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Prophylactic properties of a *Leishmania*-specific hypothetical protein in a murine model of visceral leishmaniasis

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SUMMARY

In this work, the effect of vaccination of a newly described Leishmania infantum *antigenic protein has been studied in BALB/c mice infected with this parasite species. The LiHyD protein was characterized after a proteomic screening performed with the sera from dogs suffering visceral leishmaniasis (VL). Its recombinant version was expressed, purified and administered to BALB/c mice in combination with saponin. As a result of vaccination and 10 weeks after challenge using an infective dose of* L. infantum *stationary promastigotes, vaccinated mice showed lower parasite burdens in different organs (liver, spleen, bone marrow and footpads' draining lymph nodes) than mice inoculated with the adjuvant alone or the vaccine diluent. Protected mice showed anti-*Leishmania *IgG2a antibodies and a predominant IL-12-driven IFN-γ production (mainly produced by CD4$^+$ T cells) against parasite proteins, whereas unprotected controls showed anti-*Leishmania *IgG1 antibodies and parasite-mediated IL-4 and IL-10 responses. Vaccinated mice showed an anti-LiHyD IgG2a humoral response, and their spleen cells were able to secrete LiHyD-specific IFN-γ, IL-12 and GM-CSF cytokines before and after infection. The protection was correlated with the* Leishmania*-specific production on nitric oxide. Altogether, the results indicate that the new LiHyD protein could be considered in vaccine formulations against VL.*

Keywords *BALB/c mice, experimental vaccine, hypothetical protein,* Leishmania infantum*, saponin*

INTRODUCTION

Visceral leishmaniasis (VL) caused by *Leishmania donovani* and *L. infantum*/*L. chagasi* is an important neglected disease in the world (1). About 350 million people are at risk of contracting the infection in 98 countries, and 0·2–0·5 million of new cases of VL are registered annually (1, 2). The treatment of this disease is yet mostly based on the parenteral administration of pentavalent antimonials. However, increased parasite resistance and side effects registered in the patients have been the important problems (3, 4). Alternative drugs, such as amphotericin B and its liposomal formulations, pentamidine and miltefosine, have showed encouraging results; however, their toxicity, the increase of parasite resistance and/or high cost had limited their use (5–7). A VL elimination campaign has been initiated around the world, which will focus on vector control, early diagnosis and drug treatment strategies (8, 9). However, it is presumed that a vaccine will be required for successful elimination of the disease.

The development of a type 1 T helper (Th)-mediated immunity to prevent leishmaniasis has inspired the development of prophylactic vaccination against the disease, but few have progressed beyond the experimental stage using mice models for cutaneous and VL (10–14). The induction of specific CD4$^+$ Th1 cells against parasite proteins is crucial in controlling the infection caused by *Leishmania* spp. Both T CD4$^+$ and CD8$^+$ cells are the major weapons for an anti-*Leishmania* immune response, and

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they play a crucial role through the IL-2 and IFN-γ production. These cytokines activate the effector functions of macrophages, inducing the production of nitric oxide (NO), a molecule able to destroy the intracellular amastigotes (15, 16). Progression of the disease is associated with the induction of parasite-specific TGF-β-, IL-10- and IL-13-mediated responses (17–19). In addition, control of the IL-4-mediated Th2 humoral response against *Leishmania* commonly enhances vaccine-induced protection by indirectly increasing IFN-γ production by T cells (20–23).

The search for new targets to compose a vaccine is an important strategy for leishmaniasis control, mainly when employing proteins that are recognized by patients' sera, indicating that these antigens are present in the intracellular stages of the parasites and that they are able to interact with the host immune system. In recent decades, most of the studies evaluating vaccines for leishmaniasis have focused on *Leishmania* spp. proteins possessing orthologue forms in other protozoan or mammalian species (16, 24–28). As a result of the application of proteomics for the search of new antigens, some proteins considered in the database as hypothetical (because of their lack of sequence similarity to previous described proteins) were characterized. As they were recognized by sera of mammalian hosts infected by cutaneous or viscerotropic *Leishmania* species, they have emerged as putative new vaccine candidates. Besides its antigenicity, these proteins do not exhibit a high degree of sequence conservation with any host counterparts, decreasing the possibility to induce cross-reactive responses against the host.

We have designed a study to evaluate a new vaccine candidate, a *Leishmania* spp.-specific hypothetical protein namely LiHyD. The effects of the administration of the protein obtained as a recombinant protein (rLiHyD) in BALB/c mice (combined with saponin) in VL evolution due to *L. infantum* infection have been studied. This protein was selected for the analysis, because of its reactivity with sera from dogs with active VL (29). In addition, it is conserved among different *Leishmania* species, but it is not present in other trypanosomatids or in mammalian species. The decrease in *L. infantum* infection progression and the immune correlate with protection in vaccinated mice is discussed, presenting the LiHyD hypothetical protein associated with Th1-type adjuvants as a formulation able to protect against VL.

MATERIALS AND METHODS

Ethics statement

This study was approved by Committee on the Ethical Handling of Research Animals from Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil, under the protocol number 043/2011.

Mice and parasite

Female BALB/c mice (8 weeks of age) obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, UFMG, were maintained under specific pathogen-free conditions. Experiments were carried out using the *L. infantum* (MHOM/BR/1970/BH46) strain. Parasites were grown at 24°C in Schneider's medium (Sigma, St. Louis, MO, USA), supplemented with 10% heat-inactivated foetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 200 U/mL penicillin and 100 μg/mL streptomycin, at pH 7·4. The soluble *L. infantum* antigenic extract (SLA) was prepared from 1×10^{10} stationary-phase promastigote cultures (5–7 days old), as described (30).

Obtaining the *Leishmania*-specific hypothetical protein, LiHyD

The LiHyD (LinJ.33·3150) nucleotide and amino acid sequences used in this study were obtained from TriTrypDB (http://tritrypdb.org). The local alignment of the LiHyD sequence against the available complete genomes of other organisms was performed by BLAST. For obtaining the recombinant protein (rLiHyD), its coding region was amplified by PCR using genomic *L. infantum* DNA (as *Leishmania* protein genes lacks introns) and the next primers: *forward* (5'-GGATCCATGCAGATGCAAGG CAACATG-3') and *reverse* (5'-AAGCTTATTGTTGCCG CATACTTGG-3'). The coding region was cloned into the *p*GEM®-T Vector Systems (Promega, Madison, WI, USA) and double stranded sequenced. After the DNA insert was obtained by a *BamHI* and *HindIII* double digestion (taking advance of the corresponding cut sites included in the primers [underlined]) and transferred to the same cut sites of the pQE30 plasmid (Qiagen, Hilden, Germany). Recombinant plasmid was transformed into *Escherichia coli* M15 strain where the overexpression of the recombinant protein was performed by adding 1·0 mM IPTG (isopropyl-β-D-thiogalactopyranoside, Promega, Montreal, Canada), for 3 h at 37°C. For protein purification, cells were lysed by a homogenizer after five passages through the apparatus. The product was centrifuged at 13 000 × *g* for 20 min at 4°C. The rLiHyD protein, containing a tag of 6× residues of histidine fused at its N-terminal region, was purified under nondenaturing conditions, using a 5 mL His-Trap column (GE Healthcare Bio-Sciences, Pittsburgh, USA), attached to an FPLC (GE Healthcare Life Science) system. The recombinant protein was dial-

ysed using saline as a buffer. After dialysis, rLiHyD was passed through a polymyxin–agarose column (Sigma), to remove residual endotoxin content (<10 ng of LPS per 1 mg of recombinant protein, measured by the Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000; BioWhittaker, Walkersville, USA). In addition, the purity of the recombinant protein was checked by a one-dimensional 10% SDS-PAGE as described below.

SDS-PAGE and immunoblotting analysis

To analyse the purity of the recombinant LiHyD protein, bacterial total extracts containing the expressed protein and the protein obtained at the end of the purification process (10 μg each sample) were submitted to a 10% SDS-PAGE. Gels were stained with Coomassie blue. Similar gels containing the purified were blotted onto a nitrocellulose membrane (0·2 μm pore size, Sigma). Membranes were blocked with PBS-T (phosphate-buffered saline plus Tween-20 0·05%) plus 5% BSA for 1 h and were independently incubated for 2 h with two pools of sera samples obtained from naive mice ($n = 8$) or *L. infantum*-chronically infected BALB/c mice (1 : 100 dilution in PBS-T solution). VL animals ($n = 8$) were subcutaneously infected by 1×10^7 stationary promastigotes of *L. infantum,* and sera were collected at week 10 after infection. As secondary antibody, blots were incubated with peroxidase-conjugated anti-mouse IgG (1 : 10 000; Sigma) for 2 h. Reactions were revealed by adding chloronaphthol, diaminobenzidine and H_2O_2, and stopped by adding distilled water.

Immunization and challenge infection

For immunization, three groups ($n = 8$ animals per group) were made. Vaccinated group were inoculated subcutaneously in their left hind footpad with 25 μg of rLiHyD associated with 25 μg of saponin (*Quillaja saponaria* bark saponin, Sigma). Control mice were similarly inoculated with the adjuvant (25 μg of saponin) or the vaccine diluent (PBS). Three doses were administered at 2-week intervals. Four weeks after the final immunization, animals ($n = 4$ per group) were euthanized to analyse the immune response elicited by vaccination. At the same time, the remaining animals ($n = 4$, per group) were subcutaneously infected in the right hind footpad with 1×10^7 stationary-phase promastigotes of *L. infantum*. To determine parasite burden and to evaluate the immune response post-challenge, mice were euthanized at week 10 after infection. Vaccination experiments were repeated and presented similar results. Data shown in this study represent the mean ± standard deviation of the individual data pooled from two independent experiments, which presented similar results.

Estimation of parasite load

Single-cell suspensions of the spleen, liver, draining lymph nodes (dLN) and bone marrow (BM) from mice were independently prepared for parasite quantification, following a limiting-dilution protocol (30). Briefly, the organs were weighed and homogenized using a glass tissue grinder in sterile PBS. Tissue debris were removed by centrifugation at $150 \times g$, and cells were concentrated by centrifugation at $2000 \times g$. Sediment cells were suspended in 1 mL of supplemented Schneider's insect medium (prepared as depicted above, but using 20% FBS). Ten-fold serial dilutions (from 10^{-1} to 10^{-12}) were made in the same medium using 96-well flat-bottom microtiter plates (Nunc, Nunclon®, Roskilde, Denmark). Each individual sample was plated in triplicate, and parasites' presence was analysed by microscopy after 7 days of culturing at 24°C. Pipette tips were discarded after each dilution step to avoid carrying adhered parasites from one well to another. Results are expressed as the log of the titre (*i.e.* the dilution corresponding to the last positive well) adjusted per milligram of tissue.

Cytokine and nitrite production

Splenocyte cultures were established from individual mice ($n = 4$, per group) at the time of the two described sacrifices. For this, single-cell preparations were plated in duplicate in 24-well plates (Nunc) at 5×10^6 cells per mL. Cells were incubated in complete DMEM medium (DMEM supplemented with 10% heat-inactivated foetal bovine serum, 20 mM L-glutamine, 200 U/mL penicillin and 100 μg/mL streptomycin) in the absence (negative control) or in the presence of rLiHyD protein (20 μg/mL) or *L. infantum* SLA (25 μg/mL), at 37°C in 5% CO_2 for 48 h. IFN-γ, IL-4, IL-10, IL-12 and GM-CSF levels were assessed in the supernatants by a sandwich ELISA kit (BD OptEIA TM set mouse IFN-γ (AN-18), IL-4, IL-10, IL-12 and GM-CSF; Pharmingen®, San Diego, CA, USA) following manufacturer's instructions. When indicated, and in order to block IL-12-, $CD4^+$- and $CD8^+$-mediated T-cell cytokine release, spleen cells of mice vaccinated with rLiHyD plus saponin and challenged with *L. infantum* were *in vitro* stimulated with SLA (25 μg/mL), in the absence or in the presence of 5 μg/mL of monoclonal antibodies (mAb) against mouse IL-12 (C17·8), CD4 (GK 1·5) or CD8 (53-6·7). Appropriate isotype-matched controls (rat IgG2a (R35-95) and rat IgG2b (95-1)) were employed in the assays. Antibodies (no azide/low endotoxin™) were purchased from BD (Pharmingen®). The nitrite production in the cultures supernatant was assessed by the Griess reaction (31) in the

supernatants of spleen cells cultures established from individual infected mice ($n = 4$, per group), and stimulated and cultured as above. Data were expressed as μM per 5×10^6 cells.

Evaluation of the antibody production

The humoral response was evaluated by collecting sera samples of the animals ($n = 4$, per group) after the last immunization and before infection (4 weeks after last doses), as well as at the 10th week after challenge. For this, The LiHyD- and *L. infantum* SLA-specific IgG1 and IgG2a isotypes levels were measured by an ELISA technique, as described (12). Recombinant protein was employed at 5 μg/mL, and SLA was assayed at 10 μg/mL (100 μL per well). The sera samples were diluted at 1 : 100, and the anti-mouse IgG1 and IgG2a horseradish peroxidase-conjugated antibodies (Sigma-Aldrich) were used in a 1 : 5000 dilution.

Statistical analysis

The results were processed using MICROSOFT EXCEL (version 10.0) or GRAPHPAD PRISM™ (version 6.0 for Windows). The one-way analysis of variance (ANOVA), followed by Bonferroni's post-test, was used for multiple comparisons between the groups. Differences were considered significant when $P < 0{\cdot}05$. As indicated above, individual data from two different experiments were employed for the statistical analysis.

RESULTS

Expression, purification and antigenicity of the rLiHyD

To analyse the immunological properties of the rLiHyD protein, the recombinant version of the protein was overexpressed *E. coli* (Figure 1a, lane 2) and purified by affinity chromatography (Lane 1A, lane 3, black arrow). A band of 36·0 kDa, molecular weight expected from its primary structure, was observed in a Coomassie-stained 10% SDS-PAGE gel (Figure 1a). Next, a Western blot of the purified protein was incubated with sera samples obtained from naïve BALB/c mice or sera from the same animals but chronically infected with *L. infantum*. Results shown in Figure 1(b) demonstrate that the purified protein was recognized only by the sera from the infected mice (lane 3, black arrow).

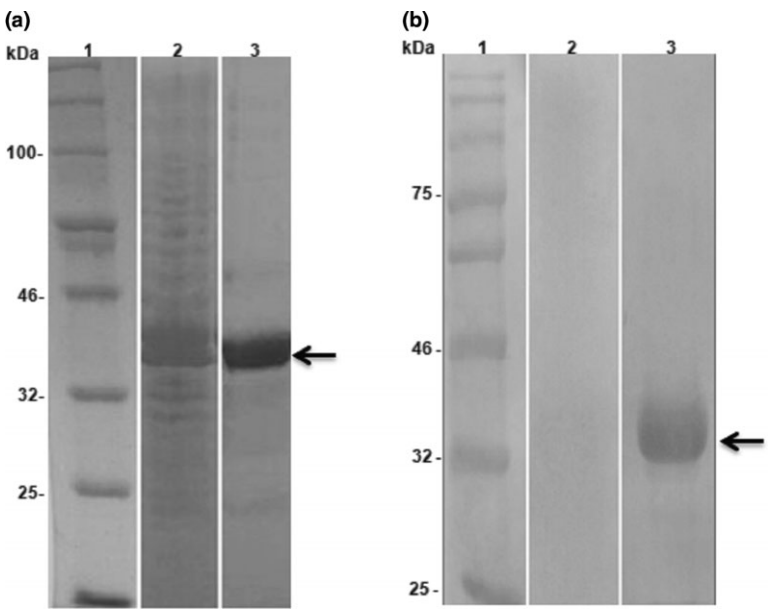

Figure 1 Antigenicity of the purified recombinant LiHyD protein. Analysis of the purification process is shown in (a). Molecular weight markers (lane 1), 10 μg of total bacterial cultures expressing the rLiHyD protein (lane 2) and protein purified by affinity chromatography (lane 3) were electrophoresed on a SDS-PAGE 10% gel. Coomassie staining of the gel is shown. In (b), similar gels loaded with molecular weight markers (lane 1) and 10 μg of the LiHyD purified protein (lanes 2 and 3) were electrophoresed and blotted onto a nitrocellulose membrane. Blots were incubated with pools of sera samples ($n = 8$ each group) from noninfected (lane 2) or chronically *Leishmania infantum*-infected mice (lane 3), and revealed with chloronaphthol. A scan from the blots is shown. Black arrows label the position of the rLiHyD protein in gels and blots.

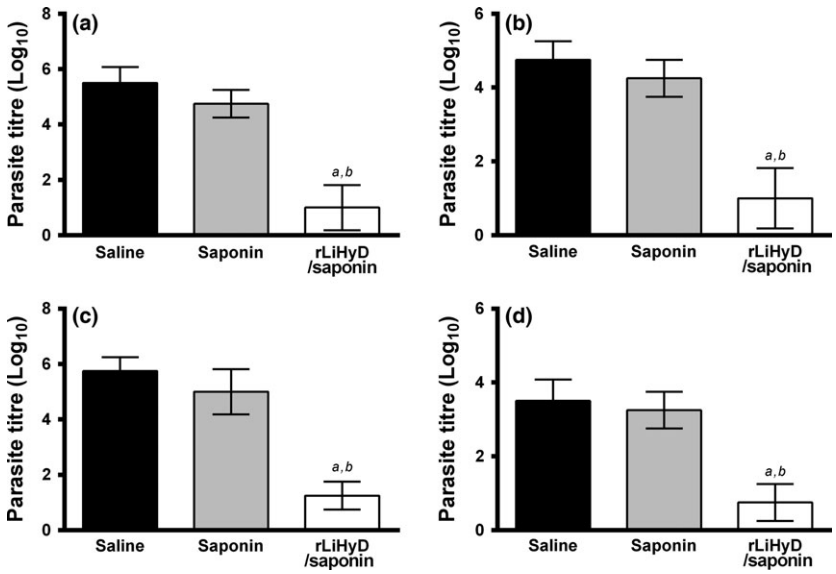

Figure 2 Protection of BALB/c mice vaccinated with rLiHyD plus saponin against *Leishmania infantum* infection. Mice inoculated with saline, with saponin or with rLiHyD plus saponin ($n = 4$, per group) were subcutaneously challenged with 1×10^7 stationary-phase promastigotes of *L. infantum*. The number of parasites in the liver (a), spleen (b), bone marrow (c) and paws' draining lymph nodes (d) was measured, 10 weeks after challenge, by a limiting-dilution technique. Mean ± standard deviation (SD) in each group is shown. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0{\cdot}001$). Data shown in this figure represent the mean ± standard deviation of two independent experiments.

Protective efficacy of a vaccine composed by rLiHyD plus saponin against *L. infantum*

To study the prophylactic properties of the rLiHyD protein, a vaccination–infection experiment was performed. For that purpose, a group of mice was immunized with the recombinant protein administered in combination with saponin. Two control groups were performed: mice inoculated with the same dose of saponin and mice inoculated with the vaccine diluent. A comparative analysis of the parasite burdens found in the spleen, liver, dLN and BM of the animals from the three groups was performed 10 weeks after a *L. infantum* challenge, performed subcutaneously in the footpads. It was observed that vaccinated mice presented significant reductions (between 4-log and 5-log) in the parasite load in the spleen (Figure 2a), liver (Figure 2b), dLN (Figure 2c) and BM (Figure 2d) when compared to both, saline and saponin groups.

The immune response generated in the animals against the parasite total proteins was analysed to compare the post-infection immune parameters found in the three groups of mice. Ten weeks after infection, vaccinated mice had a Th1 profile against a preparation of soluble leishmanial antigen (SLA). Thus, following *in vitro* stimulation with *L. infantum* SLA, spleen cells from vaccinated and infected mice produced higher levels of IFN-γ, IL-12 and GM-CSF, than those secreted by spleen cells from control mice groups (Figure 3a). As a correlate of disease progression, spleen cells from saline and saponin inoculated mice produced predominant IL-4 and IL-10 cytokine responses (Figure 3a). To evaluate the involvement of $CD4^+$ and $CD8^+$ T cells and the dependence of IL-12 in the parasite-specific IFN-γ production found in protected mice, their spleen cells were stimulated with SLA in the absence or presence of anti-IL-12, anti-CD4 or anti-CD8 monoclonal antibodies (Figure 3b). IFN-γ production was significantly inhibited when the three antibodies were employed. The highest inhibition was obtained when the IL-12 blocking antibody was used, implicating to this cytokine in the parasite-specific IFN-γ response found in the protected mice. Higher decrease was found after anti-CD4 treatment than inhibiting the stimulation of $CD8^+$ T cells with monoclonal antibodies. These data were indicating that a predominant Th1 response was found against the parasite after infection in the protected mice. This finding correlates with the presence of a predominant IgG2 parasite-specific humoral response after infection in rLiHyD plus saponin vaccinated mice, in opposition to control groups, having a predominant IgG1 humoral response against SLA (Figure 4).

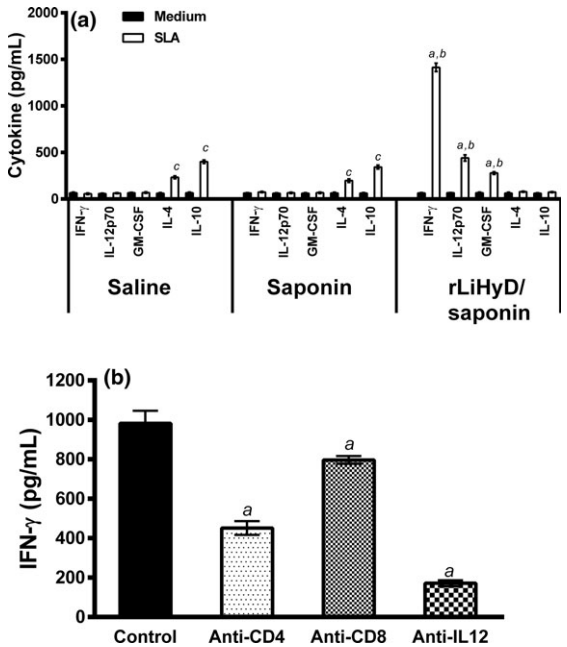

Figure 3 Cellular response against parasite proteins after *Leishmania infantum* challenge. Single spleen cells from mice that received saline or were inoculated with saponin or rLiHyD plus saponin ($n = 4$, per group) and were infected with *L. infantum* were collected, and independently cultured without stimulus (medium; negative control) or *in vitro* stimulated with SLA (25 μg/mL) for 48 h at 37°C in 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4, and IL-10 levels were measured by ELISA in the culture supernatants (panel a). (*a*) indicates statistically significant increase in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant increase in relation to the saponin group ($P < 0{\cdot}001$). (*c*) indicates statistically significant increase in relation to the rLiHyD/saponin group ($P < 0{\cdot}001$). Also, spleen cells from mice vaccinated with rLiHyD plus saponin ($n = 4$) were *in vitro* stimulated with SLA (25 μg/mL; control) and incubated in the absence (positive control) or presence of 5 μg/mL of monoclonal antibodies (mAb) against mouse IL-12, $CD4^+$ or $CD8^+$ (panel b). The levels of IFN-γ in supernatants are shown. (*a*) indicates statistically significant differences from antibody treated samples and the untreated control sample ($P < 0{\cdot}001$). Bars represent the mean ± standard deviation (SD) of the groups.

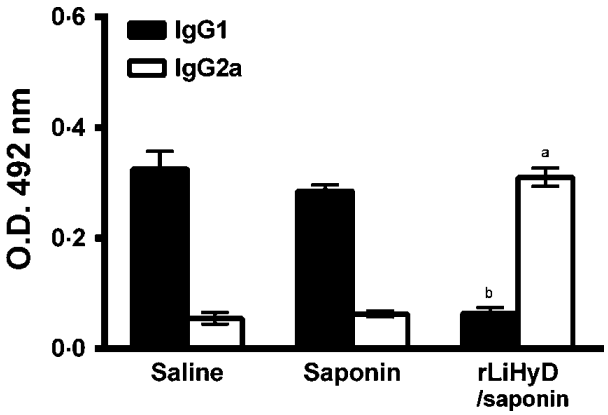

Figure 4 Anti-*Leishmania infantum* SLA humoral response after challenge. Sera samples were obtained from control and vaccinated mice ($n = 4$, per group), 10 weeks after infection with *L. infantum*. The levels of IgG1 and IgG2a isotypes against *L. infantum* SLA are shown. Bars represent the mean ± standard deviation (SD) of the groups. (*a*) indicates statistically significant increase in IgG2a levels in vaccinated group regarding the saline and saponin groups ($P < 0{\cdot}001$). (*b*) indicates statistically significant decrease in IgG1 levels between vaccinated and both control groups ($P < 0{\cdot}001$).

rLiHyD-specific cellular and humoral response elicited by immunization using rLiHyD plus saponin before and after challenge infection

Next, the immune response elicited against the parasite antigen contained in the vaccine was studied. For that, the production of cytokines in the supernatants of spleen cells cultures stimulated with rLiHyD was analysed before and 10 weeks after challenge infection (Figure 5). Before challenge, it was observed that spleen cells derived from mice vaccinated with rLiHyD plus saponin produced higher levels of rLiHyD-specific IFN-γ, IL-12 and GM-CSF cytokines, than those secreted by spleen cells from control groups (Figure 5a). In contrast, no production LiHyD-derived IL-4 and IL-10 could be observed in any experimental group. Mice that were immunized and lately challenged with *L. infantum* maintained the Th1 profile after infection, as their spleen cells cultures produced similar level of rLiHyD-specific cytokines than those secreted by spleen cells from vaccinated but uninfected mice (Figure 5b).

Evaluating the rLiHyD-specific humoral response in the vaccinated and/or infected animals (Figure 6), it was observed that before (Figure 6a) and after (Figure 6b) challenge, mice vaccinated with rLiHyD plus saponin presented higher levels of anti-rLiHyD-specific IgG1 and IgG2a isotype antibodies than the saline and saponin groups. The higher reactivity of the IgG2a antibodies found in the sera from vaccinated animals against the rLiHyD protein correlated with the Th1 cellular response determined by cytokine analysis. Although similar profiles were found for the humoral response before and after infection, the higher reactivity found post-challenge (comparing data from panel A and data shown in panel B; Figure 6) was indicating that the infection was able to boost the immune response elicited by vaccination, without changing its quality.

Analysis of the macrophage effector capacities after infection

It is well documented that macrophages stimulated by IFN-γ are able to activate NO synthesis to destroy the

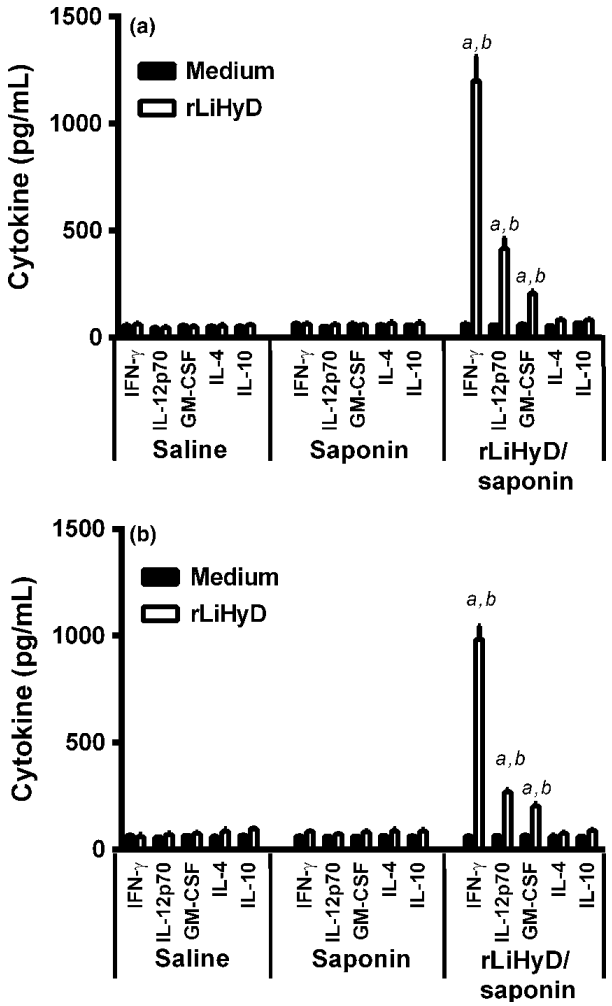

Figure 5 Vaccine-induced rLiHyD-specific cellular response. Single spleen cells of mice that received saline or were immunized with saponin or rLiHyD plus saponin ($n = 4$, per group) were collected before and after *Leishmania infantum* infection, and were nonstimulated (medium, negative control) or *in vitro* stimulated with rLiHyD protein (20 μg/mL), for 48 h at 37°C in 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4 and IL-10 levels were measured by ELISA in the culture supernatants before (a) and after (b) parasite challenge. Bars represent the mean ± standard deviation (SD) of the groups. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0{\cdot}001$).

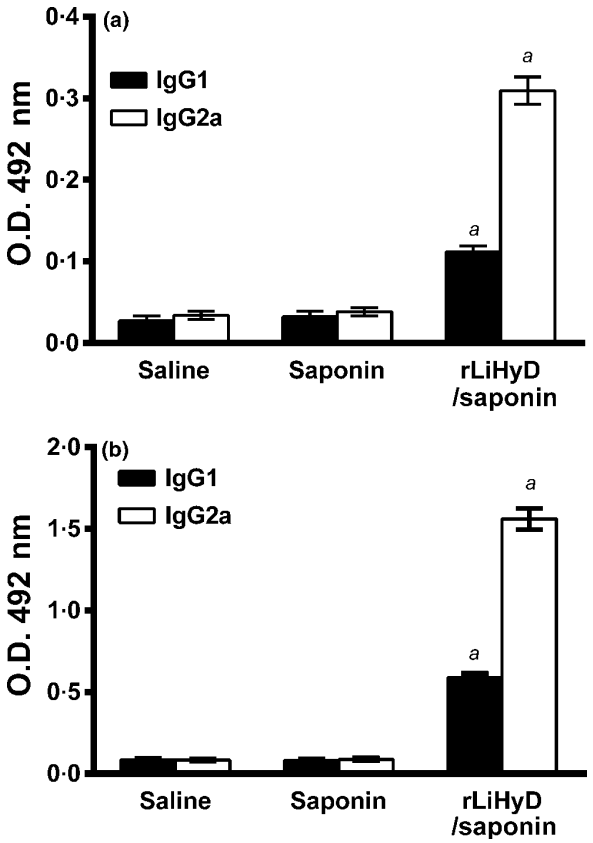

Figure 6 Vaccine-induced anti-rLiHyD humoral response. Sera samples were obtained from mice that received saline or were immunized with saponin or rLiHyD plus saponin, before ($n = 4$) (a) or after *Leishmania infantum* infection ($n = 4$) (b). Bars represent the mean ± standard deviation (SD) of IgG1 and IgG2a anti-LiHyD reactivity values from mice sera analysed in each experimental group. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0{\cdot}001$).

intracellular amastigotes. In an attempt to evaluate the parasite antigen-specific activation of macrophages in the three groups of mice, the presence of nitrite in culture supernatants was assayed as an indicator of NO production. For that purpose, spleen cells from infected mice (control and vaccinated mice groups) were cultured in the absence or in the presence of *L. infantum* SLA or the rLiHyD protein. Antigen-specific nitrite production was only detected in supernatants of the vaccinated and protected mice for both tested antigenic preparations (Figure 7).

DISCUSSION

In a recent immunoproteomic approach performed in stationary promastigote and axenic amastigote total extracts of *L. infantum* (29), different parasite proteins recognized by antibodies in sera of dogs suffering from active VL were characterized. Among them, some proteins annotated as hypothetical proteins were identified. These proteins are usually considered to have not defined functions, as they are predicted by genomics but they have not known domains or enough sequence conservation with other proteins (32). However, their antigenicity may be indicating

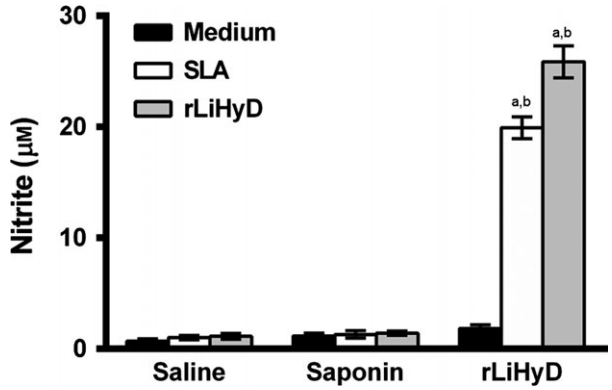

Figure 7 *Leishmania*-specific nitrite production after infection. Spleen cells from mice that received saline, saponin or rLiHyD plus saponin ($n = 4$, per group) and were challenged with *L. infantum* were collected 10 weeks after challenge. Spleen cells cultures were *in vitro* established and independently stimulated with SLA (25 μg/mL) or rLiHyD (20 μg/mL) for 48 h at 37°C in 5% CO_2. The presence of nitrite in the supernatants was analysed by the Griess method. Bars represent the mean ± standard deviation (SD) of the level of nitrites expressed in μM. (*a*) indicates statistically significant difference in relation to the saline group ($P < 0{\cdot}001$). (*b*) indicates statistically significant difference in relation to the saponin group ($P < 0{\cdot}001$).

that they are expressed in the promastigote or the amastigotes forms of the parasite during the active disease, and importantly that they are interacting with the host immune system. The LiHyD protein can be considered within this family of *Leishmania* antigens. It is recognized by sera from *L. infantum*-infected dogs (29), but also by antibodies present in the sera of *L. infantum*-chronically infected BALB/c mice. Interestingly, searches in the nucleotide or protein database have revealed that this protein is only found in the *Leishmania* genus. One gene copy exists in different *Leishmania* genomes, with identity ranging from 60% to 80%, in *L. major, L. braziliensis* and *L. mexicana*. No orthologue genes were found in the *Trypanosoma cruzi, T. brucei, T. vivax* and *T. congolense* annotated genomes. In this context, we have obtained the antigen as a purified recombinant protein and its immunoprophylactic properties have been analysed in a murine model of VL: BALB/c infected with *L. infantum*.

Although the use of murine models to test VL vaccines presents a limitation related to the organ-specific responses (parasites are cleared in the liver but infect chronically the spleen) (33), BALB/c mice infected with *L. infantum* or *L. donovani* have been widely employed with this purpose. In this sense, we have employed the subcutaneous challenge instead the intravenous infection, as similar evolution of the disease is produced (34, 35), and intravenous infection may undervalue the potential of some vaccine candidates (36). In addition, the rLiHyD protein has been combined with saponin, as it is an adjuvant able to induce cellular responses that have been employed for some canine vaccines (32, 37). Combination of parasite antigens with cellular adjuvants has resulted in the characterization of effective prophylactic tools against tegumentary and VL (16, 25, 30, 38–40).

Different previous studies evaluating vaccine candidates against leishmaniasis in murine models have showed different degree of success employing proteins evolutionary conserved (16, 27, 28, 41, 42). On the other hand, hypothetical proteins have emerged as interesting alternatives for vaccine development. As an example, Martins *et al.* (2013) evaluated the immunogenicity and protective efficacy of an amastigote-specific *L. infantum* hypothetical protein, LiHyp1, which was administered in association with saponin in BALB/c mice challenged subcutaneously with stationary promastigotes of *L. infantum* (16). The rLiHyp1/saponin vaccine induced a specific production of IFN-γ, IL-12 and GM-CSF. Interestingly, vaccinated animals showed significant reductions in the parasitism in organs such as liver, spleen, BM and dLNs, when compared to the control groups. Similarly, rLiHyD plus saponin-based vaccine was also able to induce a Th1 response against the hypothetical protein. This response did not change after challenge in quality, but it was boosted as a result of infection, as demonstrated by the higher anti-LiHyD immunoglobulin reactivity found in mice after challenge. The immune response elicited by the vaccine was able to diminish parasite numbers in all the evaluated organs (liver, spleen, BM and dLN). This decrease was correlated with high changes in the immune response elicited against the infective agent. Protected mice showed a pro-inflammatory profile against parasite antigens, showing an IL-12-dependent production of IFN-γ, whereas unvaccinated controls present parasite-specific IL10- and IL-4-driven responses. It was concluded that the induction of cellular inflammatory responses against the LiHyD was able to change the immune response against the parasite.

In the present study, the involvement of the $CD4^+$ and $CD8^+$ T cells in the IFN-γ production was evaluated using monoclonal antibodies in the *in vitro* cultures, a technical protocol previously described by us (12, 16, 24, 39) and others (43, 44). This strategy has permitted to evaluate the contribution of both $CD4^+$ and $CD8^+$ T cells in the production of this cytokine in experimental vaccine models, based on the block antigen presentation. Data indicated here shown that $CD4^+$ T cells were the main source of IFN-γ in the vaccinated and infected mice, as the *in vitro* deactivation of these cells using the anti-CD4 monoclonal antibody significantly abrogated the parasite-dependent production of this cytokine. In agreement, $CD8^+$ T cells

also contribute, although in a less extension, to the production of parasite-specific IFN-γ-mediated response.

Our results also showed that the spleen cells from protected mice produced higher levels of parasite-specific GM-CSF than controls, a cytokine related with macrophage activation and related to protection against different *Leishmania* species in murine models (12, 38, 45, 46). Macrophage activation was also demonstrated by the *in vitro* production of nitrites after stimulation with the recombinant protein and total parasite extracts. According to these data, it could be speculated a possible activation of anti-*Leishmania* effector mechanisms mediated by IFN-γ production, as previously described by others (39, 47, 48), mainly the induction of the synthesis of the NO effector molecule.

Visceralizing and noncuring forms of leishmaniasis have been evaluated on extensive works in BALB/c mice, and disease progression has been generally thought to be associated with a Th2-type response and, in particular, with an early and sustained production of IL-4. Indeed, elevated levels of IL-4, IL-10 and/or IL-13 have been associated with VL progression (49, 50). Many authors have also related the induction of IL-4-dependent production of IgG1 antibodies, to be associated with the disease progression due to different *Leishmania* species, such as *L. amazonensis* (38, 51) and *L. infantum* (12, 16, 25, 39). In this context, a high production of IL-4 could induce to high levels of parasite-specific IgG1 isotype antibodies in the infected animals, this being an indicator of the polarization of Th2 response. On the other hand, IFN-γ has been implicated with the switch of antibody isotype on BALB/c mice to the IgG2a production. In consequence, high levels of this cytokine can induce a higher production of parasite-specific IgG2a isotype antibodies, being this humoral response an indicator of the development of a Th1 immune response in the animals (12, 25, 30).

Recombinant protein-based vaccines, although offering advantages in terms of safety and production costs, must be supplemented with immune adjuvants, to improve their immunogenicity (Cerpa-Cruz *et al.*, 2013). This situation has been observed in several studies that evaluate recombinant proteins, also considered effective vaccine candidates to protect against tegumentary and VL, but whose the association of an immune adjuvant is also considered critical to vaccine efficacy (16, 25, 29, 30, 38, 40, 52). In this context, saponins are natural glycosides derived from steroid or triterpene, which exhibit distinct biological and pharmacological activities. Notably, they can activate the mammalian immune system, which has led to their use as immune adjuvants in vaccines. Their unique capacity to stimulate both the Th1 immune response and the production of cytotoxic T cells makes the saponins ideal for use in vaccine compositions to protect against intracellular pathogens, as is the case of the *Leishmania* parasite (53, 54). Although they are considered toxic for use in humans, due to the possibility to cause local and granulomatous reactions, haemolysis and local pain (55); they are one of the few products authorized to use in dogs, being the adjuvant employed in a commercial Brazilian vaccine to protect against canine VL, namely Leish-Tec® (56).

In the present study, vaccinated mice were able to down-regulate the *Leishmania*-specific IL-10-mediated responses generated in the control nonprotected mice. The immuno-modulatory effects of IL-10, making macrophages unresponsive to IFN-γ, is another determinant factor for disease progression caused by viscerotropic *Leishmania* species (57–59). This would imply that the changes observed in the present work regarding the balance between IFN-γ and IL-10 during infection in protected vs. nonprotected mice are particularly important for the VL control as previously reported (60). Overall, our results validate the possibility to employ LiHyD, a *Leishmania*-specific protein, as a vaccine candidate against VL.

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DECLARATION OF INTEREST

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

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