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Immunogenicity and protective efficacy of a new *Leishmania* hypothetical protein applied as a DNA vaccine or in a recombinant form against *Leishmania infantum* infection

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ABSTRACT

Vaccination is one the most important strategies for the prevention of visceral leishmaniasis (VL). In the current study, a new *Leishmania* hypothetical protein, LiHyP, which was previously showed as antigenic in an immunoproteomic search in canine VL, was evaluated regarding its immunogenicity and protective efficacy against *Leishmania infantum* infection. The effects of the immunization using LiHyP were evaluated when administered as a DNA plasmid (DNA LiHyP) or recombinant protein (rLiHyP) associated with saponin. The immunity elicited by both vaccination regimens reduced the parasitism in liver, spleen, bone marrow and draining lymph nodes, being associated with high levels of IFN-γ, IL-12, GM-CSF, and specific IgG2a antibody, besides low production of IL-4, IL-10, and protein and parasite-specific IgG1 antibodies. $CD4^+$ T cells contributed more significantly to IFN-γ production in the rLiHyP/saponin group, while $CD8^+$ T cells were more important in the production of this cytokine in the DNA LiHyP group. In addition, increased IFN-γ secretion, along with low levels of IL-10, were found when PBMCs from treated VL subject and healthy individuals were stimulated with the recombinant protein. In conclusion, when administered either as a DNA plasmid or recombinant protein, LiHyP can direct the immune response towards a Th1 immune profile, protecting animals against *L. infantum* infection; therefore, it can be seen as a promising immunogen against human VL.

1. Introduction

Leishmaniasis is a disease complex that affects more than 12 million people worldwide, with another 350 million at risk of infection by *Leishmania* parasites, and nearly 2 million new cases reported annually (WHO, 2016). Visceral leishmaniasis (VL) is a fatal disease if the diagnosis and treatment are not promptly performed (Roatt et al., 2014). It is caused by *Leishmania infantum* species in the Americas, but is assuming importance in other regions in the world (Portela et al., 2017). The treatment of VL presents problems, since the currently available drugs, *i.e.*, pentavalent antimonials, amphotericin B, miltefosine, and pentamidine can cause elevated toxicity requiring prolonged hospitalization, or present high cost in the case of liposomal formulations (Sundar and Chakravarty, 2013; Sundar and Singh, 2017). As a

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consequence, the development of preventive measures to avoid the spreading of VL is a priority (Martins et al., 2017; Ponte-Sucre et al., 2017; Travi et al., 2018).

The search for new vaccine candidates has been made possible by the understanding of immune mechanisms associated with the protection against *Leishmania* infection (Amit et al., 2017; Barbosa-Santos et al., 2017; Oliveira et al., 2018). Current knowledge has shown a close relation between the development of Th1 cell response and resistance against *L. infantum* infection, an event characterized by predominance of $CD4^+$ and $CD8^+$ T cells producing cytokines such as IFN-γ, IL-12, GM-CSF, among others that induce the nitric oxide production by infected phagocytic cells (Reed et al., 2016; Dias et al., 2018). On the other hand, Th2 response based on the production of the cytokines such as IL-4, IL-10, IL-13, and specific IgG1 antibodies, has been associated with the susceptibility to the infection (Palatnik-de-Sousa, 2008; Srivastava et al., 2016).

By the advancements from recombinant technology, new parasite molecules have been identified. Between them, uncharacterized hypothetical proteins presenting unknown function have been recognized in different *Leishmania* species, such as *L. infantum* (Coelho et al., 2012), *L. amazonensis* (Magalhães et al., 2014), L. donovani (Gupta et al., 2007), and *L. braziliensis* (Duarte et al., 2015). These molecules can be of prime concern understanding functional aspect, their role in pathogenicity, drug resistance, disease control, and parasite intracellular survival. As a consequence, they can prove importance not only for diagnosis but also for vaccine development and drug targets against the disease (Fernandes et al., 2012; Jamal et al., 2017).

The candidates tested as vaccine against VL are incorporated in non-pathogenic live parasites, bacteria, protein-encoding DNA plasmids, and administered as recombinant proteins in mammalian models, which after are challenged with parasite promastigotes (Bruhn et al., 2012; Chhajer and Ali, 2014; Gannavaram et al., 2014; Zahedifard et al., 2014). In studies using murine or canine models, purified proteins are associated with Th1 adjuvants and administered in hosts as vaccine against infection (Dias et al., 2017; Khabazzadeh et al., 2016; Schaut et al., 2016). Adjuvant molecules are necessary to enhance the immunogenicity of antigens, provide antigen-dose sparing to accelerate the immune response, reduce the need for several immunizations, and/or increase the duration of protection (Srivastava et al., 2016). Otherwise, DNA vaccines are also considered, since they present intrinsic adjuvant properties, inducing cell-mediated immune response, and can result in long-lasting immunity (Campos et al., 2015). These vaccines are also simpler and inexpensive to produce than recombinant proteins, and effectively engage both major histocompatibility class (MHC) I and MHC II pathways in the vaccinated hosts, thereby inducing both $CD4^+$ and $CD8^+$ T cells (Amit et al., 2017).

Although several antigens have being tested in mammalian models, such as mice and dogs, no human vaccine is available. In this context, new candidates to be tested as immunogens against human VL are still under investigation. In the present study, a *Leishmania* hypothetical protein, LiHyP (LINJ_35_2700), which was recently showed to be antigenic in the canine VL by an immunoproteomic study (Coelho et al., 2012), was evaluated regarding its immunogenicity and protective efficacy against VL. The effects of the administration of this molecule obtained as a recombinant protein associated with saponin (rLiHyP/saponin) or as a DNA vaccine (DNA LiHyP) in *L. infantum*-infected BALB/c mice was studied, besides immunofluorescence assays were performed to identify LiHyP in this parasite strain. Aiming to develop a future human antileishmanial vaccine, rLiHyP was also used to *in vitro* stimulate the peripheral blood mononuclear cells (PBMCs) collected from VL patients and healthy subjects, when the cytokine profile generated after the stimulus was investigated.

2. Materials and methods

2.1. Ethics statement and blood samples

The present work was approved by the Ethics Committee of Federal University of Minas Gerais (UFMG) with the protocol number CAAE–32343114.9.0000.5149. All subjects received a copy of the study policy before collection of their sample, which was reviewed by an independent person, and all participants gave their consent form in Portuguese. Then, peripheral blood samples were collected by venipuncture of medial vein in heparinized tubes, and they were used to prepare the PBMC cultures. Samples were collected from active VL patients (n = 8, including 5 males and 3 females, with ages ranging from 22 to 54 years) living in an endemic area of disease (Belo Horizonte, Minas Gerais, Brazil). Patients were diagnosed by means of clinical examination and demonstration of *L. infantum* kDNA in bone marrow aspirates by PCR assay. One sample (10 mL) was collected before and other sample (10 mL) was collected six months after the end of the treatment sessions. All patients were submitted to the same therapeutic regimen using pentavalent antimonials (Sanofi Aventis Farmacêutica Ltda., Suzano, São Paulo, Brazil), at a dose of 20 mg Sb^{+5} per kg during 30 days, and none of them suffered from any other infections or had any pre-existing disease. In addition, when all of them had the treatment completed, no parasite DNA was found in aspirates of the spleen and bone marrow, and patients were free of any symptom of VL. Blood samples (10 mL) were also collected from healthy individuals living in an endemic area of disease (n = 8, including 5 males and 3 females, with ages ranging from 25 to 48 years; Belo Horizonte). These subjects did not show clinical signal of disease, and presented negative serology result for leishmaniasis by using the Kalazar Detect™ Test (InBios® International, USA).

2.2. Mice and parasite

BALB/c mice (female, 8 weeks age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, UFMG, and were maintained under specific pathogen-free conditions. The study was approved by the Committee on the Ethical Handling of Research Animals of UFMG, with the protocol number 333/2015. *L. infantum* (MHOM/BR/1970/BH46) strain was used. Parasites were grown at 24 °C in complete Schneider's medium (Sigma-Aldrich, USA), which was composed by the Schneider´s medium plus 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin, at pH 7.4. The soluble *Leishmania* antigenic extract (SLA) was prepared from stationary promastigotes according previously described (Martins et al., 2017).

2.3. Cloning and purifying the recombinant LiHyP protein (rLiHyP)

The LiHyP (LINJ_35_2700) codifying gene (2,004 base pairs) was cloned from *L. infantum* genomic DNA using the following primers: 5´-GGCTGAATTCACCATGGGATGCGAGACGTCGCCGTCT-3´ (*forward*) and 5´-TGATGCGGCCGCTCAGCGTTGCATGGTCTCAC-3´ (*reverse*), and the *EcoRI* and *Not1* restriction enzymes. The DNA fragment was purified and linked into pGEM®-T vector (Promega, USA), and the recombinant plasmid was used to transform *E. coli* XL1-Blue competent cells. DNA fragments obtained from digestion of pGEM-LiHyP plasmid were ligated into a pET28a-TEV vector, and BL21 cells were transformed with 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 rLiHyP (71.5 kDa), cells were induced using 1.0 µM isopropyl-β-D-thiogalactopyranoside (IPTG, Promega, Montreal, Canada), and cultures were incubated for 3 h at 37 °C, at which time cells were ruptured by six cycles of ultrasound of 30 s each (38 MHz), followed by six cycles

of freezing and thawing. After, cell debris were removed by centrifugation, and the recombinant protein (~71.5 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 (NMWL, Millipore, Germany), and further purified on a Superdex™ 200 gel-filtration column (GE Healthcare, USA). Then, the rLiHyP protein was 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).

2.4. Preparation of DNA LiHyP vaccine

To perform the DNA LiHyP vaccine construction, the LiHyP coding sequence was PCR amplified, and the DNA fragment was ligated into the pVAX1 vector (Thermo Fischer Scientific, USA) to obtain the pVAX1-LiHyP plasmid. The insert size and orientation were checked by double digestion using restriction enzymes, and the final plasmid was sequenced. Empty pVAX1 plasmid was used as a vector control. Then, pVAX1 and pVAX1-LiHyP plasmids were purified from transformed *E. coli* DH5α cells using the Qiagen Endofree Plasmid Maxi Kit (Qiagen Ltda., Crawley, UK), with pyrogen-free material, and the final pellet was resuspended in pyrogen-free phosphate-buffered saline (PBS 1x) according to the manufacturer's instructions. Plasmid quality was evaluated by 1% agarose gel electrophoresis, and the ratio between the optical density values obtained at 260 and 280 nm ranged between 1.8 to 2.0.

2.5. Purification of anti-rLiHyP IgG antibody

To obtain the anti-protein IgG antibody, BALB/c mice (n = 6) were immunized subcutaneously in their left hind footpad with 10 μg rLiHyP associated with 10 μg saponin (*Quillaja saponaria* bark saponin, Sigma). Two doses were administered at 2-week interval, and seven days after the last immunization, blood was drawn and serum was collected. Next, 1 mg rLiHyP was covalently bound to a cyanogen bromide (CNBr)-activated Sepharose 4B column (GE Healthcare, USA), which was previously equilibrated with 20 mM phosphate buffer at pH 7.4. Coupling and blocking were carried out according to manufacturer instructions. Then, 2 mL sera pool was passed through protein column, and the specific antibody was eluted by using 0.1 M glycine, pH 2.8. The preparation was equilibrated to pH 7.5 with 1 M Tris/HCl buffer, and the solution of the antibody was restored to the original volume of the pooled sera (2 mL). An enzyme linked immunosorbent assay (ELISA) and sodium dodecyl sulfate-12% polyacrylamide gel (SDS-12% PAGE) were performed to verify the reactivity and quality of the purified antibody, respectively (data not shown).

2.6. Visualization of LiHyP in L. infantum parasites

Immunofluorescence assay in *L. infantum* promastigotes was performed as described elsewhere (Faria et al., 2016). Briefly, parasites were double-labeled with the anti-rLiHyP IgG antibody (at a 1:100 dilution), and incubated with a secondary antibody conjugated to an Alexa Fluor® 488 Goat anti-mouse IgG antibody (1:1000; Life Technologies, USA) and Hoechst 33,258 (1 μg/mL, Life Technologies), for 1 h at room temperature. Next, samples were washed 3 times in PBS 1x for 10 min, and mounted with hydromount (Electron Microscopy Sciences). 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), with an oil 63 times 1.4 NA objective lens, and a GaAsP (Gallium arsenide phosphide) detector apparatus. Samples were excited at 405 nm and observed at 415–480 nm to detect Hoechst, and at 510–540 nm to detect Alexa 488. The Zeiss Efficient Navigation (ZEN) software was used for orthogonal projections (XY, XZ, YZ), and image adjustments were performed according to the negative controls.

2.7. Western-blotting assay

For immunoblottings, rLiHyP (10 μg) was submitted to a SDS-12% PAGE and blotted onto a nitrocellulose membrane (0.2 μm pore size, Sigma-Aldrich, USA). Then, membranes were blocked with a solution composed by PBS 1x 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 naïve, DNA pVAX1-LiHyP or rLiHyP/saponin-vaccinated mice sera pool (all 1:100 diluted in PBS-T). Membranes were washed with PBS-T and an anti-mouse IgG horseradish-peroxidase conjugated antibody (diluted 1:10,000 in PBS-T, Sigma-Aldrich, USA) was added, at which time a new incubation was developed for 1 h at 37 °C. Reactions were developed by adding 12.5 mg chloronaphtol, 25.0 mg diaminobenzidine, and 20 μL H_2O_2 30 vol., and stopped by adding 10 mL distilled water.

2.8. Vaccine experiments: immunization, parasite challenge and parasite load

BALB/c mice (n = 16 per group) were immunized with either three subcutaneous doses of rLiHyP (10 μg) plus saponin (10 μg) two weeks apart or by an intramuscular route using 50 μg DNA pVAX1-LiHyP diluted in 50 μL of sterile PBS 1x, and boosted two weeks later by a second injection. Control groups included the empty pVAX vector, saponin and saline. Half of the animals (n = 8 per group) were euthanized 30 days after the last vaccine dose, when their sera samples and spleen were collected to perform immunological analyses. In the remaining animals (n = 8 per group), a subcutaneous injection using 10^7 *L. infantum* stationary promastigotes was performed and, 60 days after infection, animals were euthanized and liver, spleen, bone marrow (BM), and paws' draining lymph nodes (dLN) were collected to evaluate the parasite burden, through a limiting-dilution technique or RT-PCR assay. Briefly, organs were homogenized using a glass tissue grinder in sterile PBS 1x, and tissue debris were removed by centrifugation at 150 × *g*. Cells were concentrated and pellets were resuspended in 1 mL of Schneider's insect medium supplemented with 20% FBS. Then, 220 μL was plated onto 96-well flat-bottom microtiter plates (Nunc, Nunclon®, Roskilde, Denmark) and diluted in log-fold serial dilutions in Schneider's medium with a 10^{-1} to 10^{-12} dilution. Each sample was plated in triplicate, at 24 °C, and read seven days after the beginning of the culture. Results were expressed as the negative log of the titer adjusted per milligram of organ. The splenic parasite load was also evaluated by RT-PCR technique (Duarte et al., 2016). Parasite quantification for each sample was calculated by interpolation from the standard curve, performed in duplicate, and converted into number of parasites per nucleated cells (multiplied by one thousand to facilitate visualization).

2.9. Cellular response

2.9.1. Capture ELISA

Splenocytes (5 × 10^6 cells) of the animals were collected 30 days after the last immunization and 60 days post-infection (n = 8 per group in each), and they were incubated in complete RPMI medium (medium, background control), which was composed by the medium plus 20% FBS, 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, at pH 7.4, or stimulated with rLiHyP or *L. infantum* SLA (10 and 25 μg/mL, respectively) for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70, and GM-CSF levels were assessed in the culture supernatant by an ELISA capture (BD OptEIA TM set mouse, Pharmingen®, San Diego, CA, USA), following manufacturer's instructions. In addition, the involvement of $CD4^+$ and $CD8^+$ T cells in the IFN-γ

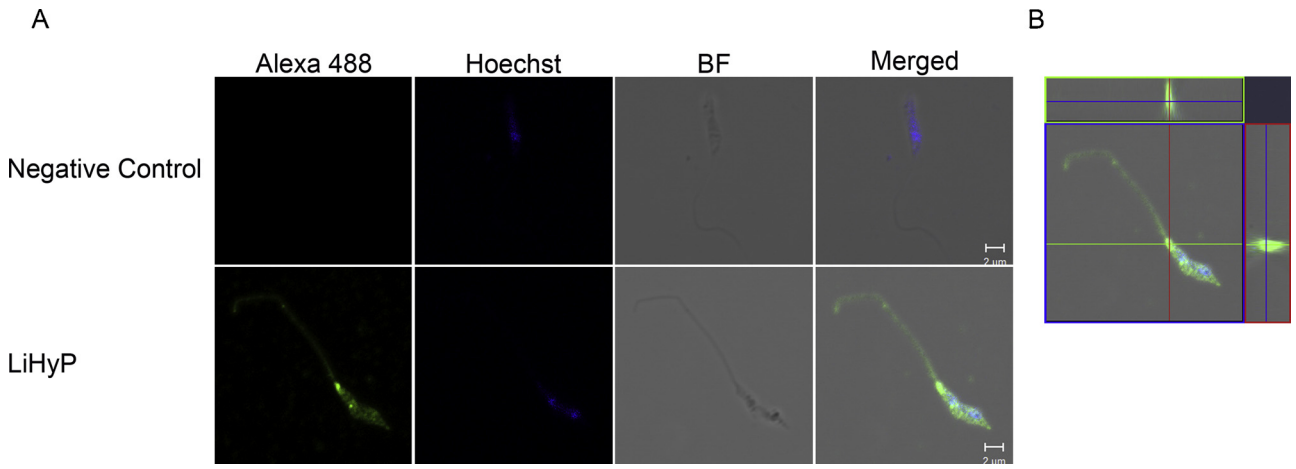


Fig. 1. Cell location of LiHyP protein. Confocal fluorescence images were obtained using a negative control (upper panel), and the location of LiHyP in *L. infantum* stationary promastigotes is shown (green color) stained with Hoechst (blue color, lower panel). BF represents the bright field. The merged image shows the location of LiHyP (A). The three-dimensional reconstruction is shown at the top, and sections are shown at the right for each image (B). Images are representative of two independent experiments. Scale bar: 2 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

production was evaluated in protein or SLA-stimulated cultures, which 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 µg/mL. Appropriate isotype-matched controls (no azide/low endotoxin™), rat IgG2a (R35-95), and rat IgG2b (95-1) were used (Pharmingen®, USA).

2.9.2. Flow cytometry

A flow cytometry assay was also performed, 30 days after the last immunization and 60 days post-infection (n = 8 per group in each), in order to evaluate the IFN-γ, IL-2, TNF-α and IL-10-producing $CD4^+$ and $CD8^+$ T cells frequency. For this, spleen cells (5×10^6 cells) were incubated in complete RPMI 1640 medium in polypropylene tubes (Pharmingen®), and they were unstimulated (medium) or stimulated with *L. infantum* SLA (25 µg/mL) for 48 h at 37 °C in 5% CO_2. Then, cells were incubated with phorbol myristate acetate (PMA, 25 ng/mL) and ionomycin (1 µg/mL), which were diluted in complete RPMI 1640 medium, and cultures again were incubated. The IFN-γ, IL-2, TNF-α and IL-10-producing $CD4^+$ and $CD8^+$ T cells frequency was evaluated following an analysis based on their relative flow cytometry size (forward laser scatter – FSC) and granularity (side laser scatter – SSC) graphs. After the selection of the interest region R1 containing FSCLow and SSCLow phenotype cells, graphs of density plot distribution of CD4/FL1 or CD8/FL1 *versus* IFN-γ/$FL2^+$, IL-2/$FL2^+$, TNF-α/$FL2^+$, and IL-10/$FL2^+$ cells were performed to determine the IFN-γ^+, IL-2^+, TNF-α^+, and IL-10^+ T cells frequency in the stimulated cultures. Results were expressed as indexes, which were calculated by the ratio between the cytokine-producing $CD4^+$ and $CD8^+$ T cells percentage *versus* the values obtained in the unstimulated cultures (SLA/CC ratio).

2.10. Nitrite secretion and humoral response

Sixty days post-infection, cell supernatant was also used to evaluate the nitrite production by Griess reaction. For this, 100 µL of protein or parasite-stimulated culture supernatants were mixed with an equal volume of Griess reagent (Sigma-Aldrich, USA). After 30-min incubation at room temperature, nitrite concentration was calculated using a standard curve of known concentrations, and the results were expressed as µM (Green et al., 1982). To evaluate the humoral response before and after infection, serum samples were collected 30 days after the last immunization and 60 days post-infection. The rLiHyP and parasite-specific IgG1 and IgG2a antibody levels were evaluated by ELISA (Martins et al., 2017). Briefly, the recombinant protein and SLA (0.5 and 1.0 µg per well, respectively) were added as antigen in the plates, 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 1:5,000 or 1:7,500 dilutions, which were performed in PBS-T, respectively.

2.11. Human specific-rLiHyP cytokine assay

The rLiHyP-specific IFN-γ and IL-10 production was evaluated in human PBMCs purified from blood samples collected from healthy subjects and active or treated VL patients. Cells (1×10^7) were cultured in 48-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA), with each well containing RPMI 1640 medium (background control) with or without rLiHyP or SLA (10 or 25 µg/mL, respectively), during 5 days at 37 °C in 5% CO_2. After, supernatants were collected, and IFN-γ and IL-10 levels were measured using commercially available ELISA kits (Human IFN-γ and IL-10 ELISA, BD Biosciences, USA), according to manufacturer's instructions.

2.12. Statistical analysis

The results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed by 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 comparisons between groups. Differences were considered significant with $P < 0.05$. The vaccination experiments were repeated and the results were similar between them. Data showed in this study are representative of the first experiment.

3. Results

3.1. Cell localization and immunoblotting using anti-rLiHyP IgG antibody

Aiming to identify LiHyP in *L. infantum*, an immunofluorescence experiment was performed using parasite promastigotes and an anti-rLiHyP IgG antibody. Results by confocal microscopy showed that the protein is located in the parasite cytoplasm (Fig. 1). Immunoblottings were also performed using the anti-protein antibody, and a specific reactivity was found when the purified recombinant protein was present in the membranes. In addition, DNA LiHyP or rLiHyP/saponin-immunized mice sera were also reactive with the recombinant protein, showing the expression of this antigen in the DNA plasmid-vaccinated animals (Fig. 2).

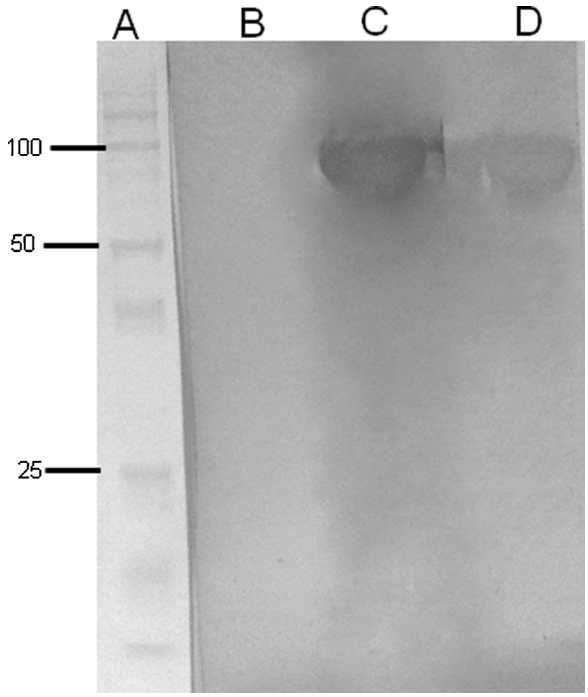


Fig. 2. Western-blotting assays using anti-rLiHyP IgG antibody. Immunoblottings were performed using 10 µg rLiHyP (71.5 kDa), which were electrophoresed in SDS-12% PAGE gel and stained with colloidal Coomassie Brilliant Blue G-250. A low range protein ladder standard (Invitrogen™, Life Technologies, USA) was used (lane A). Non-immunized and non-infected mice (n = 6) sera pool (lane B), as well as sera from rLiHyP/saponin- and DNA LiHyP-vaccinated animals (n = 6 in each, lanes C and D, respectively) were employed in the analyses. Sera samples were prepared and pooled at a 1:200 dilution. Results were derived from three independent experiments, and one representative preparation was shown in this study.

3.2. Immunogenicity of DNA LiHyP and rLiHyP/saponin vaccine in BALB/c mice, before and after L. infantum infection

The immunogenicity of LiHyP administered in a recombinant form or as DNA vaccine was evaluated in BALB/c mice before and after infection. Regarding the cellular response before infection, significantly higher levels of IFN-γ, IL-12 and GM-CSF were found in the DNA LiHyP- and rLiHyP/saponin-vaccinated animals groups, when compared to results found in the saline, saponin and pVAX groups, which were used as controls (Fig. 3A). Concerning the humoral response, mice vaccinated with the two LiHyP vaccines showed higher anti-protein and anti-parasite IgG2a/IgG1 ratios in comparison to control groups; showing a Th1 response in the LiHyP-vaccinated animals (Fig. 3B). The rLiHyP/saponin-vaccinated mice showed higher levels of Th1 cytokines and higher IgG2a/IgG1 ratio, when compared to the DNA LiHyP group, but this difference did not reach statistical significance.

The immunogenicity of LiHyP was then evaluated after challenge infection. When compared to controls, DNA LiHyP and rLiHyP/saponin-vaccinated animals showed a specific Th1 response, as evidenced by significantly higher levels of rLiHyP and SLA-specific IFN-γ, IL-12 and GM-CSF in the culture supernatant, contrasting to the lower levels of IL-4 and IL-10 (Fig. 4A). In addition, ratios between the IFN-γ/IL-10 and IL-12/IL-10 levels were calculated and are also showed (Fig. 4B). The humoral response profile was also similar to the one shown before infection in the vaccinated animals, since higher IgG2a/IgG1 ratios were found in the DNA LiHyP and rLiHyP/saponin groups (Fig. 4C). Nitrite production was evaluated as a macrophage's activation marker, and there were significantly higher levels of this molecule in the DNA LiHyP and rLiHyP/saponin groups, when compared to the controls (Fig. 4D). Again, no significant difference was found between the groups

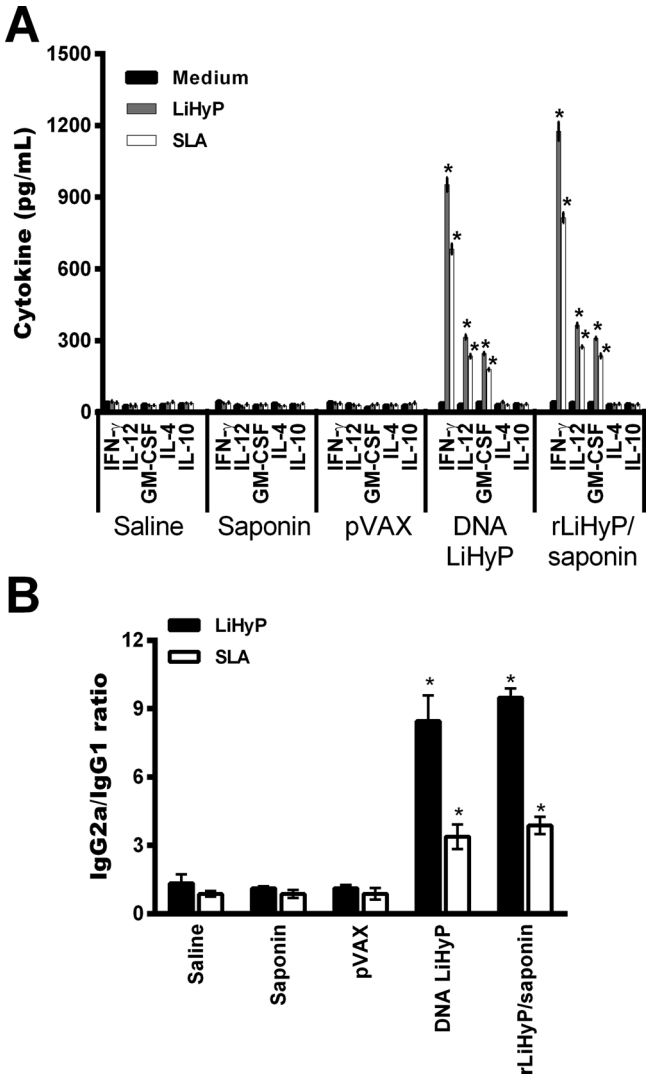


Fig. 3. Cellular and humoral responses in LiHyP-vaccinated mice. Thirty days after the last immunization, spleen and sera samples were obtained of mice (n = 8 per group). Splenocytes were unstimulated (medium) or separately stimulated with rLiHyP or *L. infantum* SLA (10 and 25 µg/mL, respectively), for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70, and GM-CSF were measured in culture supernatants by a capture ELISA (A). The anti-rLiHyP and anti-SLA IgG2a and IgG1 isotypes antibody levels were obtained and ratios between IgG2a/IgG1 values were calculated, and are shown (B). Bars represent the mean ± standard deviation of the groups. *indicates statistically significant difference in relation to the saline, saponin and pVAX groups ($P < 0.0001$).

immunized with recombinant protein or DNA after infection. Together these results indicate the induction of a homologous immune response by the two vaccines employing LiHyP.

The participation of $CD4^+$ and $CD8^+$ T cells in IFN-γ production in the DNA LiHyP and rLiHyP/saponin groups was also evaluated. There was significant reduction in the production of IFN-γ when anti-CD4 and anti-CD8 antibodies were added in the cultures. Noteworthy, more significant reductions were found when anti-CD8 antibody was added into the cell cultures of DNA LiHyP group (Fig. 5A), while more significant reductions in the IFN-γ production were found when anti-CD4 antibody was added in the cell culture of rLiHyP/saponin group (Fig. 5B). We further analyzed the cytokine-producing splenic T-cell phenotype by flow cytometry after immunization using DNA or recombinant protein and *L. infantum* infection. The immunization with DNA LiHyP or rLiHyP/saponin induced an increase in the percentage of IFN-γ, IL-2 and TNF-α^+-producing $CD4^+$ and $CD8^+$ T cells in both

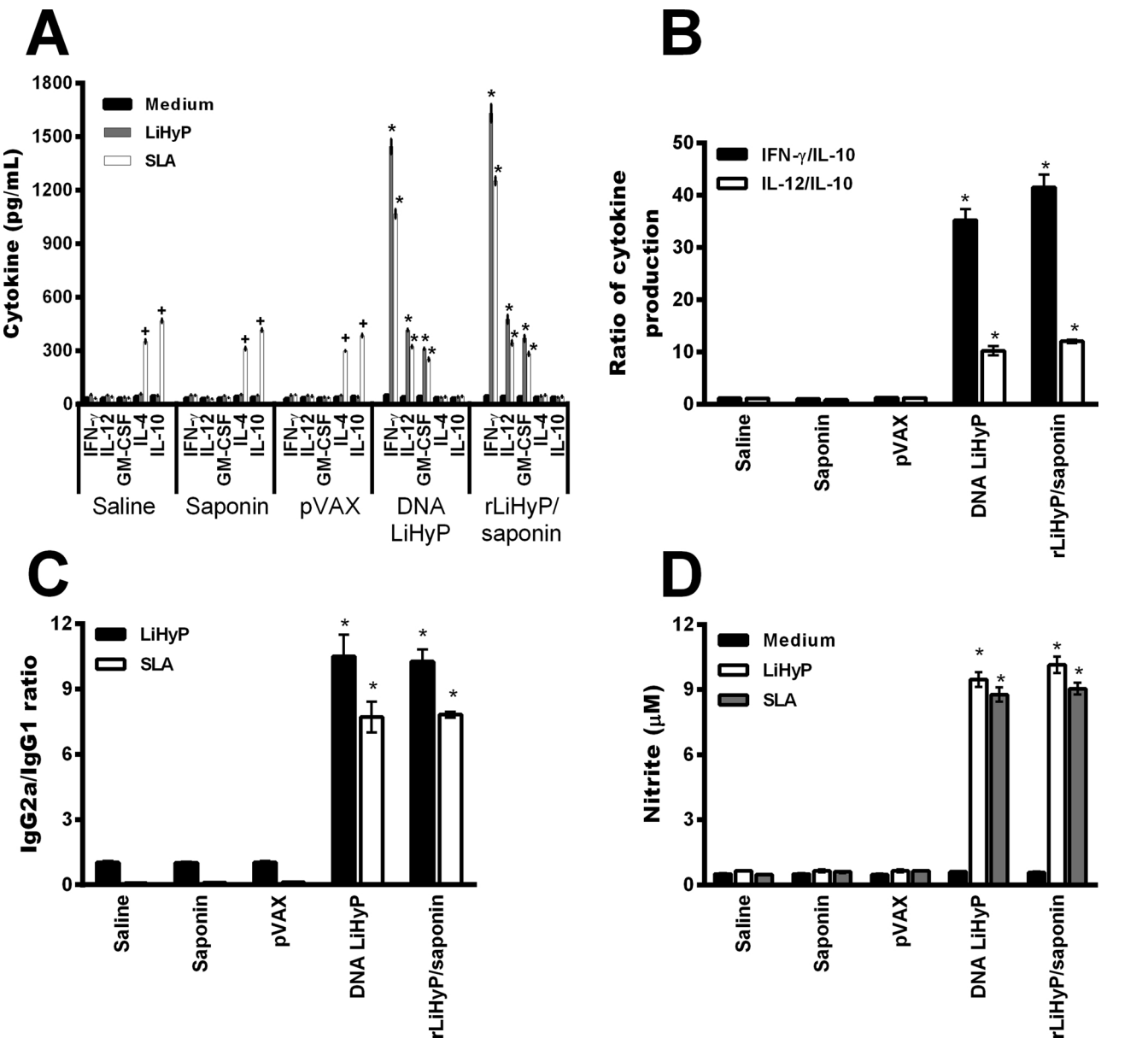


Fig. 4. Immune response induced after *Leishmania infantum* infection. Sixty days after infection, spleen and sera samples were obtained of mice (n = 8 per group), and splenocytes were unstimulated (medium) or stimulated with rLiHyP or SLA (10 and 25 μg/mL, respectively), for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70, and GM-CSF were measured in culture supernatants by capture ELISA (A). The ratios between the IFN-γ/IL-10 and IL-12/IL-10 levels were calculated and are shown (B). The anti-rLiHyP and anti-SLA IgG2a and IgG1 isotypes levels were obtained and ratios between IgG2a/IgG1 values were also calculated, and they are shown (C). Employing the cell supernatant used to evaluate cytokine profile, the anti-protein and antileishmanial nitrite production was analyzed by the Griess reaction (D). Bars represent the mean ± standard deviation of the groups. *indicates statistically significant difference in relation to the saline, saponin and pVAX groups ($P < 0.0001$). +indicates statistically significant difference in relation to the DNA LiHyP and rLiHyP/saponin groups ($P < 0.0001$).

conditions (Fig. 6). In addition, vaccinated mice presented lower levels of antileishmanial IL-10$^+$-producing T cells when compared to controls; confirming the induction of Th1 immune response in mice vaccinated with LiHyP by two independent vaccine strategies.

3.3. Protection against L. infantum infection

The protective efficacy of LiHyP vaccines was evaluated against *L. infantum* infection. DNA LiHyP or rLiHyP/saponin-immunized mice and later infected showed significant reductions in the parasite load in all evaluated organs, *i.e.*, liver (Fig. 7A), spleen (Fig. 7B), BM (Fig. 7C) and dLN (Fig. 7D), when compared to controls. No significant difference was found among the saline, saponin and pVAX groups, or between the DNA LiHyP and rLiHyP/saponin groups. Otherwise, when the recombinant protein was administered without association of saponin, no significant difference was found when compared to results obtained in the controls (data not shown). The parasite load was also evaluated by RT-PCR technique. The immunization using DNA LiHyP or rLiHyP/saponin reduced significantly the splenic parasite load when compared to saline, saponin, and pVAX groups (Fig. 8).

3.4. Cytokine profile in PBMCs from VL patients and healthy subjects

To evaluate the immunogenicity of rLiHyP using human cells from VL patients and healthy subjects, IFN-γ and IL-10 levels were measured in the cell supernatant of protein or parasite-stimulated PBMC cultures. In the results using rLiHyP as a stimulus, IFN-γ and IL-10 levels were 755.4 ± 22.3 and 65.6 ± 9.9 pg/mL, respectively, in treated VL

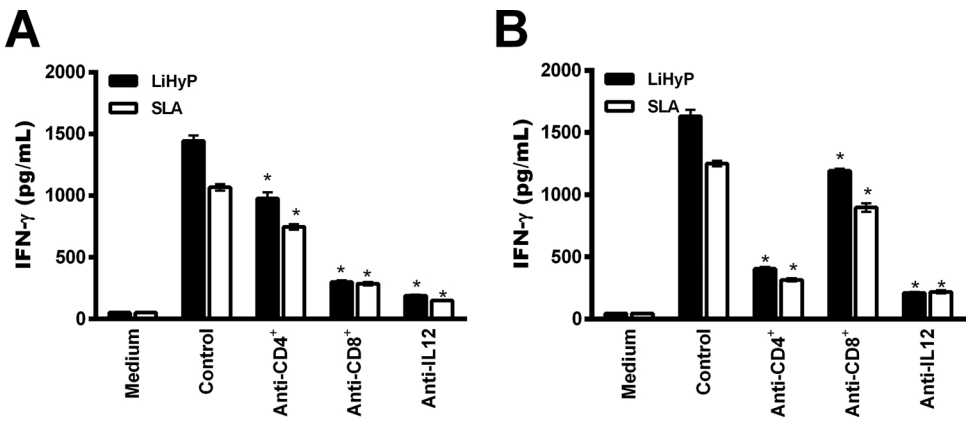


Fig. 5. Involvement of T cell subtypes in IFN-γ production after infection. Anti-CD4 and anti-CD8 monoclonal antibodies were added to the splenocyte cultures of the infected and DNA LiHyP- and rLiHyP/saponin-vaccinated animals (A and B, respectively), and IFN-γ production was measured in the cell supernatants after incubation for 48 h at 37 °C in 5% CO_2. rLiHyP and SLA were used as stimuli (10 and 25 μg/mL, 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. *indicates statistically significant difference in relation to the control group ($P < 0.0001$).

patients; and from 201.7 ± 19.4 and 79.8 ± 13.4 pg/mL, respectively, in patients with the active disease (Table 1). Using SLA as a stimulus, IFN-γ and IL-10 levels in treated patients were 146.6 ± 22.5 and 99.2 ± 11.2 pg/mL, respectively, whereas in active VL patients these values were 68.8 ± 7.8 and 582.2 ± 34.4 pg/mL, respectively. PBMCs collected from healthy subjects showed a predominance of IFN-γ production in comparison to the lower IL-10 levels encountered in the cell supernatant (Table 1).

4. Discussion

In spite of available information regarding life stages of Leishmania, species-specific diversity, heterogeneity of the immune response, among others, successful vaccine development against human disease remains a challenge. Parasite proteins have been tested in immunization protocols, such as cysteine-proteinases (Das and Ali, 2014), KMP-11 (Dikhit et al., 2017), LiHyp1 (Martins et al., 2013); LiHyD (Lage et al., 2015), among others. Although some of these molecules have showed satisfactory results in the protection against murine leishmaniasis, they have either failed to prevent natural infection or offered no immunogenicity or protection in other mammalian models. As a consequence, the search continues to identify new molecules that could also induces immune response in others mammalians than mice and protect against *Leishmania* infection.

In a previous immunoproteomics study, *L. infantum* proteins expressed in the promastigotes and amastigotes of the parasites were identified by antibodies in active VL dog sera (Coelho et al., 2012). The fact that antibodies in infected animal sera had recognized these antigens indicates that they are expressed during the active disease and are relevant to the infected host's immune system (Fernandes et al. 2012). Here, one of these proteins, LiHyP, which was identified in both *L. infantum* promastigote and amastigote extracts was cloned and evaluated by two different immunization strategies of immunization to protect against VL.

We chose to test LiHyP as a DNA vaccine since plasmid-using vaccination has been probed as an alternative to attain protection against diseases requiring cellular immunity, such as leishmaniasis (Maspi et al., 2017; Rafati et al., 2006). The use of adjuvants and/or delivery systems is necessary for almost any candidate particularly recombinant protein-containing vaccine against *Leishmania* (Badiee et al. 2013; Crowther 2013). Proteins such as LeIF (Coler et al., 2002; Maspi et al., 2016), LmSTI1 (Campos-Neto et al., 2001; Fujiwara et al., 2005), cysteine proteinases (Ahmed et al., 2009; Pascalis et al., 2003), LACK (Ghaffarifar et al., 2013; Melby et al., 2001), TSA (Coler et al., 2007; Tabatabaie et al., 2014), and A2 (Coelho et al., 2003; Zanin et al., 2007), among others, when tested in a recombinant form to protect against the disease have demonstrated low and/or limited protective efficacy without the association of these immune compounds. In contrast, when they are administered as DNA vaccines, a stronger immune response and long-lasting protection have been reached (Zanin et al., 2007).

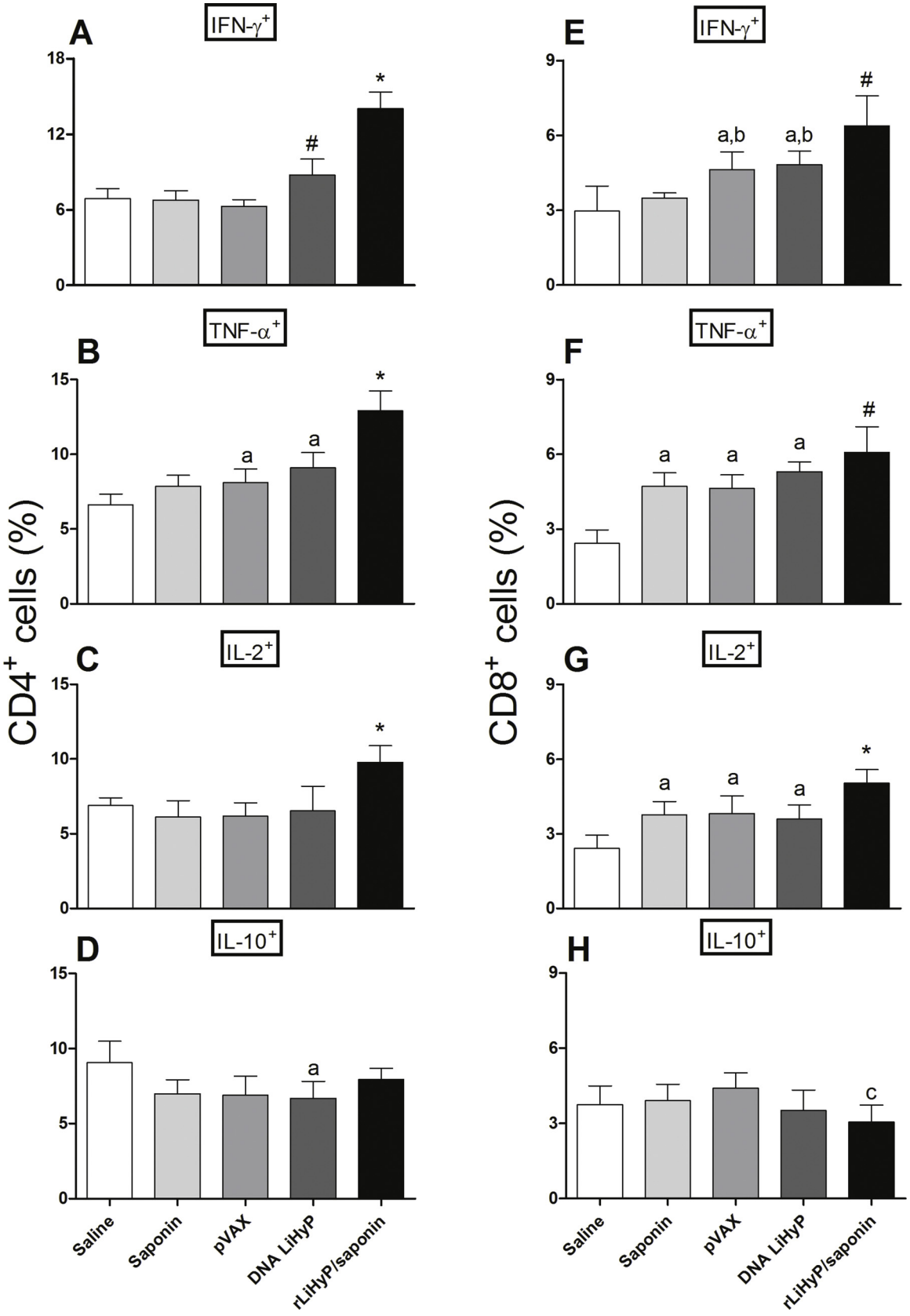
This fact is derived from the ability of DNA plasmids to engage both MHC-I and MHC-II pathways, inducing both T cell subtypes (Kumar and Samant, 2016). The immune stimulatory CpG ODNs sequences present in the plasmid lead to activation of the innate immunity, which is characterized by up-regulation of co-stimulatory molecules, production of pro-inflammatory cytokines such as IFN-γ and IL-12, and oxidant radicals by antigen-presenting cells explaining, at least in part, the efficacy of these compounds to induce the development of a protective response when incorporated with a parasite protein (Zimmermann et al., 2008; Mazumder et al., 2011).

The protective immunity against *L. donovani* and *L. infantum* is dependent of IFN-γ production, a marker of Th1 response (Gannavaram et al., 2016; Singh et al., 2012). Thus, antigens capable of induce the Th1 response can be considered as promising to protect against infection (Costa et al., 2015). In our study, we evaluated the immunogenicity induced by administration of DNA LiHyP and rLiHyP/saponin before and after *L. infantum* infection. Both immunized groups developed a specific Th1 immunity, which was characterized by higher levels of IFN-γ, IL-12 and GM-CSF along with lower production of IL-4 and IL-10.

The contribution of $CD4^+$ and $CD8^+$ T cells in the IFN-γ production was also evaluated in the protected animals, and results showed that the rLiHyP/saponin composition was able to stimulate the production of this cytokine by $CD4^+$ T cell pathway, while using DNA LiHyP the IFN-γ production was induced mainly by $CD8^+$ T cells. In fact, recombinant protein-based vaccines delivered by subcutaneous immunization are processed as extracellular antigens and associated to MHC class II molecules to be presented to $CD4^+$ T cells (Gurunathan et al., 1998). On the other hand, intracellularly processed antigens, such as proteins synthesized in cell cytosol, as those present in DNA plasmids, are presented to the host immune system in a way similar to that of a natural viral infection, that is, by MHC class I pathway for $CD8^+$ T-cell response (Bivas-Benita et al., 2005). In this context, results obtained here regarding the IFN-γ production are expected from an immunological point-of-view (Ribeiro et al., 2018).

Since the killing of parasites by Leishmania-infected macrophages in mammalian hosts is essential to achieve cure against VL (Agallou et al., 2014), in our study, we have measured the production of GM-CSF and IL-12 as macrophages' activation markers. Results showed an increased production of these molecules in the rLiHyP/saponin- and DNA LiHyP-vaccinated animals, then suggesting the induction of an antileishmanial mechanism based on the macrophage´s activity in the infected and immunized hosts, then arguing that the Leishmania killing was mediated through a NO pathway, induced by Th1 cytokines, mainly IFN-γ, in the vaccinated animals.

In agreement with the role of Th1 response in the leishmaniasis control, significant reductions in the parasite load were observed in DNA LiHyP- or rLiHyP/saponin-immunized mice. Although pVAX



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group has exhibited a reduction in their parasitism, no significant difference was found when compared to the saline and saponin groups, indicating then the necessity of the incorporation of LiHyP in the plasmid to induce protection against *L. infantum*. Like a consequence, and due to the aspects related with the production of immunogens, such as cost of production, stability, number of doses, association of

Fig. 6. Percentage of intracytoplasmic cytokine-producing T cells. BALB/c mice received saline (Saline: white rectangle) or were immunized with saponin (light grey rectangle), pVAX (medium grey rectangle), DNA LiHyP (dark grey rectangle) or rLiHyP/saponin (black rectangle), and later challenged with *L. infantum* promastigotes. Sixty days after splenocytes were cultured and *in vitro* stimulated with *L. infantum* SLA. IFN-γ, TNF-α, IL-2, and IL-10-producing $CD4^+$ and $CD8^+$ T cells percentage was calculated by means of the ratio between values obtained in the stimulated cultures *versus* those from the unstimulated cultures. Results are expressed by percentage of $CD4^+$ (A, B, C and D) or $CD8^+$ (E, F, G and H) T cells producing IFN-γ, TNF-α, IL-2 and IL-10 cytokines in the SLA-stimulated cultures. Bars represent the mean plus standard deviation of the groups. The letters *a, b* and *c* indicate significant statistically differences in relation to the saline, saponin and pVAX groups, respectively ($P < 0.05$). #indicates significant statistically difference in relation to the saline, saponin and pVAX groups ($P < 0.05$). *indicates significant statistically in relation to the saline, saponin, pVAX and rLiHyP/saponin groups ($P < 0.05$).

adjuvants, among others; one could speculate that LiHyP could be employed as a candidate to be administered as a DNA vaccine, then adding new data in the literature on the efficacy of these vaccines against VL.

It is known that PBMCs from active VL patients do not produce pro-inflammatory cytokines such as IFN-γ and IL-12 in response to *Leishmania* antigenic extracts (Gidwani et al., 2009; Kumar and Nylén, 2012; Portela et al., 2017). However, this cytokine response can be detected few months after successful completion of the treatment against the disease, as well as in individuals with subclinical or asymptomatic infection (Carvalho et al., 1992; Dayakar et al., 2016; Dias et al., 2017; Peruhype-Magalhães et al., 2005; Singh & Sundar, 2014). In our study, rLiHyP induced higher IFN-γ and lower IL-10 production in PBMC from treated patients, as well as higher levels of IFN-γ in PBMCs from active VL patients and healthy subjects, when compared to results obtained using *L. infantum* SLA. These results suggest the immunogenic potential of this protein to be administered in humans, highlighting the possibility to use it as a future immunogen against VL in this mammalian host.

The parasite elimination complete seems to be an unrealistic goal against *Leishmania* infection (Aguiar-Soares et al., 2014). In our study, saline, saponin and pVAX groups showed high parasitism in their liver, spleen, BM and dLN. Conversely, DNA LiHyP and rLiHyP/saponin-vaccinated mice presented significant reductions in the parasite load in all organs, but not complete elimination of the parasites. Some studies have demonstrated that a low number of parasites can persist in cured hosts, allowing them to maintain the immune system continuously stimulated by means of active immunological memory (Santos-Gomes et al., 2014; Selvapandiyan et al., 2012). In this context, LiHyP, when applied either in a recombinant form or as DNA vaccine, could represent a potential candidate against *L. infantum*. A word of caution is

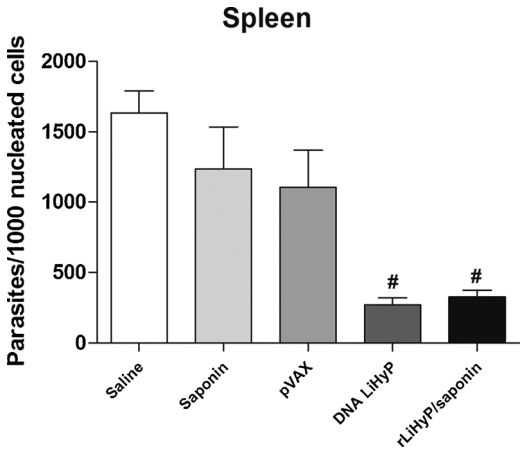

Fig. 8. Parasite burden evaluated by RT-PCR technique. Mice were inoculated with saline or immunized with saponin, DNA pVAX, DNA LiHyP or rLiHyP/saponin, and were challenged with *L. infantum* promastigotes. Then, 60 days after their spleens were collected and parasite load was evaluated by RT-PCR technique. Results were expressed as the number of parasites per 1000 nucleated cells. Bars indicate the mean plus standard deviation of the groups. #indicates statistically significant difference in relation to the saline, saponin and pVAX groups ($P < 0.05$).

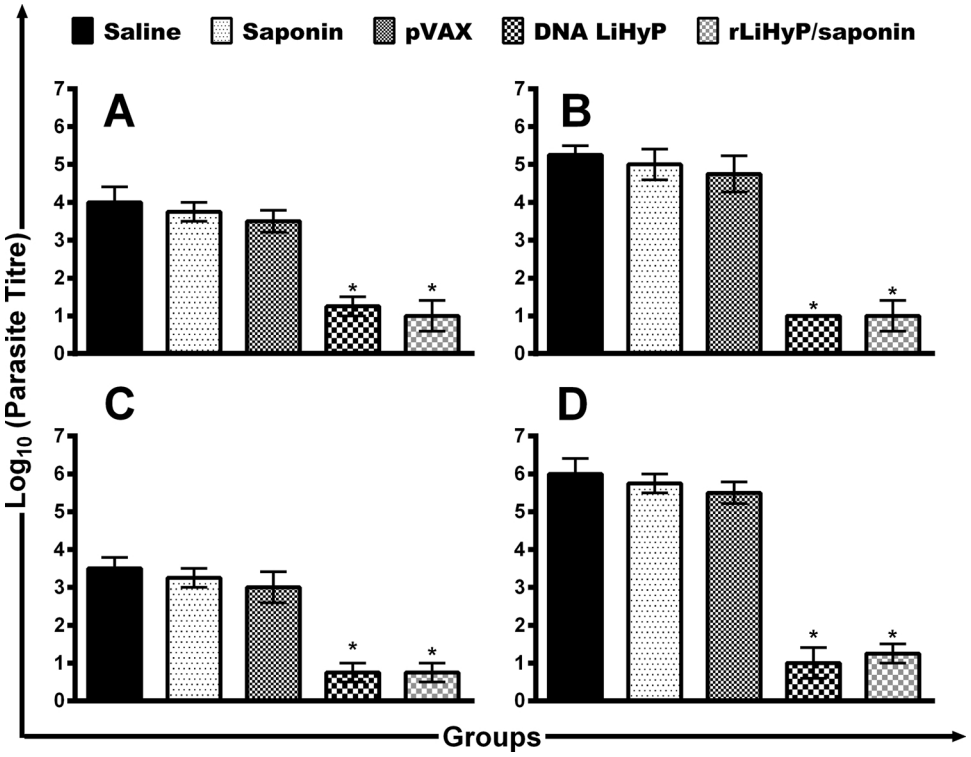

Fig. 7. Parasite burden evaluated by limiting dilution technique. Mice were inoculated with saline or immunized with saponin, DNA pVAX, DNA LiHyP or rLiHyP/saponin, and challenged with *L. infantum* promastigotes. Sixty days after infection, parasite load in the liver, spleen, bone marrow, and draining lymph nodes (A, B, C and D, respectively) was evaluated by limiting-dilution technique. Bars represent the mean ± standard deviation of the groups. *indicates statistically significant difference in relation to the control (saline, saponin and pVAX) groups ($P < 0.0001$).

Table 1
Immunogenicity in PBMCs from active and treated visceral leishmaniasis patients and healthy subjects. To evaluate the rLiHyP-specific immunogenicity in human PBMC, cells (1×10^7) were added in wells containing RPMI medium and incubated alone (medium, background control) or stimulated with rLiHyP or *L. infantum* SLA (10 and 25 μg/mL, respectively) for 5 days at 37 °C in 5% CO_2. Then, culture supernatants were collected, and IFN-γ and IL-10 levels were measured by a capture ELISA by using commercial kits. The mean ± standard deviation of the groups is shown.

Stimulus	Immunogenicity in human PBMCs (pg/mL)					
	Active VL		Treated VL		Healthy subjects	
	IFN-γ	IL-10	IFN-γ	IL-10	IFN-γ	IL-10
Medium	59.8 ± 4.4	71.1 ± 5.2	55.6 ± 4.9	61.5 ± 6.6	50.8 ± 3.7	71.9 ± 7.4
rLiHyP	201.7 ± 19.4	79.8 ± 13.4	755.4 ± 22.3	65.6 ± 9.9	877.7 ± 65.5	77.6 ± 10.9
SLA	68.8 ± 7.8	582.2 ± 34.4	146.6 ± 22.5	99.2 ± 11.2	155.4 ± 21.1	89.9 ± 9.6

necessary as without evaluating the parasite load at later infection time points, since is not possible to conclude whether LiHyP-vaccinated mice truly present infection resolution or only a delay in the parasite spreading. In this regard, further investigations should be performed.

As a limiting factor of the present study, the evaluation of vaccine efficacy was performed only one month after the last immunization. In the absence of the evaluation of the rLiHyP/saponin and DNA LiHyP compositions at longer times before challenge, it cannot be discarded that vaccinated mice could maintain or eventually decrease the vaccine´ prophylactic effect against infection. Also, parasitological and immunological analyzes were performed only 60 days after challenge, and it is not possible to infer about the long-lasting protective immunity by our vaccine candidate. In this context, further investigations are necessary to be developed, aiming to evaluate different periods of time to define the longer time to our vaccine candidate guarantee protection against infection.

In conclusion, the present study´ data could be taken as a proof-of-concept of the efficacy of a new *Leishmania* hypothetical protein to induce protection against VL, when administered as a DNA vaccine or in a recombinant form plus saponin. The results are encouraging, but certainly additional studies are necessary to test this molecule as an immunogen against human disease.

Conflict of interest

The authors declare no commercial or financial conflict of interest.

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References

Agallou, M., Athanasiou, E., Koutsoni, O., Dotsika, E., Karagouni, E., 2014. Experimental validation of multi-epitope peptides including promising MHC class I- and II-restricted epitopes of four known *Leishmania infantum* proteins. Front. Immunol. 5, 268.

Aguiar-Soares, R.D., Roatt, B.M., Ker, H.G., Moreira, N.D., Mathias, F.A., Cardoso, J.M., Gontijo, N.F., Bruna-Romero, O., Teixeira-Carvalho, A., Martins-Filho, O.A., Corrêa-Oliveira, R., Giunchetti, R.C., Reis, A.B., 2014. LBSapSal-vaccinated dogs exhibit increased circulating T-lymphocyte subsets ($CD4^+$ and $CD8^+$) as well as a reduction of parasitism after challenge with *leishmania infantum* plus salivary gland of *lutzomyia longipalpis*. Parasit. Vectors. 7, 61.

Ahmed, S.B., Touihri, L., Chtourou, Y., Dellagi, K., Bahloul, C., 2009. DNA based vaccination with a cocktail of plasmids encoding immunodominant *leishmania major* antigens confers full protection in BALB/c mice. Vaccine 27, 99–106.

Amit, A., Vijayamahantesh, D.M.R., Singh, A.K., Kumar, V., Suman, S.S., Singh, A., Kumar, A., Thakur, A.K., Das, V.R., Das, P., Bimal, S., 2017. Immunization with *leishmania donovani* protein disulfide isomerase DNA construct induces Th1 and Th17 dependent immune response and protection against experimental visceral leishmaniasis in BALB/c mice. Mol. Immunol. 82, 104–113.

Barbosa-Santos, M.L., Nico, D., Oliveira, F.A., Barreto, A.S., Palatnik-de-Sousa, I., Carrillo, E., Moreno, J., Luca, P.M., Morrot, A., Rosa, D.S., Palatnik, M., Bani-Corrêa, C., Almeida, R.P., Palatnik-de-Sousa, C.B., 2017. *Leishmania donovani* nucleoside hydrolase (NH36) domains induce T-cell cytokine responses in human visceral leishmaniasis. Front. Immunol. 8, 227.

Bivas-Benita, M., Ottenhoff, T.H., Junginger, H.E., Borchard, G., 2005. Pulmonary DNA vaccination:concepts, possibilities and perspectives. J. Control. Release 107, 1–29.

Bruhn, K.W., Birnbaum, R., Haskell, J., Vanchinathan, V., Greger, S., Narayan, R., Chang, P.L., Tran, T.A., Hickerson, S.M., Beverley, S.M., Wilson, M.E., Craft, N., 2012. Killed but metabolically active *Leishmania infantum* as a novel whole-cell vaccine for visceral leishmaniasis. Clin. Vaccine Immunol. 19, 490–498.

Campos, B.L., Silva, T.N., Ribeiro, S.P., Carvalho, K.I., Kallas, E.G., Laurenti, M.D., Passero, L.F., 2015. Analysis of iron superoxide dismutase-encoding DNA vaccine on the evolution of the *Leishmania amazonensis* experimental infection. Parasite Immunol. 37, 407–416.

Campos-Neto, A., Porrozzi, R., Greeson, K., et al., 2001. Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. Infect. Immun. 69, 4103–4108.

Carvalho, E.M., Barral, A., Pedral-Sampaio, D., Barral-Netto, M., Badaró, R., Rocha, H., Johnson, W.D.Jr., 1992. Immunologic markers of clinical evolution in children recently infected with Leishmania donovani chagasi. J. Infect. Dis. 165, 535–540.

Chhajer, R., Ali, N., 2014. Genetically modified organisms and visceral leishmaniasis. Front. Immunol. 5, 213.

Coelho, E.A.F., Tavares, C.A.P., Carvalho, F.A.A., et al., 2003. Immune responses induced by the *Leishmania (Leishmania) donovani* A2 antigen, but not by the LACK antigen, are protective against experimental *Leishmania (Leishmania) amazonensis* infection. Infect. Immun. 71, 3988–3994.

Coelho, V.T., Oliveira, J.S., Valadares, D.G., Chávez-Fumagalli, M.A., Duarte, M.C., Lage, P.S., Soto, M., Santoro, M.M., Tavares, C.A., Fernandes, A.P., Coelho, E.A., 2012. Identification of proteins in promastigote and amastigote-like *Leishmania* using an immunoproteomic approach. PLoS Negl. Trop. Dis. 6, e1430.

Coler, R.N., Skeiky, Y.A.W., Bernards, K., et al., 2002. Immunization with a polyprotein vaccine consisting of the T-cell antigens thiol-specific antioxidant, *leishmania major* stress-inducible protein 1, and *leishmania* elongation initiation factor protects against leishmaniasis. Infect. Immun. 70, 4215–4225.

Coler, R.N., Goto, Y., Bogatzki, L., Raman, V., Reed, S.G., 2007. Leish-111f, a recombinant polyprotein vaccine that protects against visceral leishmaniasis by elicitation of $CD4^+$ T cells. Infect. Immun. 75, 4648–4654.

Costa, L.E., Chávez-Fumagalli, M.A., Martins, V.T., Duarte, M.C., Lage, D.P., Lima, M.I., Pereira, N.C., Soto, M., Tavares, C.A., Goulart, L.R., Coelho, E.A., 2015. Phage-fused epitopes from *Leishmania infantum* used as immunogenic vaccines confer partial protection against *Leishmania amazonensis* infection. Parasitology 142, 1335–1347.

Das, A., Ali, N., 2014. Combining cationic liposomal delivery with MPL-TDM for cysteine protease cocktail vaccination against Leishmania donovani: evidence for antigen synergy and protection. PLoS Negl. Trop. Dis. 8, e3091.

Dayakar, A., Chandrasekaran, S., Veronica, J., Maurya, R., 2016. Leptin induces the phagocytosis and protective immune response in *Leishmania donovani* infected THP-1 cell line and human PBMCs. Exp. Parasitol. 160, 54–59.

Dias, D.S., Ribeiro, P.A.F., Martins, V.T., Lage, D.P., Ramos, F.F., Dias, A.L.T., Rodrigues, M.R., Portela, Á.S.B., Costa, L.E., Caligiorne, R.B., Steiner, B.T., Chávez-Fumagalli, M.A., Salles, B.C.S., Santos, T.T.O., Silveira, J.A.G., Magalhães-Soares, D.F., Roatt, B.M., Machado-de-Ávila, R.A., Duarte, M.C., Menezes-Souza, D., Silva, E.S., Galdino, A.S., Coelho, E.A.F., 2017. Recombinant prohibitin protein of *Leishmania infantum* acts as a vaccine candidate and diagnostic marker against visceral leishmaniasis. Cell. Immunol. S0008-8749 (17) 30196-X.

Dikhit, M.R., Kumar, A., Das, S., Dehury, B., Rout, A.K., Jamal, F., Sahoo, G.C., Topno, R.K., Pandey, K., Das, V.N.R., Bimal, S., Das, P., 2017. Identification of potential MHC class-II-restricted epitopes derived from *Leishmania donovani* antigens by reverse vaccinology and evaluation of their $CD4^+$ T-cell responsiveness against visceral leishmaniasis. Front. Immunol. 8, 1763.

Duarte, M.C., Lage, D.P., Martins, V.T., Costa, L.E., Lage, L.M., Carvalho, A.M., Ludolf, F., Santos, T.T., Roatt, B.M., Menezes-Souza, D., Fernandes, A.P., Tavares, C.A., Coelho, E.A., 2016. A vaccine combining two *Leishmania braziliensis* proteins offers heterologous protection against *Leishmania infantum* infection. Mol. Immunol. 76, 70–79.

Faria, J.A., Andrade, C., Goes, A.M., Rodrigues, M.A., Gomes, D.A., 2016. Effects of different ligands on epidermal growth factor receptor (EGFR) nuclear translocation.

Bioch. Biop. Res. Commun. 478, 39–45.
Fujiwara, R.T., Vale, A.M., Franca-da-Silva, J.C., et al., 2005. Immunogenicity in dogs of three recombinant antigens (TSA, LeIF and LmSTI1) potential vaccine candidates for canine visceral leishmaniasis. Vet. Res. 36, 827–838.
Gannavaram, S., Dey, R., Avishek, K., Selvapandiyan, A., Salotra, P., Nakhasi, H.L., 2014. Biomarkers of safety and immune protection for genetically modified live attenuated *Leishmania* vaccines against visceral leishmaniasis - discovery and implications. Front. Immunol. 5, 241.
Gannavaram, S., Bhattacharya, P., Ismail, N., Kaul, A., Singh, R., Nakhasi, H.L., 2016. Modulation of innate immune mechanisms to enhance *Leishmania*vaccine-induced immunity: role of coinhibitory molecules. Front. Immunol. 7, 187.
Ghaffarifar, F., Jorjani, O., Sharifi, Z., et al., 2013. Enhancement of immune response induced by DNA vaccine cocktail expressing complete LACK and TSA genes against Leishmania major. APMIS 121, 290–298.
Gidwani, K., Rai, M., Chakravarty, J., Boelaert, M., Sundar, S., 2009. Evaluation of leishmanin skin test in Indian visceral leishmaniasis. Am. J. Trop. Med. Hyg. 80, 566–567.
Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. Anal. Biochem. 126, 131–138.
Gupta, S.K., Sisodia, B.S., Sinha, S., Hajela, K., Naik, S., Shasany, A.K., Dube, A., 2007. Proteomic approach for identification and characterization of novel immunostimulatory proteinsfrom soluble antigens of Leishmania donovani promastigotes. Proteomics 7 (March 5), 816–823.
Gurunathan, S., Prussin, C., Sacks, D.L., Seder, R.A., 1998. Vaccine requirements for sustained cellular immunity to an intracellular parasitic infection. Nat. Med. 4, 1409–1415.
Jamal, F., Shivam, P., Kumari, S., Singh, M.K., Sardar, A.H., Pushpanjali, Murugesan S., Narayan, S., Gupta, A.K., Pandey, K., Das, V.N.R., Ali, V., Bimal, S., Das, P., Singh, S.K., 2017. Identification of leishmania donovani antigen in circulating immune complexes of visceral leishmaniasis subjects for diagnosis. PLoS One 18;12 (August (8)), e0182474.
Khabazzadeh, T.N., Mahdavi, M., Maleki, F., Zarrati, S., Tabatabaie, F., 2016. The role of montanide ISA 70 as an adjuvant in immune responses against *leishmania major* induced by thiol-specific antioxidant-based protein vaccine. J. Parasit. Dis. 40, 760–767.
Kumar, R., Nylén, S., 2012. Immunobiology of visceral leishmaniasis. Front. Immunol. 3, 251.
Kumar, A., Samant, M., 2016. DNA vaccine against visceral leishmaniasis: a promising approach for prevention and control. Parasite Immunol. 38, 273–281.
Magalhães, R.D., Duarte, M.C., Mattos, E.C., Martins, V.T., Lage, P.S., Chávez-Fumagalli, M.A., Lage, D.P., Menezes-Souza, D., Régis, W.C., Manso Alves, M.J., Soto, M., Tavares, C.A., Nagen, R.A., Coelho, E.A., 2014. Identification of differentially expressed proteins from Leishmania amazonensis associated with the loss of virulence of the parasites. PLoS Negl. Trop Dis. 3;8 (April4), e2764.
Martins, V.T., Duarte, M.C., Lage, D.P., Costa, L.E., Carvalho, A.M., Mendes, T.A., Roatt, B.M., Menezes-Souza, D., Soto, M., Coelho, E.A., 2017. A recombinant chimeric protein composed of human and mice-specific $CD4^+$ and CD8+ T-cell epitopes protects against visceral leishmaniasis. Parasite Immunol. 39, e12359.
Maspi, N., Ghaffarifar, F., Sharifi, Z., Dalimi, A., 2016. Codelivery of DNA vaccination encoding LeIF gene and IL-12 increases protection against *Leishmania major* infection in BALB/c mice. Parasite Immunol. 38, 228–235.
Maspi, N., Ghaffarifar, F., Sharifi, Z., Dalimi, A., Khademi, S.Z., 2017. DNA vaccination with a plasmid encoding LACK-TSA fusion against *Leishmania major* infection in BALB/c mice. Malays. J. Pathol. 39, 267–275.
Mazumder, S., Maji, M., Das, A., Ali, N., 2011. Potency, efficacy and durability of DNA/DNA, DNA/protein and protein/protein based vaccination using gp63 against *Leishmania donovani* in BALB/c mice. PLoS One 6, e14644.
Melby, P.C., Yang, J., Zhao, W., Perez, L.E., Cheng, J., 2001. *Leishmania donovani* p36(LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. Infect. Immun. 69, 4719–4725.
Oliveira, M.P., Martins, V.T., Santos, T.T.O., Lage, D.P., Ramos, F.F., Salles, B.C.S., Costa, L.E., Dias, D.S., Ribeiro, P.A.F., Schneider, M.S., Machado-de-Ávila, R.A., Teixeira, A.L., Coelho, E.A.F., Chávez-Fumagalli, M.A., 2018. Small myristoylated protein-3, identified as a potential virulence factor in *Leishmania amazonensis*, proves to be a protective antigen against visceral leishmaniasis. Int. J. Mol. Sci. 19, 1.
Palatnik-de-Sousa, C.B., 2008. Vaccines for leishmaniasis in the fore coming 25 years. Vaccine 26, 1709–1724.
Pascalis, H., Lavergne, A., Bourreau, E., et al., 2003. Th1 cell development induced by cysteine proteinases A and B in localized cutaneous Leishmaniasis due to Leishmania guyanensis. Infect. Immun. 71, 2924–2926.
Peruhype-Magalhães, V., Martins-Filho, O.A., Prata, A., et al., 2005. Immune response in human visceral leishmaniasis: analysis of the correlation between innate immunity cytokine profile and disease outcome. Scand. J. Immunol. 62, 487–495.
Ponte-Sucre, A., Gamarro, F., Dujardin, J.C., Barrett, M.P., López-Vélez, R., García-Hernández, R., Pountain, A.W., Mwenechanya, R., Papadopoulou, B., 2017. Drug resistance and treatment failure in leishmaniasis: A 21st century challenge. PLoS Negl. Trop. Dis. 11, e0006052.
Portela, A.S.B., Costa, L.E., Salles, B.C.S., Lima, M.P., Santos, T.T.O., Ramos, F.F., Lage, D.P., Martins, V.T., Caligiorne, R.B., Lessa, D.R., Silva, F.R., Machado, A.S., Nascimento, G.F., Gama, I.S., Chávez-Fumagalli, M.A., Teixeira, A.L., Rocha, M.O.C., Rocha, R.L., Coelho, E.A.F., 2017. Identification of immune biomarkers related to disease progression and treatment efficacy in human visceral leishmaniasis. Immunobiology S0171-2985 (17), 30184–30185.
Rafati, S., Ghaemimanesh, F., Zahedifard, F., 2006. Comparison of potential protection induced by three vaccination strategies (DNA/DNA, Protein/Protein and DNA/Protein) against*Leishmania major* infection using Signal Peptidase type I in BALB/c mice. Vaccine 24, 3290–3297.
Reed, S.G., Coler, R.N., Mondal, D., Kamhawi, S., Valenzuela, J.G., 2016. *Leishmania*vaccine development: exploiting the host-vector-parasite interface. Expert Rev. Vaccines 15, 81–90.
Ribeiro, P.A.F., Dias, D.S., Novais, M.V.M., Lage, D.P., Tavares, G.S.V., Mendonça, D.V.C., Oliveira, J.S., Chávez-Fumagalli, M.A., Roatt, B.M., Duarte, M.C., Menezes-Souza, D., Ludolf, F., Tavares, C.A.P., Oliveira, M.C., Coelho, E.A.F., 2018. A leishmania hypothetical protein-containing liposome-based formulation is highly immunogenic and induces protection against visceral leishmaniasis. Cytokine 21 (August 111), 131–139.
Roatt, B.M., Aguiar-Soares, R.D., Coura-Vital, W., Ker, H.G., Moreira, N.D., Vitoriano-Souza, J., Giunchetti, R.C., Carneiro, C.M., Reis, A.B., 2014. Immunotherapy and immunochemotherapy in visceral leishmaniasis: promising treatments for this neglected disease. Front. Immunol. 5, 272.
Santos-Gomes, G.M., Rodrigues, A., Teixeira, F., Carreira, J., Alexandre-Pires, G., Carvalho, S., et al., 2014. Immunization with the *Leishmania infantum* recombinant cyclophilin protein 1 confers partial protection to subsequent parasite infection and generates specific memory T cells. Vaccine 32, 1247–1253.
Schaut, R.G., Grinnage-Pulley, T.L., Esch, K.J., Toepp, A.J., Duthie, M.S., Howard, R.F., Reed, S.G., Petersen, C.A., 2016. Recovery of antigen-specific T cell responses from dogs infected with Leishmania (L.) infantum by use of vaccine associated TLR-agonist adjuvant. Vaccine 17;34 (October 44), 5225–5234.
Selvapandiyan, A., Dey, R., Gannavaram, S., Lakhal-Naouar, I., Duncan, R., Salotra, P., et al., 2012. Immunity to visceral leishmaniasis using genetically defined live-attenuated parasites. J. Trop. Med. 2012, 631460.
Singh, O.P., Stober, C.B., Singh, A.K., Blackwell, J.M., Sundar, S., 2012. Cytokine responses to novel antigens in an Indian population living in an area endemic for visceral leishmaniasis. PLoS Negl. Trop. Dis. 6, e1874.
Srivastava, S., Shankar, P., Mishra, J., Singh, S., 2016. Possibilities and challenges for developing a successful vaccine for leishmaniasis. Parasit. Vectors. 9, 277.
Sundar, S., Chakravarty, J., 2013. Leishmaniasis: an update of current pharmacotherapy. Expert Opin. Pharmacother. 14, 53–63.
Sundar, S., Singh, A., 2017. Chemotherapeutics of visceral leishmaniasis: present and future developments. Parasitology 7, 1–7.
Tabatabaie, F., Mahdavi, M., Faezi, S., et al., 2014. Th1 platform immune responses against *Leishmania major* induced by thiol-specific antioxidant-based DNA vaccines. Jundishapur J. Microbiol. 7, e8974.
Travi, B.L., Cordeiro-da-Silva, A., Dantas-Torres, F., Miró, G., 2018. Canine visceral leishmaniasis: diagnosis and management of the reservoir living among us. PLoS Negl. Trop. Dis. 12, e0006082.
World Health Organization, 2016. Leishmaniasis. http://www.who.int/topics/leishmaniasis/en/.
Zahedifard, F., Gholami, E., Taheri, T., Taslimi, Y., Doustdari, F., Seyed, N., Torkashvand, F., Meneses, C., Papadopoulou, B., Kamhawi, S., Valenzuela, J.G., Rafati, S., 2014. Enhanced protective efficacy of nonpathogenic recombinant *Leishmania tarentolae*expressing cysteine proteinases combined with a sand fly salivary antigen. PLoS Negl. Trop. Dis. 8, e2751.
Zanin, F.H., Coelho, E.A., Tavares, C.A., et al., 2007. Evaluation of immune responses and protection induced by A2 and nucleoside hydrolase (NH) DNA vaccines against *Leishmania chagasi* and *Leishmania amazonensis* experimental infections. Microbes Infect. 9, 1070–1077.
Zimmermann, S., Dalpke, A., Heeg, K., 2008. CpG oligonucleotides as adjuvant in therapeutic vaccines against parasitic infections. Int. J. Med. Microbiol. 298, 39–44.