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

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

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and GM-CSF, and an IgG2a isotype-based humoral response. In addition, the CD4$^+$ and CD8$^+$ T-cell subtypes contributed to IFN-γ production in the protected animals. The results showed the immunogenicity in human cells and the protective efficacy against *L. infantum* in a murine model, and well indicate that this recombinant chimera can be considered as a promising strategy to be used against human disease. (Translational Research 2018; 200:18–34)

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

AT A GLANCE COMMENTARY

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Background

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

Translational Significance

In this context, this recombinant chimera could well be considered in future studies as a prophylactic alternative to prevent against human VL.

INTRODUCTION

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

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

Evidence that patients cured from VL develop a Th1 response that protects against new infections suggest that immune cells of these subjects, such as peripheral blood mononuclear cells (PBMCs), could be employed as useful tools to identify new targets to protect against disease.[13,14] We have previously reported that *Leishmania* antigens used as recombinant proteins were protective against murine VL. One of these molecules,

LiHyS (Leishmania hypothetical protein S), a *Leishmania* conserved hypothetical protein, was shown to be immunogenic and conferred protection in BALB/c mice against *L. infantum* infection. A positive lymphoproliferative response was also found when this protein was used to stimulate PBMCs collected from treated VL patients.[15] Other antigens, small glutamine-rich tetratricopeptide repeat-containing (SGT) and prohibitin (PHB), showed also similar results on the immunogenicity in mice and humans, and an additional protective efficacy against challenge using this parasite species.[16,17] However, these molecules were tested as individual antigens against a complex parasite, and a single product grouping distinct *Leishmania* proteins could be easier to produce, cheaper and highly immunogenic, since different T-cell epitopes were present in the amino acid sequence of the protein.[18]

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

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

MATERIAL AND METHODS

Recombinant antigens production. LiHyS,[15] SGT,[16], and PHB[17] proteins were cloned, expressed, and purified as recombinant proteins. For the construction of the recombinant chimeric protein, amino acid sequences of the 3 proteins were subjected to bioinformatics assays aiming to select regions specific to murine and human T-cells haplotypes and containing immunogenic epitopes. In order to predict $CD8^+$ T-cell epitopes, the NetCTLPan program (version 1.1) was used to identify epitopes able to bind to A2, A3, and B7 alleles of human MHC class I with a peptide length of 9 amino acids, weight on C-terminal cleavage site of 0.225, weight on TAP transport efficiency of 0.025 and threshold of epitope identification of 1.0.[24] These alleles were selected since they are frequent in more than 90% of the human population of any ethnic group.[25] Epitopes with ability to bind to H-2-Kd, H-2-Ld, and H-2-Dd alleles from BALB/c mice were also predicted by NetCTLPan program. The $CD4^+$ T-cell epitopes were predicted using the NetMHCII 2.3 server with selection of epitopes with binding affinity lower than 500 nmol/L for 30% of alleles from human and I-Ad and I-Ed alleles from mice.[26] B-cell epitopes were identified using the with the BepiPred 2.0 program with threshold of 1.0 in at least 7 consecutive amino acids.[27] Protein regions containing specific T-cell epitopes from humans and mice, but without B-cell epitopes, were selected to construct the chimeric protein. The conservation of potential epitopes in this sequence was confirmed by using the same programs used to predict $CD4^+$ and $CD8^+$ T-cell epitopes.

The chimeric protein was submitted for selection by using specific codons, aiming its expression in *Escherichia coli* with the web codon optimization tool (https://www.idtdna.com/CodonOpt), and optimized to reduce the presence of intramolecular interactions of messenger RNA, being calculated with the MFOLD Program. The chimera was synthesized by GenScript, and the recombinant protein was expressed in an Artic Express strain (DE3, Agilent Technologies), by using 1 mM of IPTG for 24 hour at 12°C. After, the protein was purified in a nickel affinity column. The evaluation of the physicochemical properties of the chimera sequence was performed with the ProtParam tool in the ExPASy server.[28] The parameters computed included: molecular weight, theoretic isoelectric point, amino acid residues, positive and negative residues, extinction coefficient, instability index, aliphatic index, and grand average of hydropathicity (GRAVY). After purification, all recombinant proteins were passed on a

polymyxin-agarose column (Sigma-Aldrich) in order to remove the residual endotoxin content (<10 ng of lipopolysaccharide per 1 mg of protein, Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000, BioWhittaker).

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

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

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

Mice and vaccination schedule. BALB/c mice (female, 6−8 weeks) were obtained from the breeding facilities of the Department of Biochemistry and

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

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

Cytokine detection: capture ELISA and flow cytometry. Spleen cells were collected 30 days after the last vaccine dose, as well as 60 days after infection, when cell cultures were performed.[18] Splenocytes (5×10^6) were incubated in DMEM (background control) added with 20% FBS, 20 mM L-glutamine, 200 U/mL penicillin, and 100 μg/mL streptomycin, pH 7.4, or stimulated with rSGT, rPHB, rLiHyS, or chimera (15 μg/mL each), protein mix (5 μg/mL of each protein) or *L. infantum* SLA (25 μg/mL), for 48 hour at 37°C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12, and GM-CSF levels were measured in the supernatants by capture ELISA (BD OptEIA TM set mouse kits, Pharmingen, San Diego, CA), following manufacturer's instructions. The involvement of IL-12, and $CD4^+$, and $CD8^+$ T-cells in the IFN-γ production in the protein mix or chimera-vaccinated mice groups was evaluated by incubating spleen cells with monoclonal antibodies against mouse IL-12 (C17.8), CD4 (GK 1.5), or CD8 (53-6.7) (5 μg/mL in all cases). Appropriate isotype-matched controls − rat IgG2a (R35-95) and rat IgG2b (95-1) − were employed. Antibodies (no azide and/or low endotoxin) were purchased from BD (Pharmingen). The nitrite secretion was also evaluated in the culture supernatants by Griess method.[31] In addition, the percentage of $CD4^+$ and $CD8^+$ T-cells producing intracytoplasmic cytokines (IFN-γ, TNF-α, IL-2, and IL-10) was determined in the SLA-stimulated cultures.[15] The results were expressed as indexes which were determined by dividing the percentage of $CD4^+$ and $CD8^+$ cytokine-positive T-cells in the stimulated cultures vs the values obtained in the unstimulated cultures (control).

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

Aldrich) were used in 1:5000 and 1:10,000 dilutions, respectively.

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

RESULTS

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

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

Immune response generated after vaccination with the recombinant proteins plus saponin. We investigated if the chimeric vaccine, when associated with saponin as an adjuvant, could stimulate the development of a specific immune response in vaccinated BALB/c mice. Like controls, the rLiHyS, rSGT, and rPHB proteins were prepared in a protein mix and also applied in the animals associated with saponin. Results showed that the immunization with the recombinant chimera plus saponin or protein mix plus saponin induced significantly higher levels of IFN-γ, IL-12, and GM-CSF in the cell supernatant, which were associated with low IL-4 and IL-10 levels (Fig. 4A). The humoral response was also evaluated, and results showed that the immunization using chimera and/or saponin or protein mix and/or saponin induced significantly higher antiprotein and antiparasite IgG2a/IgG1 ratios, when compared to the control (saline and saponin) groups (Fig. 4B). Comparing the immunogenicity developed in the immunized animals, it can be observed that the chimera and/or saponin group developed a more polarized Th1 response, when both cytokine and antibody productions were evaluated. After challenge infection, the immune response profile was maintained in the chimera and/or saponin and protein mix and/or saponin groups, with the production of significantly higher levels of IFN-γ, IL-12, and GM-CSF in the stimulated cells, whereas in the control groups, a significantly higher production of antiparasite IL-4 and IL-10 was found (Fig. 5A). The antibody production showed also significantly higher IgG2a/IgG1 ratios in the chimera and/or saponin or protein mix and/or saponin groups, whereas in the control groups, the antiparasite IgG1 was higher than IgG2a isotype, then resulting in an IgG2a/IgG1 ratio lower than 1.0 (Fig. 5B). The cellular and humoral response developed in the chimera and/or saponin group was also related to a more polarized Th1 immune profile, when compared to results obtained in the protein mix and/or saponin group.

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

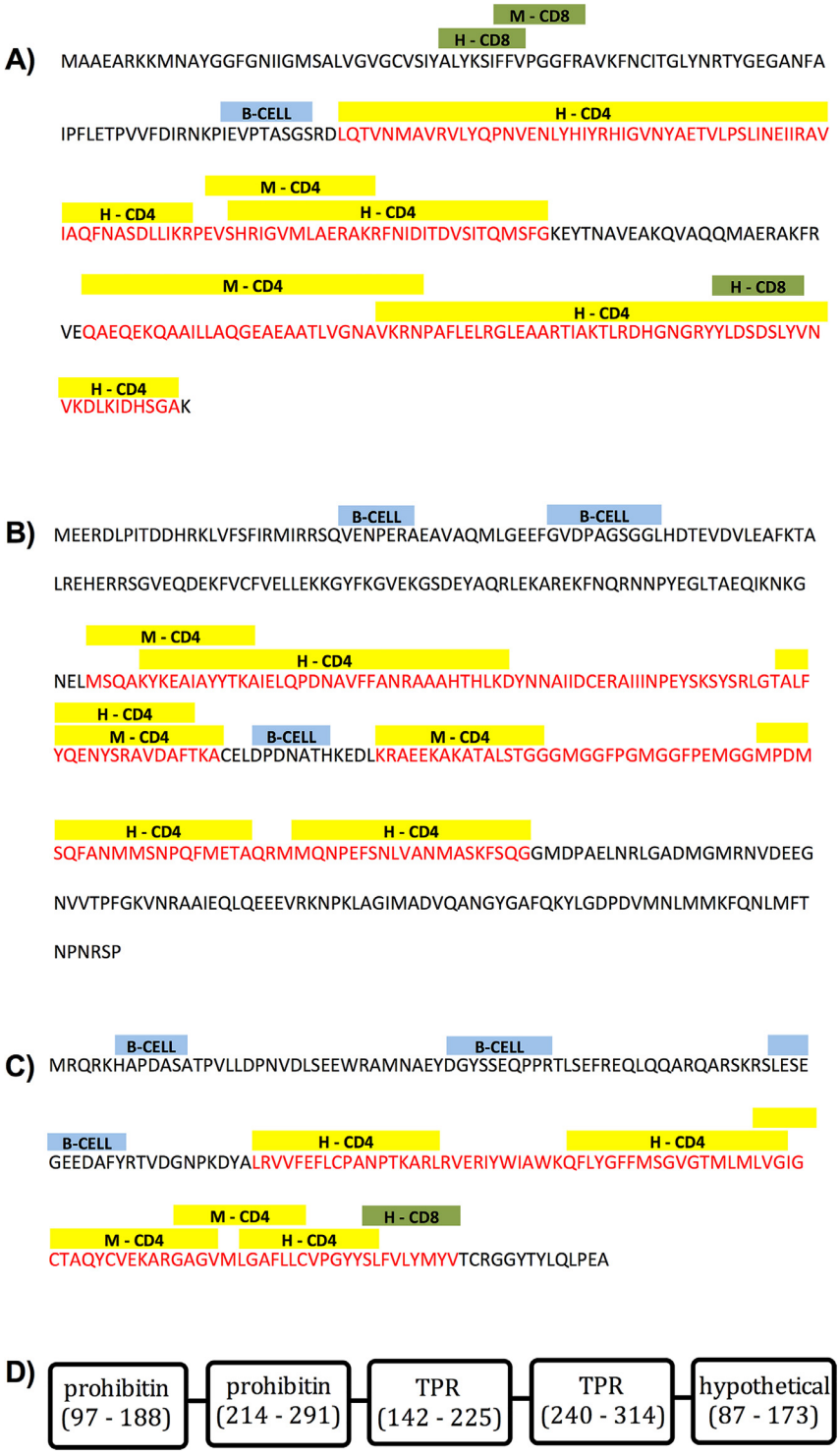

Fig. 1. Construction of the recombinant chimeric protein based on T- and B-cell epitopes prediction from distinct immunogenic *Leishmania* proteins. The prediction of B- and T cell-epitopes specific to the prohibitin (PHB; XP_001468827.1) (in **A**), SGT (XP_001467120.1) (in **B**), and LiHyS (XP_001467126.1)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

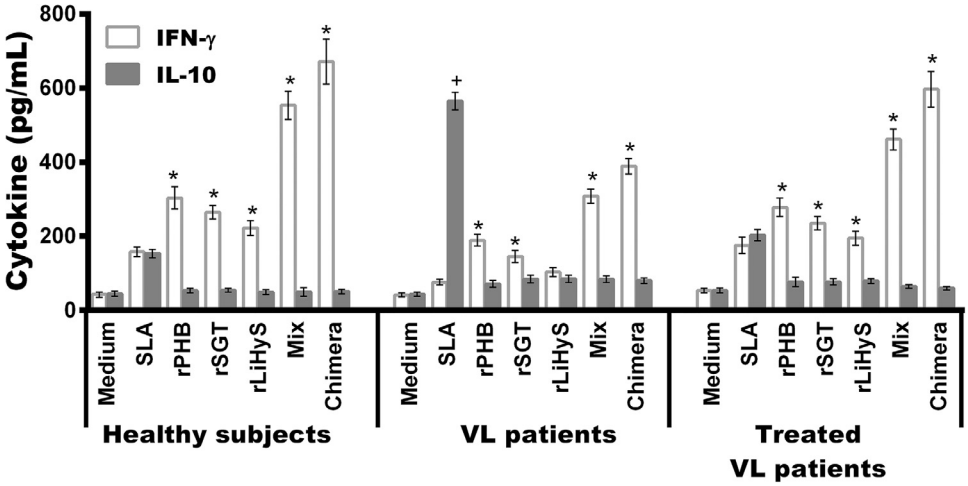

Fig. 2. Immunogenicity in human PBMCs from healthy subjects and VL patients. The PHB, SGT, LiHyS, protein mix, chimera, and SLA were individually analyzed regarding their immunogenicity in human cells. For this, PBMCs (10^7) from healthy subjects (n = 6) and treated and untreated VL patients (n = 6)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

importance of both T-cell subtypes in the protective immunity induced by the vaccines.

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

Protective efficacy in the infected and immunized mice. The protective efficacy induced by the immunizations using the protein mix and chimera plus saponin was evaluated. As controls, animals received saline or were immunized with saponin, protein mix, or chimera without adjuvant. Results showed that the immunization using the chimera and/or saponin or protein mix and/or saponin induced significant reductions in the parasite load in the spleen, liver, bone marrow (BM), and draining lymph nodes, when compared to the other groups (Fig. 9). In the absence of adjuvant, protein mix, or chimera-immunized mice did not present reductions in the parasitism, in comparison to the saline and saponin groups. Between the vaccinated animals, the immunization using chimera and/or saponin induced the reduction in the parasitism in all organs, when compared with the protein mix and/or saponin group, then demonstrating a better efficacy of the chimeric protein in protect against infection. The splenic parasite burden was also evaluated by a RT-PCR technique, and results showed that protein mix and/or saponin or chimera and/or saponin-immunized mice presented significant reductions in the parasitism in this organ, when compared to the controls (Fig. 10), then demonstrating the protective efficacy induced by the immunizations.

DISCUSSION

In spite of the vast knowledge about the immunity contributing to resistance and/or susceptibility against VL, there is no licensed vaccine to protect against human disease.[32] Current studies are mostly focused on rodent models and cannot be extrapolated to dogs or

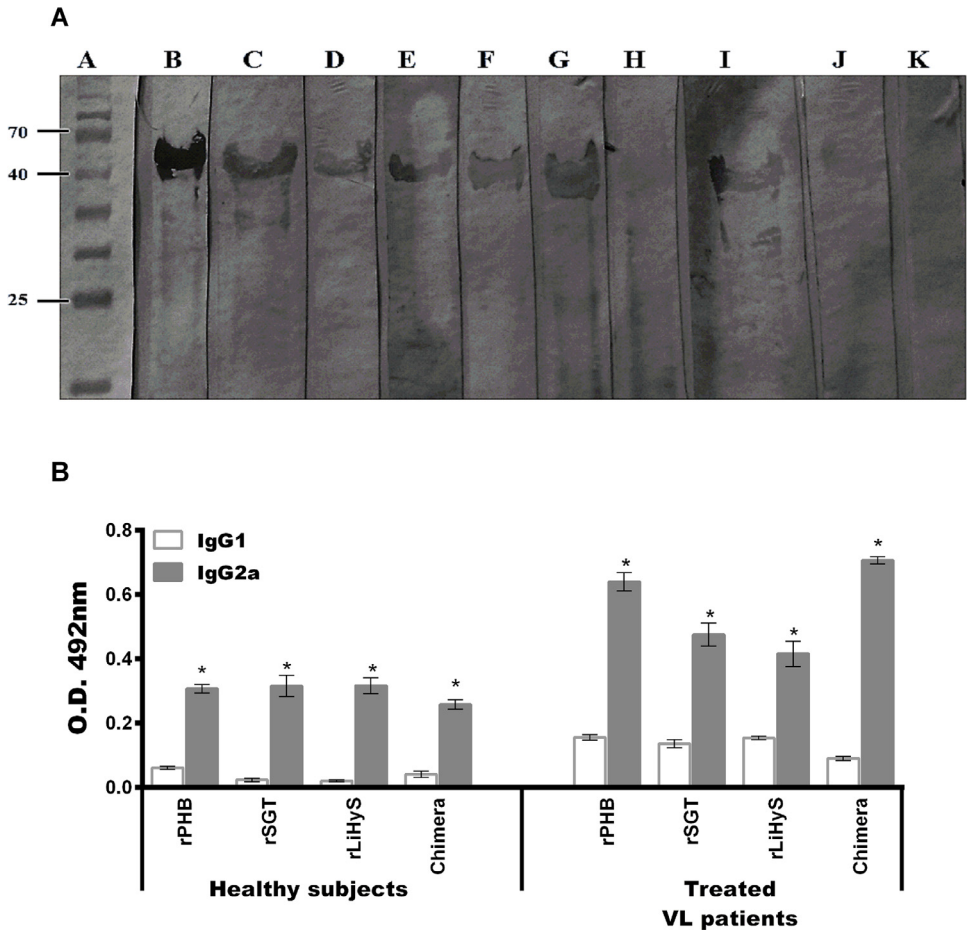

Fig. 3. Antigenicity of the recombinant chimeric protein using human sera. Immunoblottings were performed using the recombinant chimera against sera samples collected from mice, dogs, and humans. For this, the protein (10 μg) was submitted to a 12% SDS-PAGE and blotted onto nitrocellulose membrane. Results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

humans, since the majority of the candidates are either untested or present low efficacy when used to stimulate canine or human cells.[33–36] However, the fact that treated VL patients develop long-lasting protection based on the Th1 immunity against a new *Leishmania* infection supports the feasibility to develop a human vaccine.[37–39] An ideal candidate should be safe and able to induce both $CD4^+$ and $CD8^+$ T-cell responses, which could be boosted by natural infections, thus reducing the number of immunogen doses. Also, it should comprise different immunogenic parts of *Leishmania* proteins in a single product, making it more specific, immunogenic, easier, and cheaper to produce.[40,41]

In the present study, 3 recombinant *L. infantum* proteins (LiHyS, SGT, and PHB), which were previously shown to be protective against murine VL, were analyzed by distinct bioinformatics tools and a recombinant chimera was constructed. This construct was compared in the in vitro and in vivo analyses with a combination of 3 recombinant antigens. Our results

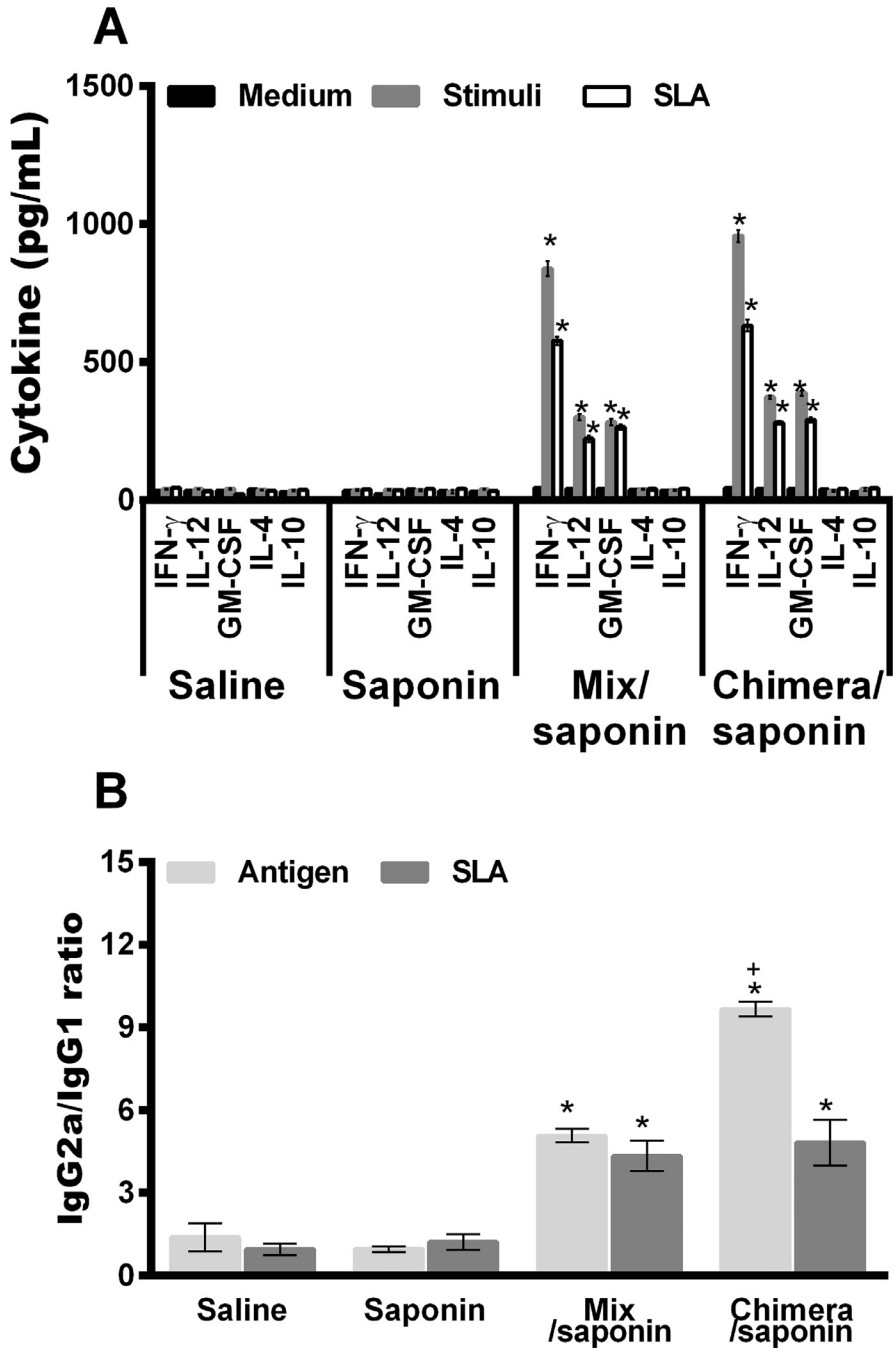

Fig. 4. Immunogenicity induced in vaccinated BALB/c mice before *L. infantum* infection. Mice (n = 8 per group) received saline or were immunized with saponin, chimera and/or saponin or protein mix and/or saponin. Thirty days after the last vaccine dose, they were euthanized and their spleen cells (5×10^6)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

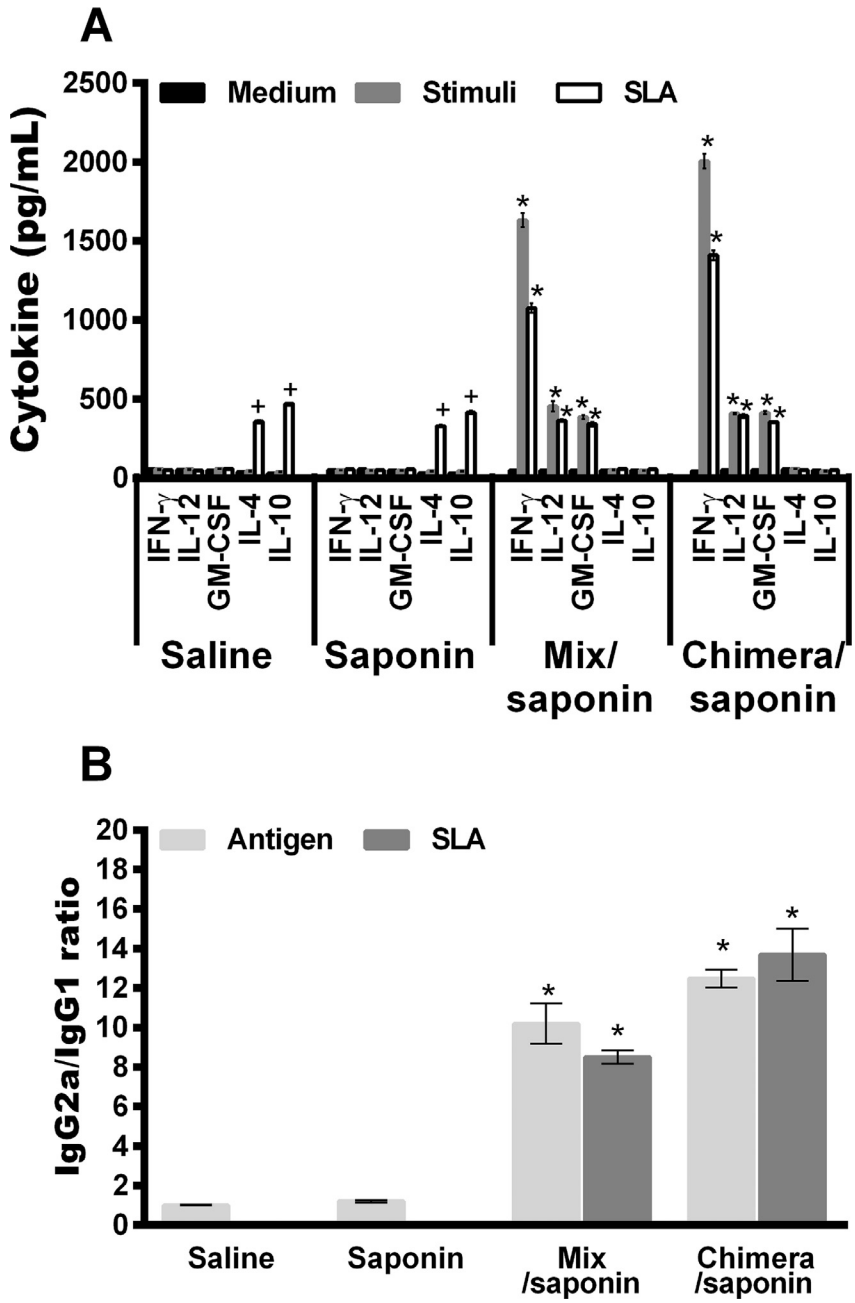

Fig. 5. Immune response developed after *L. infantum* challenge. Mice (n = 8 per group) received saline or were immunized with saponin, chimera and/or saponin or protein mix and/or saponin. Thirty days after the last dose, they were infected with *L. infantum* promastigotes. Ten weeks later, animals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

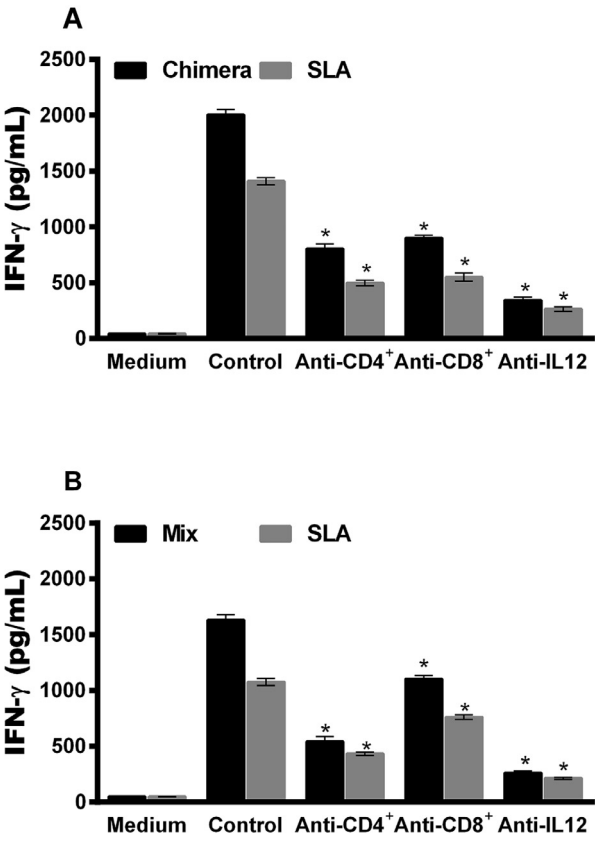

Fig. 6. Evaluation of the participation of $CD4^+$ and $CD8^+$ T-cells in the IFN-γ production in the vaccinated animals. Mice (n = 8 per group) were immunized with chimera and/or saponin or protein mix and/or saponin and challenged with *L. infantum* promastigotes. Ten weeks later, they were euthanized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

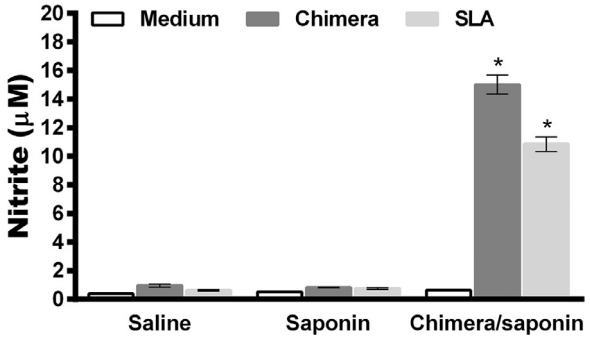

Fig. 7. Nitrite production. Splenocytes from the infected and immunized mice (n = 8 per group) were cultured to perform the dosage of cytokines, and also used to investigate the protein and parasite-specific nitrite production, by means of the Griess reaction. Bars represent the mean ± standard deviation.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

showed that the chimeric protein was better than the protein mix, when cytokine production and parasite load were evaluated in infected and vaccinated BALB/c. In addition, chimera was highly immunogenic and induced higher IFN-γ/IL-10 ratios in cells collected from healthy subjects and treated VL patients, then demonstrating the possibility to use this antigen as an immunoprophylactic target in studies against human disease.

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

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

In addition, when anti-CD4 and anti-CD8 monoclonal antibodies were used to inhibit the IFN-γ production by spleen cells from vaccinated animals, both antibodies significantly reduced the production of this

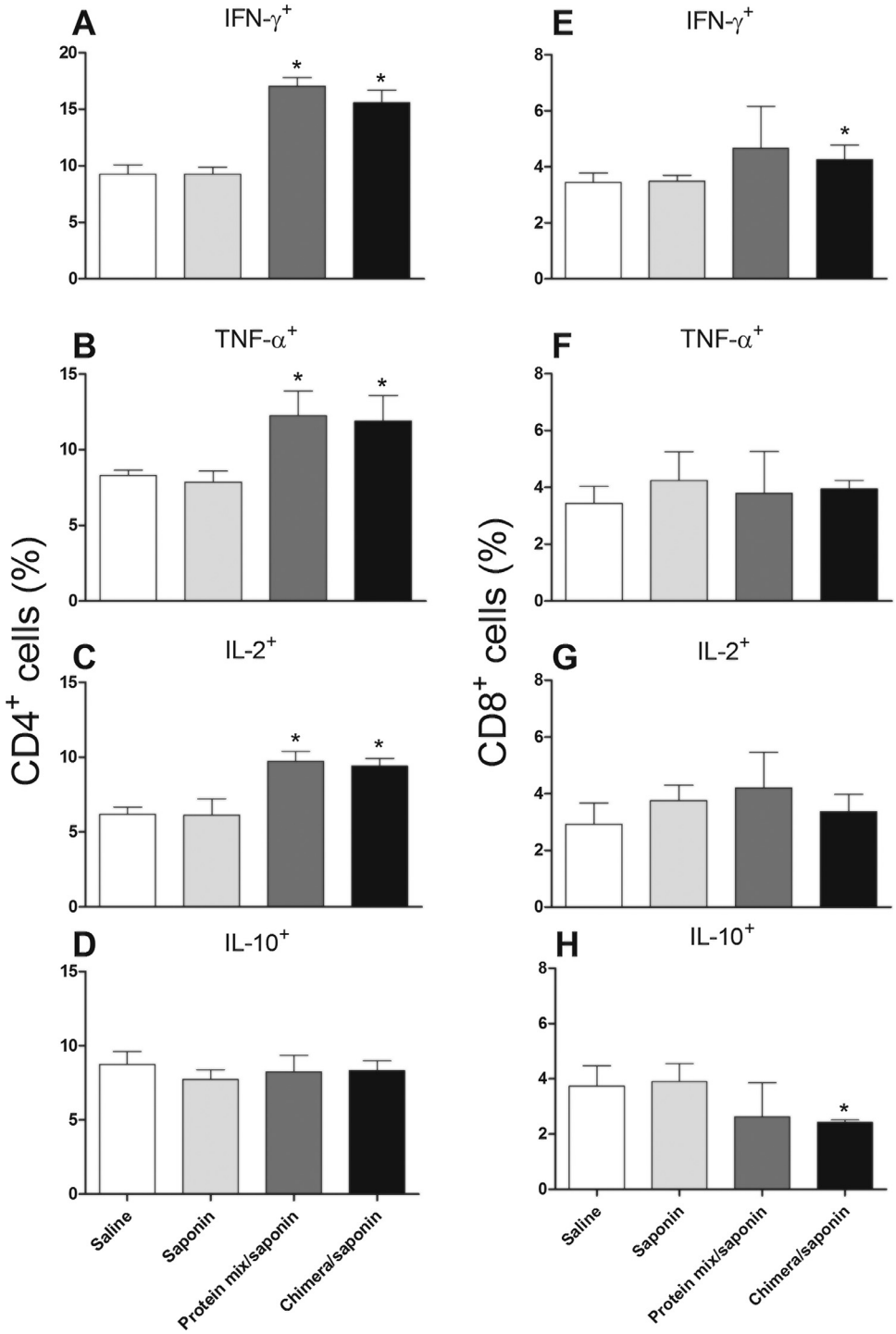

Fig. 8. Evaluation of intracytoplasmic cytokine-producing T-cells profile by flow cytometry. The profile of IFN-γ, TNF-α, IL-2, and IL-10-producing $CD4^+$ and $CD8^+$ T-cells was calculated by means of the ratio between values obtained in the SLA-stimulated cultures vs those from the unstimulated cultures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

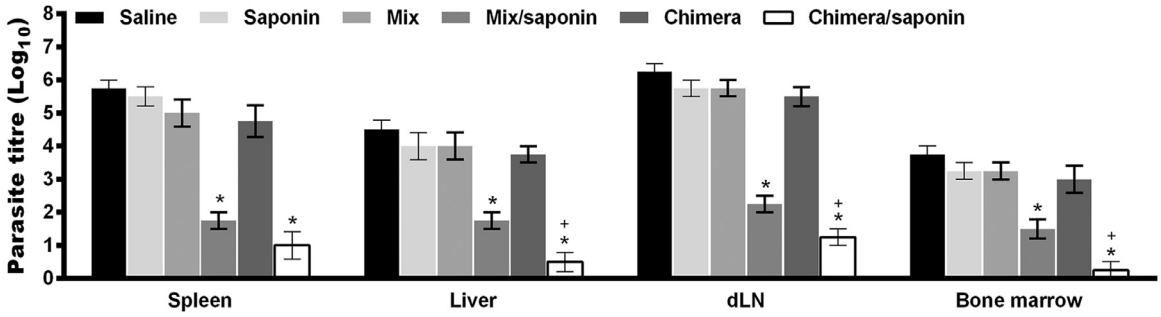

Fig. 9. Parasite burden evaluated by limiting-dilution technique. Mice (n = 8 per group) received saline or were immunized with saponin, chimera, chimera and/or saponin, protein mix or protein mix and/or saponin. Then, they challenged with *L. infantum* promastigotes. Sixty days after infection, their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

cytokine, indicating the efficacy of our molecule to develop a protective cellular response against infection based on $CD4^+$ and $CD8^+$ T-cells. A similar profile was also obtained when a flow cytometry experiment was performed, since higher percentage of IFN-γ^+, IL-2^+, and TNF-α^+-producing T-cells, which were associated with low presence of specific IL-10^+ T-cells, were found in the infected and immunized animals. These findings are in concordance with previous studies evaluating also vaccine candidates against VL, where both

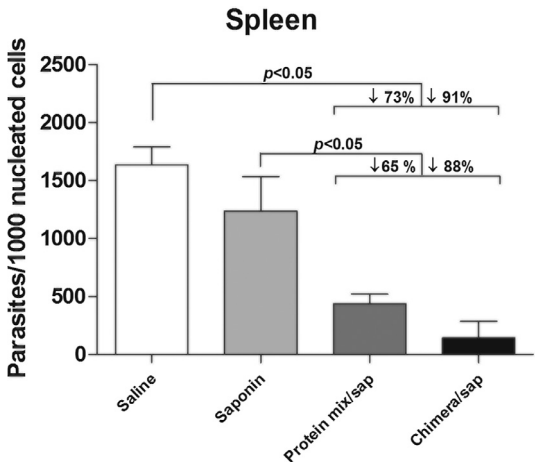

Fig. 10. Parasite burden evaluated by RT-PCR technique. Mice (n = 8 per group) received saline or were immunized with saponin, chimera and/or saponin or protein mix and/or saponin. Then, they challenged with *L. infantum* promastigotes. Sixty days after infection, the splenic parasitism was evaluated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

T-cell subtypes were considered important to protect against murine disease.[45–47]

Although our molecule was built with predicted T-cell epitopes of 3 parasite proteins, we understand that the immunization using this molecule, being associated with saponin, induces both cellular and humoral responses in the vaccinated animals. This fact is in concordance with data showed in other studies,[18] in which a humoral response was also observed to be specific against the recombinant chimera used to immunize mice. We believe that this humoral profile, mainly based on the IgG2a isotype, is related to the IFN-γ production, corroborating the protective phenotype against *L. infantum* infection achieved with the vaccine. In other studies, antiprotein antibody responses were used as biomarkers of efficacy and/or immunogenicity induced by recombinant vaccines.[48]

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

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

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

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

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

ACKNOWLEDGMENTS

Conflicts of Interest: All authors have read the journal's policy on conflicts of interest and have none to declare. All authors have read the journal's authorship agreement and approved submission of the manuscript. The manuscript, neither has been published nor is currently under consideration for publication by any other journal.

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