDA *et al.*, 2014). Além de níveis baixos de IL-12, existe um comprometimento da expressão da cadeia β2 de seu receptor (HEINZEL *et al.*, 1993; JONES *et al.*, 2000)

Em camundongos BALB/c, a infecção por *L. amazonensis* ocorre em maior nível quando há depleção de neutrófilos, em um mecanismo dependente das citocinas IL-17 e IL-10 (SOUSA *et al.*, 2014). No homem, neutrófilos infectados por formas

promastigotas do parasito as produzem “NETs” (neutrophil extracelular traps) que eliminam os parasitos (GUIMARAES-COSTA *et al.*, 2009; GUIMARAES-COSTA *et al.*, 2014). Como são as primeiras células a chegarem ao sítio de infecção, os neutrófilos apresentam papel importante no controle do parasitismo no início da infecção (CARLSEN *et al.*, 2013; GUIMARAES-COSTA *et al.*, 2014).

Uma característica marcante da infecção por *L. amazonensis* é o papel que o IFN-γ desempenha, uma vez que baixas concentrações dessa citocina induzem à proliferação de formas amastigotas em macrófagos infectados *in vitro*, assim como na infecção por *L. major*, onde o IFN-γ é crucial na ativação de macrófagos, na morte do parasito e na resolução da infecção (QI *et al*., 2004). Outra diferença na infecção por *L. amazonensis* é que as células T reguladoras parecem ter um papel de proteção, porém sua ação protetora não foi associada à produção de TGF-β e IL-10 (JI *et al.*, 2005).

## 2.7. DESENVOLVIMENTO DE VACINAS CONTRA AS LEISHMANIOSES

O fato que sugere a possibilidade para o desenvolvimento de uma vacina contra as leishmanioses é que a maioria dos pacientes que tiveram contato com o parasito pode desenvolver imunidade duradoura de longo prazo (KUMAR *et al.*, 2010; SELVAPANDIYAN *et al.*, 2012). Para que uma vacina seja considerada efetiva contra as leishmanioses a mesma deve, preferencialmente, conter antígenos que sejam compartilhados por diferentes espécies dos parasitas, ser imunogênica contra a maioria das espécies, além de não interferir na imunogenicidade induzida pelos outros antígenos componentes da vacina (GRIMALDI, TESH, 1993; COELHO *et al*., 2003, CHÁVEZ-FUMAGALLI *et al*., 2010).

A leishmanização foi uma das primeiras estratégias vacinais utilizadas no combate às leishmanioses, a 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. Entretanto, devido ao risco de causar uma infecção crônica, esse método foi abandonado, dando lugar à utilização de parasitos mortos (HANDMAN, 2001; KUMAR, ENGWERDA, 2014).

Os resultados obtidos em experimentos que utilizaram os parasitos irradiados ou autoclavados foram conflitantes e insatisfatórios para proteger contra a doença

(GRIMALDI, TESH, 1993; HANDMAN, 2001). Observou-se que a persistência do patógeno no organismo do indivíduo tivesse uma grande importância na obtenção de uma resposta, e que para isso seria então necessário administrar mais doses do parasito. Sendo assim, vacinas com parasitos vivos atenuados pudesse ser a solução para contornar tal obstáculo (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). Apesar desse tipo de vacina permitir que o sistema imune do hospedeiro seja capaz de gerar uma resposta específica contra os antígenos e que células efetoras e de memória conferira uma proteção. Uma das preocupações com a utilização desta vacina é a possível reversão da virulência dos parasitos e a produção de formas mais graves da doença. Todavia, uma alternativa para contornar este problema seria a eliminação dos genes de virulência, a utilização de parasitos mutantes sensíveis a determinadas drogas, bem como a utilização de espécies não patogênicas, como *L. tarentolae.* Porém, é necessário 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).

A utilização de frações proteicas de *Leishmania spp*. além de apresentar-se mais segura, apresenta 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 também sendo testadas com diferentes sistemas de entrega.

A formulação vacinal LBSap, baseada em extrato bruto de *L. brasiliensis* adicionada de saponina, foi administrada em cães e demonstrou ser capaz de induzir uma resposta humoral com anticorpos específicos anti-*Leishmania,* além de induzir um perfil celular ativando LT CD4 e CD8. Além disso, uma elevada produção de IFN-ɣ e IL-12 bem como uma redução na carga parasitária do baço demonstraram sua imunogenicidade e proteção contra a infecção experimental por *L. infantum* (ROATT *et al.*, 2012; RESENDE *et al*., 2013). Uma vacina similar foi também testada com extrato bruto de *L. amazonensis* e BCG, nomeada de Leishvaccine, sendo 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 Europa, proteínas de *L. infantum* secretadas e purificadas do sobrenadante da cultura foram utilizadas em uma formulação denominada LiESAP-MDP que posteriormente se tornou a vacina CaniLeish, apresentando eficácia de 92% nos animais vacinados (LEMESRE *et al*., 2007). No Brasil, a vacina Leishmune que utiliza o composto ligante fucose-manose (FML) de formas promastigotas de *L. donovani* em conjunto com um 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) sendo capaz de induzir proteção na maioria dos animais vacinados (PALATNIK-DE-SOUSA, 2012). A proteína recombinante A2 em associação com a saponina deu origem a vacina comercial denominada Leish-Tec® e provou induzir um aumento significativo de IFN-ɣ nos cães vacinados, além de reduzida produção de IL-10 (FERNANDES *et. al.,* 2008).

As técnicas de biologia molecular permitem a produção de proteínas sob a forma recombinante, tornando-se mais viáveis em relação à utilização de extratos proteicos solúveis ou totais dos parasitos (MAIA *et al.,* 2012). Desta forma, moléculas específicas de determinada espécie ou fase de vida do parasito puderam ser reproduzidas e estudadas, originando novos alvos vacinais (JOSHI *et al.,* 2014). O primeiro antígeno recombinante testado na imunização contra as leishmanioses foi a glicoproteína gp63, proteína de virulência do parasito e encontrado conservado em todas as espécies de leishmania. Tanto a proteína nativa quanto sua forma recombinante foram capazes de estimular uma resposta protetora em camundongos, demonstrando que a imunogenicidade foi relacionada ao processamento e apresentação de peptídeos e não apenas da estrutura conformacional nativa (DAS, ALI, 2012).

Por meio da técnica de imunoproteômica, Coelho e colaboradores (2012) identificou novas proteínas, algumas ainda hipotéticas, mas que podem se apresentar como potenciais candidatas à vacina, pois foram antigênicas e imunogênicas na doença canina. Dentre elas, as proteínas hipotéticas LiHyp1, LiHyD, LiHyT e LiHyR apresentaram-se como possíveis alvos vacinais 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. (MARTINS *et al*., 2013; LAGE, *et al.*,2015; MARTINS *et al*, 2016; RIBEIRO *et al.,* 2018).

O desenvolvimento de vacinas recombinantes ou mesmo de peptídeos sintéticos apresentam a vantagem de permitir a utilização de epitopos determinados e já caracterizados, (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 apresentam vantagens como 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 apresentam desvantagens como a incapacidade de alguns indivíduos responderem ao estímulo ofertado, além disso, normalmente vacinas com peptídeos isolados 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).

Por meio da técnica de *phage display,* Costa e colaboradores (2014) 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*.

Devido aos polimorfismos genéticos encontrados no sistema imune de mamíferos, vacina de quimera proteica, 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). Essas vacinas 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).

Vacinas de DNA têm se tornado atrativas para 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, YURINA, 2018, RIBEIRO *et al.*,

2018). Porém, dúvidas relacionadas à segurança deste tipo de vacina 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*.

Recentemente vacinas utilizando proteínas salivares de *Phlebotomus spp*. ou *Lutzomyia spp.*, proteínas 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- *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; THAKUR; KAUR; KAUR, 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 para uma prevenção efetiva contra as leishmanioses.

## *OBJETIVOS*

# 3. OBJETIVOS

## 3.1 OBJETIVO GERAL

Avaliar o desempenho diagnóstico das proteínas rSGT, rLiHyS, rPHB nas leishmanioses visceral e tegumentar e a eficácia vacinal das proteínas administradas nas suas formas isoladas, em associação ou como uma quimera polipeptídica contendo seus principais epitopos de células T em camundongos BALB/c contra a infecção experimental por *L. infantum.*

## 3.2 OBJETIVOS ESPECÍFICOS

- Analisar as proteínas previamente identificadas em *L. infantum* (Coelho *et al*., 2012), por meio de programas de bioinformática de predição de epitopos específicos para linfócitos T $CD4^+$ e $CD8^+$; com posterior alinhamento das sequências de aminoácidos para verificação de sua conservação entre as espécies.

- Clonar a região codificadora das proteínas selecionadas; expressar em células de *Escherichia coli* e purificar as proteínas recombinantes.

- Desenhar e sintetizar uma quimera polipeptídica contendo epitopos selecionados das proteínas.

- Avaliar o potencial diagnóstico das proteínas selecionadas rSGT, rLiHyS e rPHB contra um painel sorológico composto por amostras de soros de cães e humanos.

- Imunizar camundongos BALB/c com as proteínas recombinantes e com a quimera polipeptídica e avaliar o perfil da resposta imune celular gerada nos

animais imunizados, por meio da dosagem das citocinas IFN-γ, IL-12, GM-CSF, IL-10 e IL-4 por ELISA de captura e citometria de fluxo.

- Infectar os camundongos BALB/c imunizados com a espécie *L. infantum* e avaliar o grau de proteção nos animais imunizados e infectados, por meio da carga parasitária no baço, fígado, medula óssea e linfonodos drenantes através das técnicas de diluição limitante e por RT-PCR.

- Avaliar o perfil da resposta imune celular gerada nos animais imunizados e infectados, por meio da dosagem das citocinas IFN-γ, IL-12, TGF-β, GM-CSF, IL-10 e IL-4 por ELISA de captura e citometria de fluxo; além da dosagem de óxido nítrico e da resposta humoral.

# *METODOLOGIA E RESULTADOS*

## 4. METODOLOGIA E RESULTADOS

As seções de metodologia e resultados serão apresentadas sob a forma de artigos científicos, que foram diretamente derivados do projeto e que foram publicados em revistas internacionais de elevado impacto; conforme a Resolução nº 02/2013, de 18 de setembro de 2013 do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical.

**Artigos:**

**Artigo 1** – Recombinant small glutamine-rich tetratricopeptide repeat-containing protein of *Leishmania infantum:* Potential vaccine and diagnostic application against visceral leishmaniasis.

**Artigo 2** – Antigenicity, immunogenicity and protective efficacy of a conserved *Leishmania* hypothetical protein against visceral leishmaniasis.

**Artigo 3** – Serological diagnosis and prognostic of tegumentary and visceral leishmaniasis using a conserved *Leishmania* hypothetical protein.

**Artigo 4** – Recombinant prohibitin protein of *Leishmania infantum* acts as a vaccine candidate and diagnostic marker against visceral leishmaniasis.

**Artigo 5** – Vaccination with a $CD4^+$ and $CD8^+$ T-cell epitopes-based recombinant chimeric protein derived from *Leishmania infantum* proteins confers protective immunity against visceral leishmaniasis

## 4.1 ARTIGO 1 – BREVE INTRODUÇÃO

O primeiro artigo a ser apresentado foi intitulado como “Recombinant small glutamine-rich tetratricopeptide repeat-containing protein of *Leishmania infantum*: Potential vaccine and diagnostic application against visceral leishmaniasis” e publicado pela revista internacional *Molecular Immunology* (doi: http://dx.doi.org/10.1016/j.molimm.2017.09.017).

O presente estudo avaliou a aplicação de uma proteína recombinante contendo repetições de tetratricopeptídeo ricos em glutamina (rSGT, XP_001467120.1) da espécie *Leishmania infantum* na proteção contra infecção e no diagnóstico sorológico da leishmaniose visceral.

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Research paper

# Recombinant small glutamine-rich tetratricopeptide repeat-containing protein of Leishmania infantum: Potential vaccine and diagnostic application against visceral leishmaniasis

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ABSTRACT

Different *Leishmania* proteins have been evaluated in order to find a potential vaccine candidate or diagnostic marker capable of providing long lasting protection against infection or helping to identify infected mammalian hosts, respectively. However, just few molecules have fulfilled all the requirements to be evaluated. In the current study, we evaluated the prophylactic and diagnostic value against visceral leishmaniasis (VL) of a small glutamine-rich tetratricopeptide repeat-containing (SGT) protein from *Leishmania infantum* species. In a first step, the immune response elicited by the immunization using the recombinant protein (rSGT) plus saponin was evaluated in BALB/c mice. Immunized animals had a low parasitism in all evaluated organs. They developed a specific Th1 immune response, which was based on protein-specific production of IFN-γ, IL-12 and GM-CSF, and a humoral response dominated by antibodies of the IgG2a isotype. Both $CD4^+$ and $CD8^+$ T cells contributed to the IFN-γ production, showing that both T cell subtypes contribute to the resistance against infection. Regarding its value as a diagnostic marker, rSGT showed maximum sensitivity and specificity to serologically identify *L. infantum*-infected dog and human sera. No cross-reactivity with sera from humans or dogs that had other diseases was found. Although further studies are necessary to validate these findings, data showed here suggest immunogenicity of rSGT and its protective effect against murine VL, as well as its potential for the serodiagnosis of human and canine VL.

## 1. Introduction

Leishmaniasis is a neglected tropical disease resulting in a global mortality rate, with approximately 60,000 cases per year (WHO, 2016). Visceral leishmaniasis (VL) can be fatal if left untreated, and the disease represents a serious public health problem in countries where it is endemic (Alvar et al., 2012). American VL is mainly caused by the *L. infantum* species, and its clinical manifestations include fever, fatigue,

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weight loss, enlarged lymph nodes, hepatosplenomegaly, and pancytopenia. Parasites multiply and reside inside phagocytic cells, disseminating through the lymphatic and vascular systems and infecting new cells in the reticulum endothelial system, resulting in the development of the disease (Saporito et al., 2013).

VL is diagnosed by means of clinical evaluation associated with laboratorial tests (Sundar and Rai, 2002). These tests include parasite detection by means of conventional and molecular parasitological exams, which are considered specific but present problems of sensitivity. In the case of PCR technique, it is applied in only some centers and present a high cost (Arora et al., 2008). The serological diagnosis is also used to identify the disease; however, problems related to the sensitivity and/or specificity of the antigens in the laboratorial techniques are also registered (Boelaert et al., 2007; Niknam et al., 2014; Salam et al., 2011). There is also the concern of cross-reactivity with other diseases, making the diagnosis process complex. In this scenario, new candidates are necessary to improve the sensitivity and specificity of the diagnostic tests of VL (Salles et al., 2017).

Since the treatment against VL presents problems related to the drugś toxicity, high cost and/or parasite resistance; prophylactic vaccination could be better evaluated as an alternative measure of control of disease. In this context, studies of vaccine candidates have been developed in the last decades, although the most of them have been based on experimental mouse (Athanasiou et al., 2017; Duthie et al., 2016; Santos et al., 2017) or dog (Regina-Silva et al., 2016; Roatt et al., 2017; Schaut et al., 2016) models and cannot be extrapolated to humans. However, the fact that patients who recover from VL usually develop resistance against reinfections indicates that a protective vaccine against human disease VL is also feasible (Saha et al., 2006). The pathogenesis of disease is associated with the overproduction of the cytokines IL-4 and IL-10, whereas molecules that selectively induce the development of a Th1 response, such as IFN-γ, IL-12 and GM-CSF, are considered protective as they activate infected cells stimulating them to kill amastigotes (Singh et al., 2012; Verma et al., 2010). So far there is no human vaccine available, although several antigens have been tested in murine and canine models. In this context, new candidates to be used as effective immunogens against VL are still under investigation.

In the current study, we used a *Leishmania* protein, the small glutamine-rich tetratricopeptide repeat-containing (SGT, LinJ.30.2740), which was specifically identified in *L. infantum* promastigotes and amastigotes by antibodies in VL dogs sera (Coelho et al., 2012) as a vaccine candidate and diagnostic marker for VL. This protein was described as a co-chaperone of the HSC70 chaperones, possibly acting as a negative regulator of the protein refolding activity of the HSP70/HSP40 complex (Angeletti et al., 2002). Since molecular chaperones play a pivotal role in *Leishmania* parasites, controlling cell fate and ensuring intracellular survival, an immunological application of SGT was proposed (Ommen et al., 2010). SGT was applied as an immunogen against murine VL, as well as used to stimulate PBMCs collected from untreated and treated VL patients, aiming to evaluate the specific lymphoproliferative response and cytokine production induced in these immune cells. The antigenic role of this SGT was also evaluated employing canine and human serological panels, aiming to identify its potential as a new serological marker of VL.

## 2. Materials and methods

### 2.1. Ethics and human and canine sera

Experiments were performed according the Animal Research Ethics Committee from the Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil (protocol number 333/2015), as well as by Human Research Ethics Committee from UFMG, with the protocol number CAAE–32343114.9.0000.5149. Sera samples from VL patients (n = 20) which were diagnosed by means of clinical evaluation and demonstration of *L. infantum* kDNA in bone marrow aspirates by PCR technique were used. Also, samples from healthy individuals living in endemic (n = 20, Belo Horizonte) or non-endemic (n = 15, Poços de Caldas, Minas Gerais, Brazil) areas of VL, as well as from subjects diagnosed with paracoccidioidomycosis (n = 4), leprosy (n = 15), aspergillosis (n = 8), and Chagas's disease (n = 20) were used. None of the VL patients had been treated with antileishmanial drugs before blood sampling. Regarding canine sera, samples were collected from healthy dogs living in endemic (n = 15, Belo Horizonte) or non-endemic (n = 15, Poços de Caldas) areas of disease. Also, sera of asymptomatic (n = 8) or symptomatic (n = 15) VL dogs were used. The disease was confirmed by means of the identification of *L. infantum* kDNA in bone marrow samples by PCR technique, as well as by positive serological results by using the IFAT-LVC® and EIE-LVC® commercial kits (BioManguinhos, Rio de Janeiro, Brazil). Symptomatic animals presented three or more of the following symptoms: weight loss, alopecia, adenopathy, renal azotemia, onychogryposis, hepatomegaly, splenomegaly, exfoliative dermatitis on the nose, tail, and ear tips; whereas asymptomatic dogs presented positive parasitological and serological results, but no clinical signal of the disease. To evaluate the cross-reactivity of the antigens, samples of healthy animals vaccinated with Leish-Tec® (n = 15), and from those experimentally infected with *Ehrlichia canis* (EC, n = 10) or *Trypanosoma cruzi* (TC, n = 10) were also used.

### 2.2. SGT sequence analysis and production of the recombinant antigens

The similarity of the *L. infantum* SGT amino acid sequence (LinJ.30.2740) was evaluated by the BLAST tool and Cobalt (Constraint-based Multiple Alignment Tool) program, available at (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi). The following *Leishmania* spp. were used in the analysis: *L. major* (XP_001684878), *L. mexicana* (XP_003877417), and *L. braziliensis* (XP_001566896). A low identity in *L. donovani* was found, and this analysis was not included in the current study. In addition, the secondary structure of SGT protein was predicted by using the *L. infantum* sequence and the JPRED4 program, available at http://www.compbio.dundee.ac.uk/jpred4/index_up.html, as described elsewhere (Drozdetskiy et al., 2015). The recombinant protein was cloned from *L. infantum* kDNA using specific primers. The DNA fragment was purified and linked into a pGEM®-T vector system (Promega, USA) and the recombinant plasmid was used to transform *E. coli* XL1-Blue bacteria. Fragment obtained from digestion of pGEM-rSGT plasmid was ligated into a pET28a-TEV vector, and cells were transformed. Sequencing was performed to confirm the identity of the insert by using a MegaBace 1000 automatic sequencer apparatus (Amersham Biosciences, USA).

For the purification of the rSGT protein, cells were induced with 1.0 μM isopropyl-β-D-thiogalactopyranoside (IPTG, Promega®, Canada), and cultures were incubated for 2 h at 37 °C. Then, cells were ruptured by six cycles of ultrasonication with cycles of 30 s each (36 MHz), followed by five cycles of freezing and thawing, and debris were removed by centrifugation. The protein (45.8 kDa) was purified onto HisTrap HP affinity column (GE Healthcare Life Sciences, USA) connected to an AKTA system. The eluted fractions were concentrated in Amicon® ultra15 centrifugal filters 10,000 nominal molecular weight limit (NMWL, Millipore, Germany), and further purified on a Superdex™ 200 gel-filtration column (GE Healthcare, USA). As a control antigen, the A2 recombinant (rA2) protein was used has being purified as described elsewhere (Coelho et al., 2003). After purification, both proteins were passed through a polymyxin-agarose column (Sigma-Aldrich, USA) in order to remove residual endotoxin content (Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000 kit, BioWhittaker, MD, USA). A 12% SDS-PAGE and mass spectrometry were performed and purified protein showed purity greater than 99% (data not shown).

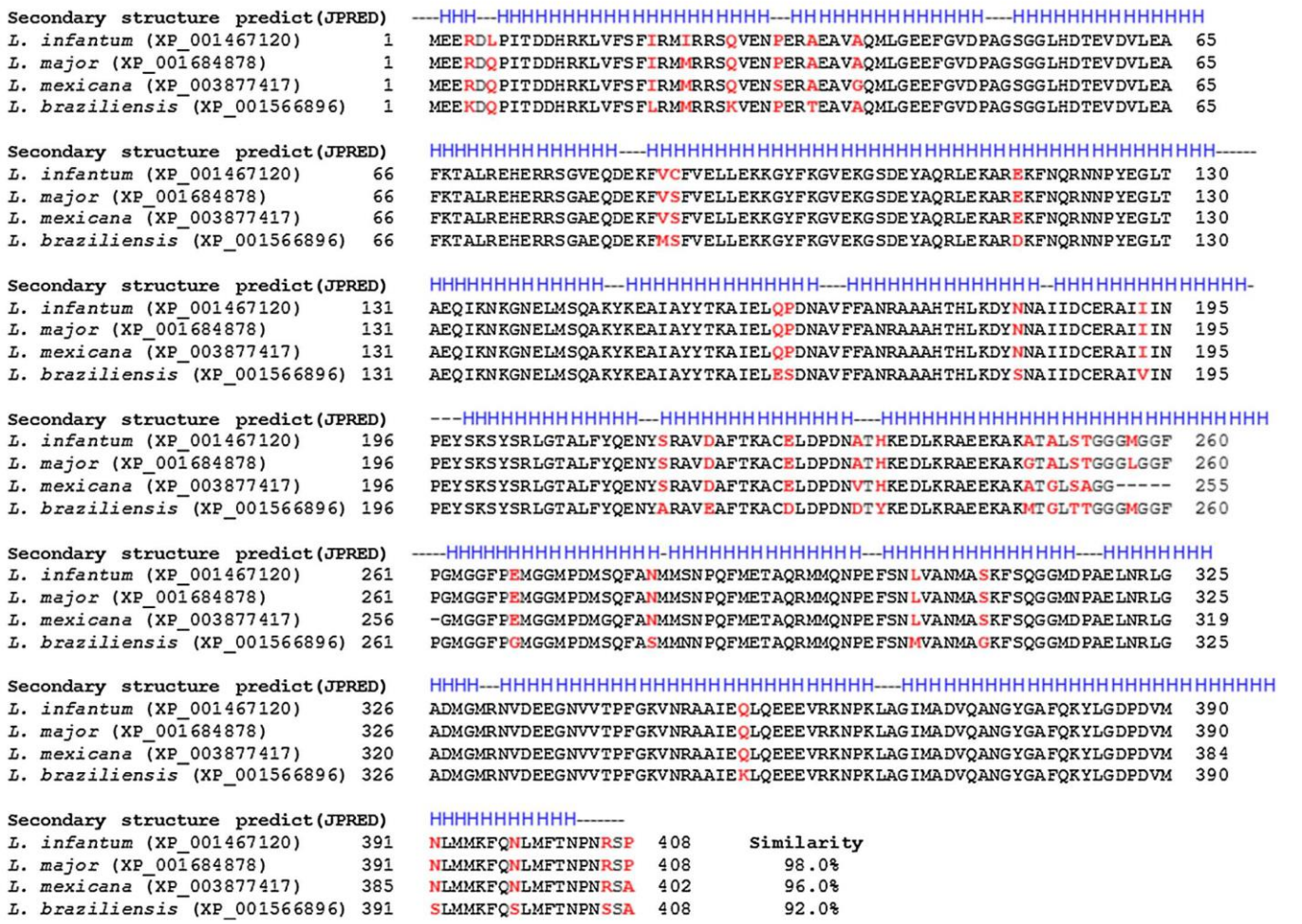


**Fig. 1.** Analysis of the amino acid sequence of the SGT protein. An *in silico* assay of the *L. infantum* SGT protein (XP_001467120.1) was performed by using the BLAST tool and Cobalt (Constraint-based Multiple Alignment Tool) program. The similarity search among *Leishmania* protein sequences was performed with the following species: *L. major* (XP_001684878), *L. mexicana* (XP_003877417), and *L. braziliensis* (XP_001566896). The distinct residues are shown in red color, as well as the identity percentage between them. The secondary structure of protein was predicted using *L. infantum* sequence and the JPRED4 program (available at http://www.compbio.dundee.ac.uk/jpred4/index_up.html). Abbreviation: H: alpha-helix. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 2.3. ELISA for the serodiagnosis of VL

Titration curves were performed to determine the most appropriate concentration of antigens and sera samples dilutions to be used in the serological assays. Then, rSGT, rA2 and *L. infantum* SLA (0.25, 1.0, and 1.0 μg per well, respectively) were added into the wells in microtiter plates (Falcon), diluted in 100 μL coating buffer (50 mM carbonate buffer, pH 9.6) for 18 h at 4 °C. Next, free binding sites were blocked using 200 μL of PBS-T (phosphate buffer saline plus Tween 20 0.05%) containing 5% casein during 1 h at 37 °C. After washing the plates five times with PBS-T, they were incubated with 100 μL of human or canine sera (1:400 and 1:200 diluted in PBS-T, respectively), for 1 h at 37 °C. Plates were subsequently washed seven times in PBS-T, and incubated with anti-human or anti-dog IgG horseradish-peroxidase conjugated antibodies (1:10,000 and 1:5000 diluted in PBS-T; catalog I5260 and A6792, respectively, Sigma-Aldrich, USA), for 1 h at 37 °C. After washing the plates seven times with PBS-T, reactions were developed by incubation with 100 μL of a solution composed by 20 μL $H_2O_2$ 30 vol., 2 mg ortho-phenylenediamine and 10 mL citrate-phosphate buffer, pH 5.0, during 30 min and in the dark. The reactions were stopped by adding 25 μL 2 N $H_2SO_4$. Optical density (O.D.) values were read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada) at 492 nm.

### 2.4. Animals, parasite, vaccination and infection

Female BALB/c mice (8 weeks age), which were obtained from the breeding facilities of the Institute of Biological Sciences, UFMG, were maintained under specific pathogen-free conditions. *Leishmania infantum* (MOM/BR/1970/BH46) strain was used. The soluble *L. infantum* antigenic extract (SLA) was prepared from $2 \times 10^9$ stationary-phase promastigote cultures (5-7 day-old) (Coelho et al., 2003). Briefly, parasites were washed three times in 5 mL of cold sterile phosphate-buffered saline (PBS 1x). After five cycles of freezing and thawing, suspension was centrifuged at 10,000 x *g* for 15 min at 4 °C; and aliquots containing SLA were collected and stored at −80 °C until use. Protein concentration was estimated by the Bradford method (Bradford, 1976). For the immunization experiments, mice (n = 16, per group) were vaccinated subcutaneously in their left hind footpad with 20 μg of rSGT with or without 20 μg saponin (*Quillaja saponaria* bark saponin, Sigma-Aldrich, USA), or received saline or saponin. Three doses were administered at 14-day intervals. Thirty days after the last dose, animals (n = 8, per group) were infected subcutaneously in their right hind footpad with $10^7$ *L. infantum* stationary promastigotes. Sixty days after infection, animals were euthanized and liver, spleen, bone marrow (BM), and paws' draining lymph nodes (dLN) were collected for the parasitological and immunological evaluations.

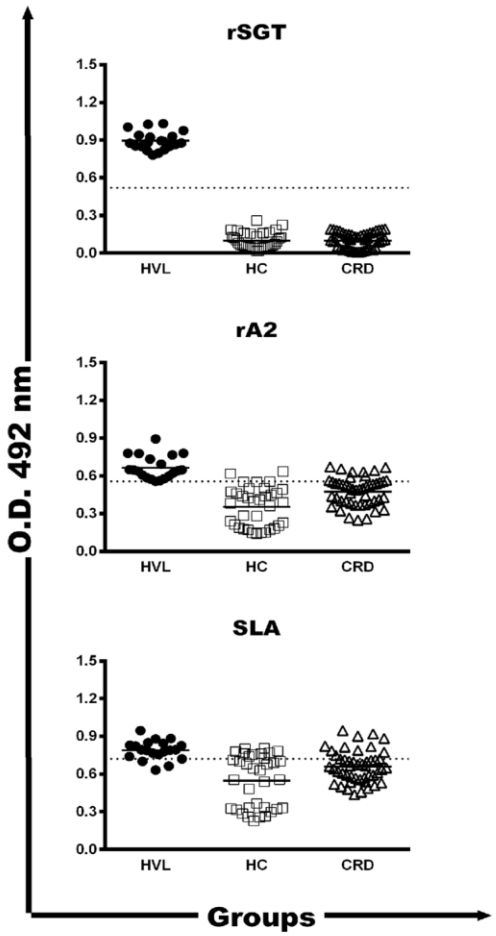


**Fig. 2.** ELISA assays using a human serological panel. The rSGT protein was evaluated as an antigen for the serodiagnosis of human VL. For this, serum samples collected from VL patients (HVL, n = 20), healthy subjects living in endemic (n = 20) or non-endemic (n = 15) areas of the disease, and classified as healthy control group (HC, n = 35), as well as from those diagnosed with paracoccidioidomycosis (n = 4), leprosy (n = 15), aspergillosis (n = 8), or Chagas disease (n = 20), which were classified as potentially cross-reactive diseases group (CRD, n = 47), were used in the serological assays. The individual O.D. values are shown, and were used to calculate the means of the groups. The dotted line represents the cut-off value calculated by a ROC analysis. rA2 and *L. infantum* SLA were used as control antigens.

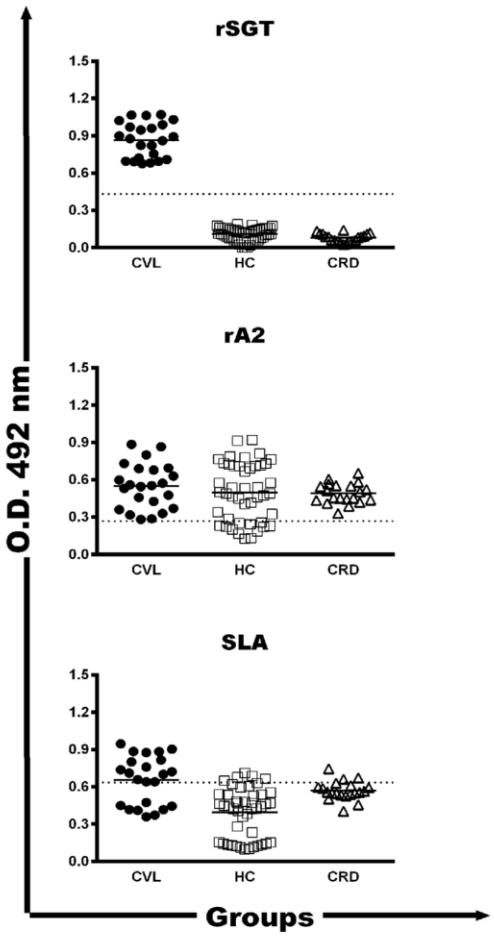


**Fig. 3.** ELISA using a canine serological panel. The rSGT protein was also evaluated as an antigen for the serodiagnosis of canine VL. For this, sera collected from asymptomatic (n = 8) or symptomatic (n = 15) VL dogs, classified as canine visceral leishmaniasis group (CVL, n = 23), as well as from healthy dogs living in endemic (n = 15) or non-endemic (n = 15) area of disease, classified as healthy control group (HC, n = 30), and those from dogs immunized with Leish-Tec® vaccine (n = 15), or from those experimentally infected with *Trypanosoma cruzi* (n = 10) or *Ehrlichia canis* (n = 10), classified as cross-reactive diseases group (CRD, n = 45), were used in the assays. The individual O.D. values are shown, and they were used to calculate the means of each group. The dotted line represents the cut-off value calculated by a ROC analysis. The rA2 and *L. infantum* SLA were used as control antigens.

**Table 1**
ELISA assays for the serodiagnosis of visceral leishmaniases. The diagnostic efficacy of rSGT, rA2 and *L. infantum* SLA was evaluated using a canine and human serological panel. ROC curves were used to determine ELISA sensitivity (Se), specificity (Sp), confidence interval (95%CI), area under curve (AUC), and Youden index of each antigen. Parameters were calculated using O.D. values obtained from 88 and 102 canine and human serum samples.

| Antigen | Human samples | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | AUC | *P* value | Cut-off | Se(%) | 95%CI | Sp(%) | 95%CI | Youden index |
| rSGT | 1.00 | < 0.0001 | 0.5203 | 100 | 83.16–100 | 100 | 95.60–100 | 1.00 |
| rA2 | 0.94 | < 0.0001 | 0.5558 | 100 | 83.16–100 | 85.4 | 75.83–92.20 | 0.85 |
| SLA | 0.84 | < 0.0001 | 0.7203 | 85.0 | 62.11–96.79 | 74.4 | 63.56–83.40 | 0.59 |
| Antigen | Canine samples | | | | | | | |
| | AUC | *P* value | Cut-off | Se(%) | 95%CI | Sp(%) | 95%CI | Youden index |
| rSGT | 1.00 | < 0.0001 | 0.4320 | 100 | 85.18–100 | 100 | 94.48–100 | 1.00 |
| rA2 | 0.57 | 0.2811 | 0.2693 | 100 | 85.18–100 | 18.5 | 9.92–30.03 | 0.18 |
| SLA | 0.74 | 0.0005 | 0.6358 | 65.2 | 42.73–83.62 | 87.7 | 77.18–94.53 | 0.53 |

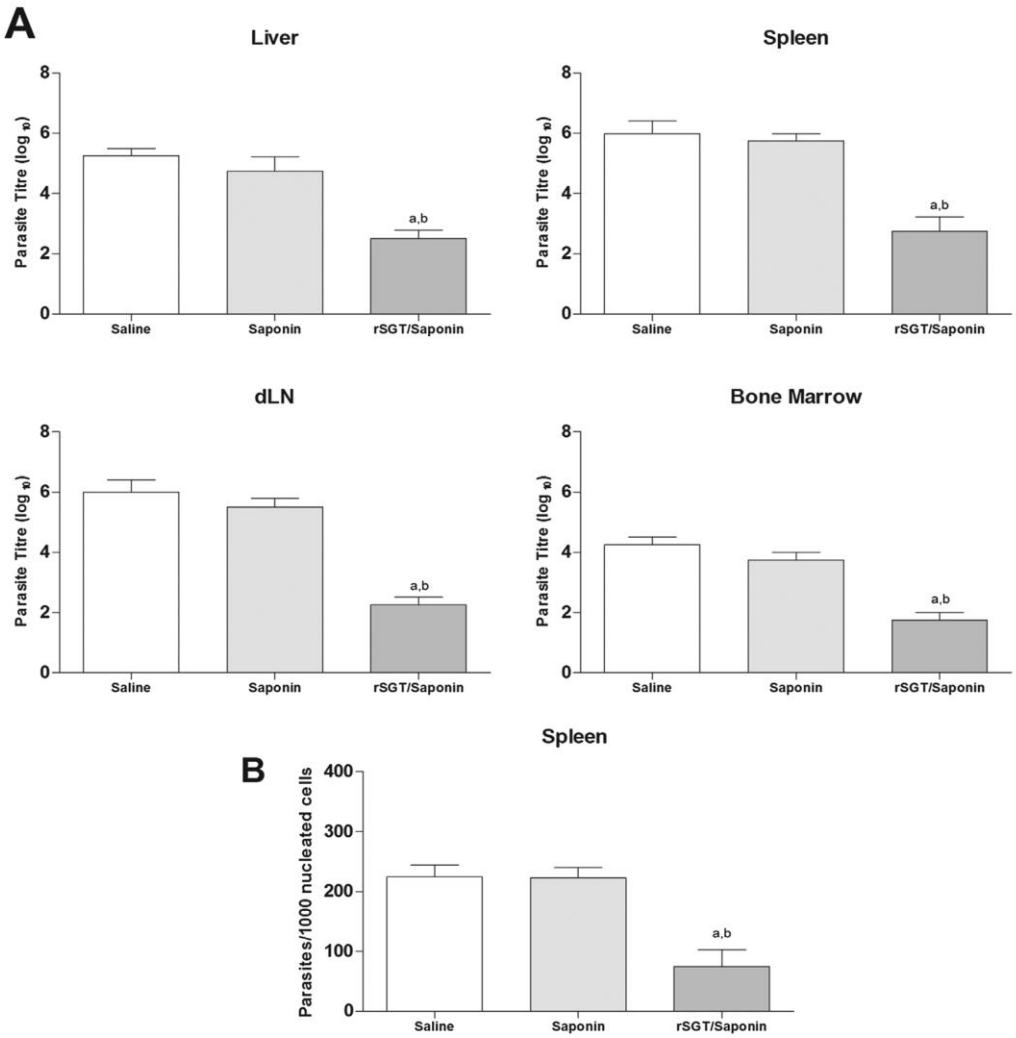


**Fig. 4.** Protection against *Leishmania infantum* infection. BALB/c mice were inoculated with saline or immunized with saponin or rSGT/saponin and 60 days after infection with $1 \times 10^7$ *L. infantum* stationary promastigotes they were euthanized (n = 8 per group). Parasite load was investigated by a limiting-dilution technique (A) and *q*PCR assay (B). For this, liver, spleen, bone marrow, and paws' draining lymph nodes (dLNs) of the animals were collected and processed. Bars represent the mean plus standard deviation. [a]indicates statistically significant difference in relation to the saline group ($P < 0.0001$). [b]indicates statistically significant difference in relation to the saponin group ($P < 0.0001$).

### 2.5. Evaluation of the parasite burden

The parasite load was evaluated by a limiting-dilution technique and quantitative PCR (*q*PCR). For this, spleen, liver, dLN and BM were macerated and concentrated by centrifugation at 2000*g*, when pellet was resuspended in 1 mL of Schneider's insect medium plus 20% FBS. Then, 220 µL were plated onto 96-well flat-bottom microtiter plates (Nunc, Nunclon®, Roskilde, Denmark), and diluted in log-fold serial dilutions in Schneider's medium ($10^{-1}$ to $10^{-12}$ dilution) using 96-well flat-bottom microtiter plates (Nunc, Nunclon®, Roskilde, Denmark). Each sample was plated in triplicate and parasiteś presence was analyzed by microscopy after 7 days of culturing at 24 °C. Results were expressed as the log of the titer (*i.e.*, the dilution corresponding to the last positive well) adjusted per milligram of organ (Coelho et al., 2003). In addition, the splenic parasite load was also evaluated by *q*PCR technique (Martins et al., 2017). Experiments were repeated twice and showed similar results.

### 2.6. Cellular and humoral response

Cytokine production and antibody response were evaluated in two moments: 30 days after the last vaccine dose and before infection, and 60 days after infection. For this, mice (n = 8 per group, in each time) were euthanized and their spleens were collected, and splenocytes were cultured ($5 \times 10^6$ cells per mL and per well) in 24-well plates (Nunc). Cells were incubated in complete RPMI medium (control), which was added with 20% FBS, 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, pH 7.4; or stimulated with rSGT or SLA (10 and 25 µg/mL, respectively) during 48 h at 37 °C, 5% $CO_2$. Then, IFN-γ, IL-4, IL-10, and IL-12 levels were measured in cell supernatants using commercial kits (Pharmingen®, San Diego, CA, USA). Nitrite production was also evaluated using cellular supernatants by the Griess method (Green et al., 1982). The involvement of the $CD4^+$ and $CD8^+$ T cells in IFN-γ production was evaluated in the spleen cells of the rSGT/saponin-vaccinated mice, in which cells were stimulated with protein or SLA in the presence of monoclonal antibodies against mouse IL-12 (C17.8), CD4 (GK 1.5), or CD8 (53-6.7), all in a concentration of 5 µg/mL. Appropriate isotype-matched controls (no azide/low endotoxin™), rat IgG2a (R35-95), and rat IgG2b (95-1) were used (Pharmingen®, USA). A flow cytometry was developed to evaluate the IFN-$\gamma^+$, TNF-$\alpha^+$ and IL-$10^+$-producing $CD4^+$ and $CD8^+$ T cells frequency (Martins et al., 2017). Results were expressed as indexes which were calculated by the ratio between the percentage of $CD4^+$ and $CD8^+$ T cells in the stimulated cultures, and the values obtained using the unstimulated cultures (SLA/CC ratio). Antibody production was evaluated in sera samples of the animals in both times: 30 days after the last vaccine dose and before infection, and 60 days after infection challenge. Protein- and parasite-specific IgG1 and IgG2a isotype levels were evaluated by an indirect ELISA. Briefly, rSGT and SLA (0.5 and 1.0 µg per well, respectively) were used as antigens in the plates, and individual sera were 1:100,

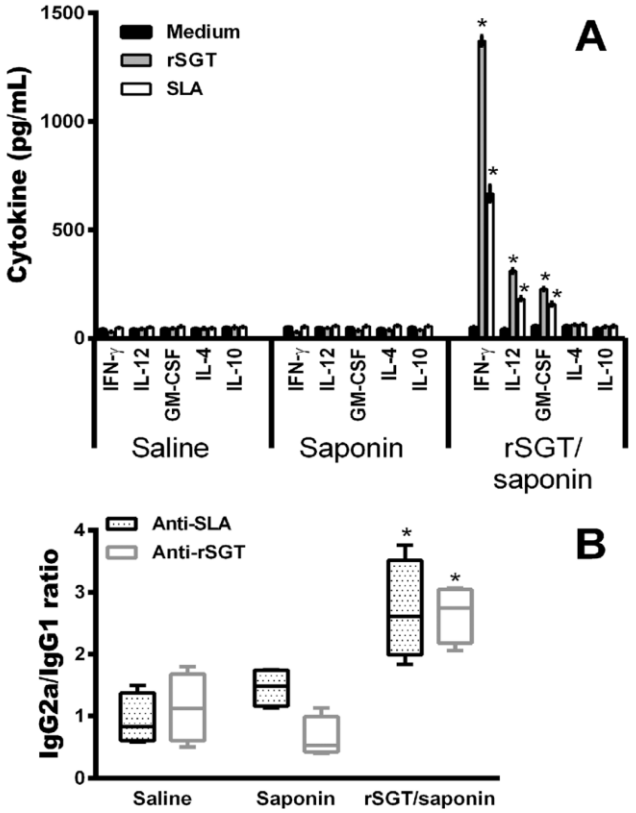


**Fig. 5.** Immune response induced before *Leishmania infantum* infection. Spleen cells of the infected and/or vaccinated mice (n = 8 per group) were collected 30 days after the last immunization, and cells were unstimulated (medium) or stimulated with rSGT or SLA (10 and 25 μg/mL, respectively), during 48 h at 37 °C in 5% $CO_2$. IFN-γ, IL-12, GM-CSF, IL-4, and IL-10 levels were measured in culture supernatants by ELISA (A). Also, serum samples were collected and the anti-protein and anti-parasite IgG1 and IgG2a isotype levels were measured. The IgG2a/IgG1 ratio was calculated in all groups as a measure of humoral response (B). Bars represent the mean ± standard deviation. *indicates statistically significant difference in relation to the saline and saponin groups ($P < 0.0001$).

diluted in PBS-T. Anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich, USA) were used in 1:5000 and 1:10,000 dilutions, which were performed in PBS-T, respectively, and reactions were developed as described above.

### 2.7. Stimulation of human PBMCs and cytokine assays

Lymphoproliferative response and IFN-γ and IL-10 production were evaluated in human PBMCs collected from untreated and treated VL patients. Patients samples (n = 10) were collected before the initiation of treatment and 6 months after the end of the treatment sessions using pentavalent antimonials (Sanofi Aventis Farmacêutica Ltda., Suzano, São Paulo, Brazil). Cells ($1 \times 10^7$) were labeled with carboxy fluorescein diacetate succinimidyl ester (CFSE), and incubated by 10 min at 37 °C, when they were centrifuged by three times during 7 min, and 50 μL were analyzed by a flow cytometry. PBMCs were cultured in 48-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA), with each well containing 800 μL of RPMI 1640 medium, and they ($1 \times 10^6$) were added in triplicate to the wells containing 100 μL of RPMI (control), being incubated alone (medium) or stimulated with rSGT or SLA (10 or 25 μg/mL, respectively) during 5 days at 37 °C in 5% $CO_2$. After, cells were collected, washed twice in fluorescence activated cell sorter (FACS) buffer, and fixed using a FACS fixing solution. Then, PBMCs were analyzed to obtain the flow cytometric acquisition and analysis which were performed using a FACScalibur® instrument (Becton Dickson − BD, USA). The Cell-Quest™ software package (Franklin Lakes, NJ, USA) was used for data acquisition and analysis based on 30,000 events per sample (Martins et al., 2017). In addition, cell supernatants were collected and IFN-γ and IL-10 levels were measured by a capture ELISA using commercial kits (Human IFN-γ and IL-10 ELISA Sets, BD Biosciences, USA), according to manufacturer's instructions.

### 2.8. Statistical analysis

Results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed by using GraphPad Prism™ (version 6.0 for Windows). ROC (receiver operating characteristic) curves were constructed to analyze the diagnostic potential of the antigens, and obtain their sensitivity, specificity, positive predictive value, negative predictive value, and likelihood ratio. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by the Bonferronís post-test for multiple comparisons between the groups. Differences were considered significant at $P < 0.05$.

## 3. Results

### 3.1. Antigenicity of the rSGT protein

The amino acid sequence of *L. infantum* SGT protein was evaluated in different *Leishmania* species. The results showed a high similarity with the protein expressed in *L. major* (XP_003722404), *L. donovani* (XP_003864600), *L. braziliensis* (XP_001568126), and *L. guyanensis* (CCM18788), with identity values higher than 92% (Fig. 1). A low sequence identity was found when SGT was evaluated in other Trypanosomatides (data not shown). As previously described (Ommen et al., 2010), SGT protein family presents a TPR domain, an all-helical structural motif affording protein–protein interactions, an N′-terminal coiled-coil motif required for dimerization, and a glutamine-rich region at the C terminus. The secondary structure of the protein from *L. infantum* shown here is in line with these findings (Fig. 1).

The diagnosis application of the recombinant protein was evaluated using human and canine serological panels. Regarding human sera, when rSGT was tested, the individual O.D. values in all VL samples were above the cut-off value (Fig. 2). Accordingly, the values of sensitivity and specificity were both of 100%. On the other hand, using rA2 protein, the values were of 100% and 85.4%, respectively, whereas using SLA, they were of 85.0 and 74.4%, respectively (Table 1).

Regarding the canine samples, the individual O.D. values of VL samples were also all above of the cut-off value (Fig. 3), and sensitivity and specificity were both of 100%, using rSGT in the experiments. On the other hand, using rA2, these values were of 100% and 18.5%, respectively, whereas using SLA; they were of 65.2% and 87.7%, respectively (Table 1).

### 3.2. Protection of rSGT/saponin-immunized BALB/c mice against L. infantum infection

The protective efficacy against *L. infantum* challenge in the vaccinated animals was evaluated 60 days post-infection by the measurement of the parasite load by two distinct techniques. First, a limiting-dilution assay was employed using spleen, liver, BM and dLN of the infected and vaccinated animals. When animals were immunized with the rSGT/saponin combination and challenged, significant reduction in the order of 2.8- and 2.3-log in spleen, 3.3- and 3.0-log in liver, 2.5- and 2.0-log in BM and 3.8- and 3.0-log in dLN were observed in comparison to the values obtained with animals receiving saline or saponin, respectively (Fig. 4A). A *q*PCR technique was also performed in the spleen of the animals. rSGT/saponin-vaccinated mice and challenged presented reduction in the order of 2.4- and 2.0-times in the parasitism when compared to the values found in the saline and saponin groups,

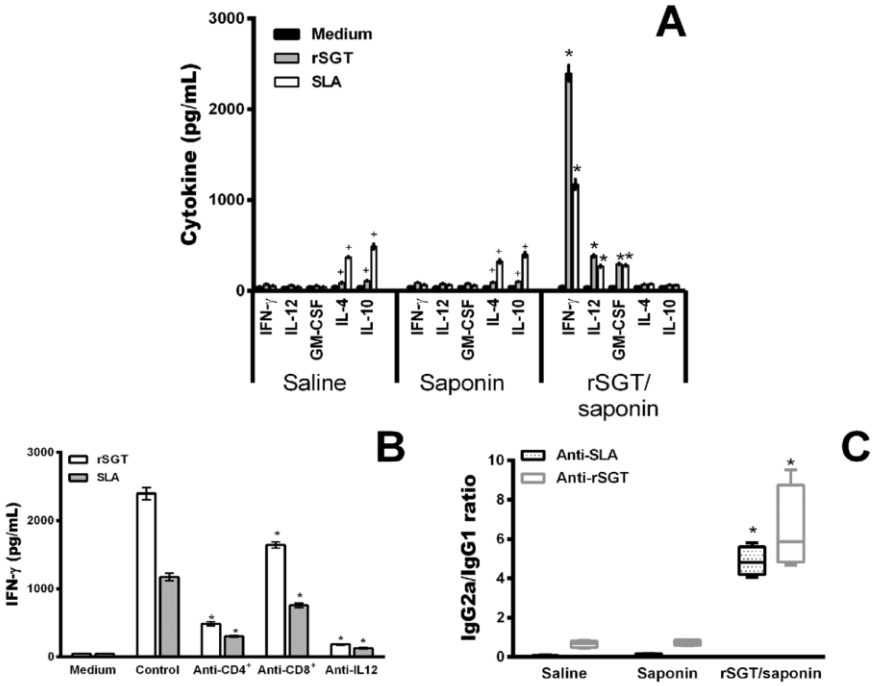


**Fig. 6.** Immune response induced after *Leishmania infantum* infection. Spleen cells of the infected and/or vaccinated mice (n = 8 per group) were collected 60 days after infection, and cells were unstimulated (medium) or stimulated with rSGT or SLA (10 and 25 μg/mL, respectively), during 48 h at 37 °C in 5% $CO_2$. IFN-γ, IL-12, GM-CSF, IL-4, and IL-10 levels were measured in culture supernatants by ELISA (A). IFN-g production was also measured in the rSGT/saponin group after addition of anti-IL-12, anti-CD4, and anti-CD8 monoclonal antibodies in the splenic cultures, followed by incubation by 48 h at 37 °C in 5% $CO_2$ (B). In addition, sera samples were collected and the anti-protein and anti-parasite IgG1 and IgG2a isotype levels were measured. The IgG2a/IgG1 ratio was calculated in all groups as a measure of humoral response (B). Bars represent the mean ± standard deviation. *indicates statistically significant difference in relation to the saline and saponin groups (A and C) and control (B) ($P < 0.0001$).

respectively (Fig. 4B).

### 3.3. Th1 immune response generated in rSGT/saponin-vaccinated mice before and after infection

Immunological response was evaluated before and after infection. Before infection, rSGT-saponin-vaccinated animals showed high levels of protein and parasite-specific IFN-γ, IL-12 and GM-CSF when compared to saline and saponin groups (Fig. 5A). Humoral response was also evaluated, and rSGT-saponin-vaccinated mice showed higher IgG2a/IgG1 ratios when rSGT and SLA were used as antigens (Fig. 5B). After infection, rSGT/saponin group mice still showed a polarized Th1 profile (Fig. 6A).

When the involvement of $CD4^+$ and $CD8^+$ T cells in the IFN-γ production was evaluated in rSGT-saponin-vaccinated animals, significant reduction in the production of this cytokine was obtained when anti-IL-CD4 and anti-CD8 monoclonal antibodies were used to deplete $CD4^+$ T cells and $CD8^+$ T cells *in vivo*, respectively (Fig. 6B). The analysis of the humoral response after infection corroborated the results found before challenge, since higher IgG2a/IgG1 ratios were observed in the vaccinated animals as compared to the values found in control groups (Fig. 6C).

As an additional parameter to evaluate the immunogenicity of the vaccine, intracytoplasmic cytokine-producing T cells frequency was investigated by a flow cytometry assay. Infected and rSGT/saponin-vaccinated mice showed significantly higher levels of IFN-$\gamma^+$-producing $CD4^+$ and $CD8^+$ T cells that were associated with significantly lower levels of both IL-$10^+$-producing T cells, indicating a Th1 profile before and after the infection (Fig. 7). Although TNF-$\alpha^+$-producing $CD4^+$ and $CD8^+$ T cells frequency was slightly higher in the rSGT/saponin group, it was not statistically different from the other groups. Nitrite secretion was also evaluated in order to investigate macrophages activation in the experimental groups. Infected and rSGT/saponin-vaccinated mice showed higher levels of nitrite as compared with saline and saponin groups (Fig. 8).

### 3.4. Lymphoproliferation and cytokine response in PBMCs from human VL patients

Aiming to evaluate the immunogenicity of rSGT protein in VL patients, lymphoproliferative response induced by this antigen was evaluated in PBMCs from untreated and treated patients. Using PBMCs from untreated patients, the proliferative indexes after stimuli using rSGT and SLA were of 1.5 ± 0.2 and 0.5 ± 0.2, respectively, however, when cells of treated patients were stimulated, higher values of lymphoproliferation were found, being results of 5.5 ± 0.3 and 0.8 ± 0.3, respectively. As a culture control, cells were unstimulated, and results were 0.3 ± 0.2 and 0.2 ± 0.1, when rSGT and SLA were as stimuli, respectively ($P < 0.0001$). After, IFN-γ and IL-10 levels were evaluated in the cellular supernatants. Using rSGT as stimulus, IFN-g and IL-10 levels were of 887.6 ± 56.5 and 70.4 ± 16.3 pg/mL, respectively, in recovered and treated VL patients, whereas in untreated patients and developing the active disease, these values were of 150.5 ± 19.6 and 106.6 ± 11.0 pg/mL, respectively. Using SLA as stimulus, IFN-g and IL-10 production in PBMCs of treated patients was of 257.5 ± 38.5 and 92.8 ± 9.7 pg/mL, respectively, whereas in untreated patients these values were of 78.5 ± 10.5 and 497.0 ± 27.4 pg/mL, respectively ($P < 0.0001$).

## 4. Discussion

Leishmaniasis is a disease complex in which a protective vaccine is possible to develop since exposure and cure from infection usually results in long-lasting immunity (Srivastava et al., 2016). In this context, the development of vaccines to prevent against disease can be considered as an alternative measure for VL control. The candidate molecules are mainly based on the parasite combined or isolated recombinant proteins which are administered in mammalian models being associated with immune adjuvants in order to induce a specific cellular response (Athanasiou et al., 2017; Baharia et al., 2015; Mortazavidehkordi et al., 2016). However, the partial efficacy of these candidates, as well as the absence of a product able to confer protection against human disease, makes the identification of new candidates a necessity.

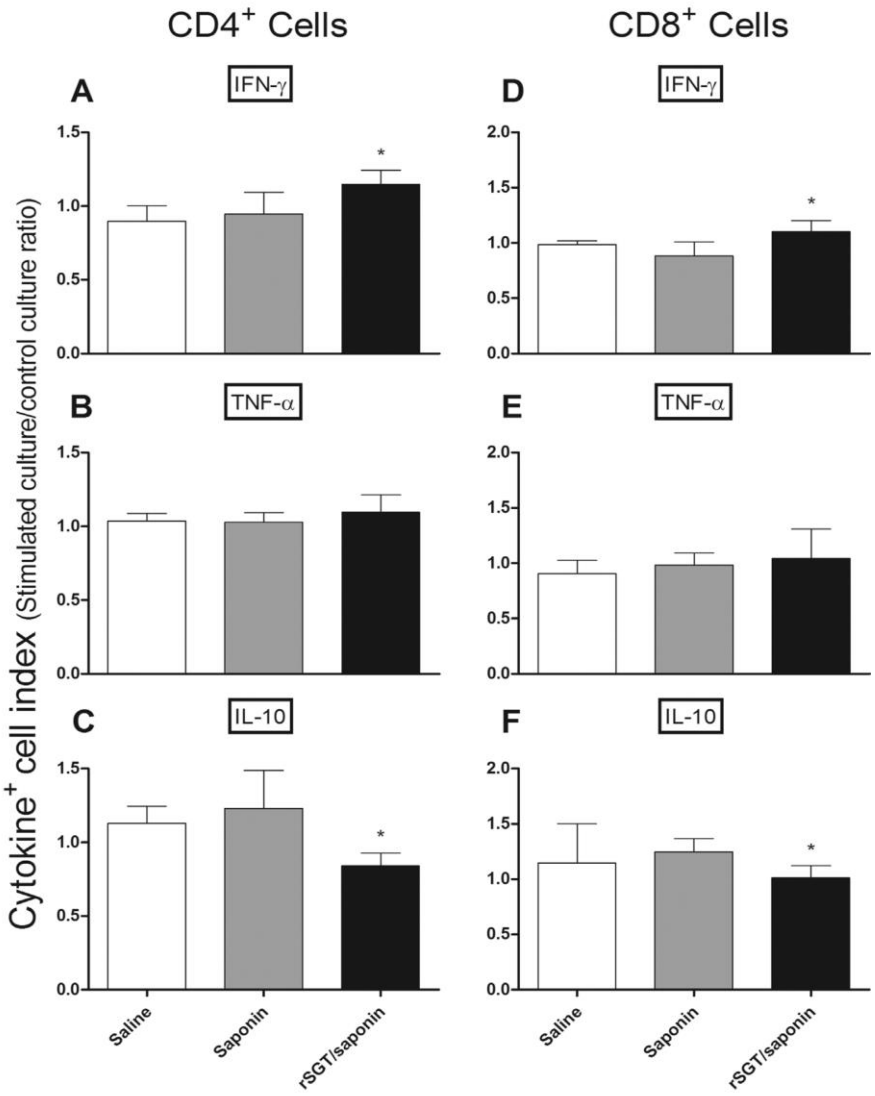


**Fig. 7.** Intracytoplasmic cytokine-producing T cells frequency in spleen cells of infected and rSGT/saponin-vaccinated mice. The cytokine-producing $CD4^+$ and $CD8^+$ T cell frequency was calculated as the ratio between the $cytokine^+$ cells obtained in the SLA-stimulated cultures and the unstimulated cultures (SLA/CC ratio). Mice (n = 8 per group) received saline (white bars) or were immunized with saponin (light grey bars) or rSGT/saponin (black bars) and later challenged with *L. infantum*. Their splenocytes were collected after 60 days and stimulated with SLA. Results were reported as cytokine indexes (Stimulated culture/unstimulated culture ratio) in A, B and C for the $CD4^+$ T cells, and in D, E and F for the $CD8^+$ T cells. Bars represent the mean ± standard deviation. *indicates statistically significant difference in relation to the saline and saponin groups ($P < 0.0001$).

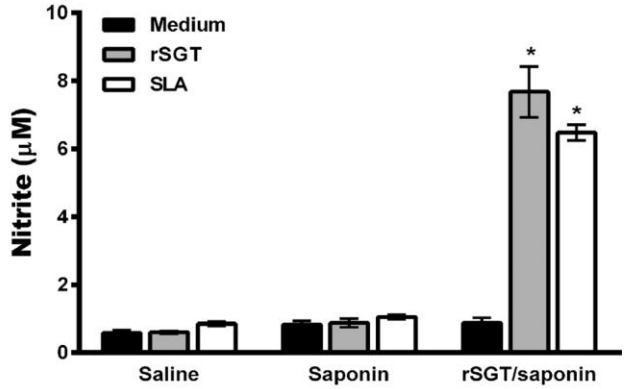


**Fig. 8.** Nitrite production in infected and vaccinated animals. Spleen cells were collected from the saline, saponin, or rPHB/saponin groups mice 60 days after infection and they were unstimulated (medium) or stimulated with rSGT or SLA (10 and 25 μg/mL, respectively), during 48 h at 37 °C in 5% $CO_2$. Nitrite production was evaluated by the Griess method. Bars represent the mean ± standard deviation. *indicates statistically significant difference in relation to the saline and saponin groups ($P < 0.0001$).

Small glutamine-rich TPR proteins are known to be involved in a variety of cellular functions, including, but not restricted to, participation in endocytosis, interaction with beta-amyloid proteins and growth hormone receptors, control of mitosis and apoptosis, cell cycle control, transcriptional repression, protein folding, and protein transport (Buchanan et al., 2007; Wang et al., 2005; Winnefeld et al., 2006; Zhang et al., 2014). In *Leishmania*, SGT protein was shown to be required for promastigotes growth and was found expressed in both parasite stages. It localizes in clusters within the *Leishmania* cytoplasm, supporting its role in the formation of leishmanial foldosome equivalents in the *L. donovani* species (Ommen et al., 2010). SGT was recently identified as expressed in both *L. infantum* promastigote and amastigote stages, and recognized by antibodies in VL dogs sera (Coelho et al., 2012). In the current study, we cloned the recombinant version of this protein and evaluated it as a vaccine candidate and diagnostic marker against VL.

Studies in experimental models using distinct *Leishmania* species have suggested that protection against infection comes from the Th1-type immune response which is initiated when IL-12 acts on antigen-presenting cells, triggering Th1 $CD4^+$ T cells to produce cytokines such as IFN-γ and TNF-α. On the other hand, the susceptibility to the disease is attributed to the Th2-type immune response with the overproduction of IL-4, IL-10 and IL-13, among others. In addition, both $CD4^+$ and

$CD8^+$ T cell subtypes are important for resistance against disease, and are involved in the production of IL-2, IFN-γ, and IL-12 (Bhowmick et al., 2014; Kaur et al., 2016). In our study, animals that were first immunized with rSGT/saponin and later infected showed a predominance of Th1 immune response not only before the infection but also after the challenge.

Accordingly, after stimuli using the protein or SLA, spleen cells of rSGT/saponin-vaccinated animals produced significantly higher levels of IFN-γ, IL-12 and GM-CSF when compared to other groups. Conversely, animals from the saline and saponin groups showed a higher production of IL-4 and IL-10, concordant with a Th2 regulatory profile in those animals that developed active infection. These results were corroborated by a flow cytometer analysis showing that rSGT/saponin group mice presented higher levels of both IFN-$\gamma^+$ and TNF-$\alpha^+$-producing $CD4^+$ and $CD8^+$ T cells in parallel with a low presence of IL-$10^+$-producing T cells. In addition, higher levels of anti-protein and anti-parasite IgG2a isotype antibodies were found in these animals, before and after infection. We also investigated macrophages, and an increased production of nitrite was observed in the rSGT/saponin group, suggesting the induction of an antileishmanial macrophage activity. Together these results suggest that *Leishmania* killing was mediated by NO pathway, possibly induced by IFN-γ. These findings were also described by others also evaluating vaccine candidates against VL (Amit et al., 2017; Islamuddin et al., 2015; Leal et al., 2015).

An important aspect to develop an antileishmanial vaccine refers to the experimental model used in the screening tests (Martins et al., 2013). Although models employing infected sand fly and hamsters seem to reflect a more natural transmission cycle, this strategy is difficult to perform since laborious technical conditions and trained personnel staff are required, and they are not widely available (Gannavaram et al., 2014). In this context, murine models have been used to evaluate candidates, since immune mechanisms and parasite load are easier to evaluate in these mammalian hosts (Requena et al., 2004). Therefore, molecules able to induce reduction in the parasitism in this experimental model could well be considered to predict future vaccine candidates to be tested against disease in dogs and humans (Duarte et al., 2016).

In a recent study, subcutaneous injection of *L. infantum* in BALB/c mice induced a similar degree of infection as compared to intravenous challenge (Oliveira et al., 2012). In our study, control (saline and saponin) groups showed a high parasitism in their liver, spleen, BM and dLN. On the other hand, rSGT/saponin-vaccinated mice presented significant reduction in the parasite load in these organs 60 days after infection. Without determining the parasite load at later infection time points, it is not possible to rule out that vaccinated mice can either present a delay in parasite spreading or even infection resolution. In this regard, further investigation must be performed.

Regarding the sterile immunity against infection, it is acceptable that a low number of *Leishmania* parasites persist in cured mammalian hosts, allowing them to maintain the immune system continuously stimulated by means of an active immunological memory. In addition, healthy people living in endemic areas of VL can develop a very mild infection caused by a low parasitism without any active disease. This fact can explain in part the resistance profile found in these subjects when compared to people non-resident in such areas which usually are infected by a low number of parasites and develop the active disease (Goto and Prianti, 2009; Niknam et al., 2014; Singh et al., 2012).

A depressed cell-mediated immunity in the human VL reveals the inability of patients PBMCs in responding to *Leishmania* antigens, then contributing to the development of the disease (Faleiro et al., 2014; Singh et al., 2012). In the current study, rSGT showed a high lymphoproliferation response in PBMCs from treated VL patients, besides of inducing high IFN-γ production and low IL-10 levels. These results showed suggest the immunogenic action of this protein in human cells, highlighting the possibility to use this molecule as a future immunogen against human VL. Others have also shown an increase in the IFN-γ secretion by antigen-stimulated PBMCs in treated VL patients, besides a low presence of IL-10 (Dayakar et al., 2016; Singh and Sundar, 2014). Activity of IL-10 in VL might render host macrophages refractory to activation signals in response to IFN-γ and other immune activating cytokines (Chamakh-Ayari et al., 2014; Niknam et al., 2014).

In the current study, an ELISA assay was performed using canine and human sera aiming to evaluate a diagnostic application of rSGT for the serodiagnosis of VL. The recombinant protein showed maximum sensitivity and specificity values. In contrast, using rA2 and *L. infantum* SLA as comparative antigens, cross-reactivity was observed, hampering the sensitivity and specificity of these antigens. These findings are in concordance with recent studies using rA2 and SLA as diagnostic antigens (Carvalho et al., 2017; Coelho et al., 2016; Lage et al., 2016). Due to the low number of samples tested as well as by the limited diversity of canine and human sera, it is still premature to conclude that rSGT is an appropriate antigen to be used in serological assays to diagnose canine and human VL. Further studies using a larger serological panel are warranted in order to validate the serodiagnostic application of this recombinant protein in the VL.

Altogether, our data showed an antigenic and immunogenic role of rSGT in VL. Given the conservation of this protein in other *Leishmania* spp., it is worth evaluating the role of rSGT as a vaccine candidate for other parasite species causing leishmaniasis. The polarized Th1 immunity and the high lymphoproliferative response found in PBMCs from VL patients using rSGT as stimulus suggest an immunological application of this protein against human disease.

## Conflict of interest

The authors declare no commercial or financial conflict of interest.

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## 4.2 ARTIGO 1 – CONCLUSÃO

Os resultados apresentados indicaram a imunogenicidade da proteína rSGT e seu efeito protetor contra a LV murina, bem como seu potencial para o sorodiagnóstico da LV humana e canina.

## 4.3 ARTIGO 2 – BREVE INTRODUÇÃO

O segundo artigo a ser apresentado foi intitulado como “Antigenicity, immunogenicity and protective efficacy of a conserved *Leishmania* hypothetical protein against visceral leishmaniasis” e publicado pela revista internacional *Parasitology* (doi: https://doi.org/10.1017/S0031182017001731).

O presente artigo avaliou a antigenicidade, imunogenicidade e eficácia protetora contra a leishmaniose visceral (LV) por parte de uma proteína hipotética recombinante nomeada LiHyS (XP_001467126.1). As técnicas de Western-Blot e ELISA foram utilizadas para analisar a antigenicidade contra soros humanos e de cães por meio de ELISA, bem como a imunogenicidade em PBMCs humanos e a eficácia protetora em modelo murino contra a infecção por *L. infantum* foram avaliadas.

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# Antigenicity, immunogenicity and protective efficacy of a conserved *Leishmania* hypothetical protein against visceral leishmaniasis

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**Research Article**

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**Abstract**

In this study, a *Leishmania* hypothetical protein, LiHyS, was evaluated regarding its antigenicity, immunogenicity and protective efficacy against visceral leishmaniasis (VL). Regarding antigenicity, immunoblottings and an enzyme-linked immunosorbent assay using human and canine sera showed high sensitivity and specificity values for the recombinant protein (rLiHyS) in the diagnosis of VL. When evaluating the immunogenicity of LiHyS, which is possibly located in the parasite's flagellar pocket, proliferative assays using peripheral blood mononuclear cells from healthy subjects or VL patients showed a high proliferative index in both individuals, when compared to the results obtained using rA2 or unstimulated cultures. Later, rLiHyS/saponin was inoculated in BALB/c mice, which were then challenged with *Leishmania infantum* promastigotes. The vaccine induced an interferon-$\gamma$, interleukin (IL)-12 and granulocyte-macrophage colony-stimulating factor production, which was maintained after infection and which was associated with high nitrite and IgG2a antibody levels, as well as low IL-4 and IL-10 production. Significant reductions in the parasite load in liver, spleen, bone marrow and draining lymph nodes were found in these animals. In this context, the present study shows that the rLiHyS has the capacity to be evaluated as a diagnostic marker or vaccine candidate against VL.

## Introduction

*Leishmania infantum* is the main causative species of the zoonotic visceral leishmaniasis (VL), with domestic dogs representing the most important parasite reservoirs. Disease control, based on the treatment of human cases, is one of the main measures used to avoid the dissemination of the disease. However, there are only a limited number of drugs available on the market today, and these can cause several side-effects in patients (Sundar and Chakravarty, 2013). In addition, lipid-based formulations, although effective and less toxic, are expensive (Mohamed-Ahmed *et al.* 2012). In this context, since life-long immunity has been reached in infected and vaccinated mammalian models, prophylactic vaccination to prevent disease has been encouraged (Duarte *et al.* 2017). The immune response associated with the effective protection against VL in humans and dogs has been largely attributed to the development of a predominant T helper (Th)1 immunity, characterized by the production of cytokines, such as interferon-$\gamma$ (IFN-$\gamma$) and interleukin (IL)-12, which subsequently mediates macrophage activation, nitric oxide (NO) production and parasite killing (Miahipour *et al.* 2016; Rodrigues *et al.* 2016).

The identification of *Leishmania* antigens containing the major histocompatibility complex class I- and/or class II-restricted epitopes is an important step towards selecting immunogens, since protection is based on the polarized and long-lasting $CD4^+$ and $CD8^+$ T-cell-dominated immune response against parasites (Agallou *et al.* 2017). As a consequence, *Leishmania* proteins have been evaluated as recombinant molecules to protect against disease (Iborra *et al.* 2008; Srivastava *et al.* 2012; Agallou *et al.* 2016). Major advantages have been associated with the use of defined immunogens, as well as by their high purity and production yield; however, these antigens present variable degrees of success, which depend on the animal model used for testing, the parasite species causing the disease, the number and concentration of the administered doses, among other factors (Rafati *et al.* 2002; Joshi and Kaur, 2014; Duarte *et al.* 2016).



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As an additional strategy used to select new candidates able to elicit protective immunity against VL, immunoproteomic approaches have been performed in *Leishmania* promastigotes and/or amastigotes using human and canine sera samples, where antileishmanial antibodies recognize antigenic proteins in parasite extracts (Kumari *et al.* 2008; Costa *et al.* 2011; Abanadés *et al.* 2012; Alcolea *et al.* 2016). These proteomic screenings have revealed a number of *Leishmania*-specific and/or conserved proteins that are present in this genus, some of which are annotated as hypothetical in *Leishmania* genome databases (Agallou *et al.* 2016). One of these candidates, namely LiHyS (LinJ_30_2800), was recognized by antibodies in VL dog's sera, but it was not identified by antibodies in sera from healthy dogs living in an endemic area of disease (Coelho *et al.* 2012). The present work evaluated the antigenicity of this protein in a recombinant version (rLiHyS) for the canine and human VL, as well as its immunogenicity and protective efficacy against *L. infantum* infection.

## Materials and methods

### Ethics statement

Experiments were performed in compliance with the Committee on the Ethical Handling of Research Animals (CEUA) of the Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil (protocol number 333/2015). The present study was also approved by the Ethics Committee of UFMG (protocol number CAAE–32343114.9.0000.5149). A written informed consent was obtained from all subjects, who received an individual copy of the study policy, which was analysed by an independent reviewer.

### Mice and parasite

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), UFMG, and were maintained under specific pathogen-free conditions. *L. infantum* (MHOM/BR/1970/BH46) was used, and parasites were grown at 24 °C in complete Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), which consisted of Schneider's medium plus 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U $mL^{-1}$ penicillin and 100 $\mu g\ mL^{-1}$ streptomycin, at pH 7·4. The soluble *Leishmania* antigenic extract (SLA) was prepared from $2 \times 10^8$ stationary-phase promastigotes of parasites, as described by Martins *et al.* (2017).

### Production of the recombinant antigens

The LiHyS (LinJ_30_2800) protein codifying gene was cloned from *L. infantum* genomic DNA using specific primers: 5′-TCTCGGATCCATGCGCCAGCGAAAGCAC-3′ (*forward*) and 5′-TGAAAAGCTTCCATGCGATCCAGTAGATG-3′ (*reverse*), and *BamHI* and *HindIII* as restriction enzymes. The DNA fragment was purified and linked into a pGEM®-T vector system (Promega, USA), and the recombinant plasmid was used to transform *Escherichia coli* XL1-Blue competent cells. DNA fragments obtained from digestion of pGEM-LiHyS plasmids were ligated into a pET28a-TEV vector, and BL21 cells were transformed into the recombinant plasmid. Sequencing was performed to confirm the identity of the insert by using a MegaBace 1000 automatic sequencer apparatus (Amersham Biosciences, USA). For the purification of rLiHyS, cells were induced with 1·0 $\mu$M isopropyl-$\beta$-D-thiogalactopyranoside (IPTG, Promega, Montreal, Canada), and cultures were incubated for 4 h at 37 °C, at which time these were ruptured by six cycles of ultrasound, in cycles of 30 s each (38 MHz), followed by six cycles of freezing (liquid nitrogen, −196 °C) and thawing (+37 °C). Then, the cell debris were removed by centrifugation, and rLiHyS (21·3 kDa) was purified onto a HisTrap HP affinity column (GE Healthcare Life Sciences, NJ, USA) connected to an AKTA system. The eluted fractions were concentrated in Amicon® ultra-15 centrifugal filters, with a 10 000 nominal molecular weight limit (Millipore, Germany), and further purified on a Superdex™ 200 gel-filtration column (GE Healthcare, USA). The A2 recombinant (rA2) protein was produced as described by Zhang *et al.* (1996). Briefly, pET16b-A2 plasmid, which was kindly provided by Dr Greg Matlashewski (Microbiology and Immunology Department, McGill University, Montreal, Quebec, Canada), was used to express and purify the recombinant protein (53 kDa). For this, transformed *E. coli* DH5$\alpha$ cells were grown in the presence of 1 mM of IPTG for 3 h (Promega, Montreal, Canada) and later disrupted by five cycles of freezing and thawing, followed by mild ultrasound treatment (five cycles of 30 s each with an ultrasound processor), in which they were centrifuged at 13·000 ***g*** for 30 min at 4 °C. After expression, the recombinant protein was purified by a HisTrap HP affinity column connected to an AKTA system. Next, rLiHyS and rA2 proteins were passed through a polymyxin-agarose column (Sigma-Aldrich, USA) to remove any residual endotoxin content (<10 ng of LPS per 1 mg of recombinant protein, measured by the Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000, BioWhittaker, MD, USA).

### Affinity purification of the anti-rLiHyS IgG antibody

The specific antibodies against the rLiHyS protein were affinity-purified from a pool of positive anti-rLiHyS sera on an antigen column. For this, BALB/c mice ($n = 6$) were vaccinated subcutaneously in their left hind footpad with 20 $\mu$g of rLiHyS associated with 20 $\mu$g of saponin (*Quillaja saponaria* bark saponin, Sigma). Two doses were administered at 14-day intervals, and 7 days after the last immunization, blood samples were collected and sera were separated. Next, 1 mg of the rLiHyS protein was covalently bound to a cyanogen bromide-activated Sepharose 4B column (GE Healthcare, USA), which was previously equilibrated with 20 mM phosphate buffer, pH 7·4. Coupling and blocking were carried out according to manufacturer instructions. Two millilitres of the sera pool were passed through the antigen column. After washing, the specific antibodies were eluted from the column with 0·1 M glycine, pH 2·8. Finally, the antibody preparation was equilibrated to pH 7·5 with 1 M Tris/HCl buffer. The solution of the antibody was restored to the original volume of the pooled sera (2 mL). An enzyme-linked immunosorbent assay (ELISA) experiment and a sodium dodecyl sulfate-12% polyacrylamide gel (SDS-12% PAGE) with rLiHyS were performed to verify the reactivity and quality of the purified antibody (data not shown).

### Sera collection

Sera samples of VL dogs ($n = 20$) were used. The disease was confirmed by means of the identification of *L. infantum* kinetoplastid DNA (kDNA) in bone marrow (BM) samples by a polymerase chain reaction (PCR) technique, as well as by positive serological results, which were evaluated by IFAT-LVC® and EIE-LVC® commercial kits (BioManguinhos®, Rio de Janeiro, Brazil). Symptomatic dogs ($n = 14$) showed three or more of the following symptoms: weight loss; alopecia; adenopathy; onychogryposis; hepatomegaly; conjunctivitis; exfoliative dermatitis on the nose, tail and ear tips; splenomegaly; lymphadenomegaly;

and renal azotemia, whereas asymptomatic animals ($n = 6$) showed positive parasitological and serological results but presented no clinical sign of disease. In addition, sera from healthy animals living in an endemic area of disease ($n = 20$) were employed. These animals were free of any clinical sign of disease and presented negative serological results. Samples from Leish-Tec®-vaccinated dogs ($n = 15$), as well as from those experimentally infected with *Ehrlichia canis* ($n = 15$) or *Babesia canis* ($n = 10$), were used in the assays. Regarding human sera, samples were obtained from VL patients ($n = 20$, including 12 males and eight females, with ages ranging from 25 to 59 years), which were collected from an endemic area of disease (Belo Horizonte, Brazil). Patients were diagnosed by means of clinical evaluation and demonstration of *L. infantum* kDNA in BM aspirates, when evaluated by PCR assay. Samples were also obtained from healthy individuals living in an endemic area of disease ($n = 20$, including 11 males and nine females, with ages ranging from 21 to 53 years, Belo Horizonte, Brazil), which showed no clinical sign of leishmaniasis, in addition to negative serological results from the Kalazar Detect™ Test (InBios® International, USA). Moreover, sera from Chagas disease patients ($n = 15$, including nine males and six females, with ages ranging from 20 to 55 years) were used, with the infection confirmed by haemoculture by means of the Chagatest® recombinant ELISA v.4·0 kit or the Chagatest® haemagglutination inhibition kit (Wiener lab., Rosario, Argentina).

### Immunoblottings and ELISA assays

For the immunoblottings, rLiHyS (10 $\mu$g) was submitted to a SDS-12% PAGE and blotted onto a nitrocellulose membrane (0·2 $\mu$m pore size, Sigma, St. Louis, USA). Next, membranes were blocked with a solution composed by phosphate buffer saline 1× plus Tween 20 0·05% (PBS-T) added with 5% albumin solution and incubated for 1 h at 37 °C before undergoing the first incubation with human or canine sera pools (1 : 400 and 1 : 200 diluted in PBS-T, respectively). Membranes were washed with PBS-T and anti-human or anti-dog IgG horseradish peroxidase-conjugated antibodies (diluted 1:10 000 and 1:5000 in PBS-T, respectively; SAB 3701282 and A6792 catalogues, respectively, Sigma-Aldrich, USA) were added, at which time a new incubation was developed for 1 h at 37 °C. Reactions were developed by adding 12·5 mg chloronaphthol, 25·0 mg diaminobenzidine and 20 $\mu$L $H_2O_2$ 30 vol., and stopped by adding 10 mL distilled water. The anti-rLiHyS polyclonal antibody was used as a positive control. For the ELISA assays, previous titration curves were performed to determine the most appropriate antigen concentration and antibody dilution to be used. Flexible microtitre immunoassay plates (Jetbiofil®, Belo Horizonte) were coated with rLiHyS, rA2, or SLA (0·5, 1·0 and 1·0 $\mu$g per well, respectively), which was diluted in 100 $\mu$L of coating buffer (50 mM carbonate buffer, pH 9·6), for 18 h at 4 °C. Next, free binding sites were blocked using 250 $\mu$L of PBS-T plus 5% casein, for 1 h at 37 °C. After washing the plates seven times with PBS-T, these were incubated with 100 $\mu$L of the individual human or canine sera (1:400 or 1:200, respectively; both diluted in PBS-T), for 1 h at 37 °C. The plates were then washed seven times in PBS-T and incubated with anti-human or anti-dog IgG horseradish peroxidase-conjugated antibodies (diluted 1:15 000 and 1:10 000 in PBS-T, respectively), for 1 h at 37 °C. After washing the plates seven times with PBS-T, reactions were developed by incubation with a solution (100 $\mu$L per well) consisting of 2 mg orthophenylenediamine, 2 $\mu$L $H_2O_2$ 30 vol. and 10 mL citrate-phosphate buffer, at a pH 5·0, for 30 min in the dark. Reactions were stopped by adding 25 $\mu$L 2 N $H_2SO_4$, and the optical density was read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada) at 492 nanometres.

### PBMCs culture and in vitro lymphoproliferation index

PBMCs were separated from the blood samples of healthy subjects ($n = 10$) and VL patients ($n = 8$), as described by Martins *et al.* (2017). For proliferation assays, cells ($1 \times 10^7$ cells) were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE). For this, after having been collected and washed, PBMCs were resuspended in pre-warmed PBS 1× containing 0·1% bovine serum albumin, at a final concentration of $1 \times 10^7$ cells mL$^{-1}$, and 2 $\mu$L of 5 mM stock Cell Trace CFSE solution (Molecular Probes, USA) was added per 1 mL of cells (at a working concentration of 10 mM). Then, the cells were incubated for 10 min at 37 °C, and the staining was quenched by adding 5 mL of RPMI 1640 plus 10% FBS for 5 min in an ice bath. The cells were then centrifuged three times for 7 min, and 50 $\mu$L were collected and analysed on a flow cytometry, aiming to evaluate the percentage of cell labelling. PBMCs were cultured in 48-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA), with each well containing 800 $\mu$L of RPMI 1640 medium. Next, cells ($1 \times 10^6$) were added in triplicate to the wells containing 100 $\mu$L of RPMI (control) and incubated alone (medium, control background) or stimulated with rLiHyS or rA2 (10 $\mu$g mL$^{-1}$, each). The incubation was carried out in a 5% $CO_2$ atmosphere for 5 days at 37 °C, after which the PBMCs were collected, washed twice in fluorescence-activated cell sorter (FACS) buffer, and fixed using a FACS fixing solution (10 g L$^{-1}$ paraformaldehyde, 10·2 g L$^{-1}$ sodium cacodylate and 6·63 g L$^{-1}$ sodium chloride, pH 7·4), which is also used in the lyses of red blood cells but not of white blood cells. The PBMCs were then stored at 4 °C, prior to flow cytometric acquisition and analysis, which were performed using a FACScalibur® instrument (Becton Dickson – BD, USA). The Cell-Quest™ software package (Franklin Lakes, NJ, USA) was used for data acquisition and analysis based on 30 000 events per sample.

### Vaccination and evaluation of the parasite load

BALB/c mice ($n = 16$, per group) were either vaccinated subcutaneously in their left hind footpad with 20 $\mu$g of rLiHyS, associated or not with saponin (20 $\mu$g), or they received saline or saponin. Three doses were administered at 14-day intervals. Thirty days after the last immunization, animals ($n = 8$, per group) were infected subcutaneously in the right hind footpad with $1 \times 10^7$ stationary-phase promastigotes of *L. infantum*; 60 days later they were euthanized, and the liver, spleen, BM) and paws' draining lymph nodes (dLNs) were collected to evaluate the parasite burden through a limiting-dilution technique, as described by Martins *et al.* (2017). Briefly, organs were homogenized using a glass tissue grinder in sterile saline, and tissue debris were removed by centrifugation at 150 ***g***. Cells were concentrated by centrifugation at 2000 ***g***, and pellets were resuspended in 1 mL of Schneider's insect medium supplemented with 20% FBS. Next, 220 $\mu$L was plated onto 96-well flat-bottom microtitre plates (Nunc, Nunclon®, Roskilde, Denmark) and diluted in log-fold serial dilutions in Schneider's medium with a $10^{-1}$–$10^{-12}$ dilution. Pipette tips were discarded after each dilution to avoid carrying adhered parasites from one well to another. Each sample was plated in triplicate, at 24 °C, and read 7 days after the beginning of the culture. Results were expressed as the negative log of the titre adjusted per milligram of organ. The parasite load was also evaluated by a quantitative PCR (*q*PCR) technique in the spleen of the animals, as described by Duarte *et al.* (2016), and the

results were expressed as the number of parasites per 1000 nucleated cells.

### *Cytokine assay by capture ELISA and flow cytometry*

Thirty days after the last immunization (Before) and 60 days post-infection (After), BALB/c mice ($n = 8$ per group) were euthanized and their spleen were collected, at which time splenocytes were cultured ($5 \times 10^6$ cells $mL^{-1}$) in 24-well plates (Nunc). Cells were incubated in RPMI medium (control) together with 20% FBS, 20 mM L-glutamine, 200 U $mL^{-1}$ penicillin and 100 $\mu$g $mL^{-1}$ streptomycin, at pH 7·4; or stimulated with rLiHyS or *L. infantum* SLA (10 and 25 $\mu$g $mL^{-1}$, respectively) for 48 h at 37 °C in 5% $CO_2$. IFN-$\gamma$, IL-4, IL-10, IL-12p70 and GM-CSF levels were assessed in the supernatants by an ELISA capture (all obtained from BD OptEIA TM set mouse, Pharmingen®, San Diego, CA, USA), following manufacturer instructions. The involvement of $CD4^+$ and $CD8^+$ T cells in IFN-$\gamma$ production was also evaluated in the spleen cells, in which cells stimulated with rLiHyS or SLA (10 and 25 $\mu$g $mL^{-1}$, respectively) were incubated in the presence of monoclonal antibodies against mouse IL-12 (C17·8), CD4 (GK 1·5), or CD8 (53-6·7), all in a concentration of 5 $\mu$g $mL^{-1}$. Appropriate isotype-matched controls (no azide/low endotoxin™), rat IgG2a (R35-95) and rat IgG2b (95-1) were used (Pharmingen®, USA). A flow cytometry assay was also performed in these spleen cells, aiming to evaluate the IFN-$\gamma$-, TNF-$\alpha$- and IL-10-producing $CD4^+$ and $CD8^+$ T-cell frequencies after *L. infantum* SLA stimulus, as described by Vieira *et al.* (2012). Results were expressed as indexes, which were calculated by dividing the cytokine-producing $CD4^+$ and $CD8^+$ T-cell percentages in the stimulated cultures by the values obtained in the unstimulated cultures (control).

### *Nitrite secretion and antibody production*

Sixty days after infection, cell supernatants were also used to evaluate the nitrite production in all experimental groups. For this, 100 $\mu$L of protein and parasite-stimulated culture supernatants were mixed with an equal volume of Griess reagent (Sigma-Aldrich, USA). After 30-min incubation at room temperature, the nitrite concentration was calculated using a standard curve of known concentrations, and the results were expressed as $\mu$M (Green *et al.* 1982). To evaluate the humoral response before and after infection, sera samples were collected from the animals, 30 days after the last immunization (Before) and 60 days post-infection (After). The protein and parasite-specific IgG1 and IgG2a antibody levels were evaluated by an ELISA assay, as described by Martins *et al.* (2017). Briefly, rLiHyS and SLA (0·5 and 1·0 $\mu$g per well, respectively) were used as antigens, and sera samples were 1:100 diluted in PBS-T. The anti-mouse IgG1 and IgG2a horseradish peroxidase-conjugated antibodies (Sigma-Aldrich, USA) were used in 1:5000 or 1:7500 dilutions, which were performed in PBS-T, respectively.

### *Confocal fluorescence microscopy*

Immunofluorescence assay was performed as described by Faria *et al.* (2016). Briefly, parasites were double-labelled with the anti-rLiHyS polyclonal IgG antibody (at a 1:50 dilution) and incubated with a secondary antibody conjugated to an Alexa Fluor® 555 Goat anti-mouse IgG antibody (1:1000 diluted, Life Technologies®, USA), together with Hoechst 33258 (1 $\mu$g $mL^{-1}$, Life Technologies, USA), for 1 h at room temperature. Next, samples were washed three times in PBS 1× for 10 min, and then mounted with hydromount (Electron Microscopy Sciences). The negative control was included in all reactions by omitting primary antibodies. Images were collected using a Zeiss LSM 880 confocal microscopy (Carl Zeiss, Jena, Germany). The Zeiss Efficient Navigation (ZEN) software was used for orthogonal projections (XY, XZ, YZ), and image adjustments were performed according to the negative controls.

### *Infection of murine macrophages using pre-treated parasites*

The inhibitory effect of anti-rLiHyS polyclonal antibody on the *L. infantum* invasion in murine macrophages was evaluated. For this, stationary-phase promastigotes of parasites ($5 \times 10^6$ cells) were pre-incubated with different concentrations (5·0, 10·0 and 50·0 $\mu$g $mL^{-1}$) of anti-rLiHyS IgG antibody or a non-immune IgG for 4 h at 24 °C. After incubation, parasites were washed three times with RPMI 1640 medium, then quantified and incubated for 24 h with macrophages (in a ratio of 10 parasites per one macrophage). As a control, the same amount of non-treated parasites was used in the infection. Then, the cells were washed, set and stained to determine the percentage of infected macrophages by counting 200 cells in triplicate. In addition, the number of amastigotes per infected cell was evaluated. Data shown are representative of two independent experiments, which presented similar results.

### *Statistical analysis*

The results were entered into Microsoft Excel (version 10·0) spreadsheets and analysed by using GraphPad Prism™ (version 6·0 for Windows). Statistical analyses were performed by one-way analysis of variance, followed by Bonferroni's post-test for comparisons between the groups. Differences were considered significant with $P < 0·05$. In addition, receiver operating characteristic (ROC) curves were constructed to obtain the sensitivity, specificity and area under the curve values for the diagnostic antigens, as well as the lower limit of positivity (cut-off). Vaccination results showed in this study are representative of two independent experiments, which presented similar results.

## Results

### *Antigenicity and immunogenicity of the rLiHyS protein*

First, the antigenicity of rLiHyS was evaluated. For this, immunoblottings were performed, and the anti-protein reactivity was observed using both asymptomatic and symptomatic VL dog sera, as well as VL patient sera. However, sera were not collected from healthy dogs or humans living in an endemic area of disease (Supplementary Fig. S1). In addition, ELISA assays were developed using a human and canine serological panel. Cut-off values for negative and positive sample discrimination were constructed using ROC curves. In the results, both *L. infantum*-infected dogs and humans (not other groups' sera) were highly reactive against rLiHyS, while the worst results of sensitivity and specificity were found when using rA2 or *L. infantum* SLA as antigens in the plates (Fig. 1). Then, the immunogenicity of rLiHyS was evaluated by means of a lymphoproliferation index in purified PBMCs from healthy subjects and VL patients (Fig. 2). In the results, this cell population showed high proliferative values when it was incubated with rLiHyS in both individuals, as compared to the results obtained when the *in vitro* cultures were unstimulated or stimulated with the rA2 antigen ($P < 0·0001$).

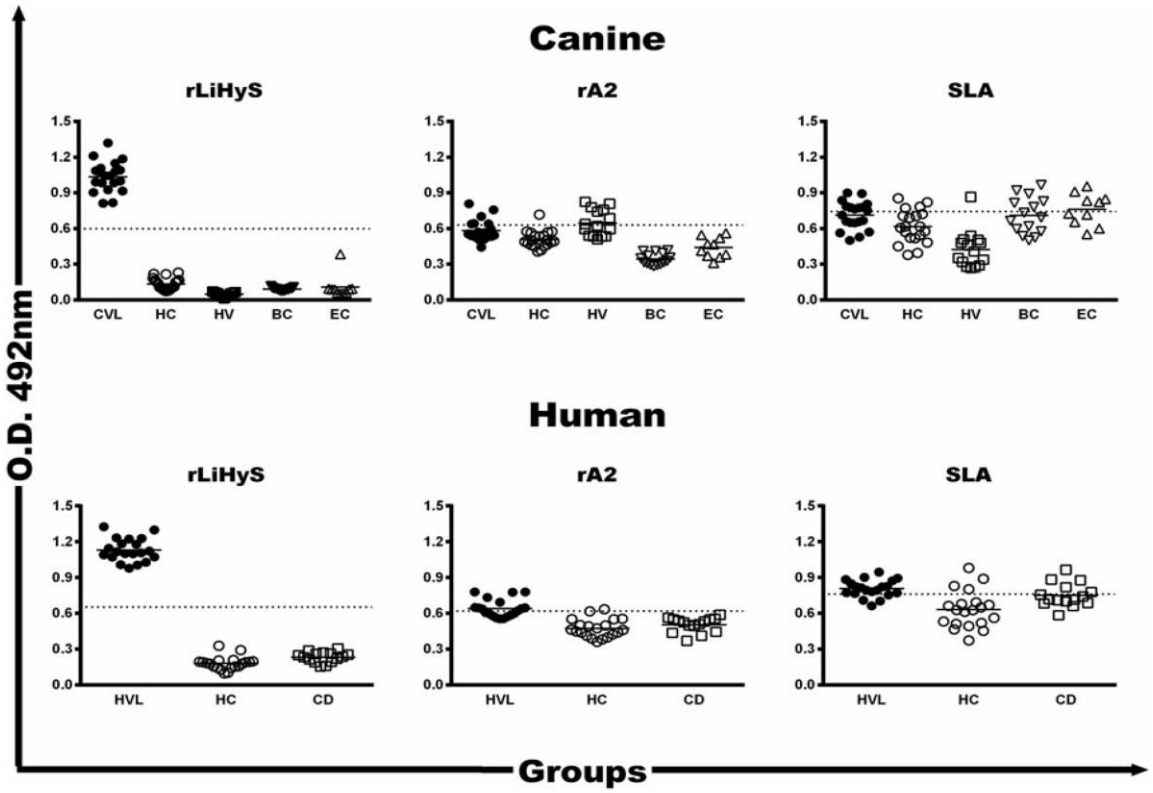


**Fig. 1.** Reactivity of the diagnostic antigens by ELISA using a serological panel. The individual optical density (O.D.) of each serum sample against rLiHyS, rA2, or *L. infantum* SLA is shown here. Sera samples of VL dogs ($n = 20$), from healthy animals living in an endemic area of disease ($n = 20$), from Leish-Tec®-vaccinated animals ($n = 15$), or those experimentally infected with *Ehrlichia canis* ($n = 15$) or *Babesia canis* ($n = 10$) were used. Sera from VL patients ($n = 20$), from healthy subjects living in an endemic area of disease ($n = 20$), and from Chagas disease patients ($n = 15$) were employed in the assays. The cut-off values (dotted lines) were calculated by using ROC curves. CVL, canine visceral leishmaniasis; HC, healthy controls; HV, healthy vaccinated animals; BC, *Babesia canis*; EC, *Ehrlichia canis*; HVL, human visceral leishmaniasis; CD, Chagas disease.

### *The rLiHyS/saponin vaccine is immunogenic in BALB/c mice*

In this case, rLiHyS was used to immunize BALB/c mice in an attempt to evaluate its protective efficacy against VL. To achieve this, animals were vaccinated three times with the protein, associated or not with saponin, and 30 days after the last vaccine dose. The cytokine production was then evaluated based on the protein- and parasite-specific IFN-$\gamma$, IL-4, IL-10, IL-12 and GM-CSF production. In the results, spleen cells from rLiHyS/saponin-vaccinated mice produced significantly higher levels of IFN-$\gamma$, IL-12 and GM-CSF than did those secreted by cells from saline and saponin group mice (Fig. 3; $P < 0{\cdot}0001$). No IL-4 and IL-10 production was detected in any experimental group. The animals were then challenged with *L. infantum*, and 60 days after infection, their splenocytes were *in vitro* stimulated with both antigens, and the cytokine production was again evaluated. The immune profile was maintained in the rLiHyS/saponin group, since higher levels of protein and parasite-specific IFN-$\gamma$, IL-12 and GM-CSF were found, which were associated with a low production of IL-4 and IL-10 (Fig. 3; $P < 0{\cdot}0001$). By contrast, spleen cells of the mice from the saline and saponin groups produced significantly higher levels of anti-parasite IL-4 and IL-10 ($P < 0{\cdot}0001$). Mice that were immunized with rLiHyS alone showed a mixed Th1:Th2 profile, which was observed before and after infection. As an additional immunological parameter, the participation of $CD4^+$ and $CD8^+$ T cells in the IFN-$\gamma$ production was evaluated in the rLiHyS/saponin group. In the results, the production of this cytokine was significantly diminished when either an anti-IL-CD4 or an anti-CD8 antibody was added to the *in vitro* cultures (Fig. 4; $P < 0{\cdot}0001$). The intracytoplasmic cytokine profile, which was evaluated by a flow cytometry assay (Fig. 5), also showed that rLiHyS/saponin-vaccinated mice presented higher levels of antileishmanial IFN-$\gamma^+$- and TNF-$\alpha^+$-producing $CD4^+$ and $CD8^+$ T cells ($P < 0{\cdot}05$), which were corroborated with the low presence of IL-$10^+$-producing T cells ($P < 0{\cdot}05$), as compared to the results found in the control groups, denoting the polarized Th1 profile found in the rLiHyS/saponin group mice.

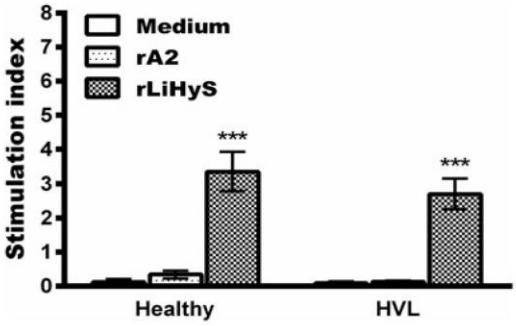


**Fig. 2.** Lymphoproliferative index in human PBMCs using different *in vitro* stimuli. Blood samples of healthy subjects ($n = 10$) and VL patients ($n = 8$) were collected; PBMCs were purified and either unstimulated (medium) or separately stimulated with rLiHyS or rA2 (10 $\mu$g $mL^{-1}$, each). The incubation was carried out in a 5% $CO_2$ atmosphere for 5 days at 37 °C, at which time the lymphoproliferation index was evaluated by flow cytometry. Results are shown as mean ± standard deviation using the different *in vitro* stimuli. ***Indicate statistically significant difference in relation to the other stimuli ($P < 0{\cdot}0001$).

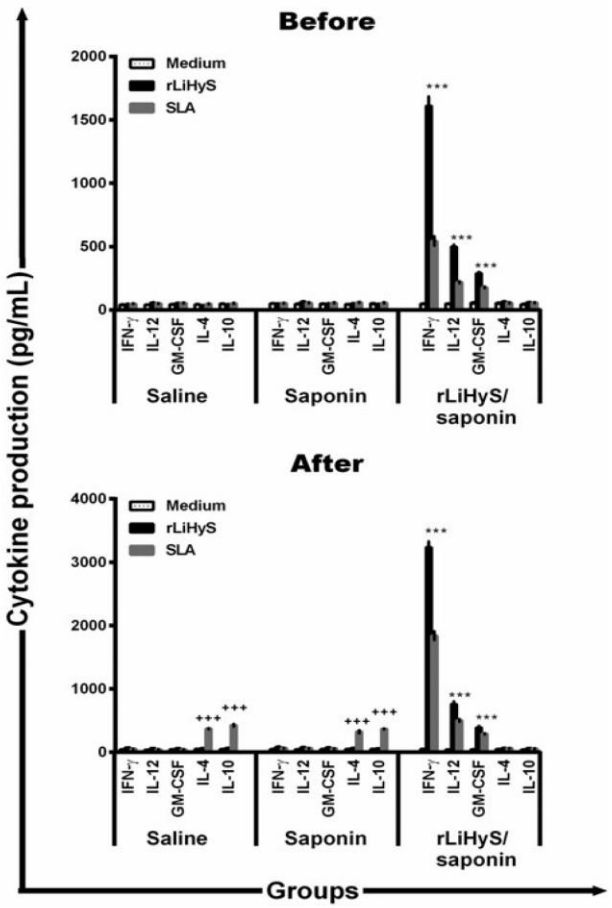


**Fig. 3.** Cell response induced in the rLiHyS/saponin-vaccinated BALB/c mice, before and after infection. Splenocytes were obtained from the spleen of mice, 30 days after the last immunization (Before) and 60 days after infection (After). Cells were unstimulated (medium) or stimulated with rLiHyS or SLA (10 and 25 $\mu$g mL$^{-1}$, respectively) for 48 h at 37 °C in 5% $CO_2$. IFN-$\gamma$, IL-12, GM-CSF, IL-4 and IL-10 levels were measured in culture supernatants by ELISA capture. Bars represent the mean ± standard deviation of the groups. ***Indicate statistically significant difference in relation to the saline and saponin groups ($P < 0{\cdot}0001$). $^{+++}$Indicate statistically significant difference in relation to the rLiHyS/saponin group ($P < 0{\cdot}0001$).

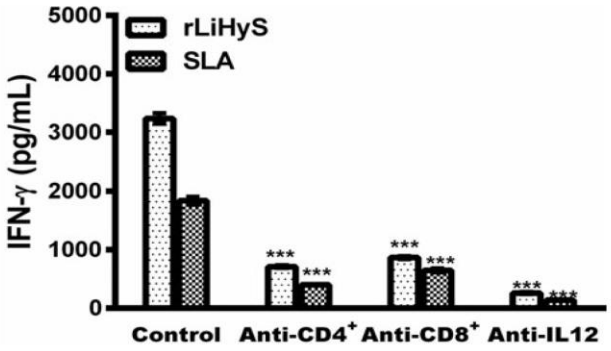


**Fig. 4.** Participation of the CD4$^+$ and CD8$^+$ T cells in the IFN-$\gamma$ production in the protected animals. The anti-IL-12, anti-CD4 and anti-CD8 monoclonal antibodies were added to the spleen cell cultures of the infected and vaccinated animals, and the IFN-$\gamma$ production was evaluated in the cell supernatants after incubation for 48 h at 37 °C in 5% $CO_2$. The rLiHyS and SLA were used as stimuli (10 and 25 $\mu$g mL$^{-1}$, respectively). Cytokine levels were measured by an ELISA capture in the absence (positive control) or presence of the monoclonal antibodies. Bars represent the mean ± standard deviation of the groups. ***Indicate statistically significant difference in relation to the control group ($P < 0{\cdot}0001$).

As an indicator of NO production, the nitrite presence was assayed in the *in vitro* cultures after challenge (Fig. 6). In the results, the nitrite secretion was significantly higher in rLiHyS/saponin-vaccinated mice, when either rLiHyS or SLA was used as a stimulus ($P < 0{\cdot}0001$), as compared to the results found in the saline or saponin groups, in which a minimum nitrite presence was verified. Regarding humoral response, the protein- and parasite-specific IgG1 and IgG2a isotype productions were evaluated before and after infection (Fig. 7), and results showed that, in both conditions, rLiHyS/saponin-vaccinated mice produced higher IgG2a levels, when compared to the IgG1 levels ($P < 0{\cdot}05$ and $P < 0{\cdot}0001$, respectively). Moreover, the ratio between the IgG2a/IgG1 isotypes was calculated, and results showed a polarized Th1 immune response ($P < 0{\cdot}05$ and $P < 0{\cdot}0001$, respectively) in the protected animals. On the other hand, isotype production in the saline and saponin groups before infection was similar when both antigens were tested, although the anti-parasite IgG1 isotype levels were higher after challenge, when compared to the IgG2a levels in these groups ($P < 0{\cdot}0001$).

### *The rLiHyS/saponin vaccine induces partial protection in BALB/c mice against* L. infantum

The protective efficacy induced by immunization with rLiHyS/saponin in BALB/c mice was investigated against an *L. infantum* challenge through the evaluation of parasite burden, 60 days after infection. In the results, using the limiting dilution technique (Fig. 8), rLiHyS/saponin-vaccinated mice showed significant reductions in the parasite load in the liver (3·3- and 2·8-log reductions, respectively), spleen (5·0- and 4·5-log reductions, respectively), BM (3·3- and 3·0-log reductions, respectively) and dLNs (5·0- and 4·8-log reductions, respectively), when compared to the saline and saponin groups ($P < 0{\cdot}0001$). Evaluating the results obtained by immunization with rLiHyS administered with or without an adjuvant, rLiHyS/saponin-vaccinated and challenged mice showed significant reductions in the parasite load in the liver, spleen, BM and dLNs (2·8-, 4·2-, 2·7- and 4·5-log reductions, respectively), as compared to the results found in the rLiHyS group ($P < 0{\cdot}0005$). Results obtained using the limiting dilution technique were corroborated by *q*PCR assay when the spleen of the animals was evaluated, since the immunization with rLiHyS/saponin reduced the parasite load in this organ in the order of 66·0% and 65·0%, when compared to the values found in the saline and saponin groups, respectively ($P < 0{\cdot}0001$).

### *Cell localization of LiHyS in the* L. infantum *parasite*

To investigate the cell location of LiHyS in *L. infantum*, an immunofluorescence experiment was performed using stationary-phase promastigotes and an anti-rLiHyS IgG antibody. In the results, confocal images showed that this protein is possibly located in the parasite's flagellar pocket (Fig. 9). In this context, it could be speculated that it would become part of the *Leishmania* flagellum machinery, and its location could be additionally investigated by using other flagellum apparatus proteins in new studies.

### *LiHyS inhibits the* Leishmania *infectivity in murine macrophages*

Aiming to show an immunotherapeutic role of LiHyS on the *Leishmania* invasion in mammalian cells, an experiment was performed using murine macrophages and parasites that were pre-

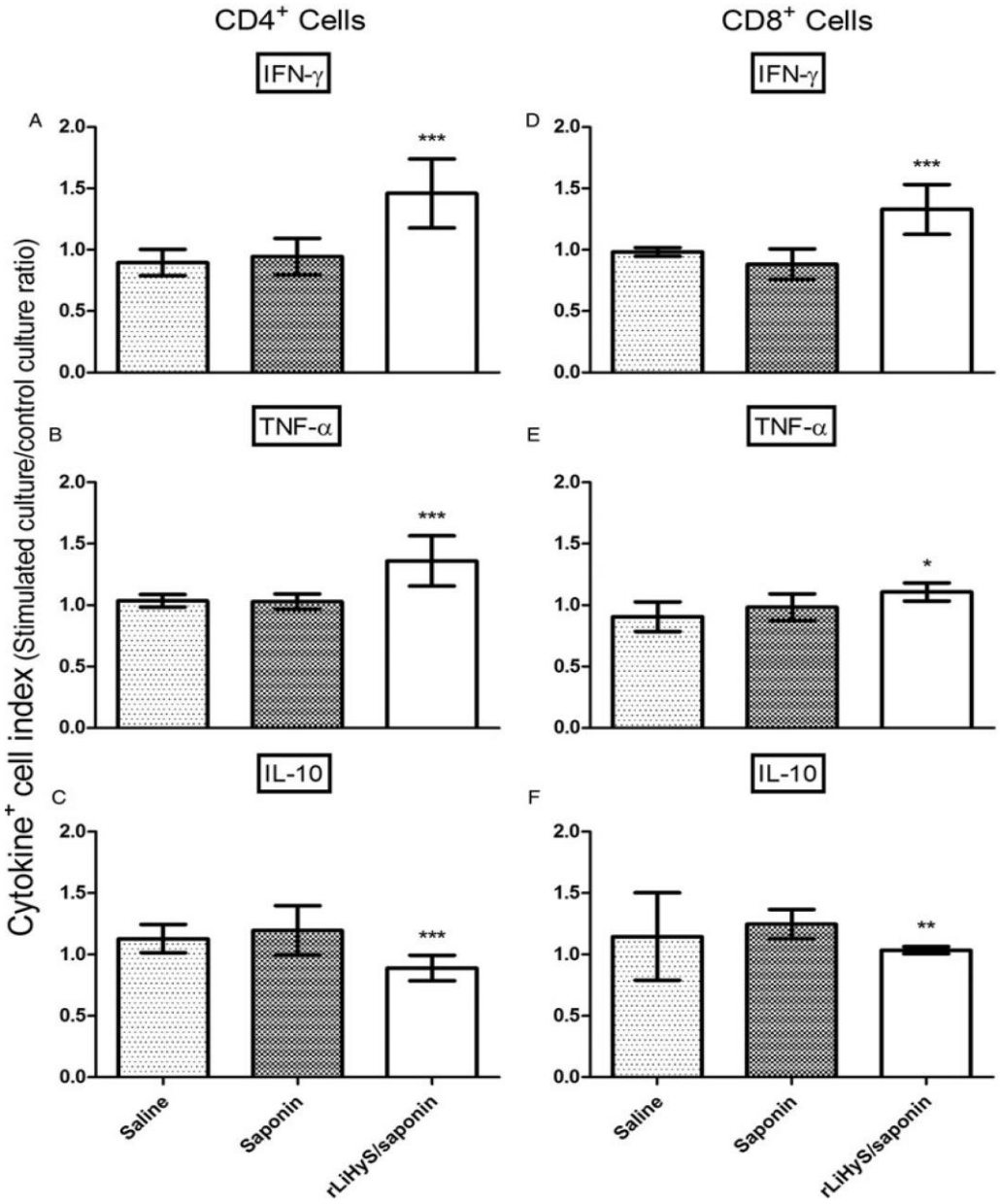


**Fig. 5.** Intracytoplasmic cytokine indexes in splenocytes after *in vitro* stimulation with the soluble *Leishmania* antigenic extract. Cytokine indexes were calculated as the ration of cytokine$^+$ cells observed in SLA-stimulated cultures divided by the control culture (SLA/CC ratio). Mice received saline (saline: white rectangle), or were immunized with saponin (saponin: light grey rectangle), or rLiHyS/saponin (rLiHyS/saponin: black rectangle), and later challenged with *Leishmania infantum* promastigotes. After 60 days, their splenocytes were collected and *in vitro* stimulated with SLA. Results were reported as cytokine indexes (stimulated culture/control culture ratio) in A, B and C for CD4$^+$ T cells and in D, E and F for CD8$^+$ T cells, and were expressed as mean ± standard deviation of the groups. *Indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}05$). **Indicate statistically significant difference in relation to the saponin group ($P < 0{\cdot}05$). ***Indicate statistically significant difference in relation to the saline and saponin groups ($P < 0{\cdot}05$).

treated with the anti-rLiHyS antibody. In the results, anti-rLiHyS IgG pre-treated promastigotes infect 46·6, 54·8 and 63·2% of the macrophages, and the number of recovered amastigotes was of $1{\cdot}0 \pm 0{\cdot}3$, $1{\cdot}5 \pm 0{\cdot}4$ and $2{\cdot}0 \pm 0{\cdot}2$, respectively, when IgG concentrations of 50·0, 10·0 and 5·0 $\mu$g mL$^{-1}$ were used. On the other hand, when using a non-immune IgG antibody, the percentage of infection was of 84·5, 88·7 and 90·6%, and the number of recovered amastigotes was of $5{\cdot}6 \pm 0{\cdot}4$, $6{\cdot}2 \pm 0{\cdot}3$ and $6{\cdot}9 \pm 0{\cdot}6$, respectively, when the same IgG concentrations were used. Parasites that were not pre-incubated with antibodies were able to infect 94·5% of the macrophages, and 7·8 amastigotes per phagocytic cell were recovered.

## Discussion

*Leishmania* antigenic proteins expressed in the *L. infantum* promastigote and amastigote stages were recently identified by an immunoproteomic approach using antibodies in VL dog sera, including some characterized as hypothetical (Coelho *et al.* 2012). The fact that antibodies in infected animal sera, but not

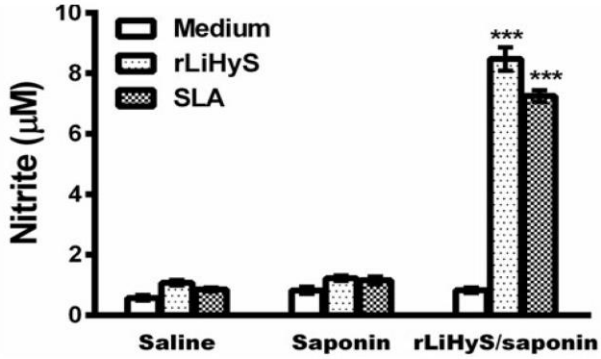


**Fig. 6.** Nitrite production. Spleen cells obtained from the saline, saponin, or rLiHyS/saponin-vaccinated mice, 60 days after challenge infection, were unstimulated (medium) or stimulated with rLiHyS or SLA (10 and 25 $\mu g\,mL^{-1}$, respectively), for 48 h at 37 °C in 5% $CO_2$. Next, nitrite production was analysed by the Griess method. Bars represent the mean ± standard deviation of the groups. ***Indicate statistically significant difference in relation to the saline and saponin groups ($P < 0{\cdot}0001$).

those from healthy animals, had recognized these molecules indicates that they are expressed by the parasites during the active disease and can be considered relevant to the infected host's immune response (Fernandes *et al.* 2012). In this light, in the present work, the LiHyS gene, which was identified in *L. infantum* promastigotes in the cited immunoproteomic study, was cloned, and its recombinant version was evaluated as a diagnostic or vaccine candidate against VL.

Studies have showed that *Leishmania* virulence factors can be found in the parasite promastigote stage (Yao *et al.* 2010; Dupé *et al.* 2015). In this line, *Leishmania* macromolecules, such as tubulins (Werbovetz *et al.* 1999), protein disulphide isomerase (Achour *et al.* 2002), sphingolipids (Heung *et al.* 2006), lipophosphoglycans (Forestier *et al.* 2015), among others, have been characterized as virulence factors. Taking this fact into account, the protective efficacy achieved by immunization with promastigote antigens often results in a more effective immune response, which will eliminate these forms soon after parasite infection by the infected sandflies. In this context, several studies have been

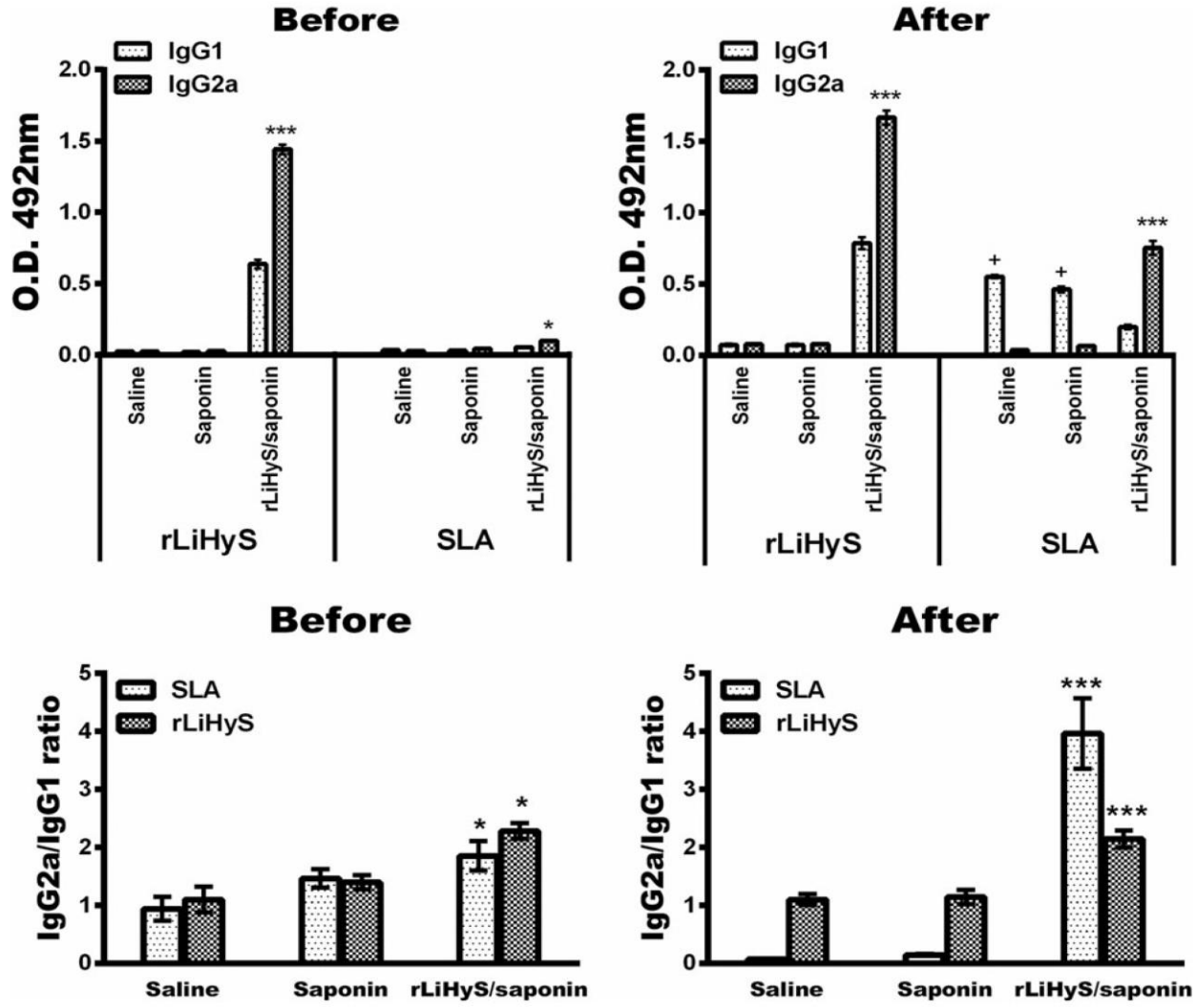


**Fig. 7.** Production of protein- and parasite-specific IgG2a and IgG1 isotype antibodies before and after infection. BALB/c mice received saline or were immunized with saponin or rLiHyS/saponin. Thirty days after the last immunization, sera samples were collected (Before). In addition, remaining animals were challenged with *Leishmania infantum* promastigotes and followed for 60 days, at which time sera samples were collected (After). The anti-protein and anti-parasite IgG2a and IgG1 isotypes antibody levels were calculated, as were the ratios between IgG2a and IgG1 values. Bars represent the mean ± standard deviation of the groups. *Indicates statistically significant difference in relation to the saline and saponin groups ($P < 0{\cdot}05$). ***Indicate statistically significant difference in relation to the saline and saponin groups ($P < 0{\cdot}0001$). $^{+}$Indicates statistically significant difference in relation to the rLiHyS/saponin group ($P < 0{\cdot}01$).

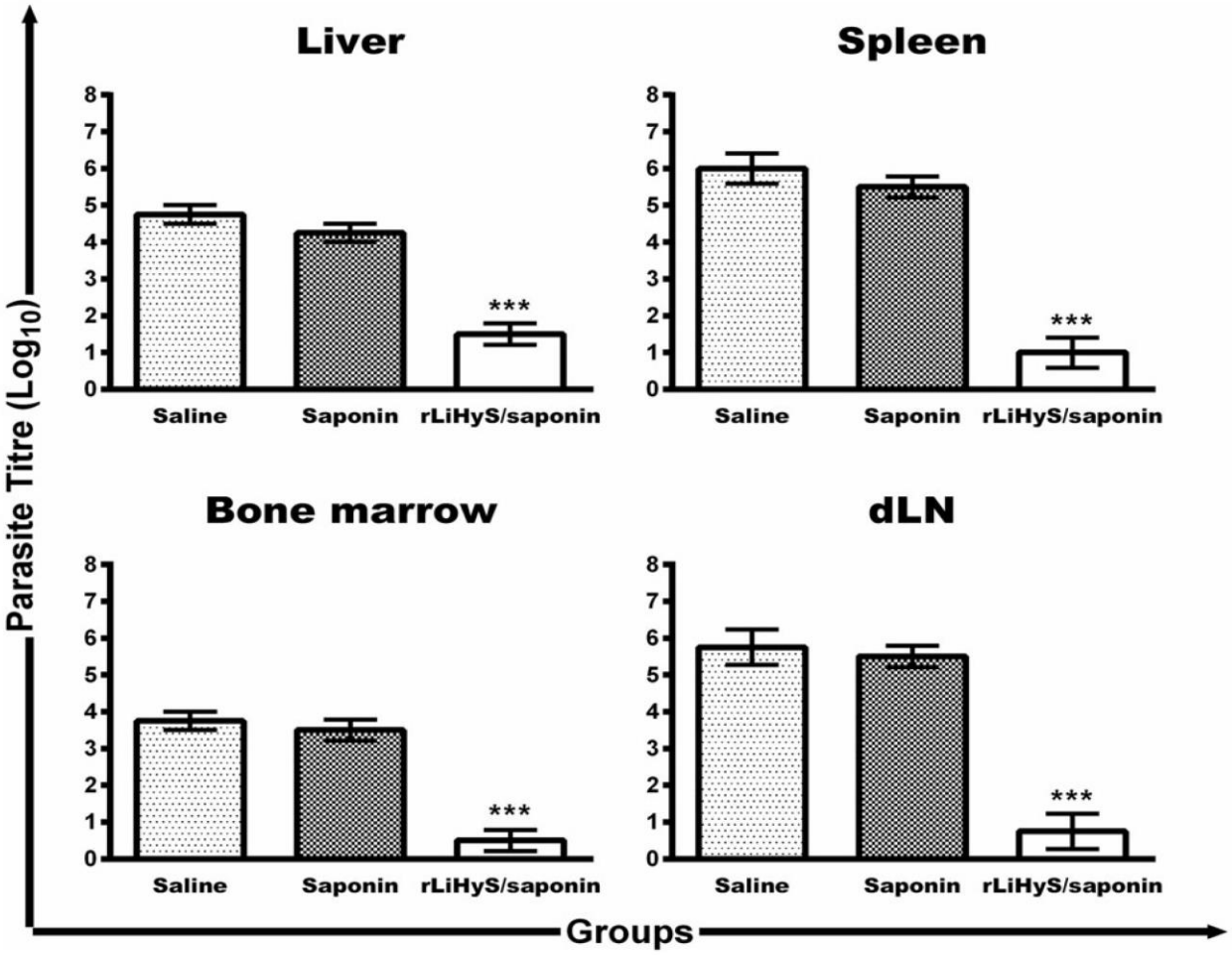


**Fig. 8.** Parasite burden evaluated in the infected and vaccinated animals. BALB/c mice inoculated with saline or immunized with saponin or rLiHyS/saponin were infected with *Leishmania infantum* promastigotes. Sixty days after, the parasite load in the liver, spleen, bone marrow (BM) and paws' draining lymph nodes (dLNs) was evaluated by a limiting-dilution technique. Bars represent the mean ± standard deviation of the groups. ***Indicate statistically significant difference in relation to the saline and saponin groups ($P < 0{\cdot}0001$).

developed using promastigote proteins as vaccine candidates against leishmaniasis (Bhowmick *et al.* 2007; Chamakh-Ayari *et al.* 2014; Thakur *et al.* 2015; Petitdidier *et al.* 2016).

In the present work, LiHyS was identified in *L. infantum* promastigotes and by an immunofluorescence assay, which proved to possibly be located in the parasite's flagellar pocket, in turn allowing one to speculate about its involvement in *Leishmania* motility and, consequently, as a parasite virulence factor. In fact, when an anti-rLiHyS IgG antibody was pre-incubated with *Leishmania* promastigotes, and these pre-treated parasites were used to infect murine macrophages, significant reductions in the infection percentage were obtained, and a lower number of amastigotes was recovered by the infected phagocytic cells, when compared with the values found when parasites were untreated or pre-treated with a non-immune IgG antibody. As a consequence, a role of rLiHyS in the *L. infantum* infectivity could be postulated, although additional studies are necessary to be performed to elucidate this protein's biological action.

It has been reported that, to achieve a protective immunity against VL, a Th1 cell response, based on the production of cytokines such as IFN-$\gamma$, IL-12, TNF-$\alpha$, GM-CSF, among others, is required (Zadeh-Vakili *et al.* 2004; Mizbani *et al.* 2009; Saljoughian *et al.* 2013; Martins *et al.* 2016; Reed *et al.* 2016). On the other hand, susceptibility to disease is associated with the reduction of this immune profile, in parallel to the development of Th2 immunity, based on the production of cytokines, such as IL-4 and IL-10 (Goto *et al.* 2011; Agallou *et al.* 2012; Gupta *et al.* 2014). In our study, the association between rLiHyS and saponin proved to be immunogenic in vaccinated mice, since higher levels of IFN-$\gamma$, IL-12 and GM-CSF were found before and after infection. These findings are related to a decrease in the parasite load in the infected and rLiHyS/saponin-vaccinated animals, represented by significant reductions in parasitaemia in the liver, spleen, BM and dLNs, when compared to results obtained in the other groups. Although rLiHyS alone was not protective against the challenge, most of the tested immunogens, when used in different mammalian hosts, normally require the association of immune adjuvants to improve their protective efficacy (Bayih *et al.* 2014; Eskandari *et al.* 2014; Thakur *et al.* 2015; Petitdidier *et al.* 2016). In this context, our antigen also fulfils the requirements to be considered as a vaccine candidate against VL.

An ideal vaccine will have the potential to protect mammalian hosts against *Leishmania* infection, as well as to reduce transmission between reservoir hosts, such as dogs and humans, in areas

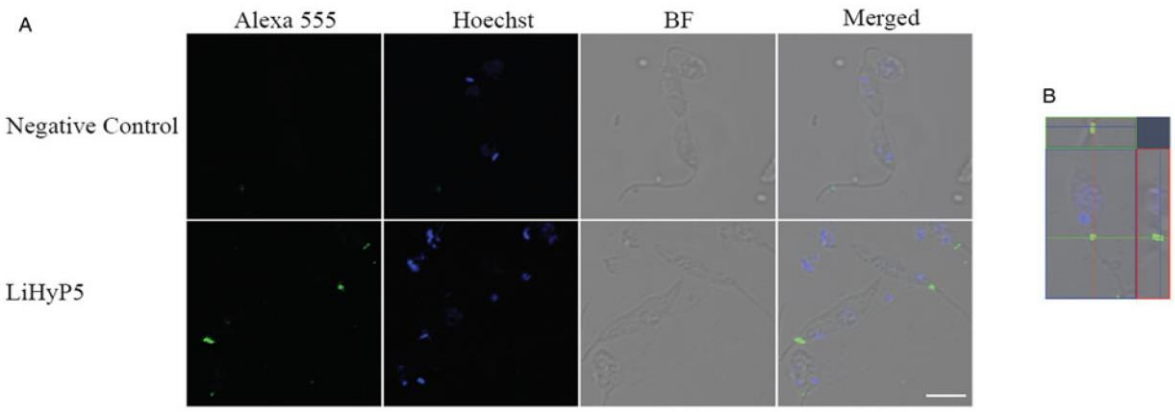


**Fig. 9.** Cell location of LiHyS protein. Confocal fluorescence images were obtained using a negative control (upper panel), and the location of LiHyS in *Leishmania infantum* promastigotes is shown (green colour), stained with Hoechst (blue colour, lower panel). BF represents the bright field. The merged image shows the location of LiHyS (A). The three-dimensional reconstruction (X–Z sections) is shown at the top, and Y–Z sections are shown at the right for each image (B). Images are representative of two independent experiments. Scale bar: 5 $\mu$m.

where parasite transmission is considered zoonotic, in addition to allowing for a more effective control of the disease (Saldarriaga *et al.* 2006; Stockdale and Newton, 2013; Sundar and Singh, 2014). In the human disease, a depressed cell-mediated immune response, characterized by the failure of patients' PBMCs to respond and proliferate when stimulated by *Leishmania* antigens, is commonly found, as is the production of cytokines, such as IFN-$\gamma$ and IL-12 (Peruhype-Magalhães *et al.* 2005). On the other hand, PBMCs from cured and treated patients usually produce pro-inflammatory cytokines when *in vitro* stimulated by parasite antigens (Singh *et al.* 2012).

The present study's data suggest that rLiHyS could be considered a potential target for an intervention in humans, due to the fact that a high lymphoproliferation index was found when PBMCs purified from healthy subjects or VL patients were *in vitro* stimulated with the recombinant protein. Although the experiments developed in this study do not allow one to infer that rLiHyS is immunogenic for human PBMCs, even though mitogen activity in cells derived from both individual classes was identified, additional studies are warranted in an attempt to clarify these findings, in turn allowing one to infer the possibility of an immunogenic action of this recombinant protein in human VL, thus opening the door to this antigen's use as a future immunogen to fight against this disease in this mammalian host.

Serological diagnosis for VL has used the rK39 antigen in clinical practice (Matlashewski *et al.* 2013; Ghosh *et al.* 2015). However, studies have shown that this protein was not highly efficient, exhibiting cross-reactivity with leishmaniasis-related pathologies, as well as with non-infected subjects living in endemic areas of disease, in turn leading to false-positive results and hampering the specificity of the serological tests (Gadisa *et al.* 2012; Aronson, 2017). In addition, since this commercial kit was developed to detect protein-specific antibodies for the *L. donovani* complex, false-negative results have been found when *L. infantum*-infected subject sera are tested (Mohammadiha *et al.* 2013; Costa *et al.* 2017). In our study, VL patient sera recognized rLiHyS with high specificity and sensitivity values, highlighting the possibility of using our antigen to diagnose disease. However, the low number of patient sera, as well as sera from leishmaniasis-related diseases developed in patients, such as malaria, tuberculosis, among others, could be considered limitations of our work, since new samples could alter the sensitivity and specificity values found to rLiHyS. As a consequence, additional experiments using a larger and more varied serological panel should be performed in an attempt to corroborate the diagnostic efficacy found in this study regarding this recombinant protein.

In conclusion, the present study's data indicated that LiHyS, a *Leishmania* conserved protein, besides its antigenicity in canine and human VL, was also immunogenic and able to confer partial protection against *L. infantum* infection in BALB/c mice. In addition, a lymphoproliferation response was found when rLiHyS was used to stimulate PBMCs from healthy subjects and VL patients, thus highlighting the possibility to evaluate it as an immunogen against human disease.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S0031182017001731.

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## 4.4 ARTIGO 2 – CONCLUSÃO

Os resultados indicaram que a proteína rLiHyS, além de sua antigenicidade na LV canina e humana, foi imunogênica e capaz de conferir proteção parcial em camundongos BALB/c contra a infecção por *L. infantum*. Além disso, uma resposta positiva de linfoproliferação foi encontrada quando rLiHyS foi usado para estimular PBMCs de indivíduos saudáveis e pacientes com LV, sugerindo assim a possibilidade de avaliá-la como um imunógeno contra a doença humana.

## 4.5 ARTIGO 3 – BREVE INTRODUÇÃO

O terceiro artigo a ser apresentado foi intitulado como “Serological diagnosis and prognostic of tegumentary and visceral leishmaniasis using a conserved *Leishmania* hypothetical protein” e publicado na revista internacional *Parasitology International* – doi: https://doi.org/10.1016/j.cellimm.2017.11.001.

O presente artigo avaliou a aplicação da proteína LiHyS (XP_001467126.1) como marcador sorológico para o diagnóstico das leishmanioses visceral e tegumentar, além de biomarcador para o prognóstico da doença.

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# Serological diagnosis and prognostic of tegumentary and visceral leishmaniasis using a conserved *Leishmania* hypothetical protein

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ABSTRACT

New candidates for serological markers against leishmaniasis are required to be identified, since the presence of high titers of anti-*Leishmania* antibodies remain detected in sera of treated and cured patients, when current antigens have being employed. In this study, the diagnostic performance of a conserved *Leishmania* hypothetical protein was evaluated against a human and canine serological panel. The serological follow-up of the patients was also evaluated, using this recombinant antigen (rLiHyS) in ELISA assays. In the results, high sensitivity and specificity values were found when rLiHyS was used in the serological tests, while when the recombinant A2 (rA2) protein or an antigenic *Leishmania* preparation were used as controls, low sensitivity and specificity were found. Regarding the serological follow-up of the patients, significant reductions in the anti-rLiHyS antibody levels were found and, one year after the treatments, the anti-protein IgG production was similar to this found in the non-infected groups, reflecting a drop of the anti-rLiHyS antibody production. In conclusion, the present study shows for the first time a new recombinant antigen used to identify tegumentary and visceral leishmaniasis, as well as being able to serologically distinguish treated and cured patients from those developing active disease.

## 1. Introduction

Leishmaniasis is a disease complex caused by parasites from *Leishmania* genus, presenting an incidence of 0.2 to 0.4 million visceral leishmaniasis (VL) cases, and 0.7 to 1.2 million of tegumentary leishmaniasis (TL) cases [45]. Tegumentary leishmaniasis (TL) exhibits distinct clinical manifestations ranging from cutaneous leishmaniasis (CL), diffuse cutaneous leishmaniasis (DCL), to mucosal leishmaniasis (ML). *Leishmania braziliensis* is the main species responsible by the cases of the disease in the Americas, while *L. infantum* is the main responsible for the VL cases [34].

Dogs are considered as important reservoirs for visceral disease transmission, due to their close relationship with humans [10]. Regarding the canine disease, animals can develop from asymptomatic infection, when they are apparently healthy, to widespread chronic infections, which can lead to the death [25,40]. Regarding TL, of which dogs are accidental hosts, marsupials, rodents, and wild canids species have been found as reservoirs of the parasites [28].

Serological tests used to diagnose leishmaniasis present variable efficacy, indicating that there is a need for new studies to reach a safer diagnostic result [18]. In addition, distinct *Leishmania* species exhibit a distribution that overlap in many geographical regions, making it

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difficult to isolate the parasite species causing the disease [16]. Also, anti-*Leishmania* antibodies in cured and treated patients usually remain positive for months and years after the treatments, making it difficult to distinguish between past, current and cured infections [23,43].

Despite diagnostic methods available for VL, some problems persist and maximum sensitivity and specificity values are not reached [11]. In addition, most of the asymptomatic animals are not identified by the serological assays. In this context, an early and reliable diagnosis could refine the identification of these animals, as well as allow to control the spread of this disease in the world [25]. The serodiagnosis of TL also presents problems, since most of the patients present low levels of antileishmanial antibodies and, consequently, false-negative results are usually found in the serological assays [32,33]. As a consequence, the search for identifying new candidates to be employed in the improvement of the diagnosis of TL still remains, aiming to improve sensitivity and specific values.

Modern laboratorial techniques have allowed the identification of recombinant antigens to be evaluated in the serodiagnosis of canine and human leishmaniasis. Recombinant proteins, such as cytochrome *c* oxidase and IgE-dependent histamine-releasing factor [9], rLiHyD [22], rKLO8 [25], rLbHyM [23], rLiHyV [24], rHSP83 [6], rA2 [1,5], among others, have been studied with this purpose. However, although they present satisfactory results to identify the active disease, their optimization is still required to obtain maximum sensitivity and specificity values aiming to identify asymptomatic cases, as well as leishmaniasis-related diseases developing patients. In addition, no antigen is currently used to serologically follow-up the evolution of the patients treatment, since antibody titers remain positive for a long period of time after their parasitological and/or clinical cure [15,19].

In this context, the search for new *Leishmania* proteins, are able to stimulate the humoral response in the infected hosts, and allow distinguishing between treated and untreated patients, should help the development of more sophisticated tests to detect the disease [13]. In this context, in the present study, a conserved parasite protein, namely LiHyS (XP_001467126.1), which was recognized by VL dogs sera, but not by sera from healthy dogs or from those infected with *Trypanosoma cruzi* [8]; was cloned and its recombinant version (rLiHyS) was evaluated for the serodiagnosis of human and canine leishmaniasis. In our work, rLiHyS was also employed as a diagnostic antigen in ELISA assays to compare its reactivity in sera samples of untreated and treated patients.

## 2. Materials and methods

### 2.1. Ethics statement

This study was approved by the Committee on the Ethical Handling of Research Animals of Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil (protocol number 0333/2015). The work was also approved by the Human Research Ethics Committee of UFMG (protocol number CAAE–32343114.9.0000.5149).

### 2.2. Canine sera

The sample used was composed by 113 domestic animals (*Canis familiaris*), and consisted of males (n = 67) and females (n = 46) of different breeds and ages. Healthy dogs (n = 21) were free of any clinical signs of disease and were selected from an endemic area of VL (Belo Horizonte). They also presented negative serological results when using a commercial kit (EIE-LVC Biomanguinhos kit, Biomanguinhos®, Rio de Janeiro, Rio de Janeiro, Brazil). VL dogs were diagnosed by parasitological exams to identify *L. infantum* kDNA in bone marrow aspirates by PCR technique, like described [31]. In addition, they presented positive serological results with two commercial tests: IFAT-LVC Bio-Manguinhos kit and EIE-LVC Bio-Manguinhos kit. The animals were classified as symptomatic (n = 25), when they presented three or more of the following symptoms: weight loss, alopecia, adenopathy, onychogryposis, hepatomegaly, conjunctivitis, exfoliative dermatitis, splenomegaly, lymphadenomegaly, and renal azotemia; or asymptomatic (n = 18), when they did not present any clinical signs of disease. To evaluate the cross-reactivity, sera samples of Leish-Tec®-vaccinated healthy dogs (n = 20), as well as from those experimentally infected with *Ehrlichia canis* (n = 13) or *Babesia canis* (n = 15) were used.

### 2.3. Human sera

Sera samples were obtained from ML (n = 23; including 15 males and 8 females with ages ranging from 22 to 55 years) or VL (n = 45, including 30 males and 15 females with ages ranging from 19 to 56 years) patients, which were collected from an endemic area of the disease (Belo Horizonte). Mucosal leishmaniasis patients were diagnosed by clinical evaluation and by the demonstration of parasites in Giemsa-stained smears of mucosal fragments, besides of a PCR assay to identify *L. braziliensis* kDNA. Visceral leishmaniasis patients were diagnosed by clinical evaluation and demonstration of *L. infantum* kDNA in bone marrow aspirates, when a PCR technique was performed. Sera samples were also obtained from healthy individuals living in an endemic area of leishmaniasis (n = 35, including 23 males and 12 females with ages ranging from 20 to 51 years). These subjects did not present any clinical signs of disease and showed negative serological results when using commercial kits. Samples were also obtained from Chagas disease patients (n = 235, including 15 males and 8 females with ages ranging from 24 to 58 years), being the infection confirmed by hemoculture using the Chagatest® recombinant ELISA v.4.0 kit or the Chagatest® hemmaglutination inhibition kit (Wiener lab., Rosario, Argentina). To evaluate the rLiHyS reactivity in patients before and after the treatment, sera samples of ML (n = 10, including 6 males and 4 females with ages ranging from 26 to 55 years) and VL (n = 10, including 7 males and 3 females with ages ranging from 22 to 58 years) patients were collected before, six and 12 months after performing the treatments. All of them were treated with pentavalent antimonials, which were administered during 20 days (Sanofi Aventis Farmacêutica Ltda., Suzano, São Paulo, Brazil). The patients were submitted to parasitological exams using the PCR technique with mucosal fragments (ML) and bone marrow aspirates (VL), 12 months after treatment, when negative results were found in all evaluations.

### 2.4. Parasites

*Leishmania infantum* (MHOM/BR/1970/BH46) and *L. braziliensis* (MHOM/BR/1975/M2903) were used. The stationary-phase promastigotes and the soluble *Leishmania* antigenic extract (SLA) were prepared according described [7].

### 2.5. Production of the recombinant antigens and evaluation of the LiHyS amino acid sequence

The LiHyS gene (XP_001467126.1) was cloned from *L. infantum* DNA using: 5′-TCTCGGATCCATGCGCCAGCGAAAGCAC-3′ (*forward*) and 5′-TGAAAAGCTTCCATGCGATCCAGTAGATG-3′ (*reverse*) primers, for the restriction enzymes *BamHI* and *HindIII*. The DNA fragment was excised from gel, purified and linked into a pGEM®-T vector system (Promega, USA). The recombinant plasmid was used to transform *E. coli* XL1-Blue competent cells, and clones were tested by restriction enzymes analysis. The DNA fragment obtained from digestion of pGEM-LiHyS plasmid was ligated into a pET28a-TEV vector, and *E. coli* BL21 cells were transformed with the recombinant plasmid. Gene insertion was confirmed by colony PCR, and the sequencing was performed in a MegaBace 1000 automatic sequencer apparatus (Amersham Biosciences, USA). For the expression and purification of rLiHyS, cells were induced with 1.0 μM IPTG and cultures were incubated for 2 h at 37 °C, shaking at 200 ×*g* per min. Then, they were ruptured by seven

cycles of ultrasonication with cycles of 30 s each (38 MHz), followed by five cycles of freezing and thawing. Cellular debris were removed by centrifugation, and rLiHyS was purified onto HisTrap HP affinity column connected to an AKTA system. The eluted fractions containing the recombinant protein (~21.3 kDa) were concentrated in Amicon® ultra15 centrifugal filters 10,000 NMWL (Millipore, Germany), and further purified on a Superdex™ 200 gel-filtration column (GE Healthcare Life Sciences, USA). The recombinant A2 (rA2) protein was purified under non-denaturing conditions according described [44]. After purification, both recombinant antigens were passed through a polymyxin-agarose column (Sigma), in order to remove any residual endotoxin content. The LiHyS amino acid sequence was compared with its homolog in the *L. brasilienzis* (XP_001566902.1) and *T. cruzi* (XP_805862.1 and XP_804031.1) species, by means of the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/) according described [37]. The B cell epitope prediction was performed by the BepiPred 1.0 server (http://www.cbs.dtu.dk/services/BepiPred/).

### 2.6. ELISA experiments

Previous titration curves were performed to determine the most appropriate antigen concentration and antibody dilution to be used. Flexible microtiter immunoassay plates (Jetbiofil®, Belo Horizonte) were coated with the rLiHyS, rA2 or *L. braziliensis* and *L. infantum* SLA (0.5, 1.0, 2.0, and 1.0 μg per well, respectively), which were diluted in 100 μL of coating buffer (50 mM carbonate buffer, pH 9.6), for 18 h at 4 °C. Next, free binding sites were blocked using 200 μL of PBS-T (phosphate buffer saline 1× plus Tween 20 0.05%) containing 5% casein, for 1 h at 37 °C. After washing the plates five times with PBS-T, they were incubated with 100 μL of human or canine sera (1:400 or 1:200, respectively; both diluted in PBS-T), for 1 h at 37 °C. Plates were washed seven times in PBS-T and incubated with anti-human or anti-dog IgG horseradish-peroxidase conjugated antibodies (1:15,000 or 1:10,000, respectively, both diluted in PBS-T), for 1 h at 37 °C. After washing the plates seven times with PBS-T, reactions were developed by incubation with a solution (100 μL per well) 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 reactions were stopped by adding 25 μL 2 N $H_2SO_4$, and the optical density (O.D.) was read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nm.

### 2.7. Statistical analysis

The results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed using GraphPad Prism™ (version 6.0 for Windows). The receiver operating characteristic (ROC) curves were constructed to obtain the sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and likelihood ratio (LR) values for the diagnostic antigens, as well as the lower limit of positivity (cut-off). Aiming to standardize the individual O.D. values, the respective mean O.D. obtained for each sample was divided by its respective cut-off value. The product of this division was called optical density index (O.D.I.). The unpaired Student *t*-test was used and significant differences were considered with $P < .05$. Tables of contingency and Fisher's exact test ($P < .05$) were also used to compare the performance of the antigens.

## 3. Results

### 3.1. Sequence evaluation and diagnostic performance of rLiHyS for canine leishmaniasis

Bioinformatics assays showed that LiHyS presents about 80% identity to its homolog in *L. braziliensis*, whereas 33% and 34% homology was found in two *T. cruzi* sequences (Fig. 1). In addition, the main B cell epitopes were found conserved in the *Leishmania* sequences, but not in the *T. cruzi* sequences, then showing a high conservation of this antigen in the *Leishmania* parasite. To evaluate the diagnostic application of rLiHyS for canine VL, ELISA assays using rA2 and *L. infantum* SLA as comparative antigens were performed, and cut-off values were determined using ROC curves. In the results, both asymptomatic and symptomatic VL dogs sera, but not those from vaccinated or leishmaniasis-related diseases developing dogs, were reactive against rLiHyS (Fig. 2A and B); while worst reactivities levels were found when rA2 (Fig. 2D and E) or SLA (Fig. 2G and H) were used in the plates. The percentage of true-positive, true-negative, false-positive and false-negative sera was calculated and results showed that rLiHyS was the antigen presenting the best diagnostic performance, since 39.2% of the animals were classified as true-positive and 60.0% as true-negative (Fig. 2C), while using rA2 (Fig. 2F) or SLA (Fig. 2I), both percentages were lower. The sensitivity values were of 100%, 55.0% and 62.0% for rLiHyS, rA2 and SLA, respectively, while the specificity values were of 99.0%, 64.0% and 73.0%, respectively (Table 1).

### 3.2. Diagnostic evaluation of rLiHyS for human leishmaniasis

The rLiHyS protein was also tested as an antigen for the serodiagnosis of human TL. For this, ROC curves were constructed to obtain the cut-off values for discrimination between negative and positive samples. In the results, antibodies in ML and VL patients sera were able to recognize the recombinant protein. However, when Chagas disease patients or healthy subjects sera were used, a low reactivity was found (Fig. 3A and B). All samples were correctly classified as true-positive (54.0%) or true-negative (46.0%) when rLiHyS was used in the experiments (Fig. 3C); however, using *L. braziliensis* SLA, a mixed reactivity was found (Fig. 3D and E), and true-positive or true-negative values were of 0.8% and 46.0%, respectively (Fig. 3F). In addition, sensitivity values were of 100% and 1.0% for rLiHyS and SLA, respectively (Table 1). When the anti-rLiHyS humoral response in patients sera obtained before and after treatment was evaluated, significant reductions in the anti-protein antibody levels were reached in both cases, when compared to the results obtained before treatment. Using sera collected 12 months after the treatment, antibody levels were similar to those found in the non-infected group. On the other hand, the anti-parasite antibody levels were similar to those observed both before and after the treatment (Fig. 4).

## 4. Discussion

The parasitological diagnosis based on *Leishmania* detection in lesion or mucosal fragments of TL patients remains as the gold standard to diagnose the disease. However, although the direct microscopic identification of the parasites is simpler, its sensitivity is hampered, mainly in the cases where parasitemia is low, such as in patients developing the chronic disease [21,35]. On the other hand, serological methods have been employed to diagnose VL, and they have been considered relevant to detect active infection. As a consequence, immunochromatographic dipstick tests employing rK39 [38], rK28 [27] and rKE16 [42] proteins have been used to diagnose symptomatic VL, although their sensitivity to detect asymptomatic cases has been variable [29].

The definition of an ideal diagnostic marker for leishmaniasis is influenced by the type of the parasite antigen used in the tests, as well as by the geographical location related to parasite species and the occurrence of clinical signs of the disease. As an example, in a study developed in an endemic area in Iran, the rK39 dipstick test showed moderate sensitivity and specificity values to identify canine VL [26]. However, positive results were reported in Brazil, India, Ethiopia and Sudan; in which these assays were used to detect canine and human leishmaniasis [14].

For CVL serodiagnosis, tests with low accuracy present

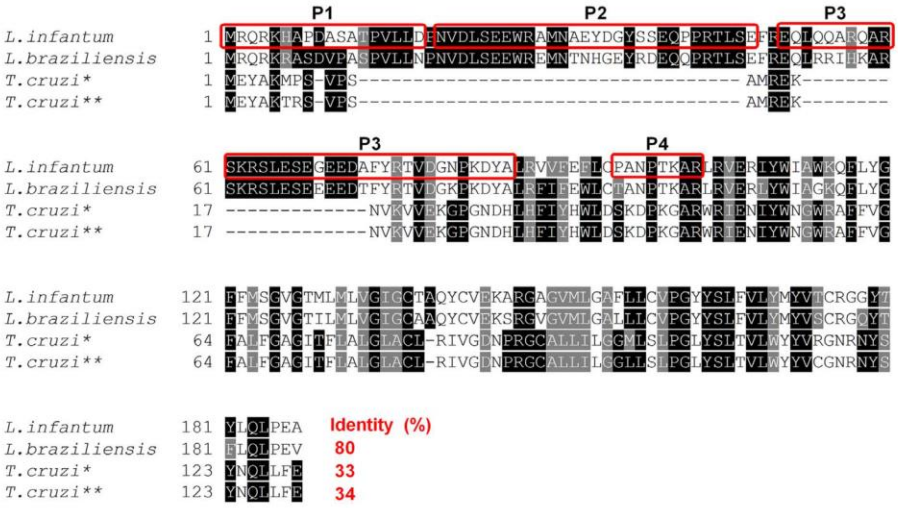


**Fig. 1.** Analysis of the LiHyS amino acid sequence. The amino acid LiHyS (XP_001467126.1) sequence was compared to those from *Leishmania braziliensis* (XP_001566902.1) and *Trypanosoma cruzi* (XP_805862.1 and XP_804031.1) species, using the Clustal Omega program. The identical residues are shown (black color), as well as the conservative (gray color) and semi-conservative (white color) substitutions. The percentage of homology between the sequences is also indicated.

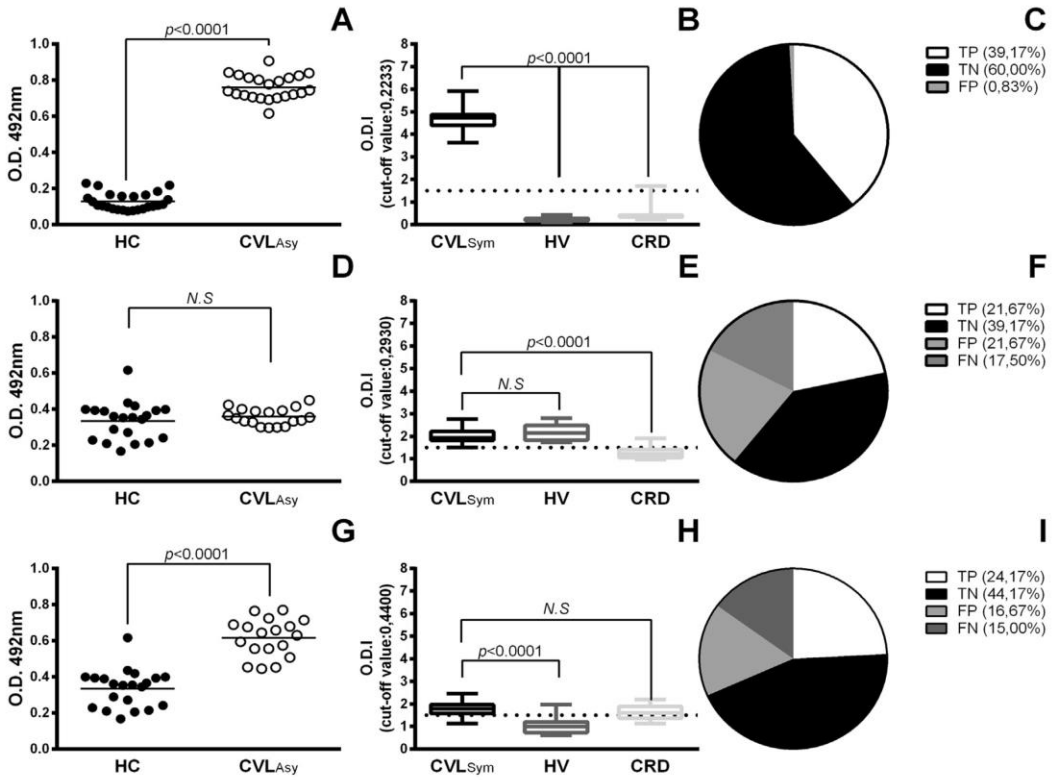


**Fig. 2.** Evaluation of the recombinant antigens for the serodiagnosis of canine leishmaniasis. ELISA assays were performed using sera samples from healthy dogs living in an endemic area of leishmaniasis (HC; n = 21), as well as sera from asymptomatic ($CVL_{Asy}$; n = 18) or symptomatic ($CVL_{Sym}$; n = 25) VL dogs, from Leish-Tec®-vaccinated dogs (HV; n = 20) and from those developing cross-reactive diseases [CRD, n = 28, which were composed by sera from animals experimentally infected with *Ehrlichia canis* (n = 13) or *Babesia canis* (n = 15)]. Individual reactions against rLiHyS (A), rA2 (D) or *L. infantum* SLA (G) are shown. Cut-off values for negative and positive samples discrimination were obtained by calculating the receiver operator curves (ROC). The values were obtained by the ratio between the optical density (O.D.) of the samples from the symptomatic VL dogs, vaccinated animals and CRD groups and their cut-off, being represented by the optical density index (O.D.I.) for the antigens: rLiHyS (B), rA2 (E) and SLA (H). The percentages of true-positive (TP), true-negative (TN), false-positive (FP) and false-negative (FN) were calculated and are shown for the antigens: rLiHyS (C), rA2 (F) and SLA (I). The statistical differences are indicated. Abbreviation: N.S.: not significant.

epidemiological consequences, such as the fact that animals classified as false-negative remain undetected, thereby maintaining the parasite dissemination in endemic areas. Also, there is the detection of false-positive animals, which can results in an unnecessary culling of the uninfected animals. In our study, rLiHyS identified 39.2% of the dogs as true-positive and 60.0% as true-negative, while rA2 showed 22.0% of them as true-positive and 39.0% as true-negative. Using SLA, these values were of 24.0% and 44.0%, respectively. In this context, rLiHyS showed the best results in discriminating positive and negative samples, when compared to two other antigens used as controls.

As recently shown [12], LiHyS was found to be present in the parasite's flagellar pocket, by means of immunofluorescence experiments using an anti-rLiHyS IgG antibody. In this context, one could speculate about the role of this protein as part of the *L. infantum*

**Table 1**
Diagnostic performance of the antigens for the serodiagnosis of canine and human leishmaniasis.

| Antigen | Sensitivity | 95% CI | Specificity | 95% CI | PPV | 95% CI | NPV | 95% CI | LR |
|---|---|---|---|---|---|---|---|---|---|
| Canine sera | | | | | | | | | |
| rLiHyS | 100% | 92.5–100 | 99.0% | 92.6–99.9 | 98.0% | 88.9–99.9 | 100% | 95.0–100 | 73.0 |
| rA2 | 55.0% | 40.1–69.8 | 64.0% | 52.3–75.3 | 50.0% | 35.8–64.2 | 69.0% | 56.7–79.8 | 1.5 |
| *L. infantum* SLA | 62.0% | 46.4–75.5 | 73.0% | 60.9–82.4 | 59.o% | 44.2–73.0 | 75.0% | 62.9–84.2 | 2.5 |
| Human sera | | | | | | | | | |
| rLiHyS | 100% | 94.7–100 | 100% | 93.8–100 | 100% | 94.7–100 | 100% | 93.8–100 | N.C. |
| *L. braziliensis* SLA | 1.0% | 0.4–7.9 | 100% | 93.8–100 | 100% | 2.5–100 | 46.0% | 37.4–55.5 | N.C. |

The canine and human serological panel was used in ELISA experiments against rLiHyS, rA2, *L. infantum* or *L. braziliensis* SLA. Results obtained were used to calculate sensitivity, specificity, confidence interval (95%CI), positive predictive value (PPV), negative predictive value (NPV), and likelihood ratio (LR). Abbreviation: N.C.: not calculated.

flagellum machinery, maybe as a parasites virulence factor, and its location could be additionally investigated using other flagellum apparatus proteins in new studies. Since the assessment of canine seroprevalence is dependent of the antigens sensitivity and specificity [3,4,17]; in our study, asymptomatic dogs were also evaluated, and results showed that rLiHyS was also able to identify this experimental group, while rA2 and SLA showed worst sensitivity values to correctly detect these samples.

The use of highly sensitive antigens that will detect the largest possible number of infected dogs, being symptomatic or asymptomatic, would be the most recommended for diagnostic actions for disease control, as well as would allow the reduction of the parasite transmission. In this point of view, the serological assays using rLiHyS that were shown in this work are adequate, since sensitivity and specificity values of 100% and 99.0%, respectively, were found when samples of both asymptomatic and symptomatic VL dogs were tested.

Regarding human TL, a number of recombinant molecules have been tested as candidates for the serodiagnosis of disease; however, the variable sensitivity of these antigens has been a problem reported [30,36,41]. In addition, the follow-up of antileishmanial antibodies after treatment has not been adequate in the majority of the studies, although this parameter should be considered as an additional control measure for the therapy success [23]. In our work, maximum sensitivity and specificity values were found when rLiHyS was used to identify TL and VL patients sera.

Also, the follow-up of rLiHyS-specific IgG antibody showed that this protein was recognized in very low levels by cured and treated patients sera, mainly when samples were evaluated 12 months after the treatment, since in this period of time a similar humoral response was observed in the patients, when compared to the controls. The persistence of anti-*Leishmania* antibodies in past VL cases is well-described when different techniques are used, such as the direct agglutination test and ELISA [19]. In our study, when SLA was used as an antigen source, a similar reactivity was also found when sera were investigated before and after the treatment against this antigen. This same profile has been observed when rK39 ELISA is used to evaluate the humoral response in treated VL patients, 4 and 12 years after the treatment [2,39]. Similarly, about 89% of VL cases were detected as positive DAT, until 8 years post-treatment [20]. Here, the low presence of anti-rLiHyS antibody in sera samples of treated VL patients could be associated with the success of the treatment, since they did not present clinical signs of disease and showed negative parasitological results, when evaluated by PCR

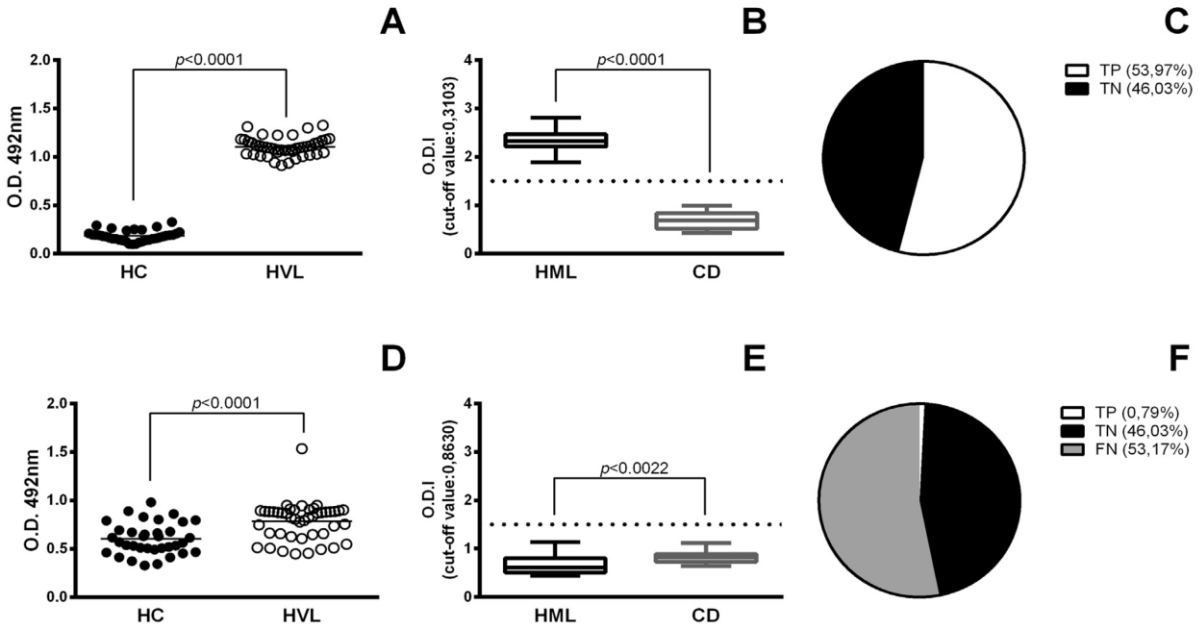


**Fig. 3.** Serological assays for the diagnosis of human leishmaniasis. ELISA experiments were performed using sera samples from healthy subjects living in an endemic area of leishmaniasis (HC; n = 35), as well as sera from mucosal (HML; n = 23) or visceral (HVL; n = 45) leishmaniasis patients, or from Chagas disease patients (CD; n = 23). Reactions against rLiHyS (A) and SLA (D) are shown. Cut-off values for negative and positive samples discrimination were calculated by receiver operator curves (ROC). Values were obtained by the ratio between the optical density (O.D.) of the samples from the HML patients, and from those with Chagas disease, and their cut-off was represented by the optical density index (O.D.I.) for the antigens: rLiHyS (B) and SLA (E). The percentages of true-positive (TP), true-negative (TN), false-positive (FP) and false-negative (FN) were calculated and are shown for the antigens: rLiHyS (C) and SLA (F). The statistical differences are indicated.

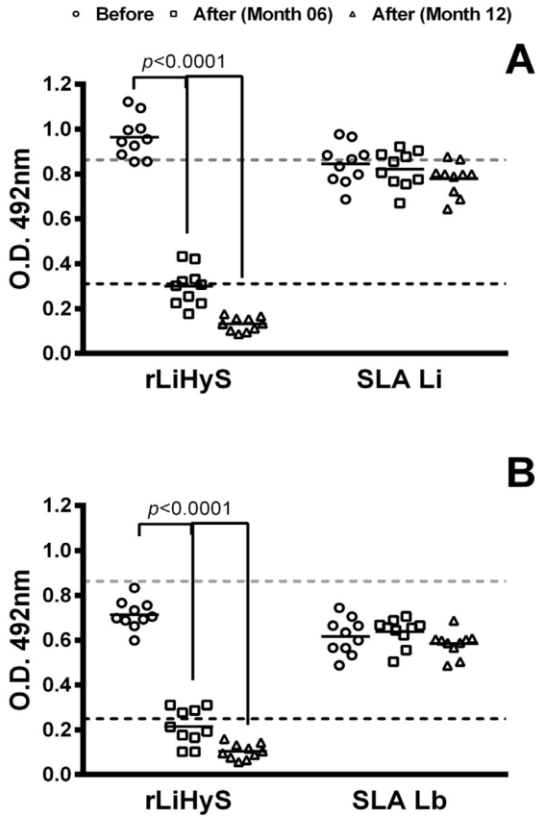


**Fig. 4.** Reactivity of rLiHyS in patients before and after the treatment. Serological assays were performed with sera samples from visceral (n = 10, in A) or mucosal (n = 10, in B) leishmaniasis patients; which were collected before, 6 and 12 months after the treatment. The optical density (O.D.) of each sample is shown, and statistical differences are indicated. The horizontal lines indicate the cut-off values obtained for rLiHyS (black color) and SLA (grey color).

technique. As a consequence, rLiHyS could be considered as an additional control strategy for a well-successful therapy against VL, when evaluated by the ELISA technique. However, new studies should be performed aiming to elucidate if the absence of significant levels of anti-rLiHyS antibodies in these treated patients will precisely reflect their clinical and parasitological cure, having been able to eliminate all parasites, or if eventually the low presence of anti-protein antibodies will reflect only in the absence of humoral reactivity against this specific molecule, since the treated patients could still present *Leishmania* parasites, once the anti-SLA reactivity still persists.

Our diagnostic antigen presents a high degree of conservation in its amino acid sequence between *L. braziliensis* and *L. infantum* species, which are responsible for TL and VL cases in the Americas, respectively. This fact could be considered relevant, since its main B cell epitopes are found also conserved between these parasite species. As a consequence, this molecule could be considerable noted to be investigated not only by ELISA technique, but also by other methodologies, aiming to diagnose leishmaniasis. Taken together, the results showed that this novel and highly accurate diagnostic antigen, could be considered for the improvement of the serodiagnosis of canine and human leishmaniasis, as well as a new biomarker for this disease prognosis.

## Conflict of interest

The authors declare no commercial or financial conflict of interest.

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## 4.6 ARTIGO 3 – CONCLUSÃO

O presente estudo mostrou um antígeno recombinante usado para a detecção sorológica das leishmanioses tegumentar e visceral, sendo capaz de distinguir pacientes tratados e curados daqueles que desenvolvem a doença ativa, dessa forma, colocando a proteína rLiHyS como um biomarcador promissor para o prognóstico das leishmanioses.

## 4.7 ARTIGO 4 – BREVE INTRODUÇÃO

O quarto artigo a ser apresentado foi intitulado “Recombinant prohibitin protein of *Leishmania infantum* acts as a vaccine candidate and diagnostic marker against visceral leishmaniasis” e publicado na revista internacional *Cellular Immunology* (doi: https://doi.org/10.1016/j.cellimm.2017.11.001).

O presente artigo teve como objetivo verificar a eficácia protetora da proteína recombinante Prohibitina (rPHB, XP_001468827.1) em camundongos BALB/c contra a infecção com *L. infantum* e seu uso como imunoestimulador de PBMCs de pacientes tratados e não-tratados com LV, bem como avaliar seu desempenho diagnostico na doença canina e humana.

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Research paper

# Recombinant prohibitin protein of *Leishmania infantum* acts as a vaccine candidate and diagnostic marker against visceral leishmaniasis

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ABSTRACT

Visceral leishmaniasis (VL) represents a serious public health problem, as *Leishmania infantum* is one of main disease causative agents in the Americas. In a previous immunoproteomic study, the prohibitin (PHB) protein was identified in *L. infantum* promastigote and amastigote extracts by antibodies in asymptomatic and symptomatic VL dog sera. This protein was found to be highly conserved between different *Leishmania* spp., but it presented a low identity with amino acid sequences of other organisms. The aim of the present study was to evaluate the cellular response induced by the recombinant PHB (rPHB) protein in BALB/c mice, as well as in PBMCs purified from untreated and treated VL patients, as well as to evaluate its protective efficacy against an infection by *L. infantum* promastigotes. Our data showed that there was a Th1 cellular response to rPHB, based on high levels of IFN-γ, IL-12, and GM-CSF in the immunized animals, as well as a proliferative response specific to the protein and higher IFN-γ levels induced in PBMCs from individuals who had recovered from the disease. The protection was represented by significant reductions in the parasite load in the animals' spleen, liver, bone marrow, and draining lymph nodes, as compared to results found in the control groups. In addition, an anti-rPHB serology, using a canine and human serological panel, showed a high performance of this protein when diagnosing VL based on high sensitivity and specificity values, as compared to results found for the rA2 antigen and the soluble *Leishmania* antigenic extract. Our data suggest that PHB has a potential application for the diagnosis of canine and human VL through antibody detection, as well as an application as a vaccine candidate to protect against disease.

## 1. Introduction

Leishmaniasis are diseases caused by parasites able to infect mammalian cells and are transmitted by hematophagous sandflies. This complex disease is a major worldwide health problem, since over 350 million people are at risk of contracting infection, with an annual incidence of more than 12 million cases [1]. The clinical manifestations are dependent upon both the parasite species and the host's immune response, and visceral leishmaniasis (VL) is the most severe form of the disease, presenting a 90% mortality rate if left untreated [2,3].

The treatment of disease is considered unsatisfactory [4], and vaccination remains an alternative method of preventing it, representing a

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relevant contribution of immunology to human health [5,6]. In animal models, immunity against *Leishmania* has been based on the Th1/Th2 paradigm. Cellular immunity is considered to be a key mediator of resistance against disease, by means of production of cytokines, such as IFN-γ, which up-regulates the production of nitric oxide, leading to oxidative bursts in infected phagocytes. By contrast, regulatory Th2 and T cells induce progressive disease in the infected hosts, while cytokines, such as IL-4 and IL-10, regulate IFN-γ-producing T cells, hampering the protection against infection [7,8]. As a consequence, a number of candidates have been tested as a vaccine in recent decades, most of which have been used in association with adjuvant systems to be administered in animal models [9,10].

Regarding VL diagnoses, despite the available methods to identify canine and human disease, there is no method to obtain maximum sensitivity and specificity values and, consequently, to allow an accurate diagnosis of disease [11,12]. Serological tests present variable reliability and accuracy, indicating that there is a need for new antigens to be used in the detection of infected subjects but not by others developing cross-reactive diseases or from those living in endemic areas of disease [13,14]. In this context, the search for *Leishmania* proteins, considered to be well-conserved and/or specific to the parasites, which will stimulate the cell and humoral response in the infected hosts, should be considered for a more effective control of the disease.

Immunoproteomic approaches have been used as a strategy to identify new *Leishmania* molecules, where antileishmanial antibodies in infected canine and human sera recognize relevant proteins in the parasite extracts [15,16]. These screenings have revealed a number of proteins with immunological potential to be applied against leishmaniasis [17]. One of these molecules, prohibitin (PHB) (XP_001468827.1), was recently identified in an immunoproteomic study by antibodies in VL dog sera but not by those from healthy dogs living in an endemic area of disease [18].

This protein is involved in events such as cell proliferation [19], ageing [20], B-cell maturation [21], and maintenance of mitochondrial integrity [22]. The presence of protein-specific immune responses during interactions between parasites and macrophages, by means of *in vitro* experiments, induces a lower degree of infection in these phagocytic cells. In addition, the presence of the specific antibodies in *Leishmania donovani*-infected patients have shown that PHB can be recognized by humans developing VL [23] and, in this context, could well be evaluated as a diagnostic marker for the disease.

In this context, in the present study, the PHB-directed immune response and protective efficacy were evaluated in BALB/c mice against *L. infantum* infection. In addition, the lymphoproliferation induced in PBMCs collected from untreated and treated VL patients, as well as the immunogenicity of this recombinant protein by means of the IFN-γ and IL-10 production were investigated. With the purpose to evaluate the diagnostic application of PHB for VL, the recombinant protein was employed in ELISA experiments against a human and canine serological panel.

## 2. Materials and methods

### 2.1. Ethics statement, mice, and parasites

Experiments were performed according the Committee on the Ethical Handling of Research Animals (CEUA) from the Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil (protocol number 333/2015). In addition, this study was approved by the UFMG Research Ethics Committee (protocol number CAAE–32343114.9.0000.5149). BALB/c mice (female, 8 weeks of 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. *Leishmania infantum* (MOM/BR/1970/BH46) was used. The parasites were grown at 24 °C in complete Schneider's medium (Sigma-Aldrich, USA), together with 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 20 mM l-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4. The soluble *Leishmania* antigenic extract (SLA) was prepared from stationary-phase promastigotes of parasites, as described [24].

### 2.2. Preparation of the recombinant proteins and sequence analysis of PHB protein

The rPHB (XP_001468827.1) protein was cloned from *L. infantum* kDNA using specific primers. The DNA fragment was purified and linked into a pGEM®-T vector system (Promega, USA) and the recombinant plasmid was used to transform *E. coli* XL1-Blue competent cells. Fragments obtained from the digestion of pGEM-rPHB plasmid were ligated into a pET28a-TEV vector, and cells were transformed. Sequencing was performed to confirm the identity of the insert, by using a MegaBace 1000 automatic sequencer apparatus (Amersham Biosciences, USA). For the purification of the recombinant protein, cells were induced with 0.5 μM isopropyl-β-d-thiogalactopyranoside (IPTG, Promega®, Canada), and cultures were incubated for 3 h at 37 °C, at which time they were ruptured by seven cycles of ultrasound, in cycles of 30 s each (36 MHz), followed by six cycles of freezing and thawing. After, debris were removed by centrifugation, and rPHB (32.3 kDa) was purified onto a HisTrap HP affinity column (GE Healthcare Life Sciences, NJ, USA) connected to an AKTA system. The eluted fractions were concentrated in Amicon® ultra15 centrifugal filters with a 10,000 nominal molecular weight limit (NMWL, Millipore, Germany), and further purified on a Superdex$^{TM}$ 200 gel-filtration column (GE Healthcare, USA). The A2 recombinant (rA2) protein was purified as described [25]. After dialysis, both proteins were passed through a polymyxin-agarose column (Sigma-Aldrich, USA) to remove residual endotoxin content (Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000 kit, BioWhittaker, MD, USA). The *in silico* analysis of the *L. infantum* PHB sequence consisted of the search for similarity among sequences deposited by a BLAST tool in non-redundant protein databases, and by comparison with the databases of other *Leishmania* species whose genomes have been sequenced or are in the phase of annotation, i.e., *L. major* (XP_003722404), *L. donovani* (XP_003864600), *L. braziliensis* (XP_001568126), *L. guyanensis* (CCM18788) (all available at www.genedb.org). The Cobalt (Constraint-based Multiple Alignment Tool) program available in (https://www.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi) was used for the alignment and analysis of the sequences.

### 2.3. Immunization, infection, and parasitological evaluation

Mice (n = 16, per group) were vaccinated in their left hind footpad with 20 μg of rPHB with or without saponin (20 μg, *Quillaja saponaria* bark saponin, Sigma-Aldrich, USA), or received saline or adjuvant. Three doses were administered at 14-day intervals, and, 30 days after the last vaccine dose, animals (n = 8, per group) were infected in the right hind footpad with $10^7$ stationary promastigotes of *L. infantum*. Forty-five days after infection, the animals were euthanized and the liver, spleen, bone marrow (BM), and paws' draining lymph nodes (dLN) were collected for parasitological assays, which were performed through a limiting-dilution assay and quantitative PCR (*q*PCR). The splenic parasite load was evaluated by the *q*PCR technique, as described [26]. For this, the mouse β-actin gene (*Forward*: CAGAGCAAGAGAGGTATCC; *Reverse*: TCATTGTAGAAGGTGTGGTGC) was used as an endogenous control to normalize (nucleated cells, single-copy-number), and evaluate the sample's integrity. Standard curves were obtained from DNA extracted from $10^8$ parasites for kDNA, and $10^8$ peritoneal macrophages for β-actin, under the same conditions used to extract the samples. Results were expressed as the number of parasites per 1000 nucleated cells in all groups. In addition, spleen, liver, dLN, and BM were macerated and concentrated by centrifugation at 2000*g*, at which

time the pellet was resuspended in 1 mL of Schneider's insect medium plus 20% FBS. Next, 220 µL were plated onto 96-well flat-bottom microtiter plates (Nunc, Nunclon®, Roskilde, Denmark), and diluted in log-fold serial dilutions in Schneider's medium ($10^{-1}$ to $10^{-12}$ dilution). Results were expressed as the negative log of the titer adjusted per milligram of organ, 7 days after the beginning of the culture, at 24 °C [26]. Experiments were repeated and presented similar results.

### 2.4. Cell response

Thirty days after the last immunization and 45 days after infection, the mice (n = 8 per group) were euthanized and their spleens collected, at which time splenocytes were cultured ($5 \times 10^6$ cells per mL per well) in 24-well plates (Nunc). Cells were incubated in RPMI medium (control) plus 20% FBS, 20 mM l-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, pH 7.4; or stimulated with rPHB or SLA (10 and 25 µg/mL, respectively), for 48 h at 37 °C in 5% $CO_2$. IFN-γ, IL-4, IL-10, and IL-12p70 levels were assessed in the supernatants by an ELISA capture using commercial kits (BD OptEIA Set Mouse, Pharmingen®, San Diego, CA, USA). The participation of the $CD4^+$ and $CD8^+$ T cells in the IFN-γ production was also evaluated in the spleen cells of the rPHB/saponin-vaccinated mice, in which cells were stimulated with rPHB or SLA in the presence of monoclonal antibodies against mouse IL-12 (C17.8), CD4 (GK 1.5), or CD8 (53-6.7), all in a concentration of 5 µg/mL. Appropriate isotype-matched controls (no azide/low endotoxin™), rat IgG2a (R35-95), and rat IgG2b (95 −1) were used (Pharmingen®, USA). The frequency of IFN-γ, TNF-α, and IL-10-producing $CD4^+$ and $CD8^+$ T cells was determined in the parasite-stimulated cultures by an analysis based on their relative flow cytometry size (forward laser scatter – FSC) and granularity (side laser scatter – SSC) graphs by flow cytometry, as described in [26]. Results were expressed as indexes, which were calculated by the ratio between the percentage of $CD4^+$ and $CD8^+$ T cells in the stimulated cultures, by means of the values obtained using the unstimulated cultures (SLA/CC ratio).

### 2.5. Nitrite and GM-CSF production and humoral response

Nitrite, TNF-α, and GM-CSF production was evaluated in all groups, before (GM-CSF and TNF-α) and 45 days after (all molecules) challenge, employing the same supernatants used to quantify the other cytokines. Results of the nitrite levels were expressed as µM, which were evaluated by the Griess method [27]. To evaluate the antibody production before and after infection, sera samples were collected from the animals in the experimental groups, and the protein- and parasite-specific IgG1 and IgG2a isotype antibody levels were evaluated by ELISA [26]. Briefly, rPHB and SLA (0.5 and 2.0 µg per well, respectively) were used as antigens in the plates, and individual sera were 1:100 diluted in PBS-T. The anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich, USA) were used in 1:5000 and 1:10,000 dilutions, which were performed in PBS-T, respectively. Reactions were developed by incubation with a solution consisting of $H_2O_2$, orthophenylenediamine and citrate-phosphate buffer (100 µL per well), pH 5.0, for 30 min and in the dark. After, these reactions were halted by adding 25 µL 2 N $H_2SO_4$, and optical density (O.D.) values were read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada) at 492 nm. In addition, controls were included in all plates. Two ELISA experiments were performed with the same samples tested in independent replicates, producing results similar.

### 2.6. Lymphoproliferative response and cytokine assay

To evaluate the rPHB-specific proliferative response in PBMCs, blood samples were collected from untreated or treated VL patients (n = 8, each group), and cells ($1 \times 10^7$) were labeled with carboxy fluorescein diacetate succinimidyl ester (CFSE) and incubated for 10 min at 37 °C. Next, the samples were centrifuged three times for 7 min, and 50 µL was collected and analyzed by flow cytometry. PBMCs were cultured in 48-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA), with each well containing 800 µL of RPMI 1640 medium. Next, cells ($1 \times 10^6$) were added in triplicate to the wells containing 100 µL of RPMI (control) and incubated alone (medium) or stimulated with rPHB or SLA (10 or 25 µg/mL, respectively). The incubation was performed for 5 days at 37 °C in 5% $CO_2$, after which time they were collected, washed twice in fluorescence activated cell sorter (FACS) buffer, and fixed using a FACS fixing solution. The PBMCs were then stored at 4 °C, prior to flow cytometric acquisition and analysis, which were performed using 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 per sample, as described in [26]. In addition, cell supernatants were collected and IFN-γ and IL-10 production was measured by a capture ELISA, by using commercial kits (Human IFN-γ and IL-10 ELISA Sets, BD Biosciences, USA) according to manufacturer's instructions.

### 2.7. Biological samples and patients

Sera samples were collected from healthy dogs living in endemic (n = 15, Belo Horizonte) or non-endemic (n = 15, Poços de Caldas, Minas Gerais, Brazil) areas of leishmaniasis, which presented no clinical signs of disease. Asymptomatic (n = 8) or symptomatic (n = 15) VL dog sera were used. The disease was confirmed by means of the identification of *L. infantum* kDNA in bone marrow samples, as well as by positive serological results, using the IFAT-LVC® and EIE-LVC® commercial kits (BioManguinhos, Rio de Janeiro, Brazil). Symptomatic animals showed three or more of the following symptoms: weight loss, alopecia, adenopathy, renal azotemia, onychogryposis, hepatomegaly, splenomegaly, and exfoliative dermatitis on the nose, tail, and ear tips; whereas asymptomatic dogs presented positive parasitological and serological results, but no clinical sign of disease. Sera samples from healthy animals vaccinated with Leish-Tec® (n = 15) and from those experimentally infected with *Ehrlichia canis* (EC, n = 10) or *Trypanosoma cruzi* (TC, n = 10) were also used. Regarding human sera, samples of healthy individuals living in endemic (n = 20, Belo Horizonte) or non-endemic (n = 15, Poços de Caldas) areas of disease, as well as from those developing VL (n = 20), paracoccidioidomycosis (n = 4), leprosy (n = 15), aspergillosis (n = 8), or Chagas disease (n = 20), were used. In addition, none of the patients had been previously treated with antileishmanial drugs before their samples were collected. To evaluate the lymphoproliferative response before and after treatment, blood samples were collected from treated VL patients (n = 8), before beginning treatment and 6 months after the end of the treatment sessions, which were performed using pentavalent antimonials (Sanofi Aventis Farmacêutica Ltda., Suzano, São Paulo, Brazil).

### 2.8. ELISA for VL serodiagnosis

For the ELISA assays, titration curves were performed to determine the most appropriate concentration of antigens and sera sample dilutions to be used in the experiments. After, rPHB, rA2, and SLA (0.5, 1.0, and 1.0 µg per well, respectively) were added into the wells in microtiter plates (Falcon), diluted in 100 µL coating buffer (50 mM carbonate buffer, pH 9.6) for 16 h at 4 °C. Next, free binding sites were blocked using 200 µL of PBS-T (phosphate buffer saline plus Tween 20 0.05%) containing 5% albumin for 1 h at 37 °C. After washing the plates three times with PBS-T, they were incubated with 100 µL of canine or human sera (1:100 and 1:200, diluted in PBS-T), for 1 h at 37 °C. Plates were subsequently washed five times in PBS-T, and incubated with anti-dog or anti-human IgG horseradish-peroxidase conjugated antibodies (1:5000 and 1:10,000 diluted in PBS-T; catalog A6792 and I5260, respectively, Sigma-Aldrich, USA) for 1 h at 37 °C. After washing the plates five times with PBS-T, reactions were developed as previously

described.

### 2.9. Statistical analysis

Results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed using GraphPad Prism™ (version 6.0 for Windows). Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by the Bonferroni's post-test for multiple comparisons between the groups. Differences were considered significant with $P < .05$. Receiver operating characteristic (ROC) curves were constructed to analyze the diagnostic antigens, and obtain their sensitivity, specificity, positive predictive value, negative predictive value, and likelihood ratio.

## 3. Results

### 3.1. Immunogenicity in rPHB/saponin-vaccinated BALB/c mice before and after infection

The rPHB protein was administered in BALB/c mice (n = 16 per group) with or without saponin as an adjuvant, and the immunogenicity was evaluated 30 days after the last vaccine dose (n = 8 per group). In the remaining animals, an infection using *L. infantum* promastigotes was performed and, 45 days after challenge, the immune profile was also investigated. In the results, the rPHB/saponin vaccine exhibited elevated levels of protein and parasite-specific IFN-γ and IL-12 in the animals before and after infection, since significantly higher levels of these cytokines were found in the cell supernatant of the splenocyte cultures of these animals (Fig. 1). Animals that were immunized only with the recombinant protein also showed a higher production of IFN-γ and IL-12 as compared to the values found in saline and saponin groups mice; however, these levels were significantly lower in comparison to results obtained in the rPHB/saponin group. Regarding IL-4 and IL-10 production before challenge, no significant production was detected in any experimental group (Fig. 1A); however, after infection, significantly higher levels of these cytokines were found in saline and saponin groups mice (Fig. 1B). The involvement of $CD4^+$ and $CD8^+$ T cells in IFN-γ production was evaluated in infected and rPHB/saponin-vaccinated mice and, in the results, when either the anti-CD4 or anti-CD8 antibody was added to the *in vitro* cultures, a significant reduction in IFN-γ production was found, demonstrating a role of both T cells in the development of antileishmanial Th1 response (Fig. 2). The frequency of intracytoplasmic cytokine-producing T cells was also investigated by a flow cytometry assay, and results showed that infected and rPHB/saponin-vaccinated mice, when compared to the other groups, presented higher levels of both T cell subtypes producing IFN-γ and TNF-α, which was associated with low levels of IL-10-producing T cells, although no significant difference was found between the experimental groups (data not shown).

Nitrite, TNF-α, and GM-CSF production was evaluated in order to evaluate macrophage activation in the experimental groups (Fig. 3). In the results, nitrite (Fig. 3A), GM-CSF (Fig. 3B), and TNF-α (Fig. 3C) levels were significantly higher in the infected and rPHB/saponin-vaccinated mice, as compared to the values found in the saline, saponin and rPHB group mice, when either the recombinant protein or SLA were used as stimuli. These results indicate an activation of the macrophages in this experimental group possibly induced by immunization. Then, the protein and SLA-specific IgG1 and IgG2a isotype antibody production was evaluated, and results showed that rPHB/saponin-vaccinated mice produced significantly higher anti-protein and anti-parasite IgG2a isotype levels, when compared to the IgG1 levels, before and after infection (Fig. 4).

### 3.2. Lymphoproliferation and cytokine response in human PBMCs

The immunogenicity of rPHB protein was also evaluated in the human VL. Initially, the lymphoproliferation was evaluated in PBMCs cultures that were unstimulated or *in vitro* stimulated with the recombinant protein or SLA. In the results, treated VL patients' cells showed a higher proliferative index when stimulated with rPHB, but not with SLA, with values of 4.2 ± 0.4 and 0.6 ± 0.2, when rPHB and SLA were used as stimuli, respectively. In untreated patients, these values were of 1.8 ± 0.3 and 0.4 ± 0.1, respectively. On the other hand, unstimulated cells showed results of 0.2 ± 0.1 and 0.1 ± 0, respectively. After, the immunogenicity of rPHB was investigated in VL, when the IFN-γ and IL-10 production was evaluated in the cellular supernatant. In the results, using the recombinant protein as a stimulus, IFN-γ and IL-10 levels were of 788.6 ± 49.8 and 68.3 ± 9.9 pg/mL, respectively, in treated patients, and of 144.6 ± 33.4 and 88.9 ± 8.6 pg/mL, respectively, in untreated patients (Table 1). Using *L. infantum* SLA as a stimulus, IFN-γ and IL-10 levels were of 199.4 ± 23.5 and 89.4 ± 8.9 pg/mL, respectively, in treated patients,

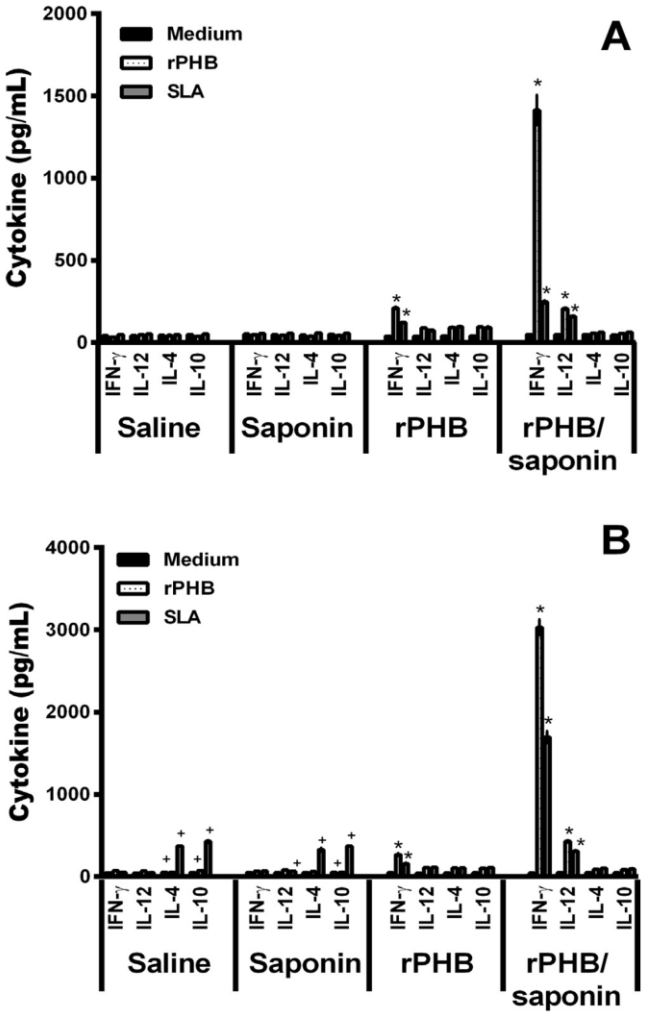


**Fig. 1.** Cellular response induced before and after infection. Spleen cells of the infected and/or vaccinated mice (n = 16 per group) were collected 30 days after the last immunization (n = 8) and 45 days after *L. infantum* infection (n = 8). Cells were unstimulated (medium) or separately stimulated with rPHB or SLA (10 and 25 μg/mL, respectively) for 48 h at 37 °C in 5% $CO_2$. IFN-γ, IL-12p70, IL-4, and IL-10 levels were measured in culture supernatants by an ELISA capture, before (in A) and after (in B) challenge. Bars represent the mean ± standard deviation of the groups. *indicates a statistically significant difference in relation to the saline and saponin groups ($P < .0001$). +indicates a statistically significant difference in relation to the rPHB/saponin group ($P < .0001$).

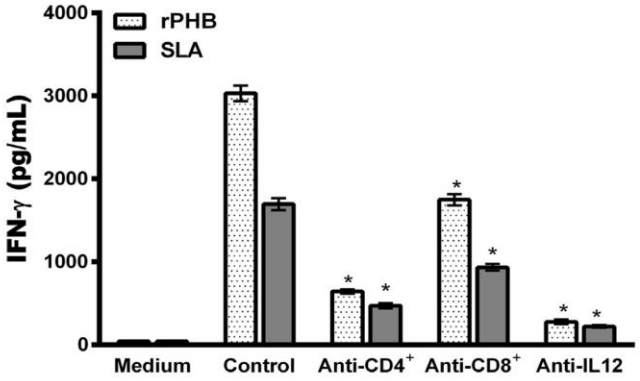


**Fig. 2.** Involvement of the $CD4^+$ and $CD8^+$ T cells in the IFN-γ production in the rPHB/saponin group. The IFN-γ production was measured in the infected and immunized animals (n = 8) after addition of anti-IL-12, anti-CD4, and anti-CD8 monoclonal antibodies in the splenic cultures, followed by incubation for 48 h at 37 °C in 5% $CO_2$. The rPHB and *L. infantum* SLA were used as stimuli (10 and 25 μg/mL, respectively), and the cytokine levels were measured by an ELISA capture. In addition, cells were not incubated with monoclonal antibody (control). Bars represent the mean ± standard deviation of the groups. *indicates a statistically significant difference in relation to the control group ($P < .001$).

and of 69.9 ± 7.7 and 433.6 ± 33.4 pg/mL, respectively, in untreated patients ($P < .0001$). In the unstimulated cultures, IFN-γ and IL-10 production was of 66.7 ± 6.0 and 61.3 ± 5.4 pg/mL, respectively, in treated VL patients, and of 61.8 ± 5.4 and 54.5 ± 3.8 pg/mL, respectively, in untreated VL patients (Table 1).

### 3.3. Protection against L. infantum infection

A parasitological evaluation used to determine the parasite burden was performed in all experimental groups 45 days after infection. In the results, associated with the immune profile developed in the rPHB/saponin-vaccinated animals before infection, and maintained after challenge, significant reductions in the parasite load were found in these animals in all evaluated organs, when both a limiting dilution and *q*PCR techniques were performed. In this context, rPHB/saponin-vaccinated mice, as compared to the saline, saponin and rPHB group mice (Fig. 5), showed significant reductions in the parasite load in the liver (3.5-, 3.3-, and 2.9-log reductions, respectively), spleen (4.8-, 4.5-, and 4.1-log reductions, respectively), dLNs (4.0-, 3.8-, and 3.5-log reductions, respectively), and BM (3.3-, 3.0-, and 2.7-log reductions, respectively). A *q*PCR assay developed in the spleen of the animals showed also significant reductions in the parasite load in the order of 61.0%, 55.0%, and 49.0% in the vaccinated mice, when compared to the values obtained in the saline, saponin and rPHB groups, respectively.

### 3.4. Analysis and application of rPHB protein for the serodiagnosis of VL

The amino acid sequence of *L. infantum* PHB was evaluated and it showed a high homology between distinct *Leishmania* species, with all B and T cell epitopes being found conserved among species able to cause tegumentary and visceral leishmaniasis, with identity values higher than 98% (Fig. 6). In addition, a low sequence similarity was found when *Leishmania* PHB was evaluated in other Trypanosomatides and human proteins, as also shown by others [23]. Next, the serodiagnosis application of rPHB in the canine and human VL was evaluated using a serological panel, and results based on individual O.D. values of the canine and human samples were obtained and are shown (Figs. 7and 8, respectively). ROC curves were constructed to obtain cut-off values, as well as parameters to evaluate the efficacy of the antigens. The rPHB protein showed sensitivity and specificity values of 100% and 96.9%, respectively, to diagnose canine VL, and of 100% and 98.8%, respectively, to diagnose human VL (Table 2). A maximum sensitivity value

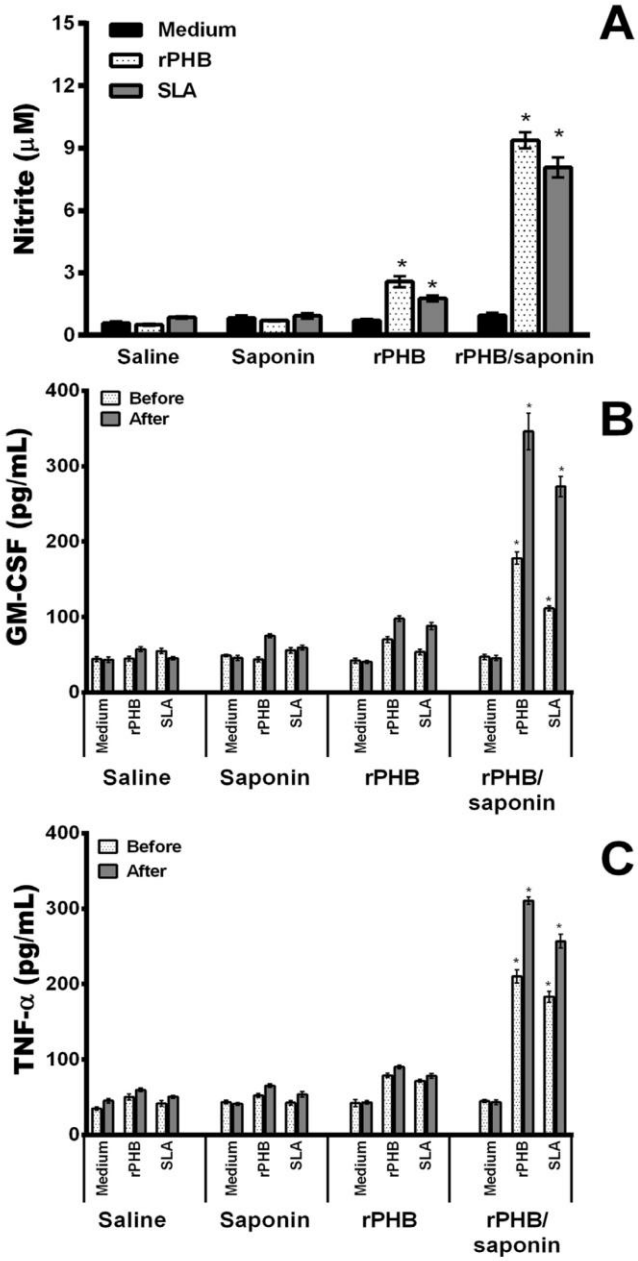


**Fig. 3.** Nitrite and GM-CSF production. Spleen cells were obtained from the saline, saponin, or rPHB/saponin-vaccinated mice, before (n = 8 per group) and after (n = 8) challenge using *L. infantum* promastigotes. The GM-CSF production was evaluated before and after infection, whereas the nitrite secretion was evaluated after challenge. For this, splenocytes were unstimulated (medium) or stimulated with rPHB or SLA (10 and 25 μg/mL, respectively) for 48 h at 37 °C, 5% $CO_2$. Bars represent the mean ± standard deviation of the groups. *indicates a statistically significant difference in relation to the saline and saponin groups ($P < .0001$).

was obtained when rA2 and SLA were used to diagnose canine VL, although their specificity was of 18.5% and 23.1%, respectively. Using human sera, the sensitivity values for these antigens were of 65.0% and 40.0%, respectively, whereas the specificity values were of 91.5% and 90.2%, respectively.

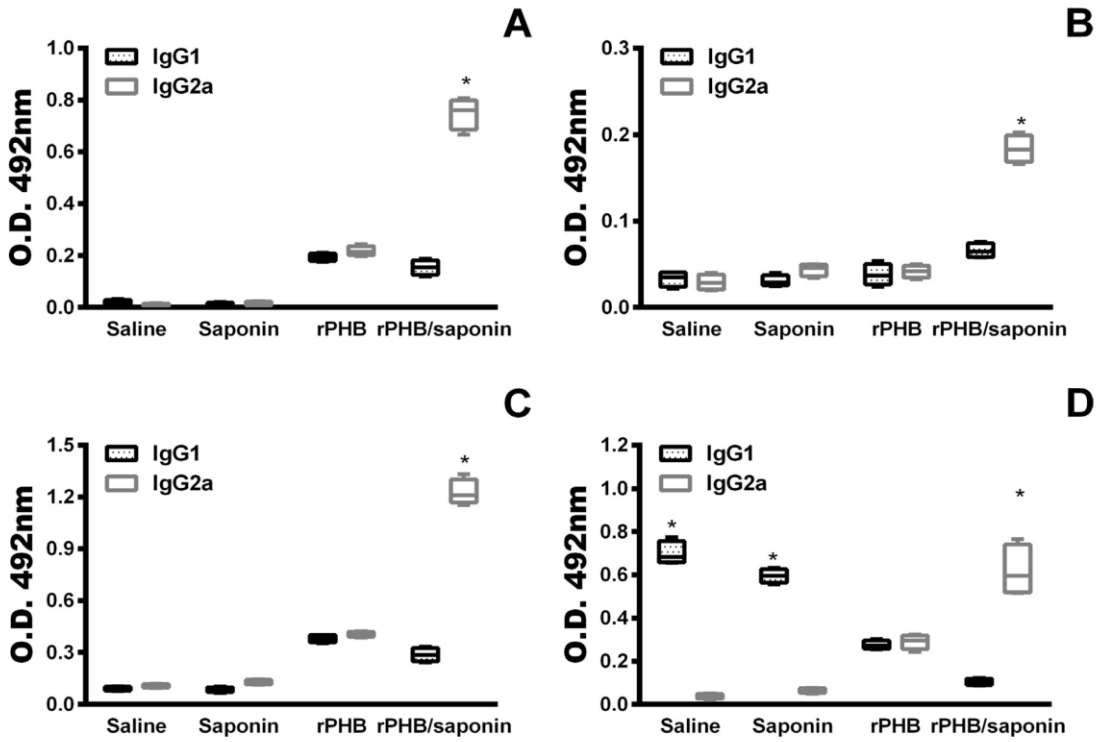


**Fig. 4.** Antibody production in the infected and/or vaccinated animals. Animals (n = 16 per group) received saline or were immunized with saponin or rPHB/saponin and, 30 days after the last dose, were euthanized (n = 8), and sera samples were collected. The others (n = 8) were challenged by a subcutaneous route with *L. infantum* promastigotes and, 45 days after infection, were euthanized and sera samples were collected. The anti-protein and anti-parasite IgG2a and IgG1 isotype antibody levels were evaluated before (A and B, respectively) and after (C and D, respectively) challenge infection. Bars represent the mean ± standard deviation of the groups. *indicates a statistically significant difference in relation to the saline and saponin groups ($P < .0001$).

**Table 1**
Lymphoproliferative index and immunogenicity in PBMCs from untreated and treated visceral leishmaniasis patients. To evaluate the rPHB-specific proliferative response and immunogenicity in human PBMCs, cells ($1 \times 10^6$) were added in wells containing RPMI medium and incubated alone (medium) or stimulated with recombinant prohibitin (rProhibitin) protein or *L. infantum* SLA (10 or 25 μg/mL, respectively). The incubation was performed for 5 days at 37 °C in 5% $CO_2$, after which time they were collected, washed and flow cytometric acquisition and analysis were performed in a FACScalibur® instrument. Also, culture supernatants were collected and IFN-γ and IL-10 production was measured by a capture ELISA using commercial kits.

| Stimulus | Lymphoproliferation index in PBMCs | |
|---|---|---|
| | Untreated VL | Treated VL |
| Medium | 0.1 ± 0 | 0.2 ± 0.1 |
| rProhibitin | 1.8 ± 0.3 | 4.2 ± 0.4 |
| *L. infantum* SLA | 0.4 ± 0.1 | 0.6 ± 0.2 |

| Stimulus | Immunogenicity in PBMCs (in pg/mL) | | | |
|---|---|---|---|---|
| | Untreated VL | | Treated VL | |
| | IFN-γ | IL-10 | IFN-γ | IL-10 |
| Medium | 61.8 ± 5.4 | 54.5 ± 3.8 | 66.7 ± 6.0 | 61.3 ± 5.4 |
| rProhibitin | 144.6 ± 33.4 | 88.9 ± 8.6 | 788.6 ± 49.8 | 68.3 ± 9.9 |
| L. infantum SLA | 69.9 ± 7.7 | 433.6 ± 33.4 | 199.4 ± 23.5 | 89.4 ± 8.9 |

## 4. Discussion

In recent decades, studies have aimed to develop new measures of control of leishmaniasis and many of them have focused on the development of vaccines to prevent against disease. In different researches, studies have used killed or attenuated parasites, recombinant antigens, synthetic peptides, recombinant bacteria expressing the parasite antigens, plasmid DNA-encoding protein antigens, and recombinant viral vaccines, which have been employed in distinct animal models [28–30]. Nonetheless, despite considerable efforts, the use of different molecules and the exploitation of different administration routes, the development of an effective vaccine remains an unresolved challenge [31]. This fact could be due to the species-specific protection usually found in many of these tested antigens or by low expression and/or conservation of these molecules in different *Leishmania* species, as well as in both parasite promastigote and amastigote stages [32].

In the present study, the PHB protein, which was previously identified in an immunoproteomic study in both *Leishmania* stages and by VL dog sera [18] was evaluated as a vaccine candidate and diagnostic marker against VL. Prohibitins have been described as tumour suppressor agents [33], as mitochondrial chaperones, or as playing a role in mitochondrial biogenesis [34]. Apart from their presence in the mitochondria of higher eukaryotes, mammalian intestinal epithelial cells were reported to use surface prohibitin bound to polysaccharides of *Salmonella typhi* [35]. *Leishmania* prohibitin is found conserved within the genus; however, it remains relatively distant from the human proteins, where a 40% similarity is found [23]. Little is known about the function of prohibitin in parasites, although due to its high conservation the study of this protein class would be beneficial. In this context, works performed in *Trypanosoma brucei*, *L. donovani*, and *Caenorhabditis elegans* have shown that prohibitin is likely to be involved in the induction of the immune response in the infected hosts, programmed cell death in parasites, and mitochondrial morphology in *C. elegans* [34,36].

The recombinant technology leading to a well-defined vaccine formulation increases the safety, stability, and reproducibility of this product by decreasing batch to batch variation, when compared to the first generation vaccines. This strategy decreases the risk of adverse

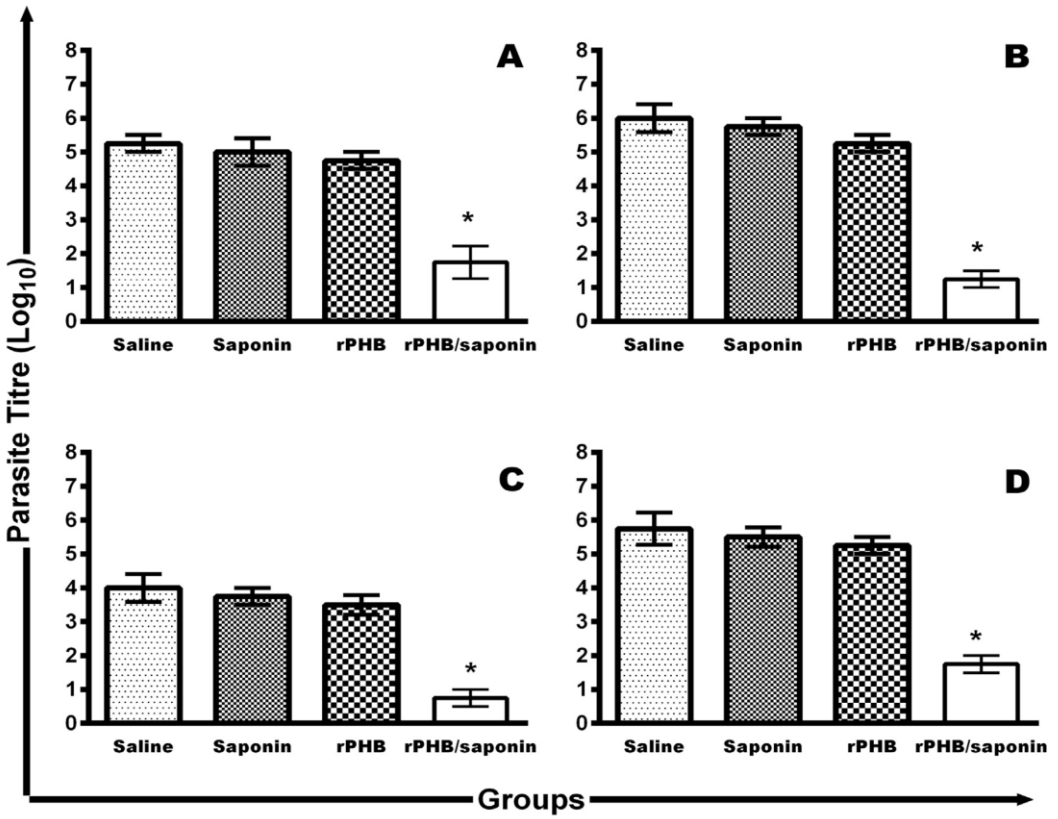


**Fig. 5.** Parasite burden evaluated by a limiting dilution technique. BALB/c mice inoculated with saline or immunized with saponin or rPHB/saponin were challenged with *L. infantum* promastigotes and, 45 days after, were euthanized (n = 8 per group), and the parasite load was investigated. For this, parasitism was evaluated in the animals' liver (A), spleen (B), bone marrow (C) and paws' draining lymph nodes (D) by a limiting-dilution technique. Bars represent the mean plus standard deviation of the groups. *indicates a statistically significant difference in relation to the saline, saponin and rPHB groups ($P < .0001$).

```
L. infantum (XP_001468827)      1    MAAEARKKMNAYGGFGNIIGMSALVGVGCVSIYALYKSIFFVPGGFRAVKFNCITGLYNRTYGEGANFAI 70
L. donovani (XP_003864600)      1    MAAEARKKMNAYGGFGNIIGMSALVGVGCVSIYALYKSIFFVPGGFRAVKFNCITGLYNRTYGEGANFAI 70
L. major (XP_003722404)         1    MAAEARKKMNAYGGFGNIIGMSALVGVGCVSIYALYKSVFFVPGGFRAVKFNCITGLYNRTYGEGANFAI 70
L. braziliensis (XP_001568126)  1    MAAEARKKMNAYGGFGNIVGMSALVGVGCVSIYALYKSVFFVPGGFRAVKFNSITGLYNRTYGEGANFAI 70
L. guyanensis (CCM18788)        1    MAAEARKKMNAYGGFGNIVGMSALVGVGCVSIYALYKSVFFVPGGFRAVKFNSITGLYNRTYGEGANFAI 83

L. infantum (XP_001468827)      71   PFLETPVVFDIRNKPIEVPTASGSRDLQTVNMAVRVLYQPNVENLYHIYRHIGVNYAETVLPSLINEIIR 140
L. donovani (XP_003864600)      71   PFLETPVVFDIRNKPIEVPTASGSRDLQTVNMAVRVLYQPNVDNLYHIYRHIGVNYAETVLPSLINEIIR 140
L. major (XP_003722404)         71   PFLETPVVFDIRNKPIEVPTASGSRDLQTVNMAVRVLYQPNVENLYHIYRHIGVNYAETVLPSLINEIIR 140
L. braziliensis (XP_001568126)  71   PFLETPVVFDIRNKPIEVPTASGSRDLQTVNMAVRVLYQPNVENLYHIYRHIGINYAETVLPSLINEIIR 140
L. guyanensis (CCM18788)        84   PFLETPVVFDIRNKPIEVPTASGSRDLQTVNMAVRVLYQPNVENLHHIYRHIGINYAETVLPSLINEIIR 153

L. infantum (XP_001468827)      141  AVIAQFNASDLLIKRPEVSHRIGVMLAERAKRFNIDITDVSITQMSFGKEYTNAVEAKQVAQQMAERAKF 210
L. donovani (XP_003864600)      141  AVIAQFNASDLLIKRPEVSHRIGVMLAERAKRFNIDITDVSITQMSFGKEYTNAVEAKQVAQQMAERAKF 210
L. major (XP_003722404)         141  AVIAQFNASDLLIKRPEVSHRIGVMLAERAKRFNIDITDVSITQMSFGKEYTNAVEAKQVAQQMAERAKF 210
L. braziliensis (XP_001568126)  141  AVIAQFNASDLLIKRPEVSHRIGVMLAERAKRFNIDITDVSITQMSFGKEYTNAVEAKQVAQQMAERAKF 210
L. guyanensis (CCM18788)        154  AVIAQFNASDLLIKRPEVSHRIGVMLAERAKRFNIDITDVSITQMSFGKEYTNAVEAKQVAQQMAERAKF 223

L. infantum (XP_001468827)      211  RVEQAEQEKQAAILLAQGEAEAATLVGNAVKRNPAFLELRGLEAARTIAKTLRDHGNGRYYLDSDSLYVN 280
L. donovani (XP_003864600)      211  RVEQAEQEKQAAILLAQGEAEAATLVGNAVKRNPAFLELRGLEAARTIAKTLRDHGNGRYYLDSDSLYVN 280
L. major (XP_003722404)         211  RVEQAEQEKQAAILLAQGEAEAATLVGNAVKRNPAFLELRGLEAARTIAKTLRDHGNGRYYLDSDSLYVN 280
L. braziliensis (XP_001568126)  211  RVEQAEQEKQAAILLAQGEAEAATLVGNAVKRNPAFLELRGLEAARTIAKTLRDHGNGRYYLDSDSLYVN 280
L. guyanensis (CCM18788)        224  RVEQAEQEKQAAILLAQGEAEAATLVGNAVKRNPAFLELRGLEAARTIAKTLRDHGNGRYYLDSDSLYVN 293

                                                                  Similarity
L. infantum (XP_001468827)      281  VKDLKIDHSGAK  292          100%
L. donovani (XP_003864600)      281  VKDLKIDHSGAK  292           99%
L. major (XP_003722404)         281  VKDLKIDHSGTK  292           99%
L. braziliensis (XP_001568126)  281  VKDLKIDHSGTK  292           98%
L. guyanensis (CCM18788)        294  VKDLKIDHSGTK  305           98%
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**Fig. 6.** Analysis of the amino acid sequence of the prohibitin expresses in different *Leishmania* spp. The *in silico* analysis of the *L. infantum* prohibitin (XP_001468827.1) sequence was performed by using the BLAST tool, where a search for similarity among sequences deposited in non-redundant protein databases was performed by comparison with the databases of the following *Leishmania* species: *L. major* (XP_003722404), *L. donovani* (XP_003864600), *L. braziliensis* (XP_001568126), and *L. guyanensis* (CCM18788). The amino acid sequences were aligned and the Cobalt (Constraint-based Multiple Alignment Tool) program was used for the analysis. The distinct residues are shown (red color).

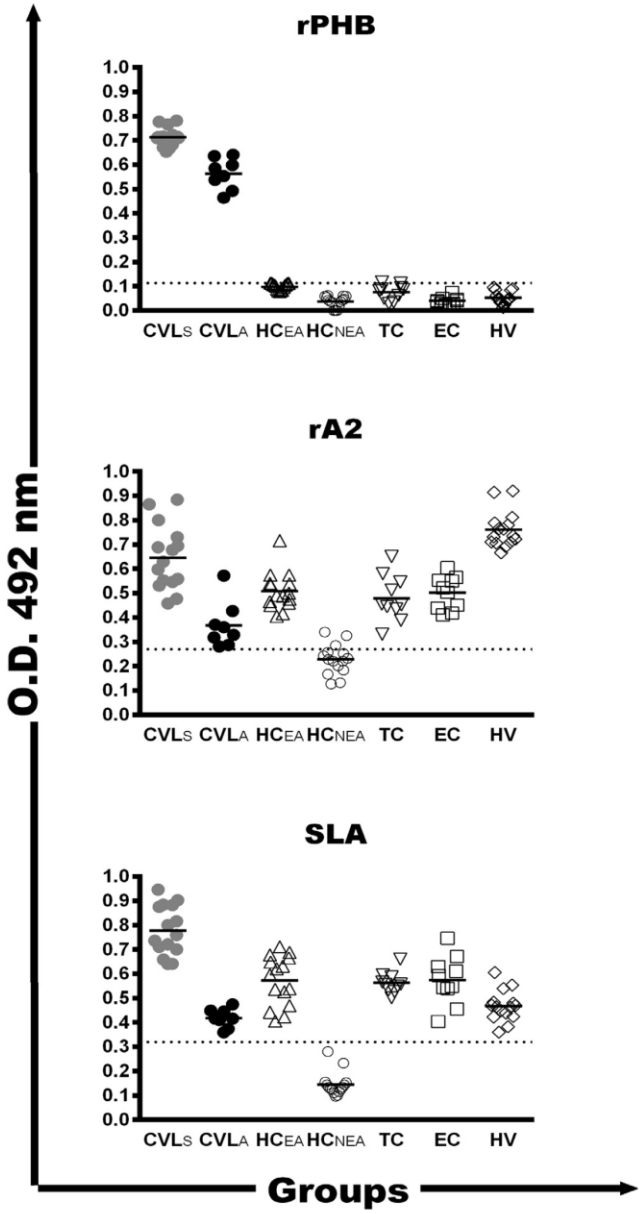


**Fig. 7.** Evaluation of ELISA reactivity using rPHB against canine sera. The rPHB protein was evaluated in ELISA experiments against sera samples collected from asymptomatic ($CVL_A$, n = 8) or symptomatic ($CVL_S$, n = 15) VL dogs, from healthy dogs living in endemic ($HC_{EA}$, n = 15) or non-endemic ($HC_{NEA}$, n = 15) areas of disease, from dogs immunized with Leish-Tec® vaccine (HV, n = 15), or from those experimentally infected with *Trypanosoma cruzi* (TC, n = 10) or *Ehrlichia canis* (EC, n = 10). The individual O.D. values are shown, which were used to calculate the means of the groups. The dotted line represents the cut-off value calculated by a ROC analysis. The rA2 and *L. infantum* SLA were used as control antigens.

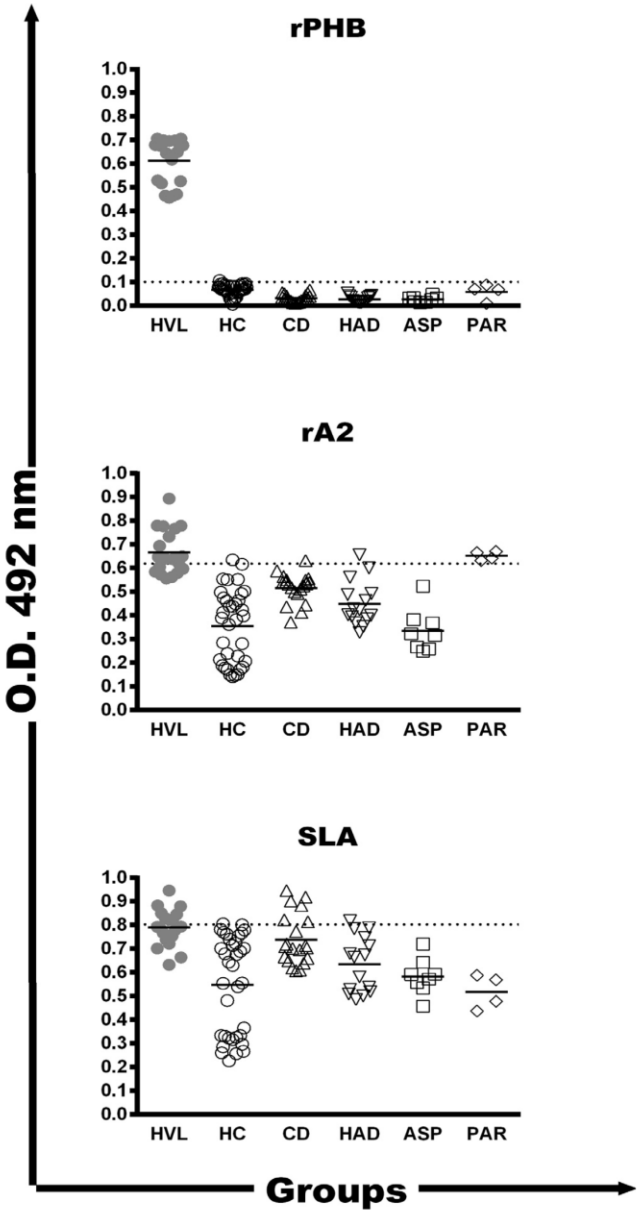


**Fig. 8.** ELISA reactivity against rPHB using a human serological panel. The rPHB protein was also evaluated by ELISA experiments against human sera. For this, samples collected from VL patients (HVL, n = 20), from healthy subjects living in endemic or non-endemic (HC, n = 35) areas of disease, as well as from those diagnosed with paracoccidioidomycosis (PAR, n = 4), leprosy (HAD, n = 15), aspergillosis (ASP, n = 8), or Chagas disease (CD, n = 20), were used in the assays. The individual O.D. values are shown, and were used to calculate the means of the groups. The dotted line represents the cut-off value calculated by a ROC analysis. The rA2 and *L. infantum* SLA were used as control antigens.

reactions, in addition to administering only the immunogenic protein directed toward a focused immune response able to induce protection against infection. In this context, the recombinant version of PHB was administered in BALB/c mice, and a challenge with *L. infantum* promastigotes was performed in these animals. In the present study, we have analyzed the immune profile developed in the animals that were immunized with the rPHB/saponin combination, since the development of a T cell-mediated response, which includes the stimulation of cells producing cytokines such as IFN-γ, IL-12, among others, as well as a positive lymphoproliferative response, is desirable for the protection and/or healing of VL [37].

BALB/c mice that were immunized showed a predominance of a protein and parasite-specific Th1 immune response, both before and after infection, which was based on higher IFN-γ, IL-12, and GM-CSF levels found in the supernatants of the stimulated splenocyte cultures, corroborated with a low presence of IL-4 and IL-10. In addition, the predominance of anti-protein and anti-parasite IgG2a isotype antibodies was observed in these animals, both before and after challenge,

**Table 2**
Performance of the diagnostic antigens in canine and human visceral leishmaniasis. The diagnostic efficacy of rPHB, rA2, and *L. infantum* SLA was evaluated by using a canine and human serological panel. Regarding canine sera, samples of asymptomatic (n = 8) and symptomatic (n = 15) VL dogs, from healthy animals living in endemic (n = 15) or non-endemic (n = 15) areas of disease, from Leish-Tec®-vaccinated animals (n = 15) or experimentally infected with *Babesia canis* (n = 10) or *Ehrlichia canis* (n = 15) were used. Regarding human samples, sera of VL patients (n = 20); from healthy subjects living in endemic (n = 20) or non-endemic (n = 15) areas of disease (n = 20); or from paracoccidioidomycosis (n = 4), leprosy (n = 15), aspergillosis (n = 8), or Chagas disease (n = 20) patients were used. ROC curves were used to evaluate the sensitivity (Se), specificity (Sp), positive predictive value (PPV), negative predictive value (NPV), and likelihood ratio (LR) of the antigens.

| Antigen | Se(%) | 95%CI | Sp(%) | 95%CI | PPV | 95%CI | NPV | 95%CI | LR |
|---|---|---|---|---|---|---|---|---|---|
| *Canine sera* | | | | | | | | | |
| rPHB | 100 | 85.18–100 | 96.92 | 89.32–99.63 | 92.00 | 73.97–99.02 | 100 | 94.31–100 | 32.50 |
| rA2 | 100 | 85.18–100 | 18.46 | 9.92–30.03 | 30.26 | 20.25–41.87 | 100 | 73.54–100 | 1.23 |
| SLA | 100 | 85.18–100 | 23.08 | 13.53–35.19 | 31.51 | 21.10–43.44 | 100 | 78.20–100 | 1.30 |
| *Human sera* | | | | | | | | | |
| rPHB | 100 | 83.16–100 | 98.78 | 93.39–99.97 | 95.24 | 76.18–99.88 | 100 | 95.55–100 | 82.00 |
| rA2 | 65.00 | 40.78–84.61 | 91.46 | 83.20–96.50 | 65.00 | 40.78–84.61 | 91.46 | 83.20–96.50 | 7.61 |
| SLA | 40.00 | 19.12–63.95 | 90.24 | 81.68–95.69 | 50.00 | 24.65–75.35 | 86.05 | 76.89–92.58 | 4.10 |

which was correlated with a low presence of the IgG1 isotype. The killing of intracellular *Leishmania* parasites by macrophages is essential to achieve a cure against VL [8]. Here, we have measured the production of GM-CSF, TNF-α and nitrite in splenocytes, aiming to investigate the macrophages' activation. In the results, an increased production of these molecules was found in the rPHB/saponin-vaccinated mice, suggesting the induction of an antileishmanial macrophage activity in these infected and immunized animals. This further argues that *Leishmania* killing was mediated through a NO pathway, induced by Th1 cytokines, mainly IFN-γ.

Regarding anti-*Leishmania* humoral immunity, evidences have showed that high levels of parasite-specific IgG2 isotype antibodies are considered to be a measure of the T cell-mediated response and protection against disease [38,39]. In the present study, anti-protein and anti-parasite IgG2 levels were significantly higher in the vaccinated mice group, when compared to the control mice, then associating the vaccine with the development of a protective response. On the other hand, parasite-specific IgG1 isotype antibodies were found in higher levels in the saline and saponin groups, which led to the conclusion that protection against *L. infantum* infection was induced by a Th1 response mediated by the presence of IFN-γ, IL-12, GM-CSF, TNF-α and nitrite, besides the presence of anti-parasite IgG2 isotype antibodies.

During the early stages of VL in BALB/c mice, parasites develop in the liver of the animals; however, once the infection becomes chronic, hepatic parasite burden tends to decrease, while splenic and BM parasitism tends to increase [40,41]. Once in the liver, the development of a cell-mediated immune response is essential for the clearance of parasites. By contrast, the spleen ultimately becomes the site of parasite persistence [42,43], suggesting that it is more susceptible to *L. infantum* infection than the liver [44]. The development of disease is mainly mediated by the loss of cell populations and changes to the local tissue microenvironments, which ultimately decrease the induction of an effective immune response and by the inability of splenic macrophage populations to generate antileishmanial mechanisms and to recruit other cells to eliminate the parasites. In our study, a parasitological evaluation performed in the infected animals showed that infected and rPHB/saponin-vaccinated animals presented significant reductions in the parasite load in all evaluated organs, demonstrating the protection induced by this vaccine combination, when compared to the other groups, in which a high parasitism was found in the liver and spleen of the animals.

Since *T*-cell proliferation is impaired in active VL, and the control of parasite growth and healing of the disease accompanies the development of a cell-mediated immune response, based on the *T*-cell proliferation, NO production, and DTH response [45,46], we further checked for a *T*-cell proliferation index in PBMCs collected from untreated and treated VL patients, as well as by IFN-γ and IL-10 production in the cell culture supernatants. Our findings showed a significant response in recovered and treated patients using rPHB to stimulate *in vitro* cultures, since a positive lymphoproliferation and higher IFN-γ levels were obtained when this protein was used as a stimulus in PBMCs of these patients, in comparison to the values found in the untreated patients and those who developed an active disease. These results indicate that our antigen could well be associated with a protective immune profile in the cured patients and suggest that it could be tested as an immunogen to protect against human disease.

Understanding the critical factors mediating protective immunity against infectious diseases such as leishmaniasis, and whether this immune response can be mediated by memory T cells, will contribute to a long-term protection against parasite infection by means of the generation of protective vaccines, since naturally acquired resistance to reinfection by *Leishmania* coincides with an ongoing primary infection [47]. Studies have showed that T cells with the functional attributes of memory cells are maintained in the absence or low presence of parasites, and this fact could represent long-lasting protection against reinfection [48–50]. In our study, immunological memory induced by the immunization using the rPHB/saponin combination was not evaluated in the vaccinated animals. However, it will be certainly performed in future works aiming to investigate the protective potential of this immunogen in animals against a longer infection. These aspects will certainly contribute for an improved design of vaccine candidates with respect to their ability to raise memory response aiming to improve their protective performance against *Leishmania* infection.

It has been shown that *Leishmania* proteins expressed in the promastigote and/or amastigote stages of the parasites can generate antibodies in dogs and humans suffering from leishmaniasis [51]. One of these proteins, rk39, a kinesin-related protein, is able to generate specific antibodies in VL patients, which is the basis for using it as a diagnostic marker, mainly in the cases where *L. donovani* species causes the pathology [52,53]. Our results showed an excellent performance of rPHB in diagnosing canine and human VL, with sensitivity and specificity values higher than 95% in both cases. The presence of anti-rPHB antibodies in VL patient sera shows the immunogenicity of this protein, providing an opportunity to test it as a possible diagnostic marker for canine and human VL caused by *L. infantum*, the main *Leishmania* species responsible for cases of VL in the Americas, where the disease is a zoonosis, with the domestic dog serving as the main reservoir of the parasite [54].

As limiting factors in our serological study, the low number of tested samples, the absence of other disease groups also related to leishmaniasis, and the evaluation of samples collected from untreated and treated VL patients to evaluate the progress of the anti-PHB humoral response in VL make the serological assays developed here as a proof-of-concept of the use of rPHB as a diagnostic marker for the canine and human disease.

In this context, the present study's data pointed out the distinct immunological applications of rPHB as a vaccine candidate or diagnostic marker against VL. The employment of products able to offer

protection and be used as markers to identify active disease added support to further investigation, thus employing rPHB, focusing on perspectives of the rational improvement of the diagnostic conditions and vaccine formulation that might have a positive impact upon the management of this important but neglected disease.

## Conflict of interest

The authors declare no commercial or financial conflict of interest.

## Acknowledgments

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## 4.8 ARTIGO 4 – CONCLUSÃO

O presente estudo sugeriu que a proteína rPHB tem um potencial para a aplicação diagnóstica da LV canina e humana, bem como aplicação como vacina contra a infecção com *L. infantum.*

## 4.9 ARTIGO 5 – BREVE INTRODUÇÃO

O quinto artigo a ser apresentado foi intitulado “Vaccination with a $CD4^+$ and $CD8^+$ T-cell epitopes-based recombinant chimeric protein derived from *Leishmania infantum* proteins confers protective immunity against visceral leishmaniasis”, e publicado na revista internacional *Translational Research* (doi: https://doi.org/10.1016/j.trsl.2018.05.001).

O presente artigo teve como objetivo avaliar o desempenho vacinal de uma quimera proteica recombinante construída a partir da predição de epitopos específicos de moléculas de MHC de classe I e II da sequência de aminoácidos das moléculas: rSGT), uma proteína hipotética (rLiHyS) e a proteína prohibitina (PHB), proteínas essas já mostradas antigênicas e imunogênicas nas leishmanioses.

# Vaccination with a CD4$^+$ and CD8$^+$ T-cell epitopes-based recombinant chimeric protein derived from *Leishmania infantum* proteins confers protective immunity against visceral leishmaniasis

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**Vaccination seems to be the best approach to control visceral leishmaniasis (VL). Resistance against infection is based on the development of a Th1 immune response characterized by the production of interferons-$\gamma$ (IFN-$\gamma$), interleukin-12 (IL-12), granulocyte-macrophage-colony-stimulating factor (GM-CSF), and tumor necrosis factor-$\alpha$ (TNF-$\alpha$), among others. A number of antigens have been tested as potential targets against the disease; few of them are able to stimulate human immune cells. In the present study, 1 prediction of MHC class I and II molecules-specific epitopes in the amino acid sequences of 3 *Leishmania* proteins: 1 hypothetical, prohibitin, and small glutamine-rich tetratricopeptide repeat-containing proteins, was performed using bioinformatics tools, and a T-cell epitopes-based recombinant chimeric protein was constructed, synthetized and purified to be evaluated in in vitro and in vivo experiments. The purified protein was tested regarding its immunogenicity in peripheral blood mononuclear cells (PBMCs) from healthy subjects and VL patients, as well as to its immunogenicity and protective efficacy in a murine model against *Leishmania infantum* infection. Results showed a Th1 response based on high IFN-$\gamma$ and low IL-10 levels derived from in chimera-stimulated PBMCs in both healthy subjects and VL patients. In addition, chimera and/or saponin-immunized mice presented significantly lower parasite burden in distinct evaluated organs, when compared to the controls, besides higher levels of IFN-$\gamma$, IL-2, IL-12,**

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**and GM-CSF, and an IgG2a isotype-based humoral response. In addition, the CD4$^+$ and CD8$^+$ T-cell subtypes contributed to IFN-$\gamma$ production in the protected animals. The results showed the immunogenicity in human cells and the protective efficacy against *L. infantum* in a murine model, and well indicate that this recombinant chimera can be considered as a promising strategy to be used against human disease. (Translational Research 2018; 000:1–17)**

**Abbreviations:** BM = Bone marrow; CD4 = Cluster of differentation 4;CD8 = Cluster of differentation 8;dLN = Draining lymph nodes;DO = Optical density;ELISA = Enzyme-linked immunosorbent assay;GM-CSF = Granulocyte-macrophage colony-stimulating factor;GRAVY = aliphatic index and grand average of hydropathicity;HASPB = Leishmania hydrophilic acylated surface protein B;HRF = IgE-dependent histamine-releasing factor;IFN- = interferon-gamma;IL = Interleukin; IPTG = Isopropyl--D-thiogalactopiranoside;KMP-11 = Kinetoplastid membrane protein-11;LiHyS = Leishmania hypothetical protein S;LiHyV = Leishmania hypothetical protein V;LiHyp1 = Leishmania hypothetical protein 1;LiHyp6 = Leishmania hypothetical protein 6;MHC = Major histocompatibility complex; PBMCs = peripheral blood mononuclear cells; PBS = Phosphate-buffered saline; PBS-T = Phosphate-buffered saline and Tween 20 0.05%; PCR = Polymerase chain reaction;PHB = prohibitin;PKDL = Post-kala-azar dermal leishmaniasis;RPMI = Roswell Park Memorial Institute medium; RT-PCR = Reverse transcription polimerase chain reaction; SGT = small glutamine-rich tetratricopeptide repeat-containing protein; SLA = Soluble Leishmania antigen extract;TGF- = Transforming growth factor beta;Th1 = T helper 1;TNF- = Tumor necrosis factor alpha; UFMG = Federal University of Minas Gerais; VL = visceral leishmaniasis; 12% SDS-PAGE = 12% sodium dodecyl sulphate polyacrilamide gel electrophoresis

## At a Glance Commentary

In this study, we have employed distinct bioinformatics tools to identify relevant CD4$^+$ and CD8$^+$ T-cell epitopes specific to humans and mice from three immunogenic proteins of *Leishmania infantum* parasites, aiming to design a single gene codifying a multi-immunogenic chimera, and use it as a vaccine candidate against visceral leishmaniasis (VL). Results showed that this new construct was immunogenic in human cells from healthy subjects and VL patients, as well as was highly effective in protect mice against *Leishmania infantum* infection. In this context, this recombinant chimera could well be considered in future studies as a prophylactic alternative to prevent against human VL.

### Background and Translational Significance

Leishmaniasis is a tropical disease complex caused by protozoan parasites of the genus *Leishmania*. The treatment against disease causes toxicity, presents high cost and parasite resistance to conventional drugs has been observed. In this context, the development of alternative control measures to prevent disease is a priority. Several studies have been developed using a number of different parasite proteins against visceral leishmaniasis (VL), although few of them have been tested or able of stimulate canine or human immune cells, so, no human vaccine is available. Here, we developed a new recombinant chimeric protein, which was constructed by means of prediction of T-cell epitopes of three immunogenic proteins of the parasites, and tested it in human cells and in a murine model against VL. We observed that this new product was immunogenic to human cells, then supporting the possibility to use it as a vaccine candidate against human disease, as well as it was protective against a challenge infection performed using *Leishmania infantum* parasites in BALÇB/c mice, then demonstrating its protective efficacy as vaccine. We hope that this product, developed by bioinformatics tools using parasite proteins, and tested in in vitro and in vivo models, can be used in a near future as a vaccine to protect against human VL.

## INTRODUCTION

Leishmaniasis is a vector-borne disease complex that is caused by protozoan parasites of the genus *Leishmania*. Approximately 350 million people in 98 countries are at risk of infection, while 12 million people are clinically affected by the disease, and 0.5 million new cases of visceral leishmaniasis (VL) are registered per year.[1,2] The first-line drugs to treat against disease present problems, such as the toxicity, high cost and/or parasite resistance.[3–5] In this scenario, the development of alternative control measures to

prevent leishmaniasis is a high priority to avoid the spread of disease.

Vaccination to protects against disease is possible, since parasite proteins that induce the development of a specific immune Th1 response in the immunized hosts, based on the activation of $CD4^+$ and/or $CD8^+$ T-cells and the subsequent production of the cytokines such as interferons-$\gamma$ (IFN-$\gamma$), interleukin-2 (IL-2), interleukin-12 (IL-12), granulocyte-macrophage-colony-stimulating factor (GM-CSF), and tumor necrosis factor-$\alpha$ (TNF-$\alpha$) protect animals against *Leishmania* infection.[6,7] On the other hand, cytokines such as IL-4, IL-10, IL-13, and TGF-$\beta$, among others, produced by immune cells play in the development of the active disease.[8,9] As a consequence, a number of different antigens have been tested against murine VL, although few of them have been able of stimulate canine or human immune cells.[10–12]

Evidence that patients cured from VL develop a Th1 response that protects against new infections suggest that immune cells of these subjects, such as peripheral blood mononuclear cells (PBMCs), could be employed as useful tools to identify new targets to protect against disease.[13,14] We have previously reported that *Leishmania* antigens used as recombinant proteins were protective against murine VL. One of these molecules, LiHyS (Leishmania hypothetical protein S), a *Leishmania* conserved hypothetical protein, was shown to be immunogenic and conferred protection in BALB/c mice against *L. infantum* infection. A positive lymphoproliferative response was also found when this protein was used to stimulate PBMCs collected from treated VL patients.[15] Other antigens, small glutamine-rich tetratricopeptide repeat-containing (SGT) and prohibitin (PHB), showed also similar results on the immunogenicity in mice and humans, and an additional protective efficacy against challenge using this parasite species.[16,17] However, these molecules were tested as individual antigens against a complex parasite, and a single product grouping distinct *Leishmania* proteins could be easier to produce, cheaper and highly immunogenic, since different T-cell epitopes were present in the amino acid sequence of the protein.[18]

Although prophylactic vaccines to protect against human disease represent the ultimate goal and are likely to have a significant impact on public health, the development for such products is highly complex.[19,20] Accordingly, some groups have developed therapeutic vaccines. For instance, a third-generation antileishmanial vaccine composed by $CD8^+$ T-cell epitopes from the KMP-11 and HASPB proteins, namely ChAd63-KH, was administered in healthy volunteers as an adenoviral vaccine. This vaccine was safe when administered by intramuscular route and was able to induce immunity characterized by IFN-$\gamma$ production and activation of dendritic cells, then supporting the feasibility to use it as a third-generation vaccine against VL and post-kala-azar dermal leishmaniasis.[20]

The development of a vaccine against human VL remains challenging.[21–23] In the current study, we evaluated the immunogenicity and protective efficacy against VL of a recombinant chimeric protein, which was constructed by means of predicted T-cell epitopes of 3 proteins: LiHyS (XP_001467126.1), SGT (XP_001467120.1), and PHB (XP_001468827.1). The chimeric protein was firstly tested regarding its capacity to stimulate PBMCs from healthy individuals and untreated or treated VL patients. Results showed that this molecule induced high IFN-$\gamma$ and low IL-10 levels after *in vitro* stimulus. Then, BALB/c mice were vaccinated with the recombinant protein plus saponin combination, and the immunogenicity and protective efficacy were evaluated against *L. infantum* challenge. The combination of the recombinant protein plus saponin induced a specific Th1 response in vaccinated animals, which was associated with significant reductions in the parasite burden in different organs, indicating a protective effect against VL.

## MATERIAL AND METHODS

**Recombinant antigens production.** LiHyS,[15] SGT,[16], and PHB[17] proteins were cloned, expressed, and purified as recombinant proteins. For the construction of the recombinant chimeric protein, amino acid sequences of the 3 proteins were subjected to bioinformatics assays aiming to select regions specific to murine and human T-cells haplotypes and containing immunogenic epitopes. In order to predict $CD8^+$ T-cell epitopes, the NetCTLPan program (version 1.1) was used to identify 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.[24] These alleles were selected since they are frequent in more than 90% of the human population of any ethnic group.[25] Epitopes with ability to bind to H-2-Kd, H-2-Ld, and H-2-Dd alleles from BALB/c mice were also predicted by NetCTLPan program. The $CD4^+$ T-cell epitopes were predicted using the NetMHCII 2.3 server with selection of epitopes with binding affinity lower than 500 nmol/L for 30% of alleles from human and I-Ad and I-Ed alleles from mice.[26] B-cell epitopes were identified using the with the BepiPred 2.0 program with threshold of 1.0 in at least 7 consecutive amino acids.[27] Protein regions containing

specific T-cell epitopes from humans and mice, but without B-cell epitopes, were selected to construct the chimeric protein. The conservation of potential epitopes in this sequence was confirmed by using the same programs used to predict $CD4^+$ and $CD8^+$ T-cell epitopes.

The chimeric protein was submitted for 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. The chimera was synthesized by GenScript, and the recombinant protein was expressed in an Artic Express strain (DE3, Agilent Technologies), by using 1 mM of IPTG for 24 hour at 12°C. After, the protein was purified in a nickel affinity column. The evaluation of the physicochemical properties of the chimera sequence was performed with the ProtParam tool in the ExPASy server.[28] The parameters computed included: molecular weight, theoretic isoelectric point, amino acid residues, positive and negative residues, extinction coefficient, instability index, aliphatic index, and grand average of hydropathicity (GRAVY). After purification, all recombinant proteins were passed on a polymyxin-agarose column (Sigma-Aldrich) in order to remove the residual endotoxin content (<10 ng of lipopolysaccharide per 1 mg of protein, Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000, BioWhittaker).

**Human and canine patients.** The present study was approved by the Ethics Committee from Federal University of Minas Gerais (UFMG; Belo Horizonte, Minas Gerais, Brazil), with protocol number CAAE−32343114.9.0000.5149. Peripheral blood samples were collected from VL patients (n = 6, including 4 males and 2 females, with ages ranging from 27 to 53 years), before and 6 months after treatment using pentavalent antimonials (Sanofi Aventis Farmacêutica Ltda, Suzano, São Paulo, Brazil). Infection was confirmed by PCR technique targeting *L. infantum* kDNA in aspirates from spleen and/or bone marrow of the patients. All of them were submitted to the same therapeutic schedule at a dose of 20 mg $Sb^{+5}$ per kg during 30 days, and none of them presented any other infection or had any pre-existing medical condition. At the end of the treatment, no parasite deoxyribonucleic acid (DNA) was found in aspirates of the spleen or bone marrow, and patients were free of disease-related symptoms. Blood samples were also collected from healthy subjects living in an endemic area of VL (n = 6, including 2 males and 4 females, with ages ranging from 20 to 43 years; Belo Horizonte). These subjects did not present any clinical sign of VL and exhibited negative serologic results by Kalazar Detect Test (InBios International). For the immunoblotting experiments, canine sera were also used. VL-positive animals (n = 8) presented positive parasitological and serologic results, which were based on identification of *L. infantum* kDNA in blood samples by PCR technique, and by EIE-LVC kit (BioManguinhos, Rio de Janeiro, Brazil). Noninfected dogs were selected from an endemic area of VL (n = 8, Belo Horizonte, Minas Gerais, Brazil), and they presented negative serologic results and were free of any clinical signal of VL at the moment of the samples collection. The study was also approved by the Animal Research Ethics Committee of UFMG (protocol number 333/2015).

**Antigen-specific cellular and humoral response in healthy subjects and VL patients.** To evaluate the cellular response in healthy subjects (n = 6) and VL patients (n = 6), PBMCs were purified as described elsewhere.[29] Then, cells ($10^7$) were cultured in 48-well flat-bottomed tissue culture plates (Costar, Cambridge, MA) in RPMI medium (background control, medium) or separately stimulated with each recombinant protein (10 $\mu$g/mL each), protein mix (5 $\mu$g/mL each), chimera (10 $\mu$g/mL), or *L. infantum* soluble liver antigen (SLA) (25 $\mu$g/mL). Incubation was carried out for 5 days at 37°C in 5% $CO_2$, after which the cell supernatant was collected. Then, IFN-$\gamma$ and IL-10 levels were measured by a capture enzyme-linked immunosorbent assay (ELISA) by using commercial kits (Human IFN-$\gamma$ and IL-10 ELISA Sets, BD Biosciences), according to manufacturer's instructions. To evaluate the humoral response, sera samples were also collected from healthy subjects and treated VL patients, and IgG1 and IgG2 subclasses were evaluated through an indirect ELISA. For this, flexible microtiter plates (Jet Biofil, Belo Horizonte) were coated with each recombinant protein (1.0 $\mu$g each) or the chimera (1.0 $\mu$g), all diluted in 100 $\mu$L coating buffer (50 mM carbonate buffer) pH 9.6, and incubated for 16 hour at 4°C. Serum samples were 1:400 diluted in PBS 1× and added in the plates, when incubation for 1 hour at 37°C was performed. Plates were washing and antihuman IgG1 and IgG2 subclasses peroxidase-conjugated antibodies (1:5000 and 1:10,000, respectively, from I2513, and I5635 catalogs, Sigma-Aldrich) were added in the plates, for 1 hour at 37°C. Reactions were developed by addition of $H_2O_2$, ortho-phenylenediamine and citrate-phosphate buffer, at pH 5.0, for 30 minute and in the dark, and stopped by adding 2 N $H_2SO_4$. The optical density (OD) values were read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nm.

**Antigenicity of chimera by Western-Blot assay.** To validate the antigenicity of the recombinant chimera,

immunoblottings were performed using the purified chimera (10 $\mu$g) which was submitted to a 12% SDS-PAGE, and blotted onto a nitrocellulose membrane (0.2 $\mu$m pore size, Sigma-Aldrich). Sera samples from rLiHyS, rSGT, rPHB, protein mix or chimera-immunized mice, as well as those from dogs or humans developing VL were separately added to the membranes. As controls, sera from naive (noninfected and nonvaccinated) mice and noninfected dogs and humans were used. The technical protocol was performed as previously described.[15] Briefly, mice (n = 6 per group), canine (n = 8 per group), and human (n = 6 per group) sera pools (1:100, 1:200, and 1:400 diluted in PBS-T, respectively) were added in the membranes. After washing, they were incubated with antimouse, antidog or antihuman IgG horseradish-peroxidase conjugated antibodies (diluted 1:5000, 1:5,000, and 1:10,000 in PBS-T, respectively), at which time a new incubation was developed for 1 hour at 37°C. Reactions were developed by adding 12.5 mg chloronaphtol, 25.0 mg diaminobenzidine, and 20 $\mu$L $H_2O_2$ 30 vol., and stopped by adding 10 mL distilled water.

**Mice and vaccination schedule.** BALB/c mice (female, 6−8 weeks) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, UFMG. For vaccine experiments, animals (n = 16 per group) were vaccinated subcutaneously in their left hind footpad with 15 $\mu$g of protein mix (containing 5 $\mu$g of rSGT, rPHB, and rLiHyS), or 15 $\mu$g of recombinant chimera, all associated with 15 $\mu$g of saponin (*Quillaja saponaria* bark saponin, Sigma-Aldrich). Additional mice (n = 16 per group) received saponin (15 $\mu$g) or saline. We used the chimera at the dosage of 15 $\mu$g per dose to match the use of 5 $\mu$g of each recombinant protein in the mix. Three doses were administered at 14-day intervals. Thirty days after the last immunization, mice (n = 8 per group) were euthanized, and serum samples and spleen were collected for immunologic evaluations. This study was approved by the Committee on the Ethical Handling of Research Animals of UFMG (code number 333/2015).

**Parasite, infection and determination of parasite burden.** *L. infantum* (MHOM/BR/1970/BH46) was used. Stationary promastigotes were grown at 24°C in Schneider's medium (Sigma-Aldrich, St. Louis, MO) added with 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich), 20 mM L-glutamine, 100 U/mL penicillin, and 50 $\mu$g/mL streptomycin, at pH 7.4. The SLA of the parasites was prepared as previously described.[30] Sixty days after the last vaccine dose, animals (n = 8 per group) were subcutaneously infected in their right hind footpad with $10^7$ stationary-phase promastigotes of *L. infantum*. They were followed by 60 days, when they were euthanized and organs were removed for parasitological and immunologic analyses. To evaluate the parasite load, spleen, liver, bone marrow (BM), and draining lymph nodes (dLN) of the infected and vaccinated animals were collected and analyzed by a limiting-dilution technique.[18] Results were expressed as the log of the titer (*ie*, the dilution corresponding to the last positive well), adjusted per milligram of organ. In addition, splenic parasite load was also evaluated by RT-PCR technique.[6] Briefly, spleen DNA was extracted using Wizard Genomic DNA Purification Kit (Promega Corporation), according to the manufacturer's instructions. The resulting DNA was resuspended in 100 $\mu$L of milli-Q $H_2O$. The parasite burden was estimated using the following primers: *forward* (CCTATTTTACACCAACCCCCAGT) and *reverse* (GGGTAGGGGCGTTCTGCGAAA), and the mouse $\beta$-actin gene was used as an endogenous control to normalize nucleated cells and to verify sample integrity. Standard curves were obtained from DNA extracted from $1 \times 10^8$ parasites for kDNA and $1 \times 10^8$ peritoneal macrophages for $\beta$-actin. PCR was performed on StepOne Instrument (48 wells-plate; Applied Biosystems) using 2X SYBR Select Master Mix (5 $\mu$L; Applied Biosystems), with 2 mM of each primer (1 $\mu$L) and 4 $\mu$L of DNA (25 ng/$\mu$L). The samples were incubated at 95°C for 10 minute, and submitted to 40 cycles of 95°C for 15 s and 60°C for 1 minute, and during each time, fluorescence data were collected. Parasite quantification for each spleen sample was calculated by interpolation from the standard curve, performed in duplicate, and converted into number of parasites per nucleated cells (multiplied by 1000 to facilitate visualization).[6]

**Cytokine detection: capture ELISA and flow cytometry.** Spleen cells were collected 30 days after the last vaccine dose, as well as 60 days after infection, when cell cultures were performed.[18] Splenocytes ($5 \times 10^6$) were incubated in DMEM (background control) added with 20% FBS, 20 mM L-glutamine, 200 U/mL penicillin, and 100 $\mu$g/mL streptomycin, pH 7.4, or stimulated with rSGT, rPHB, rLiHyS, or chimera (15 $\mu$g/mL each), protein mix (5 $\mu$g/mL of each protein) or *L. infantum* SLA (25 $\mu$g/mL), for 48 hour at 37°C in 5% $CO_2$. IFN-$\gamma$, IL-4, IL-10, IL-12, and GM-CSF levels were measured in the supernatants by capture ELISA (BD OptEIA TM set mouse kits, Pharmingen, San Diego, CA), following manufacturer's instructions. The involvement of IL-12, and $CD4^+$, and $CD8^+$ T-cells in the IFN-$\gamma$ production in the protein mix or chimera-vaccinated mice groups was evaluated by incubating spleen cells with monoclonal antibodies against mouse IL-12 (C17.8), CD4 (GK 1.5), or CD8 (53-6.7) (5 $\mu$g/mL in all cases). Appropriate isotype-

matched controls — rat IgG2a (R35-95) and rat IgG2b (95-1) — were employed. Antibodies (no azide and/or low endotoxin) were purchased from BD (Pharmingen). The nitrite secretion was also evaluated in the culture supernatants by Griess method.[31] In addition, the percentage of $CD4^+$ and $CD8^+$ T-cells producing intracytoplasmic cytokines (IFN-$\gamma$, TNF-$\alpha$, IL-2, and IL-10) was determined in the SLA-stimulated cultures.[15] The results were expressed as indexes which were determined by dividing the percentage of $CD4^+$ and $CD8^+$ cytokine-positive T-cells in the stimulated cultures vs the values obtained in the unstimulated cultures (control).

**Protein and parasite-specific antibody production.** To evaluate the humoral response, serum samples were collected of the animals, 30 days after the last vaccine dose and before infection, as well as 60 days after challenge. The IgG1 and IgG2a isotype levels were evaluated against rSGT, rPHB, rLiHyS, and recombinant chimera (0.5, 0.5, 1.0, and 0.5 $\mu$g per well, respectively), protein mix (0.25 $\mu$g/mL of each protein) or *L. infantum* SLA (1.0 $\mu$g per well), according described.[29] Serum samples were diluted at 1:100, and both antimouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich) were used in 1:5000 and 1:10,000 dilutions, respectively.

**Statistical analysis.** The results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed using GraphPad Prism (version 6.0 for Windows). The statistical analysis was performed by 1-way analysis of variance (ANOVA), followed by Bonferroni's post-test, which was used for multiple comparisons. Experiments were repeated twice, and the results are representative of one of them. Differences were considered significant when $P < 0.05$.

## RESULTS

**Construction and characterization of the recombinant chimeric protein.** In this study, a chimeric protein was constructed based on the prediction from main $CD4^+$ and $CD8^+$ T-cell epitopes of 3 immunogenic *Leishmania* proteins. To build this construct, the NetMHCII 2.3 and NetCTLPan servers were used to select the best $CD4^+$ and $CD8^+$ T-cell epitopes, respectively, in the amino acid sequences of the 3 proteins. Identification of T-cell epitopes derived from the PHB (Fig. 1A), SGT (Fig. 1B), and LiHyS (Fig. 1C) proteins was performed, and the chimeric protein was constructed as presenting 422 amino acid residues (Fig. 1D), an estimated molecular weight of 47.27 kDa, and isoelectric point of 8.87. In addition, the instability index, aliphatic index, and GRAVY values were, respectively, 39.87, 84.93, and −0.080.

**Immunogenicity and antigenicity induced in human cells.** To evaluate the immunogenicity of each recombinant antigen in human cells, the individual proteins used in the chimera construction were employed as stimuli of PBMC cultures collected from healthy subjects and treated and untreated VL patients. Results showed that rPHB, rSGT, rLiHyS, protein mix, and chimera induced significantly higher levels IFN-$\gamma$, when compared to the unstimulated or SLA-stimulated cultures in both healthy individuals and VL patients (Fig. 2). The chimeric protein stimulus was this in which higher IFN-$\gamma$ production was reached in the cell supernatant. On the other hand, IL-10 levels were low and similar after the use of the stimuli, although PBMCs from healthy subjects and VL patients had produced higher levels of this cytokine after *L. infantum* SLA-stimulus. The antigenicity of chimeric protein was evaluated by an immunoblotting assay, and results showed that the protein was recognized by sera from mice immunized with the individual or combined (mix) proteins, as well as with VL dogs and patients' sera. However, none reactivity was found when healthy dogs or human sera, or those derived from noninfected nonimmunized (naive) mice were used in the assays (Fig. 3A). The antiprotein IgG1 and IgG2 subclasses in healthy subjects and treated VL patients' sera were also evaluated, and higher IgG2 and lower IgG1 levels were found when the individual and chimeric proteins were used in the plates (Fig. 3B).

**Immune response generated after vaccination with the recombinant proteins plus saponin.** We investigated if the chimeric vaccine, when associated with saponin as an adjuvant, could stimulate the development of a specific immune response in vaccinated BALB/c mice. Like controls, the rLiHyS, rSGT, and rPHB proteins were prepared in a protein mix and also applied in the animals associated with saponin. Results showed that the immunization with the recombinant chimera plus saponin or protein mix plus saponin induced significantly higher levels of IFN-$\gamma$, IL-12, and GM-CSF in the cell supernatant, which were associated with low IL-4 and IL-10 levels (Fig. 4A). The humoral response was also evaluated, and results showed that the immunization using chimera and/or saponin or protein mix and/or saponin induced significantly higher antiprotein and antiparasite IgG2a/IgG1 ratios, when compared to the control (saline and saponin) groups (Fig. 4B). Comparing the immunogenicity developed in the immunized animals, it can be observed that the chimera and/or saponin group developed a more polarized Th1 response, when both cytokine and antibody

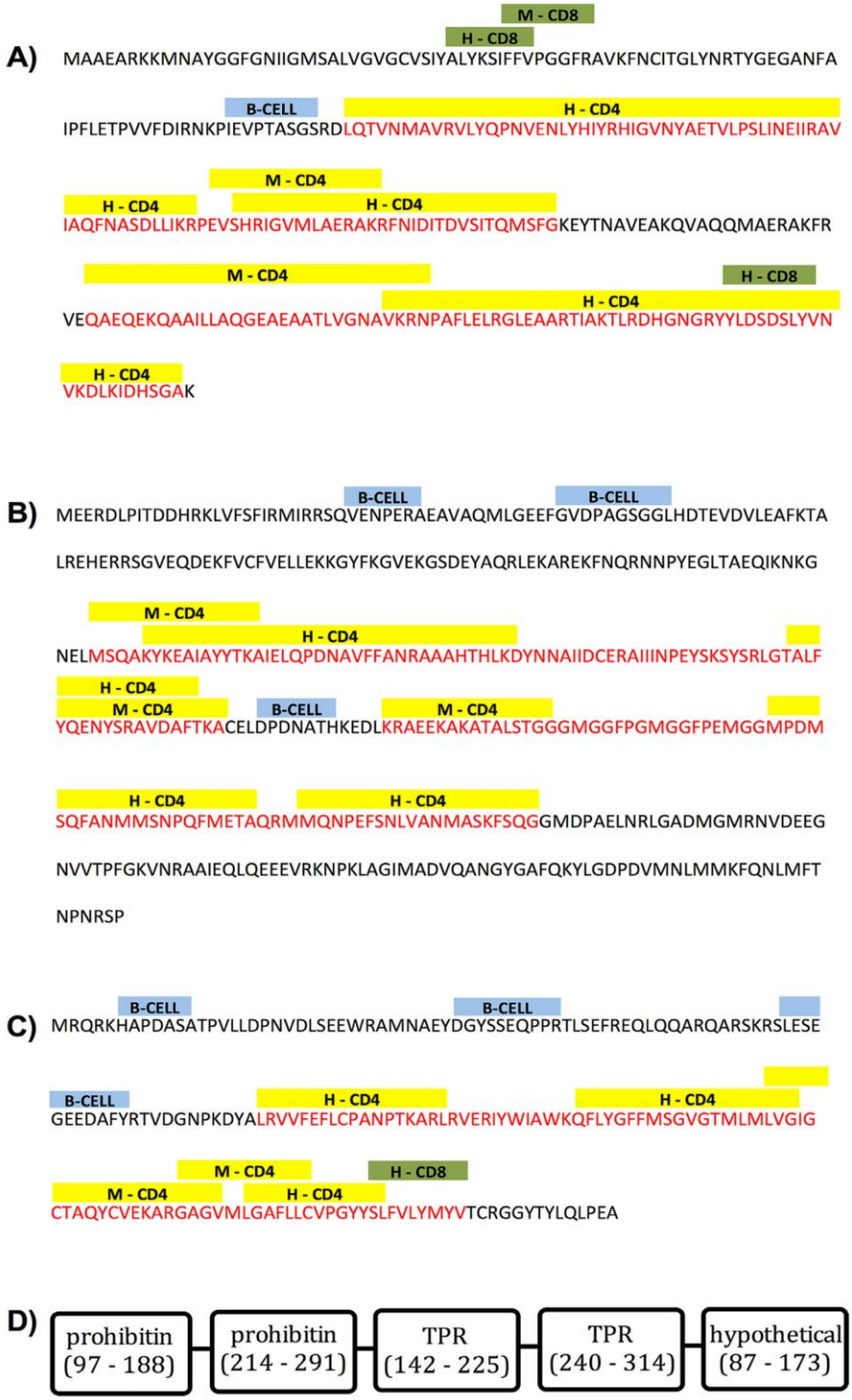


**Fig. 1.** Construction of the recombinant chimeric protein based on T- and B-cell epitopes prediction from distinct immunogenic *Leishmania* proteins. The prediction of B- and T cell-epitopes specific to the prohibitin (PHB; XP_001468827.1) (in **A**), SGT (XP_001467120.1) (in **B**), and LiHyS (XP_001467126.1) (in **C**) protein sequences were performed, and the chimeric protein construct is shown (in **D**). Red letters represent regions of the proteins selected to chimeric sequence, as well as the amino acids coordinates of the regions included inside brackets (green and yellow colors): H-CD4, human $CD4^+$ T-cell epitopes; M-CD4, mouse $CD4^+$ T-cell epitopes; H-CD8, human $CD8^+$ T-cell epitopes; M-CD8, human $CD8^+$ T-cell epitopes. PHB, prohibitin; SGT, small glutamine-rich tetratricopeptide repeat-containing.

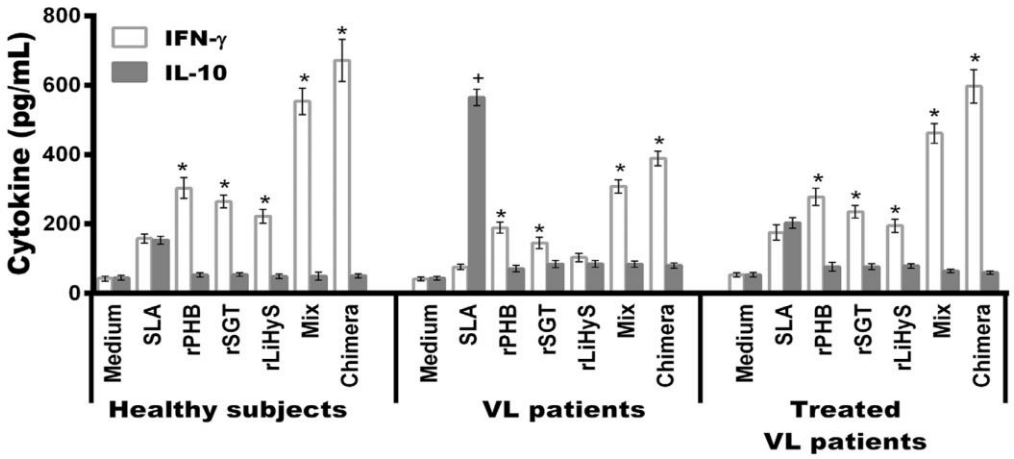


**Fig. 2.** Immunogenicity in human PBMCs from healthy subjects and VL patients. The PHB, SGT, LiHyS, protein mix, chimera, and SLA were individually analyzed regarding their immunogenicity in human cells. For this, PBMCs ($10^7$) from healthy subjects (n = 6) and treated and untreated VL patients (n = 6) were nonstimulated (medium) or separately stimulated with each recombinant protein (10 $\mu$g/mL each), protein mix (5 $\mu$g/mL each), chimera (10 $\mu$g/mL) or *L. infantum* SLA (25 $\mu$g/mL), for 5 days at 37°C in 5% $CO_2$. Then, IFN-$\gamma$ and IL-10 levels were measured in the culture supernatant by capture ELISA. White and grey bars indicate the mean ± standard deviation of the IFN-$\gamma$ and IL-10 levels, respectively. (*) indicates statistically significant difference in relation to the unstimulated control (medium; $P < 0.0001$). (+) indicates statistically significant difference in relation to the stimulus using the recombinant protein ($P < 0.0001$). ELSA, enzyme-linked immunosorbent assay; IFN-$\gamma$, interferons-$\gamma$; IL-10, interleukin-10; PBMCs, peripheral blood mononuclear cells; PHB, prohibitin; SGT, small glutamine-rich tetratricopeptide repeat-containing; SLA, soluble liver antigen; VL, visceral leishmaniasis.

productions were evaluated. After challenge infection, the immune response profile was maintained in the chimera and/or saponin and protein mix and/or saponin groups, with the production of significantly higher levels of IFN-$\gamma$, IL-12, and GM-CSF in the stimulated cells, whereas in the control groups, a significantly higher production of antiparasite IL-4 and IL-10 was found (Fig. 5A). The antibody production showed also significantly higher IgG2a/IgG1 ratios in the chimera and/or saponin or protein mix and/or saponin groups, whereas in the control groups, the antiparasite IgG1 was higher than IgG2a isotype, then resulting in an IgG2a/IgG1 ratio lower than 1.0 (Fig. 5B). The cellular and humoral response developed in the chimera and/or saponin group was also related to a more polarized Th1 immune profile, when compared to results obtained in the protein mix and/or saponin group.

The involvement of $CD4^+$ and $CD8^+$ T-cells in the IFN-$\gamma$ production was evaluated by adding monoclonal antibodies in the cell cultures of the chimera and/or saponin and protein mix and/or saponin groups. Results showed that when anti-CD4 or anti-CD8 antibodies were added to the in vitro cultures, significant reductions in the production of this cytokine were found in both experimental groups (Fig. 6), denoting the importance of both T-cell subtypes in the protective immunity induced by the vaccines.

The nitrite production was also evaluated as a marker of the macrophage activation in the experimental groups. Results showed that mice immunized with chimera and/or saponin or protein mix and/or saponin produced significantly higher levels of antileishmanial nitrite, when compared to the results obtained in the controls (Fig. 7). The intracytoplasmic cytokine profile was also evaluated by a flow cytometry experiment, and results showed that chimera and/or saponin or protein mix and/or saponin-vaccinated mice presented higher antileishmanial IFN-$\gamma^+$, IL-$2^+$, and TNF-$\alpha^+$-producing $CD4^+$ and $CD8^+$ T-cell percentages, which was associated with lower presence of antiparasite IL-$10^+$ T-cells, when compared to the control groups (Fig. 8). Overall, the immunogenicity induced by the vaccine using chimera and/or saponin induced more polarized Th1 immune response in the animals, when compared to administration of protein mix and/or saponin, before and after infection.

**Protective efficacy in the infected and immunized mice.** The protective efficacy induced by the immunizations using the protein mix and chimera plus saponin was evaluated. As controls, animals received saline or were immunized with saponin, protein mix, or chimera without adjuvant. Results showed that the immunization using the chimera and/or saponin or protein mix and/or saponin induced significant reductions in the

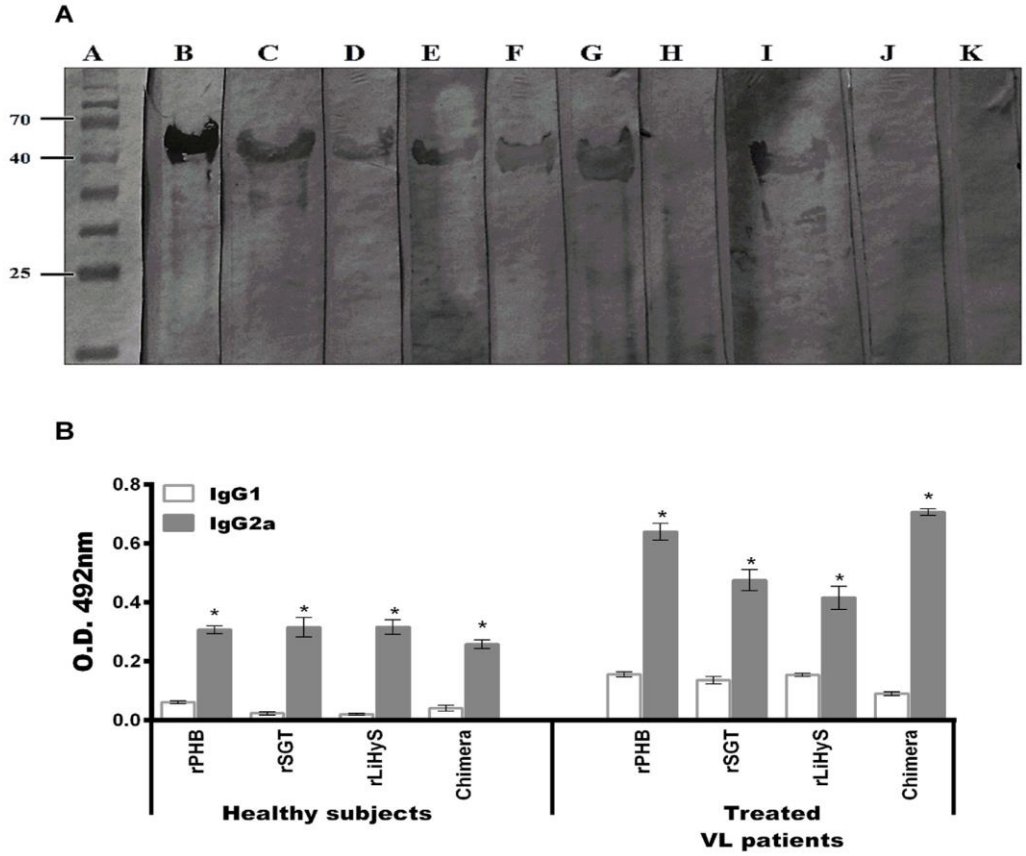


**Fig. 3.** Antigenicity of the recombinant chimeric protein using human sera. Immunoblottings were performed using the recombinant chimera against sera samples collected from mice, dogs, and humans. For this, the protein (10 $\mu$g) was submitted to a 12% SDS-PAGE and blotted onto nitrocellulose membrane. Results are shown in the panel **A**. A low range protein ladder (Invitrogen, Life Technologies) was used (A). Membranes were incubated with chimera and/or saponin (B), protein mix and/or saponin (C), rLiHyS/saponin (D), rSGT/saponin (E), and rPHB/saponin-immunized (F) mice sera, as well as with samples from VL and healthy dogs (G and H, respectively), sera from VL patients and healthy subjects (I and J, respectively), and from noninfected nonimmunized (naive) mice (K). Immunoblottings were derived from 3 independent experiments, and 1 representative preparation is shown. In addition, the humoral reactivity against the recombinant chimera was also evaluated using sera samples from healthy subjects and treated VL patients. Results are shown in the panel **B**. The rPHB, rSGT, rLiHyS, and chimera-specific IgG1 and IgG2 subclasses (white and grey bars, respectively) production was measured, and optical density (DO) values for each recombinant antigen are shown. Bars indicate the mean ± standard deviation of the groups. (*) indicates statistically significant difference in relation to the IgG1 levels ($P < 0.0001$). PHB, prohibitin; SGT, small glutamine-rich tetratricopeptide repeat-containing; VL, visceral leishmaniasis

parasite load in the spleen, liver, bone marrow (BM), and draining lymph nodes, when compared to the other groups (Fig. 9). In the absence of adjuvant, protein mix, or chimera-immunized mice did not present reductions in the parasitism, in comparison to the saline and saponin groups. Between the vaccinated animals, the immunization using chimera and/or saponin induced the reduction in the parasitism in all organs, when compared with the protein mix and/or saponin group, then demonstrating a better efficacy of the chimeric protein in protect against infection. The splenic parasite burden was also evaluated by a RT-PCR technique, and results showed that protein mix and/or saponin or chimera and/or saponin-immunized mice presented significant reductions in the parasitism in this organ, when compared to the controls (Fig. 10), then demonstrating the protective efficacy induced by the immunizations.

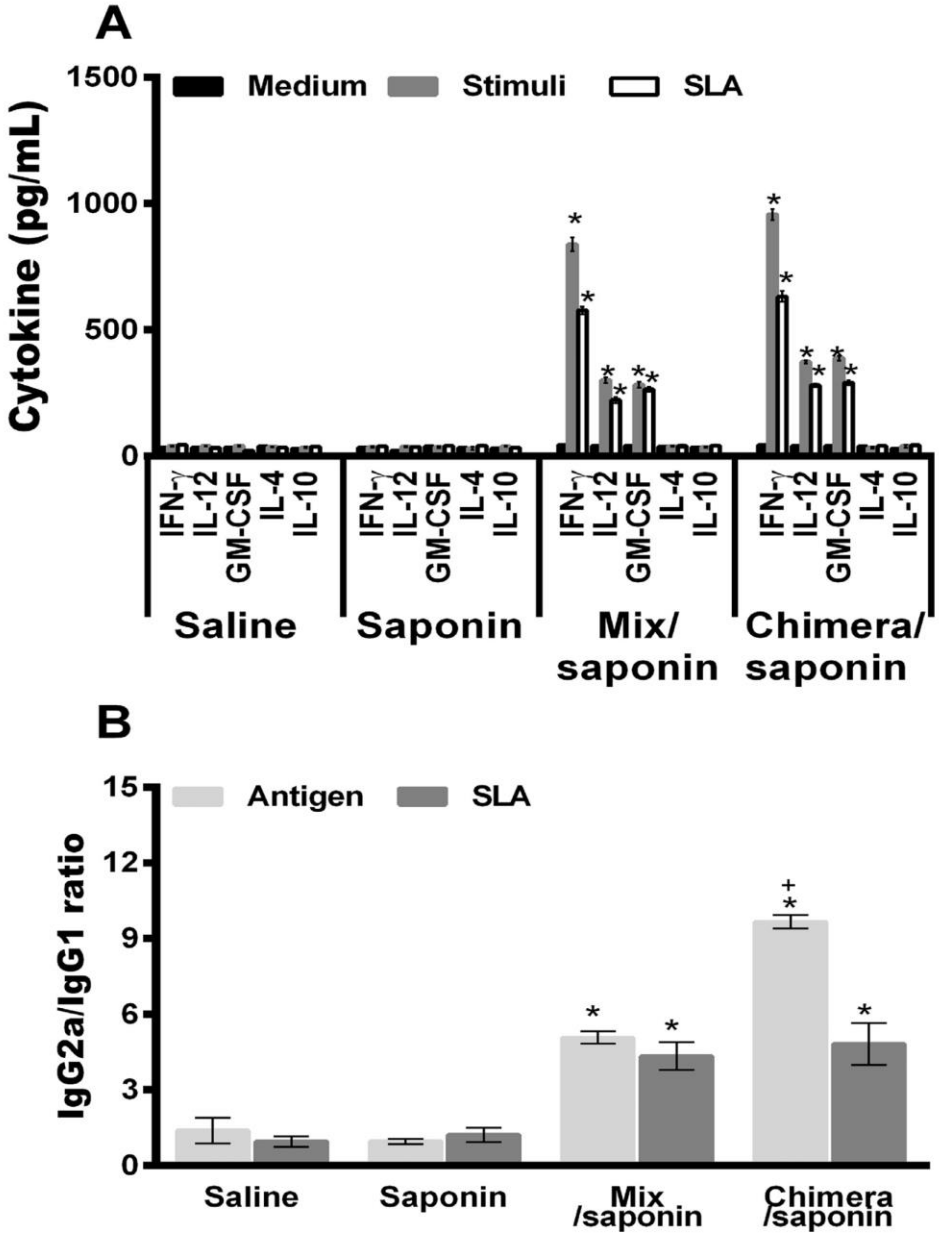


**Fig. 4.** Immunogenicity induced in vaccinated BALB/c mice before *L. infantum* infection. Mice (n = 8 per group) received saline or were immunized with saponin, chimera and/or saponin or protein mix and/or saponin. Thirty days after the last vaccine dose, they were euthanized and their spleen cells ($5 \times 10^6$) were collected, and cultured in DMEM and unstimulated (medium) or stimulated with the protein mix (saline, saponin, and protein mix and/or saponin groups), chimera (chimera and/or saponin group) or SLA (15, 10, and 25 $\mu$g/mL, respectively), for 48 hour at 37°C in 5% $CO_2$. IFN-$\gamma$, IL-12, GM-CSF, IL-4, and IL-10 levels were measured by capture ELISA (in **A**). In addition, sera samples were collected from the animals, and the protein and parasite-specific IgG1 and IgG2a isotypes production was evaluated. The ratios between the IgG2a/IgG1 levels were calculated and are shown (**B**). Bars indicate the mean ± standard deviation of the groups. (*) indicates significant difference in relation to the saline and saponin groups ($P < 0.0001$). (+) indicates statistically significant difference in relation to the protein mix/saponin group ($P < 0.01$). ELSA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage-colony-stimulating factor; IFN-$\gamma$, interferons-$\gamma$; IL-4, interleukin-4; IL-10, interleukin-10; IL-12, interleukin-12; SLA, soluble liver antigen.

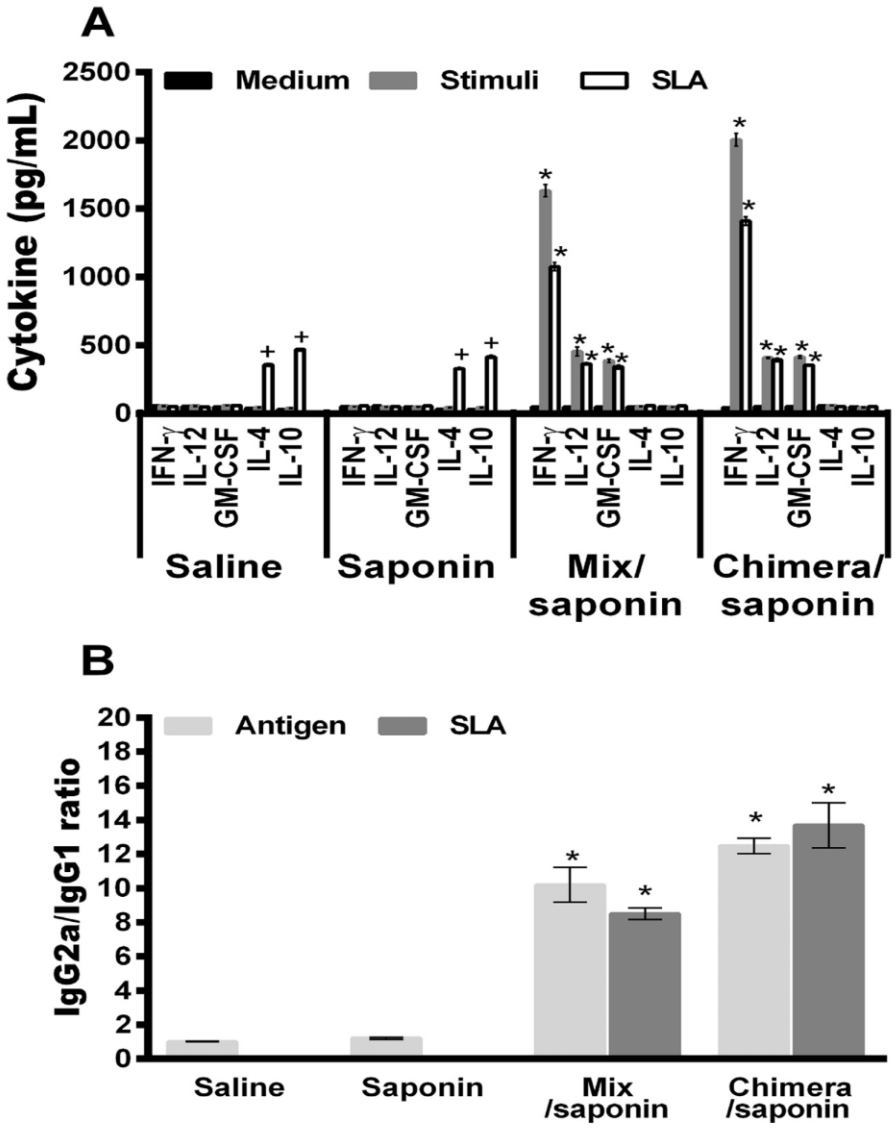


**Fig. 5.** Immune response developed after *L. infantum* challenge. Mice (n = 8 per group) received saline or were immunized with saponin, chimera and/or saponin or protein mix and/or saponin. Thirty days after the last dose, they were infected with *L. infantum* promastigotes. Ten weeks later, animals were euthanized, and their spleen cells ($5 \times 10^6$) were collected. Then, they were cultured in DMEM and unstimulated (medium) or stimulated with the protein mix (saline, saponin, and protein mix and/or saponin groups), chimera (chimera and/or saponin group) or SLA (15, 10, and 25 $\mu$g/mL, respectively), for 48 hour at 37°C in 5% $CO_2$. IFN-$\gamma$, IL-12, GM-CSF, IL-4, and IL-10 levels were measured by capture ELISA (in **A**). Sera samples were also collected from the animals, and the IgG1 and IgG2a isotypes production was evaluated. The ratios between the IgG2a/IgG1 levels were calculated and are also shown (**B**). Bars indicate the mean ± standard deviation of the groups. (*) indicates significant difference in relation to the saline and saponin groups ($P < 0.0001$). (+) indicates significant difference in relation to the chimera/saponin and protein mix and/or saponin groups ($P < 0.0001$). (+) indicates statistically significant difference in relation to the protein mix and/or saponin and chimera and/or saponin groups ($P < 0.01$). ELSA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage-colony-stimulating factor; IFN-$\gamma$, interferons-$\gamma$; IL-4, interleukin-4; IL-10, interleukin-10; IL-12, interleukin-12, SLA, soluble antigen extract.

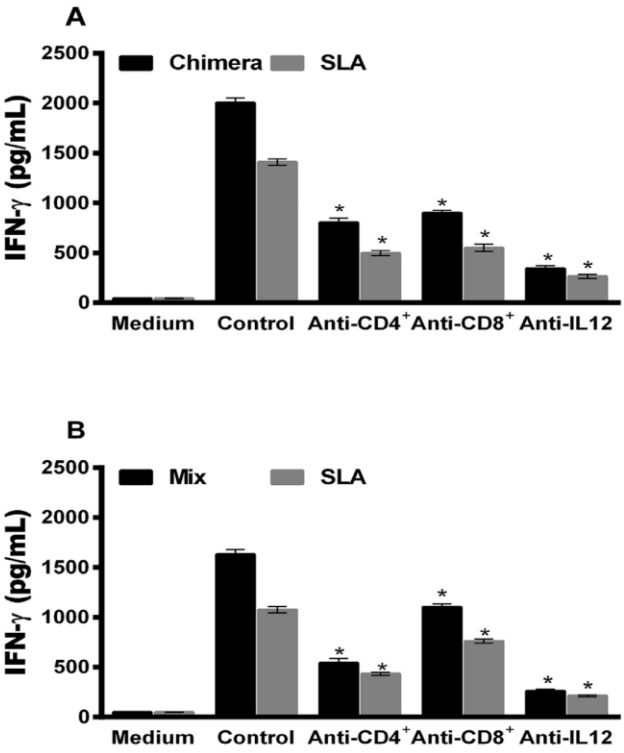


**Fig. 6.** Evaluation of the participation of $CD4^+$ and $CD8^+$ T-cells in the IFN-$\gamma$ production in the vaccinated animals. Mice (n = 8 per group) were immunized with chimera and/or saponin or protein mix and/or saponin and challenged with *L. infantum* promastigotes. Ten weeks later, they were euthanized and their spleens were collected, cultured ($5 \times 10^6$ cells per well) and stimulated with the protein mix, chimera or SLA (15, 10, and 25 $\mu$g/mL, respectively), in the absence (positive control) or presence of anti-CD4, anti-CD8, and anti-IL-12 monoclonal antibodies, for 48 hour at 37°C in 5% $CO_2$. Then, the IFN-$\gamma$ production was evaluated in the cell supernatant of the chimera and/or saponin and protein mix and/or saponin groups (**A** and **B**, respectively). Bars indicate the mean ± standard deviation of the groups. (*) indicates statistically significant difference in relation to the (positive) control ($P < 0.0001$). IFN-$\gamma$, interferons-$\gamma$; IL-2, interleukin-2; SLA, soluble liver antigen.

## DISCUSSION

In spite of the vast knowledge about the immunity contributing to resistance and/or susceptibility against VL, there is no licensed vaccine to protect against human disease.[32] Current studies are mostly focused on rodent models and cannot be extrapolated to dogs or humans, since the majority of the candidates are either untested or present low efficacy when used to stimulate canine or human cells.[33–36] However, the fact that treated VL patients develop long-lasting protection based on the Th1 immunity against a new *Leishmania* infection supports the feasibility to develop a human vaccine.[37–39] An ideal candidate should be safe and able to induce both $CD4^+$ and $CD8^+$ T-cell responses, which could be boosted by natural infections, thus reducing the number of immunogen doses. Also, it should comprise different immunogenic parts of *Leishmania* proteins in a single product, making it more specific, immunogenic, easier, and cheaper to produce.[40,41]

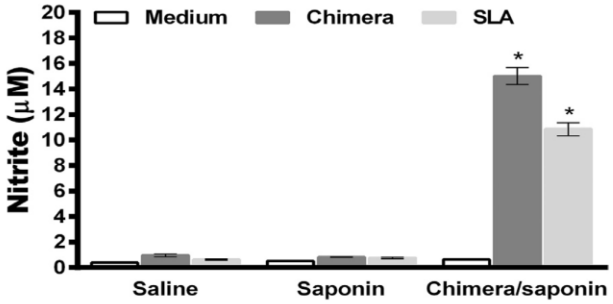


**Fig. 7.** Nitrite production. Splenocytes from the infected and immunized mice (n = 8 per group) were cultured to perform the dosage of cytokines, and also used to investigate the protein and parasite-specific nitrite production, by means of the Griess reaction. Bars represent the mean ± standard deviation. (*) indicates statistically significant difference in relation to the saline and saponin groups ($P < 0.0001$).

In the present study, 3 recombinant *L. infantum* proteins (LiHyS, SGT, and PHB), which were previously shown to be protective against murine VL, were analyzed by distinct bioinformatics tools and a recombinant chimera was constructed. This construct was compared in the in vitro and in vivo analyses with a combination of 3 recombinant antigens. Our results showed that the chimeric protein was better than the protein mix, when cytokine production and parasite load were evaluated in infected and vaccinated BALB/c. In addition, chimera was highly immunogenic and induced higher IFN-$\gamma$/IL-10 ratios in cells collected from healthy subjects and treated VL patients, then demonstrating the possibility to use this antigen as an immunoprophylactic target in studies against human disease.

Distinct *Leishmania* proteins induce potent antibody and T-cell responses against parasites. In a recent study, Martins et al.[18] used bioinformatics tools to investigate the amino acid sequences of 4 proteins, LiHyp1, LiHyp6, LiHyV, and histamine-releasing factor (HRF), aiming to identify the main $CD4^+$ and $CD8^+$ T-cell epitopes of these molecules, and a recombinant chimera was produced. This construct was evaluated as a vaccine candidate in a murine model and it was protective against *L. infantum* infection. In that study, no human immune cells or human serum samples were used to evaluate the immunogenicity and antigenicity, respectively, of the individual and chimeric proteins.

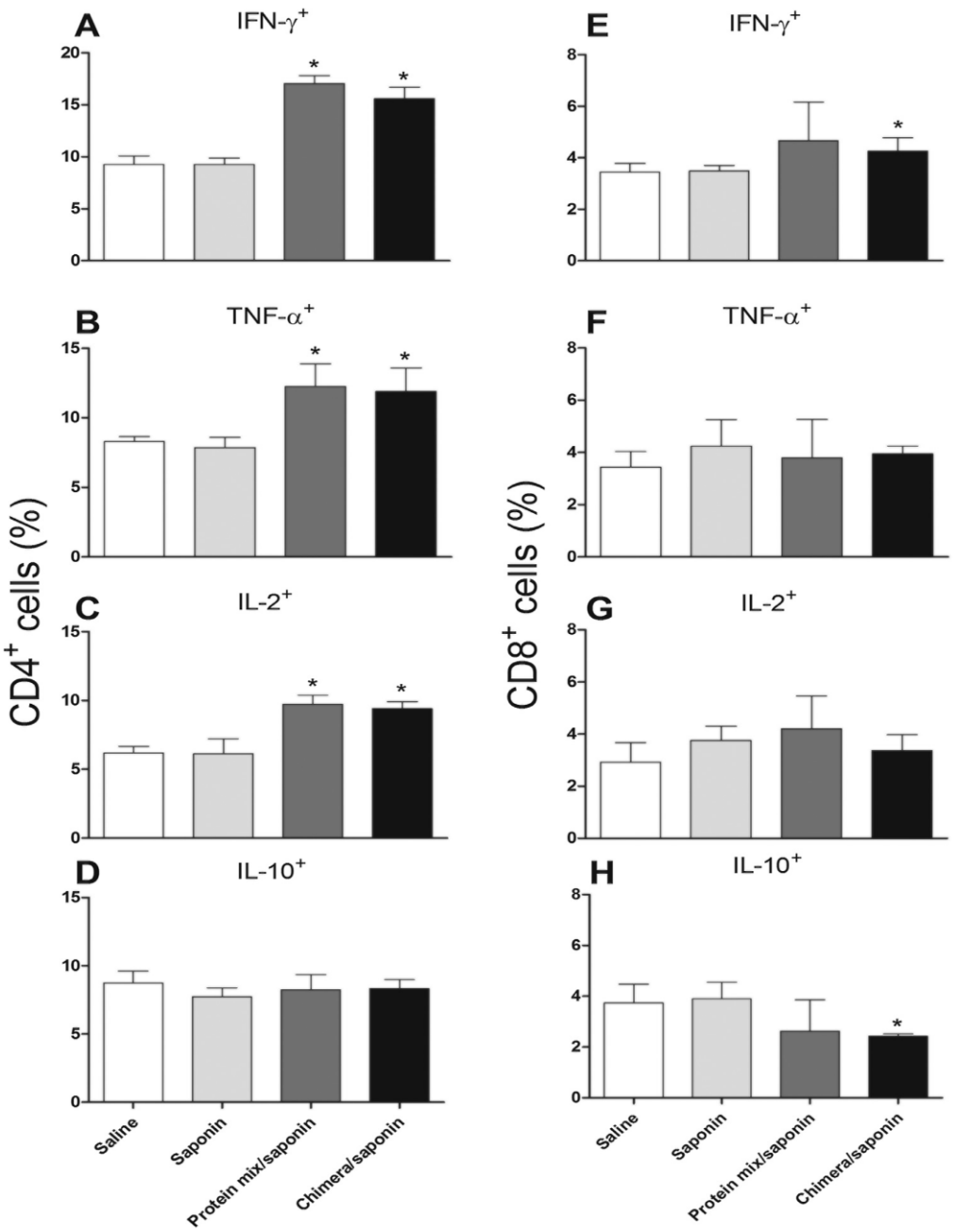


**Fig. 8.** Evaluation of intracytoplasmic cytokine-producing T-cells profile by flow cytometry. The profile of IFN-$\gamma$, TNF-$\alpha$, IL-2, and IL-10-producing $CD4^+$ and $CD8^+$ T-cells was calculated by means of the ratio between values obtained in the SLA-stimulated cultures vs those from the unstimulated cultures (control). For this, BALB/c mice received saline (white rectangle) or were immunized with saponin (light grey rectangle), protein mix and/or saponin (dark grey rectangle) or chimera and/or saponin (black rectangle), and later challenged with *L. infantum* promastigotes. Sixty days after infection, their spleens were collected and *in vitro* stimulated with SLA (25 $\mu$g/mL), when the IFN-$\gamma$, TNF-$\alpha$, IL-2, and IL-10-producing $CD4^+$ (**A, B, C**, and **D**) and $CD8^+$ (**E, F, G**, and **H**) T-cell percentages was evaluated. Bars represent the mean plus standard deviation of the groups. (*) indicates statistically significant difference in relation to the saline and saponin groups ($P < 0.05$). IFN-$\gamma$, interferons-$\gamma$; IL-2, interleukin-2; IL-10, interleukin-10, SLA, soluble antigen extract; TNF-$\alpha$, tumor necrosis factor-$\alpha$.

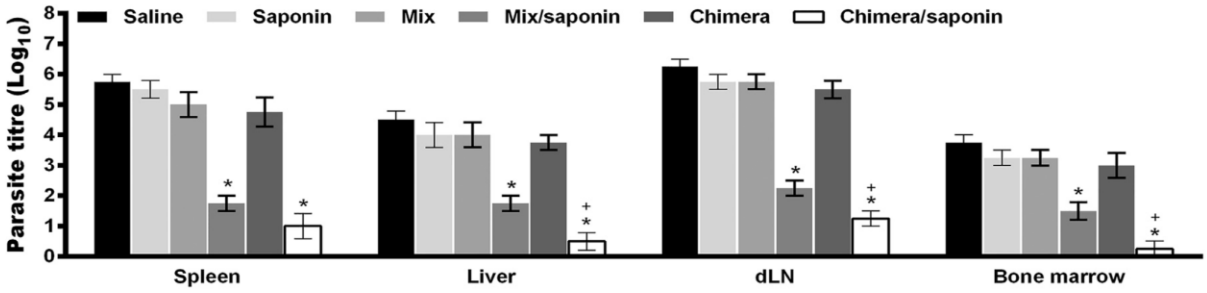


**Fig. 9.** Parasite burden evaluated by limiting-dilution technique. Mice (n = 8 per group) received saline or were immunized with saponin, chimera, chimera and/or saponin, protein mix or protein mix and/or saponin. Then, they challenged with *L. infantum* promastigotes. Sixty days after infection, their liver, spleen, bone marrow, and draining lymph nodes were collected to evaluate the parasite load through a limiting-dilution assay. Results were expressed as the negative log of the titer adjusted per milligram of organ. The parasite load in spleen, liver, draining lymph nodes (dLN), and bone marrow are shown. Bars represent the mean ± standard deviation of the groups. (*) indicates statistically significant difference in relation to the saline group ($P < 0.0001$). ($^+$) indicates statistically significant difference in relation to the protein mix and/or saponin group ($P < 0.01$).

In contrast, in the current study, human samples, *ie*, PBMCs and sera samples of both healthy subjects and VL patients were used, showing the potential of this new molecule as a protective strategy against human disease.

The presentation of antigens through the MHC I and MHC II pathways is necessary to induce the development of specific Th1 cells, which can control parasite infections.[42] The ability to produce cytokines such as IFN-$\gamma$, IL-2, IL-12, GM-CSF, TNF-$\alpha$, among others, has been proposed as a reasonable predictor of protection against intracellular pathogens, such as *Leishmania*.[43,44] In this study, we showed that mice vaccinated with the recombinant chimera plus saponin and challenged with *L. infantum* promastigotes increased the production of these cytokines, but not of IL-4 and IL-10, suggesting the development of Th1-biased response in these immunized animals. In addition, the parasite-specific nitrite production was higher in the cell supernatant of these animals, when compared to the others, then demonstrating the activation of the macrophages and the development of cellular protective response.

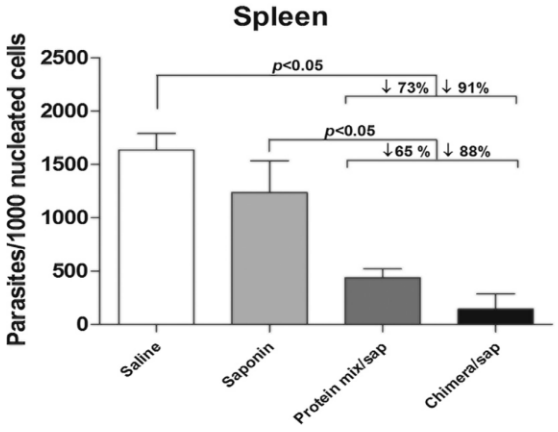


**Fig. 10.** Parasite burden evaluated by RT-PCR technique. Mice (n = 8 per group) received saline or were immunized with saponin, chimera and/or saponin or protein mix and/or saponin. Then, they challenged with *L. infantum* promastigotes. Sixty days after infection, the splenic parasitism was evaluated through a RT-PCR technique. Results were expressed as the number of parasites per 1000 nucleated cells. Bars represent the mean plus standard deviation of the groups. Statistical differences ($P < 0.05$) and percentage of reduction of the parasite burden between the groups are showed.

In addition, when anti-CD4 and anti-CD8 monoclonal antibodies were used to inhibit the IFN-$\gamma$ production by spleen cells from vaccinated animals, both antibodies significantly reduced the production of this cytokine, indicating the efficacy of our molecule to develop a protective cellular response against infection based on $CD4^+$ and $CD8^+$ T-cells. A similar profile was also obtained when a flow cytometry experiment was performed, since higher percentage of IFN-$\gamma^+$, IL-$2^+$, and TNF-$\alpha^+$-producing T-cells, which were associated with low presence of specific IL-$10^+$ T-cells, were found in the infected and immunized animals. These findings are in concordance with previous studies evaluating also vaccine candidates against VL, where both T-cell subtypes were considered important to protect against murine disease.[45–47]

Although our molecule was built with predicted T-cell epitopes of 3 parasite proteins, we understand that the immunization using this molecule, being associated with saponin, induces both cellular and humoral responses in the vaccinated animals. This fact is in

concordance with data showed in other studies,[18] in which a humoral response was also observed to be specific against the recombinant chimera used to immunize mice. We believe that this humoral profile, mainly based on the IgG2a isotype, is related to the IFN-$\gamma$ production, corroborating the protective phenotype against *L. infantum* infection achieved with the vaccine. In other studies, antiprotein antibody responses were used as biomarkers of efficacy and/or immunogenicity induced by recombinant vaccines.[48]

In previous studies, the rLiHyS, rSGT, and rPHB proteins induced lymphoproliferative response in human PBMCs, although cytokine production was not evaluated. Here, we performed these analyses and all antigens induced a Th1 response based on high IFN-$\gamma$ and low IL-10 levels in the stimulated PBMCs. The recombinant chimera induced higher IFN-$\gamma$/IL-10 ratios in comparison with individual proteins, showing a more polarized Th1 response. When the protein mix was evaluated, high levels of IFN-$\gamma$ were also induced in human cells. However, the production of recombinant proteins is expensive and laborious, and a vaccine composed by 3 different molecules could significantly increase the final cost of the product. Taking into account that the recombinant chimera is a unique molecule composed by distinct T-cell epitopes, this product will be much simpler to produce and a less expensive market product.

The use of murine models to evaluate vaccine candidates against *Leishmania* represents a useful tool to investigate initial aspects related to the parasite-host interactions, and this model has significantly contributed to the understanding of immune mechanisms related with the protection or susceptibility against disease.[49–54] However, data generated in these models usually cannot be extrapolated to predict a protective phenotype in other mammalian hosts, such as humans, and additional experiments are necessary to be developed to find these answers. The fact that VL patients treated and cured from *L. donovani* or *L. infantum* infection are usually protected against subsequent infections indicates the possibility to develop products to protect against disease in this mammalian host.[55,56] Here, the recombinant chimera was shown to be immunogenic in PBMCs collected from both healthy individuals and VL patients, showing that this protein is immunogenic for the human immune system. In addition, immunoblotting and ELISA experiments also showed that the recombinant chimera is recognized in VL patients, and the humoral response is based on a Th1 response, since higher IgG2/IgG1 ratios were found. As a consequence, it can be inferred that the recombinant chimera could be tested as an effective immunogen against human VL, and results of protection against parasites will be promising about the vaccine efficacy.

In previous studies, the rLiHyS, rSGT, and rPHB induced lymphoproliferative response in human PBMCs, although the cytokine production was not evaluated. Here, we performed these analyses and all antigens induced a Th1 response based on high IFN-$\gamma$ and low IL-10 levels in the stimulated PBMCs. The recombinant chimera also induced higher IFN-$\gamma$/IL-10 ratios, which showed values significantly higher to the others, then demonstrating the more polarized Th1 response developed by the use of this product. In fact, when the protein mix was evaluated, proteins also induced high levels of IFN-$\gamma$ in human cells. However, the production of recombinant proteins is expensive and laborious, and a vaccine composed by 3 different molecules could increase in significant levels the final cost of the product. By the fact that the recombinant chimera is an unique molecule, although composed by distinct T-cell epitopes of three proteins, this product will be much simpler to produce and less expensive as a final product to the market.

Although different *Leishmania* species cause a broad range of clinical diseases, there is a large degree of genomic conservation among parasite species.[57] As a consequence, it may be possible to generate an effective vaccine candidate to protect against disease.[58] Despite all effort dedicated to this endeavor, there is no licensed vaccine to protect against human VL. The need to use less toxic adjuvants and the number of doses to guarantee immunogenicity are also hurdles. Therapeutic vaccines could also be developed to increase cure rate in post-kala-azar dermal leishmaniasis patients and protect populations against ongoing VL transmission, being this promising strategy for the design of novel products to control VL.[59,60]

In conclusion, results presented here show a new recombinant molecule composed from distinct immunogenic parts of 3 parasite proteins already implicated in protection against *L. infantum*. This chimera was immunogenic in healthy and treated humans, as well as able to induce protection against murine VL. Furthermore, this new antigen based on multiple epitopes was more effective to induce immune response and protection than the mixed recombinant proteins. In this context, the chimera is a promising candidate to protect against VL in other mammalian hosts such as in humans.

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Conflicts of Interest: All authors have read the journal's policy on conflicts of interest and have none to declare. All authors have read the journal's authorship

agreement and approved submission of the manuscript. The manuscript, neither has been published nor is currently under consideration for publication by any other journal.

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## 4.10 ARTIGO 5 - CONCLUSÃO

Os resultados mostraram a imunogenicidade da quimera recombinante em PBMCs humanos e a eficácia protetora contra *L. infantum* no modelo murino, indicando que esta proteína pode ser considerada como um biomarcador contra a doença humana.

# *CONCLUSÃO GERAL*

## 5. CONCLUSÃO GERAL

Em conclusão, os resultados desta tese apresentam novos candidatos à vacina e diagnóstico para as leishmanioses tegumentar e visceral. Também, uma nova molécula, que foi baseada em uma quimera recombinante composta por partes imunogênicas das três proteínas do parasito *Leishmania*, rSGT, rLiHyS e rPHB, que foi capaz de ofertar proteção significativa contra a infecção por *L. infantum*. A quimera demonstrou também ser promissora para uma possível vacina humana, uma vez que ela foi imunogênica em PBMCs humanos de pacientes e indivíduos sadios residentes em área endêmica da doença.

***PERSPECTIVAS***

## 6 PERSPECTIVAS

- Avaliar o potencial imunogênico e o grau de proteção induzido pela quimera recombinante em modelo de hamster (*Mesocricetus auratus*) contra a infecção com *L. infantum*.

- Avaliar a imunogenicidade e eficácia protetora (estudos de fase 2) da quimera recombinante no modelo canino.

- Avaliar a aplicabilidade das proteínas na Imunoterapia.

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# 8. ANEXO

## 8.1. PATENTE

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL

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

**Número do Processo:** BR 10 2017 025621 9

**Dados do Depositante (71)**

**Depositante 1 de 2**

**Nome ou Razão Social:** UNIVERSIDADE FEDERAL DE MINAS GERAIS
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PETICIONAMENTO ELETRÔNICO

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 29/11/2017 às 11:46, Petição 870170092495

Petição 870170092495, de 29/11/2017, pág. 1/34

**Depositante 2 de 2**

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**Dados do Pedido**

**Natureza Patente:** 10 - Patente de Invenção (PI)
**Título da Invenção ou Modelo de Utilidade (54):** "PROTEÍNA QUIMÉRICA RECOMBINANTE, VACINA CONTRA LEISHMANIOSES E USO"
**Resumo:** A presente tecnologia trata de uma proteína quimérica recombinante, definida pela SEQ ID N°1, composta por epitopos específicos de linfócitos T CD4+ e CD8+ derivados de proteínas de Leishmania infantum para tratamento e prevenção contra as leishmanioses visceral e tegumentar no homem e cão, além de uma vacina compreendida pela proteína em questão, e seus usos.
**Figura a publicar:** 1