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*Co-senior authors.

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Author for correspondence:
Eduardo A. F. Coelho, E-mail: eduardoferrazcoelho@yahoo.com.br

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High-through identification of T cell-specific phage-exposed mimotopes using PBMCs from tegumentary leishmaniasis patients and their use as vaccine candidates against *Leishmania amazonensis* infection

Gerusa B. Carvalho[1], Lourena E. Costa[1], Daniela P. Lage[1], Fernanda F. Ramos[1], Thaís T. O. Santos[1], Patrícia A. F. Ribeiro[1], Daniel S. Dias[1], Beatriz C. S. Salles[1], Mariana P. Lima[1], Lívia M. Carvalho[2], Ana C. S. Dias[3], Patrícia T. Alves[3], Michelle L. Franklin[1], Renata A. M. Silva[1], Mariana C. Duarte[1,4], Daniel Menezes-Souza[1,4], Bruno M. Roatt[2], Miguel A. Chávez-Fumagalli[1], Luiz Ricardo Goulart[3,5,*], Antonio L. Teixeira[1,6,7,*] and Eduardo A. F. Coelho[1,4,*]

[1]Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Av. Prof. Alfredo Balena, 190, Belo Horizonte, 30130-100, Minas Gerais, Brazil; [2]Laboratório de Imunopatologia, Núcleo de Pesquisas em Ciências Biológicas, Universidade Federal de Ouro Preto, 35.400-000, Ouro Preto, Minas Gerais, Brazil; [3]Laboratório de Nanobiotecnologia, Instituto de Genética e Bioquímica, Universidade Federal de Uberlândia, Av. Amazonas s/n, Campus Umuarama, Bloco 2E, Sala 248, 38400-902, Uberlândia, Minas Gerais, Brazil; [4]Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Belo Horizonte, Av. Antônio Carlos, 6627, Pampulha, 31270-901, Minas Gerais, Brazil; [5]Department of Medical Microbiology and Immunology, University of California-Davis, Davis, CA 95616, USA; [6]Laboratório Interdisciplinar de Investigação Médica, Faculdade de Medicina, Universidade Federal de Minas Gerais, Av. Prof. Alfredo Balena, 190, Belo Horizonte, 30130-100, Minas Gerais, Brazil and [7]Neuropsychiatry Program, Department of Psychiatry and Behavioral Sciences, McGovern Medical School, The University of Texas Health Science Center at Houston, 1941 East Road, Houston, TX 77041, USA

Abstract

In the current study, phage-exposed mimotopes as targets against tegumentary leishmaniasis (TL) were selected by means of bio-panning cycles employing sera of TL patients and healthy subjects, besides the immune stimulation of peripheral blood mononuclear cells (PBMCs) collected from untreated and treated TL patients and healthy subjects. The clones were evaluated regarding their specific interferon-γ (IFN-γ) and interleukin-4 (IL-4) production in the *in vitro* cultures, and selectivity and specificity values were calculated, and those presenting the best results were selected for the *in vivo* experiments. Two clones, namely A4 and A8, were identified and used in immunization protocols from BALB/c mice to protect against *Leishmania amazonensis* infection. Results showed a polarized Th1 response generated after vaccination, being based on significantly higher levels of IFN-γ, IL-2, IL-12, tumour necrosis factor-α (TNF-α) and granulocyte-macrophage colony-stimulating factor (GM-CSF); which were associated with lower production of specific IL-4, IL-10 and immunoglobulin G1 (IgG1) antibodies. Vaccinated mice presented significant reductions in the parasite load in the infected tissue and distinct organs, when compared with controls. In conclusion, we presented a strategy to identify new mimotopes able to induce Th1 response in PBMCs from TL patients and healthy subjects, and that were successfully used to protect against *L. amazonensis* infection.

Introduction

Leishmaniases are diseases caused by protozoan parasites belonging to the genus *Leishmania*. This disease complex is characterized by high morbidity and mortality, and it is present in 98 countries in three continents (Asia, Africa and South and Central America), with 380 million people at risk of infection (WHO, 2010; Hirve *et al*., 2017). In addition, the geographic distribution of disease is expanding throughout the Western hemisphere, and it has been already found in North America, reaching as far as Southern Canada (Duarte *et al*., 2016).

The treatment against leishmaniasis is inadequate, since limitations such as side-effects, high cost and/or parasite resistance are registered (Sundar and Chakravarty, 2013; Sundar and Singh, 2016). The main clinical manifestations of disease are tegumentary (TL) and visceral (VL) leishmaniasis. TL is the most common clinical form, and it is found in Africa and Asia being caused by *Leishmania major* species, whereas in the Americas the *L. mexicana*, *L. braziliensis* and *L. amazonensis* species are the main responsible by disease (Dias *et al*., 2017; Lima *et al*., 2017). Among different parasite species causing leishmaniasis in mammalian hosts, *L. amazonensis* is particularly interesting, due to the large spectrum of clinical disease

caused by it, varying since TL to VL (Honoré *et al.*, 1998; Deak *et al.*, 2010; Lage *et al.*, 2015; Martins *et al.*, 2017*a*, 2017*b*).

Murine models have been used to understand the host–parasite relationships, and also contributing to elucidate the role of T cells in resistance and/or susceptibility to *Leishmania* infection (Garde *et al.*, 2018). The resistance against disease is associated with the development of an antiparasite $CD4^+$ and $CD8^+$ T cell-mediated Th1 immunity, which is characterized by the production of interferon-γ (IFN-γ), interleukin-2 (IL-2), IL-12 and granulocyte-macrophage colony-stimulating factor (GM-CSF), among other pro-inflammatory cytokines; while the secretion of IL-4, IL-10 and IL-13, among other anti-inflammatory molecules, contribute to the susceptibility to the infection. BALB/c mice develop a Th2 response after *L. amazonensis* infection, presenting high levels of parasite-specific IL-4 and IL-10 along with elevated antileishmanial immunoglobulin G1 (IgG1) antibody production (Coelho *et al.*, 2003; Ramírez *et al.*, 2014; Campos *et al.*, 2015).

Prophylactic vaccination can contribute to control the spread of disease, being less expensive to develop than the discovery of new antileishmanial drugs (DebRoy *et al.*, 2017; Ponte-Sucre *et al.*, 2017). An ideal candidate should be safe, able to induce both $CD4^+$ and $CD8^+$ T cell responses and long-term immune memory, which could be boosted by natural infections, thus reducing the number of vaccine doses required (Amit *et al.*, 2017; Oliveira *et al.*, 2018). In addition, it should not require the association of immune adjuvants to increase its immunogenicity, since there are few of these licensed products for the use in dogs and humans (Reed *et al.*, 2016). So far, most of the parasite proteins tested as recombinant vaccines against *L. amazonensis* infection needed the association of adjuvants (Coelho *et al.*, 2003; Ramírez *et al.*, 2014; Martins *et al.*, 2017*a*, 2017*b*; Ribeiro *et al.*, 2017). Another candidate, the Leishvacin®, which is composed of total *L. amazonensis* antigenic preparations, was tested as vaccine against infection by the parasites. However, although the safety and capacity to induce IFN-γ production was demonstrated, this product failed in clinical trials (Velez *et al.*, 2005; Pratti *et al.*, 2016), thus demonstrating the need to identify and develop new candidates to protect against TL.

Nanotechnological tools have been applied for the improvement of the conditions of prevention, diagnosis and/or prognosis of diseases (Goulart *et al.*, 2010, 2017). In this context, phage display is a high-throughput proteomic technology, in which phage-surface exposed mimotopes recognize specific target ligands. By means of this technology, large repertoires of random sequences offer the advantage that high numbers of peptide sequences can be screened in a short period of time (Kuhn *et al.*, 2016). The principle of the selection procedures, namely bio-panning cycles, consists of isolating phages that bind specifically to the target molecule, identifying later their insert by sequencing (Manoutcharian, 2005). Phage display has been used to identify mimotopes applied as biological agents in a variety of studies, and employed as new diagnostic markers, vaccine candidates and/or immunotherapeutic targets on the leishmaniasis (Costa *et al.*, 2013, 2015; Toledo-Machado *et al.*, 2015; Costa *et al.*, 2017; Link *et al.*, 2017).

In the current study, phage display was used to screen specific mimotopes against IgG antibodies from TL patients, which were subtracted from antibodies from healthy subjects. After bio-panning cycles, selected phage clones were used to stimulate peripheral blood mononuclear cells (PBMCs) from healthy individuals and treated TL patients, when the specific IFN-γ and IL-4 production was measured. The cytokine values were used to calculate the selectivity and specificity of each clone, and those presenting the best values represented by higher IFN-γ/IL4 ratios were selected for the *in vivo* experiments. Two clones, A4 and A8, were then selected and used to immunize BALB/c mice, which were later challenged with *L. amazonensis* promastigotes. This selection strategy can be considered the first proof-of-concept trial for the discovery of novel immunogens against TL able to induce specific Th1 response using phage-displayed mimotopes.

Materials and methods

Blood samples

Blood samples were collected from healthy subjects ($n = 8$, with five males and three females presenting ages ranging from 22 to 45 years) and from TL patients ($n = 12$, including eight males and four females, with ages ranging from 26 to 52 years), which were collected from an endemic area of disease (Belo Horizonte). The healthy subjects did not present clinical signal of disease, and showed negative serological results by using a commercial kit (Kalazar Detect™ Test, InBios International, Seattle, WA, USA). Regarding TL patients, the diagnosis was confirmed by means of clinical evaluation, by direct demonstration of the parasites in Giemsa-stained smears of mucosal fragments and polymerase chain reaction (PCR) identifying the *L. braziliensis* kinetoplastid DNA. The patients were treated with pentavalent antimonials (Sanofi Aventis Farmacêutica Ltda., Suzano, São Paulo, Brazil). Blood sampling was collected before treatment and 6 months after the end of the treatment sessions.

Bio-panning cycles

To perform the bio-panning cycles, the purification of IgG antibodies from sera of TL patients ($n = 12$) and healthy subjects ($n = 8$) was performed as described previously (Costa *et al.*, 2015). Briefly, 10^{12} viral particles of the bacteriophage library (Ph.D.®-C7C library, New England BioLabs, Ipswich, MA, USA) were diluted in 250 μL of 50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Tween 20 buffer (TBS-T). The mixture was incubated for 30 min, at room temperature, with microspheres coupled to the IgG antibodies purified from healthy subjects for subtraction, which were magnetically captured. The remaining phages in the supernatant were recovered and transferred to a new tube, which was subjected to positive selection using IgG from TL patients. The supernatant was removed and the bound phages were washed five times in 1 mL TBS-T buffer, and were eluted in 500 μL of 0.2 M glycine buffer, pH 2.0. Next, 75 μL of 1 M Tris-base pH 9.0 were added to neutralize the acidic pH of the solution. After the selection, 58 clones were isolated and individually picked to a sterile culture microplate (96-well microtest TM plate, BD Falcon™ clear, Corning, New York, USA), by using LB medium. The sequencing was performed by capillary electrophoresis on ABI 3130 equipment, with BigDye v 3.1 and POP7 polymer (Myleus Biotechnology®, Belo Horizonte, Brazil). For analyses of AB1 output files, the sequence scanner software (Applied Biosystems Inc., Foster City, CA, USA) was employed. From isolated 58 clones, nine showed valid and non-repeated amino acid sequences and were used to stimulate human PBMCs.

In vitro PBMC culture and cytokine production

To perform the *in vitro* cultures and evaluate the cytokine production specific to the pre-selected clones, PBMCs from treated TL patients ($n = 12$) and healthy subjects ($n = 8$) were purified by density centrifugation through Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), as described previously (Ramos *et al.*, 2017). Briefly, cells (10^7) were cultured in complete RPMI 1640 medium, which was composed of the medium plus 20% inactivated fetal bovine serum (FBS, Sigma-Aldrich, St.

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Louis, MO, USA), 2 mM L-glutamine, 200 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate and 1× non-essential amino acid. Then, they were plated in 48-well flat-bottom tissue culture plates (Costar, Cambridge, MA, USA) and incubated in medium (background control) or stimulated with the individual clones (10^{10} phages, each) or *L. amazonensis* SLA (25 μg mL^{-1}) for 48 h at 37 °C in 5% CO_2. A wild-type clone (WTP), which did not express foreign peptide, and a random non-specific phage (RP), which express a *Leishmania* non-related peptide, were used as controls (10^{10} phages, each). The supernatants were collected, and IFN-γ and IL-4 production was evaluated by capture enzyme-linked immunosorbent assay (ELISA) using commercial kits (Human IFN-γ and IL-10 ELISA Sets, BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. Results were interpolated from a standard curve using recombinant cytokines (in pg mL^{-1}).

Vaccination and challenge infection

L. amazonensis (IFLA/BR/1967/PH-8) strain was cultured at 24 °C in complete Schneider's medium (Sigma), which was composed of the medium plus 20% FBS, 20 mM L-glutamine, 200 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, pH 7.4. The soluble *L. amazonensis* antigenic extract (SLA) was prepared as described elsewhere (Coelho *et al.*, 2003). Mice ($n = 16$ per group) were inoculated subcutaneously in their left hind footpad with WTP, RP, A4 or A8 clones (10^{10} phages, each) or received saline. Three doses were administered at 14 day intervals. Thirty days after the last vaccine dose, animals ($n = 8$ per group) were euthanized for the analysis of the immune response elicited by vaccination. At the same time, the remaining mice ($n = 8$ per group) were infected subcutaneously in their right hind footpad with 10^6 *L. amazonensis* stationary promastigotes. They were followed for 10 weeks, and the course of disease was monitored at weekly intervals by measuring footpad thickness with a metric caliper, and expressed as the increase in thickness of the infected footpad compared with the uninfected footpad. Experiments were repeated and presented similar results.

Cellular response evaluated by capture ELISA and flow cytometry

The immunogenicity of the vaccines was evaluated before and after infection. Spleen cells (5×10^6) were incubated (in 24-well plates, Nunc) in duplicate in the absence (medium and background control) or presence of each clone used in the immunization (10^{10} phages, each) or *L. amazonensis* SLA (25 μg mL^{-1}), for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70 and GM-CSF levels were measured in cell supernatants using commercial kits (Pharmingen®, USA). Nitrite production was also measured in the supernatants by the Griess reaction, and results were expressed as μM. The participation of CD4$^+$ and CD8$^+$ T cells in IFN-γ production in the phage-immunized animals was evaluated by the incubation with monoclonal antibodies (mAbs) against mouse IL-12 (C017.8), CD4 (GK 1.5) or CD8 (53–6.7) (5.0 μg mL^{-1} each). Appropriate isotype-matched controls [rat IgG2a (R35-95) and rat IgG2b (95-1)] were used (Pharmingen®, USA). A flow cytometry assay was also performed in the spleen cells of the saline, A4 and A8 groups, aiming to evaluate the parasite-specific IFN-γ, IL-2, tumour necrosis factor-α (TNF-α) and IL-10-producing CD4$^+$ and CD8$^+$ T cell profiles (Duarte *et al.*, 2016). Results were expressed as indexes which were calculated by the ratio between the cytokine-producing T cell subtype frequencies in the SLA-stimulated cultures *vs* the values found in the unstimulated (control) cultures.

Humoral response evaluated by an ELISA protocol

Antibody production was evaluated in infected and/or immunized animals. For this, sera samples were collected and phage and parasite-specific IgG1 and IgG2a antibody levels were measured by an ELISA protocol as described elsewhere (Dias *et al.*, 2017). Briefly, A4 and A8 phages and SLA were used as antigens in the plates (10^9 phage and 1.0 μg of SLA per well), and sera were 1:100 diluted. The anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich, St. Louis, MO, USA) were employed, both in a 1:10 000 dilution, and the optical density was read using an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Concord, Canada) at 492 nm.

Parasite load evaluated by limiting dilution technique and RT-PCR

To evaluate the parasite burden in infected and vaccinated animals, the infected footpad, liver, spleen, draining lymph nodes (dLN) and bone marrow (BM) were collected 10 weeks after infection, and a limiting dilution technique was performed (Martins *et al.*, 2017*a*, 2017*b*). Briefly, tissue and organs were weighed and homogenized using a glass tissue grinder in sterile phosphate buffered saline (PBS 1×). Tissue debris were removed by centrifugation at 150 ***g***, and cells were concentrated by centrifugation at 2000 ***g***. Pellets were resuspended in 1 mL of complete Schneider's insect medium, and 220 μL were plated onto 96-well flat-bottom microtitre plates (Nunc), and diluted in log-fold serial dilutions (10^{-1}–10^{-12}). Each sample was plated in triplicate and read 7 days after the beginning of the culture, at 24 °C. Results were expressed as the negative log of the titre (i.e. the dilution corresponding to the last positive well), which was adjusted per milligram of tissue or organ. Splenic parasite load was also evaluated by RT-PCR as described previously (Dias *et al.*, 2017), and results were expressed as the number of parasites per 1000 nucleated cells.

Bioinformatics, cloning and purification of dihydroorotate dehydrogenase protein

The constrained PhD library used for peptide selection presents conformational peptides with cysteine residues in the borders, therefore, analyses were performed with the 7-mer peptide sequence and the amino acid sequences (AC-XXXXXXX-CGGGS) contained in the fusion with the pIII bacteriophage capsid protein, as described elsewhere (Alves *et al.*, 2014). The putative protein function was predicted by BLAST search alignment in the UNIPROT server (http://www.uniprot.org/). Physicochemical properties of the peptides (molecular weight and pI) were predicted using the Compute pI/Mw tool at the Expasy server (http://web.expasy.org/computepi/). The hydrophobicity and net charge of the peptides were predicted using the antimicrobial peptide database server (http://aps.unmc.edu/AP/prediction/predictionmain.php) (Wang *et al.*, 1990). The conformational alignment was predicted by using PepSurf software (http://pepitope.tau.ac.il/) and the immunogenic regions in protein structures were evaluated by using the Epitopia server (http://epitopia.tau.ac.il/). Bioinformatics assays indicated the dihydroorotate dehydrogenase protein (XP_003722973.1, PDB: 4WZH_A) as expressing both A4 and A8 phage-exposed mimotopes (YLLCISP and GSRCYPR, respectively). In this context, this protein was cloned using the *L. braziliensis* (MHOM/BR/75/M2904 strain) kDNA, and the recombinant version was expressed in *Escherichia coli* M15 strain by adding 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG, Promega, Montreal, Canada), for

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2 h at 37 °C. For purification, bacteria were lysed, product was centrifuged at 12 000 ***g*** for 30 min at 4 °C, and the recombinant protein was purified under non-denaturing conditions using a His-Trap column (GE Healthcare Life Science, Pittsburgh, PA, USA), attached to an FPLC (GE Healthcare Life Science) system.

ELISA and immunoblotting assay

The recombinant protein was tested in ELISA experiments by using 1.0 μg per well in Flexible microtitre immunoassay plates (Jetbiofil®, Belo Horizonte, Brazil), aiming to confirm its reaction with sera from A4 and A8 phage-vaccinated mice. Samples from non-vaccinated (naive) or WTP- or RP-clone immunized mice (all 1:100 diluted) were used as controls. An anti-mouse IgG horseradish-peroxidase conjugated antibody (1:10 000 diluted) was used and reactions were developed and stopped as described above. For immunoblotting, the recombinant protein (10 μg) was subjected to a SDS-12% PAGE and blotted onto a nitrocellulose membrane (0.2 μm pore size, Sigma, St. Louis, USA). Then, they were blocked with a solution composed of PBS 1× plus Tween 20 0.05% (PBS-T) added with 5% albumin solution, and incubated for 1 h at 37 °C before undergoing the first incubation with naive or A4- and A8-immunized mice sera pools (all 1:200 diluted in PBS-T). Membranes were washed and an anti-mouse IgG horseradish-peroxidase conjugated antibody was added into the plates, at which time a new incubation was developed for 1 h at 37 °C. Reactions were developed adding chloronaphthol, diaminobenzidine and H_2O_2 30 vol., and stopped by adding distilled water.

Statistical analysis

Results were entered into Microsoft Excel (version 10.0) spreadsheets, and analysed with GraphPad Prism™ (version 6.0 for Windows). Statistical analysis were performed by one-way analysis of variance (ANOVA) followed by the Bonferroni's post-test. Differences were considered significant with $P < 0.05$. The immunization experiments were repeated, and results were similar between them. Data shown in this study are representative of the first experiment.

Results

Selection of the phage clones using human PBMCs

Initially, bio-panning cycles were performed using IgG antibodies derived from healthy subjects and treated TL patients. A total of 58 clones were identified and pre-selected, but only nine of them exhibited valid and non-repeated amino acid sequences, and were used to stimulate human PBMCs. These sequences were deduced by the Expasy server, and an alignment showed no consensus motifs among them. Also, none of these clones were non-specific binders to the reagents used in the bio-selection cycles. Then, these molecules were used to stimulate human cells from healthy subjects and treated TL patients, and the IFN-γ and IL-4 production was measured. Our experimental strategy was based on a previous work developed by our group, where immunogenic mimotopes were identified and successfully tested as protective against experimental VL (Ramos *et al.*, 2017). As described in that previous study, in the current work, the selectivity was presented as the ability of clone to bind to its target based on the mixture of different molecules. Values were calculated by determining the ratio between the IFN-γ and IL-4 levels, which were obtained after stimulation of PBMCs from treated TL patients, through the values obtained using the RP stimulus. A ratio was calculated and results were defined as the selectivity of each clone. On the other hand, the specificity was defined as the ability of clone to bind to its target based on the presence of phage surface-displayed peptide. Values were calculated by determining the ratio between IFN-γ and IL-4 levels, which were obtained after the stimulation of PBMCs from healthy subjects, through the values obtained using the WTP stimulus. A ratio was calculated and results were defined as the specificity of each clone (Fig. 1). With the results, two clones (A4 and A8) were selected based on their higher selectivity and specificity values, and they were used for the immunization experiments in BALB/c mice.

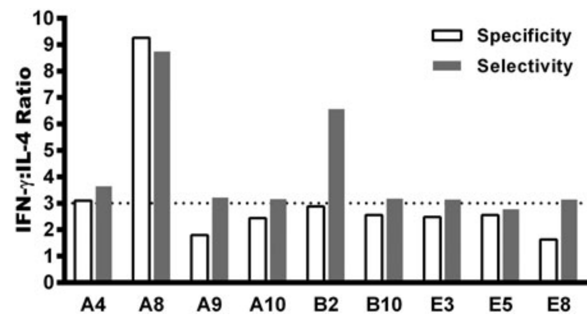

Fig. 1. Selectivity and specificity of the phage clones based on their specific IFN-γ and IL-4 production. Human PBMCs were collected from treated TL patients and healthy subjects, and cells (10^7) were cultured in complete RPMI 1640 medium in 48-well flat-bottom tissue culture plates, and non-stimulated (medium) or stimulated with each phage clone (10^{10} phages, each) or *Leishmania amazonensis* SLA (25 μg mL^{-1}), for 48 h at 37 °C in 5% CO_2. A WTP and a random phage (RP) were used as controls (10^{10}, each). Supernatants were collected and IFN-γ and IL-4 levels were measured by capture ELISA. Black bars indicate the specificity of each clone, which was calculated by the ratio between the IFN-γ and IL-4 values obtained from each clone through respective cytokine values obtained after the WTP stimulus and using PBMCs from healthy subjects. With the new values, the ratio between the IFN-γ and IL-4 levels with these results was calculated, and the specificity of clone was defined and is shown. White bars indicate the selectivity, which was calculated by dividing the IFN-γ and IL-4 levels obtained from each clone through their cytokine values, which were obtained after the RP stimulus and using PBMCs from treated TL patients. With the new values, the ratio between the IFN-γ and IL-4 levels was calculated, and the selectivity of clone was defined and is shown.

Immunogenicity induced by A4 and A8 clones, before and after infection

BALB/c mice were immunized with A4 or A8 clones. Thirty days after the last vaccine dose, their spleen cells were collected. As controls, animals were immunized with WTP or RP phages or received saline. Immunization using A4 or A8 clones induced higher levels of IFN-γ, IL-12 and GM-CSF, when compared with the control groups (Fig. 2A), whereas no significant production of IL-4 and IL-10 was observed. The humoral response showed higher production of phage and parasite-specific IgG2a isotype antibodies in the A4- and A8-immunized mice, while a low and similar production of IgG1 and IgG2a isotypes was found in other groups (Fig. 2B). The ratios between these antibody isotypes were calculated, and results showed higher IgG2a/IgG1 values in the A4- and A8-immunized mice, when compared with the controls, confirming the induction of the Th1 immune profile in these vaccinated animals.

After infection, the immunogenicity was maintained in the A4- and A8-immunized mice, since higher phage- and parasite-specific IFN-γ, IL-12 and GM-CSF levels were found. In contrast, in the controls, a significantly higher production of IL-4 and IL-10 was observed (Fig. 3A). The antibody profile was maintained in the A4 and A8 groups, since higher IgG2a and lower IgG1 production specific to phages and parasites was found, while in the control groups, higher IgG1 levels were found, corroborating the Th2 response typically reported in *L. amazonensis*-

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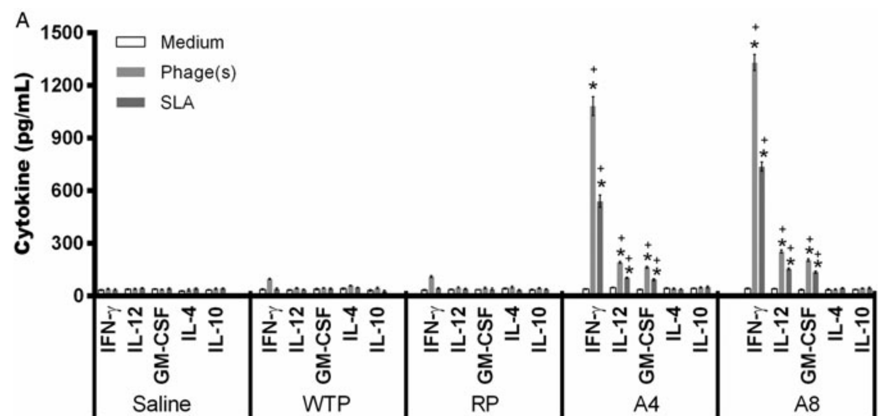

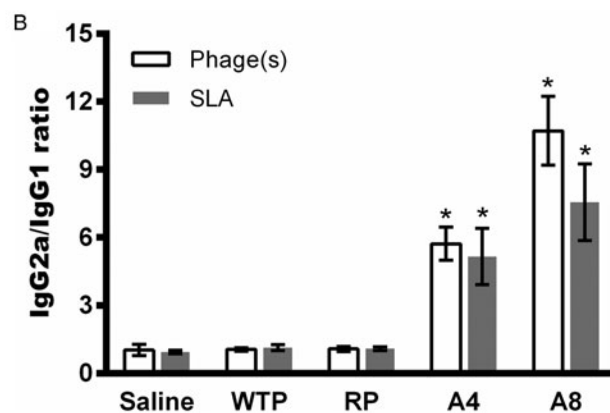

Fig. 2. Immunogenicity induced in BALB/c mice by the A4 and A8 phage clones. Spleen cells were obtained from mice ($n = 8$ per group) 30 days after the last immunization. Cells (5×10^6) were non-stimulated (control) or separately stimulated with the A4, A8, WTP or non-related phage (RP) (10^{10} phages, each), for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4 and IL-10 levels were measured in culture supernatants by ELISA (A). In addition, sera samples were collected from the animals and the anti-phage and anti-parasite IgG2a and IgG1 isotype antibody levels were determined, and the ratios between IgG2a and IgG1 production were calculated and shown (B). In both cases, 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 WTP and RP groups ($P < 0.01$).

infected BALB/c mice (Fig. 3B). A4- and A8-immunized animals and later challenged with *L. amazonensis* produced significantly higher levels of nitrite in comparison with controls (Fig. 4), indicating that high levels of IFN-γ possibly stimulated the production of nitric oxide (NO) and other molecules by phagocytic cells, thereby assisting in the parasite control in these animals.

To characterize the origin of IFN-γ in the immunized animals, the participation of $CD4^+$ and $CD8^+$ T cells in the secretion of this cytokine was evaluated by means of a cellular inhibition experiment, in which mAbs directed against $CD4^+$ and $CD8^+$ molecules were added into the *in vitro* cultures. In both immunization protocols using A4 and A8 phages, the involvement of $CD8^+$ T cells in the IFN-γ production was significantly higher when compared with results found using the anti-CD4 mAb (Fig. 5). The intracytoplasmic cytokine profile was investigated in the stimulated spleen cells by flow cytometry, and results showed that the immunization with the A4 and A8 clones induced higher levels of parasite-specific IFN-γ^+, IL-2^+ and TNF-α^+ producing both subtype T cells, which were associated with lower levels of IL-10^+ T cells, when compared with the control groups (Fig. 6).

Protection against L. amazonensis *infection*

The effect of immunization with the A4 and A8 clones was evaluated against *L. amazonensis* infection. Weekly measurements of the lesion development were performed in the infected footpads of the animals. A4- or A8-vaccinated mice showed significant reductions in the lesion development, in the order of 5.8 and 6.8 mm, respectively, when compared with the saline group; 5.0 and 6.0 mm, respectively, when compared with the WTP group and 5.3 and 6.3 mm, respectively, when compared with the RP group (Fig. 7A). Ten weeks after infection, animals were euthanized and the parasitism was evaluated in different organs. Using a limiting dilution technique, A4- and A8-vaccinated mice presented significant reductions in the parasite burden in all the evaluated tissue and organs, when compared with controls (Fig. 7B). When the splenic parasitism was evaluated by a RT-PCR technique, results also showed that the immunization with A4 or A8 clones induced significant reduction in the parasitism, when compared with results found in the control groups (Fig. 8). No significant difference was found between the A4 or A8 groups.

Bioinformatics and mapping the A4 and A8 mimotopes in Leishmania *proteins*

Bioinformatics assays showed that A4 phage-exposed peptide (YLLCISP) presents net charge, hydrophobicity and molecular weight of 0, 50.0% and 1343.6, respectively; whereas A8 phage-exposed peptide (GSRCYPR) presents net charge, hydrophobicity and molecular weight of +2, 28.0% and 1373.5, respectively. We also found that A4 and A8 phage-exposed mimotopes were present in the dihydroorotate dehydrogenase protein amino acid sequence. The A4 phage-exposed peptide was also identified in the 6-phosphogluconolactonase protein sequence, while A8 phage-exposed peptide was identified in the chain A, crystal structure of the mitochondrial peroxidoxin from *L. braziliensis* protein sequence. Aiming to corroborate such findings, the dihydroorotate dehydrogenase protein was cloned, and its recombinant version (~33.9 kDa) was used in ELISA experiments to verify the reactivity against antibodies from sera of A4 and A8 phage-immunized mice. Results showed OD values (mean ± standard deviation) of 0.467 ± 0.026 and 0.565 ± 0.055, respectively, for A4-immunized mice, and 0.588 ± 0.035 and 0.663 ± 0.041, respectively, for A8-immunized mice. Sera from naive-,

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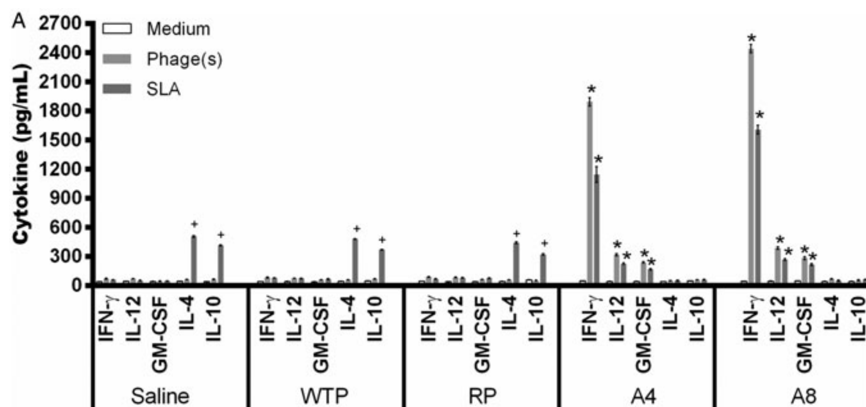

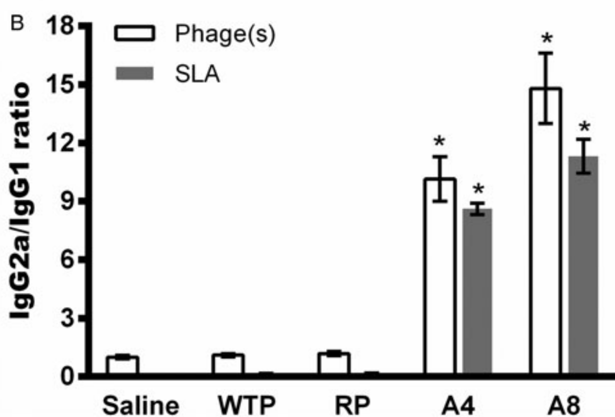

Fig. 3. Immune response generated after *Leishmania amazonensis* infection. BALB/c mice ($n = 8$ per group) were immunized and later challenged using *L. amazonensis* promastigotes. Ten weeks after infection, their spleens were collected and splenocytes (5×10^6) were non-stimulated (control) or stimulated with the A4, A8, WTP or RP (1×10^{10} molecules, each) for 48 h at 37 °C in 5% CO_2. IFN-γ, IL-12, GM-CSF, IL-4 and IL-10 levels were measured in culture supernatants by ELISA (A). Sera samples of the infected and immunized animals were also collected for evaluation of phage- and parasite-specific IgG2a and IgG1 isotype antibody production, and the IgG2a/IgG1 ratios were calculated (B). Bars represent the mean ± standard deviation of the groups. * indicates statistically significant difference in relation to the saline, WTP and RP groups ($P < 0.0001$). + indicates statistically significant difference in relation to the A4 and A8 groups ($P < 0.0001$).

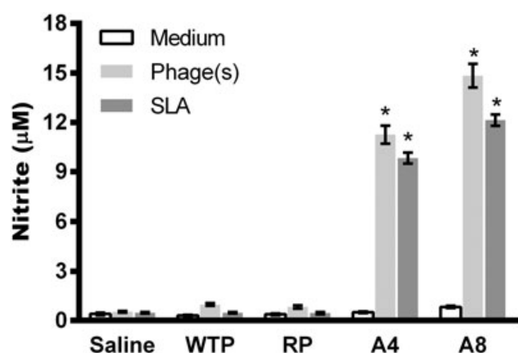

Fig. 4. Macrophage activation assessed by nitrite production. Spleen cells from mice ($n = 8$ per group) that received saline or were immunized with WTP, RP, A4 or A8 phages, and later infected with *Leishmania amazonensis* promastigotes were *in vitro* stimulated with the respective clone (10^{10} phages, each) or SLA (25 μg mL^{-1}) for 48 h at 37 °C in 5% CO_2. Nitrite production was evaluated in cell supernatants. Bars represent the mean ± standard deviation of the groups. * indicates statistically significant difference in relation to the saline, WTP and RP groups ($P < 0.0001$).

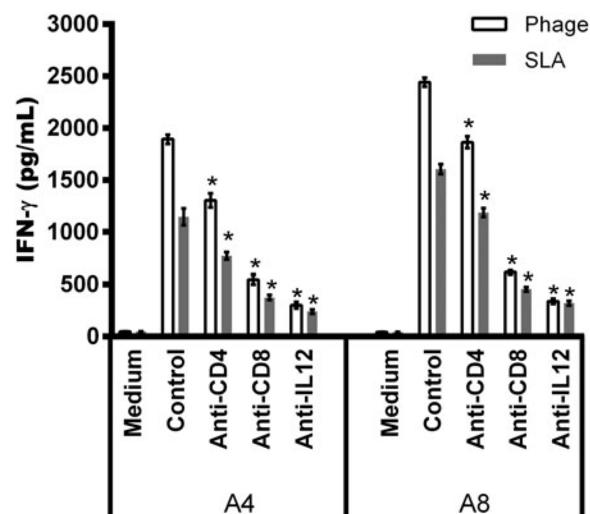

Fig. 5. Involvement of CD4+ or CD8+ T cells in IFN-γ production in infected and immunized animals. BALB/c mice ($n = 8$ per group) were immunized with the A4 and A8 clones and later challenged using *Leishmania amazonensis* promastigotes. Ten weeks after infection, their spleen cells (5×10^6) were non-stimulated (control) or stimulated with A4, A8, WTP or RP (1×10^{10}, each) in the absence or presence of mAbs against mouse IL-12, CD4+ or CD8+ (5 μg mL^{-1}, each). IFN-γ levels in cell supernatants were measured by ELISA. Bars indicate the mean ± standard deviation of the different stimuli and groups. * indicates statistically significant difference in relation to the control group ($P < 0.0001$).

WTP- or RP-vaccinated mice presented a protein-specific antibody production of 0.031 ± 0.006, 0.051 ± 0.011 and 0.062 ± 0.010, respectively. In addition, a Western-blotting assay was performed, and results showed a specific reactivity from antibodies in sera of A4- or A8-immunized mice against the recombinant protein (Supplementary Fig. S1), whereas no reaction was found using naive-, WTP- or RP-vaccinated mice sera. In addition, an alignment of the conformational structure of the dihydroorotate dehydrogenase protein was performed aiming to identify the A4 and A8 peptide sequences, and results showed that they were grouped in the protein cluster region, presenting a high immunogenicity accordingly predicted by the Epitopia server (Supplementary Fig. S2).

Discussion

Leishmaniasis control measures are mainly based on therapeutic interventions in infected humans and in the vector control, but they have shown unsatisfactory results (Singh *et al.*, 2014). New

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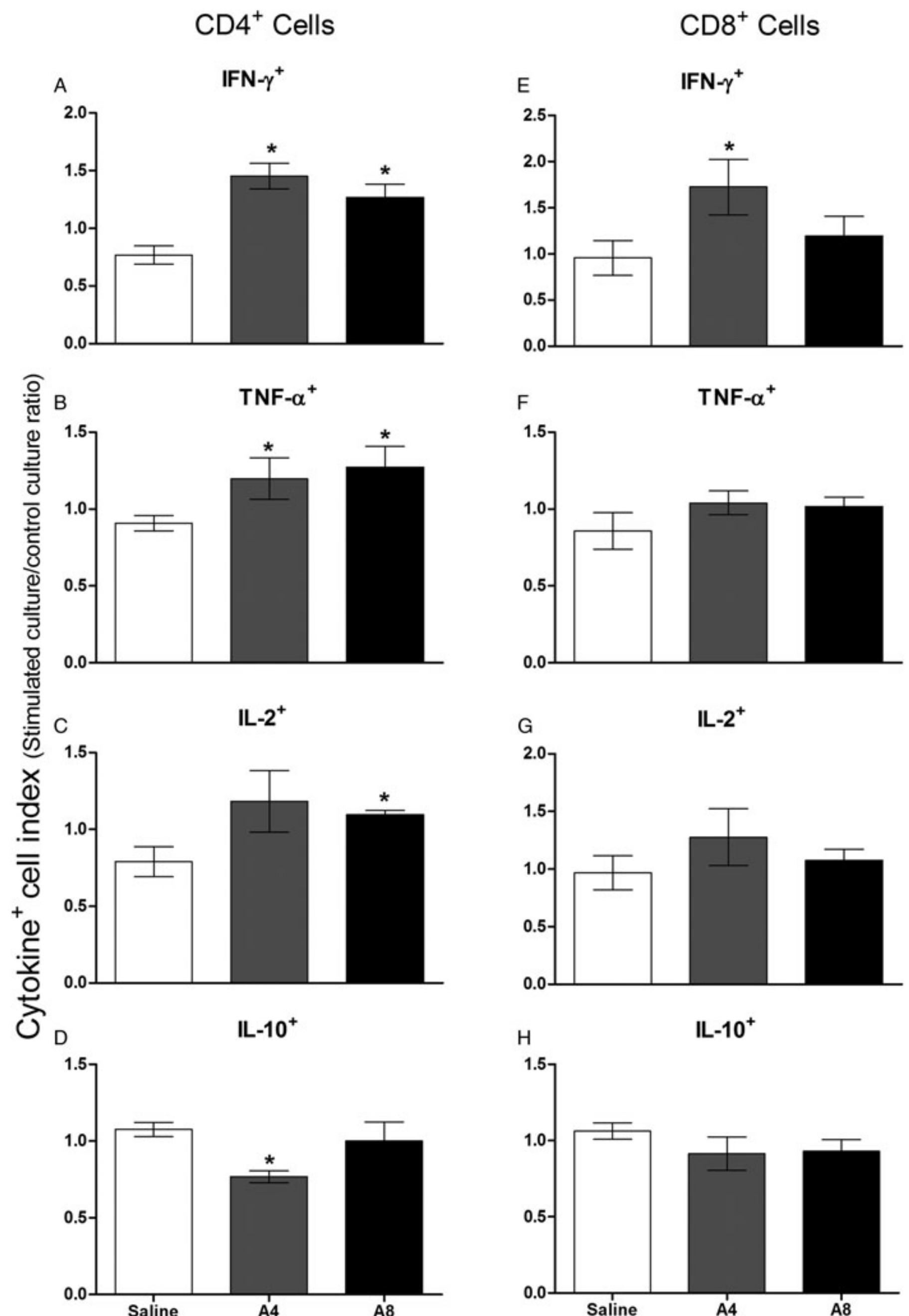

Fig. 6. Intracytoplasmic cytokine-producing CD4+ and CD8+ T cells frequency in infected and immunized animals. Mice received saline (white rectangle) or were immunized with A4 (dark grey bars) or A8 (black bars) clones. Then, they were infected with *Leishmania amazonensis* promastigotes and, 10 weeks later, their splenocytes were *in vitro* stimulated with SLA (25 μg mL^{-1}). Results were expressed as cytokine indexes (stimulated culture/control culture) for IFN-γ, IL-2, TNF-α and IL-10 in (A), (B) and (C) for CD4+ T cells, and in (D), (E) and (F) for CD8+ T cells. Bars indicate the mean plus standard deviation of the groups. * indicates statistically significant difference in relation to the saline group ($P < 0.05$).

therapeutic drugs are challenging to develop, mainly due to the high cost involved, and by the fact that leishmaniases are considered neglected diseases (Mendonça *et al.*, 2018). In this scenario, prophylactic vaccination can be an interesting way to solve this healthy public problem in countries where the disease is endemic. For TL, protection against infection is associated with the development of a parasite-specific Th1 immunity, based on the production of cytokines, such as IFN-γ, IL-2, IL-12 and GM-CSF. In contrast, IL-4, IL-10, IL-13 and transforming growth factor-β (TGF-β), among other anti-inflammatory molecules are associated with susceptibility to the infection in the mammalian hosts (Coelho *et al.*, 2003; Costa *et al.*, 2015; Duarte *et al.*, 2017).

In this context, the screening of phage-displayed peptide libraries by means of bio-panning cycles represents an attractive approach to identify new biological targets (Somers *et al.*, 2002; Feliciano *et al.*, 2014; Coelho *et al.*, 2015; Schieferdecker *et al.*, 2016). Our group recently identified two phage-exposed mimotopes that were immunogenic in PBMCs from VL patients, and they were well-successfully tested as vaccine candidates against experimental disease (Ramos *et al.*, 2017). In the current study, using a similar experimental strategy, but still not applied for TL, we identified two immunogenic mimotopes to be tested as vaccine candidates against this disease. The approach can be considered interesting, since the most bio-panning procedures used in experimental trials have employed ELISA readings to select the best phage clones in their studies (Alban *et al.*, 2014; Alves *et al.*, 2014; Costa *et al.*, 2015; Toledo-Machado *et al.*, 2015). However, since the cellular response based on the production of IFN-γ, IL-12 and GM-CSF, among others, is also required for protection against TL; we have choice to use cytokine dosage in these stimulated immune cells as a strategy to define the best immunogens able to induce a more prominent Th1 immune response in mammalian hosts, and eventually protect against infection. With the IFN-γ and IL-4 values, the selectivity and specificity of each clone were calculated, and results showed that A4 and A8 clones were those presenting the best results, and then these molecules were tested in a murine model to protect against *L. amazonensis* infection.

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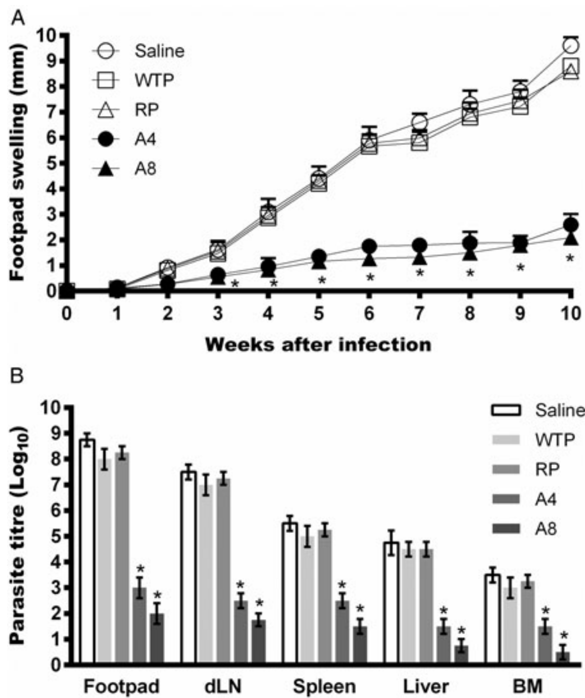

Fig. 7. Protective efficacy against *Leishmania amazonensis* infection with the immunization using A4 and A8 phage clones. BALB/c mice ($n = 8$ per group) were inoculated with saline or immunized with WTP, RP, A4 or A8 phages (1×10^{10} phages, each). Three doses were administered at 2-week intervals, and 4 weeks after the last immunization; mice were subcutaneously infected with 10^6 *L. amazonensis* stationary promastigotes. The course of the infection was monitored at weekly intervals and expressed as the increase in thickness of the infected footpad compared with the uninfected footpad (A). * indicates statistically significant difference in relation to the saline, WTP and RP groups ($P < 0.0001$). In addition, the parasite load was evaluated in the infected footpad, liver, spleen, dLN and BM of the animals by a limiting-dilution technique (B). Bars indicate the mean ± standard deviation of the groups. * indicates statistically significant difference in relation to the saline, WTP and RP groups ($P < 0.0001$).

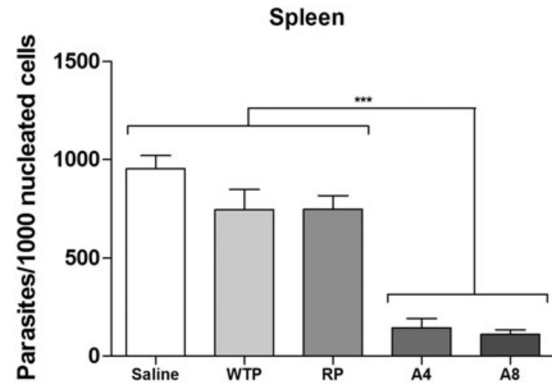

Fig. 8. Parasite burden evaluated by the RT-PCR technique. At the same period of time when infected and immunized mice ($n = 8$ per group) were euthanized and the parasite load was evaluated by a limiting-dilution technique, their spleens were also used to evaluate the parasitism by a RT-PCR technique. Results are shown as the number of parasites per 1000 nucleated cells. Bars indicate the mean ± standard deviation of the groups. *** indicates statistically significant difference in relation to the saline, WTP and RP groups ($P < 0.0001$).

Phage-displayed mimotopes present advantages to be applied as vaccines, such as (i) phages can be taken up by host cells and processed efficiently enabling antigen presentation by MHC classes I and II molecules; (ii) the production of these particles is easier and less expensive than conventional peptide synthesis or recombinant protein production and (iii) the final product consists of multiple virus copies providing high level of mimotope exposure to the host's immune system (Costa *et al*., 2015; Cano *et al*., 2017). In addition, phages are not pathogenic to humans, although they can replicate inside phagocytic cells and potentiate the immune response in the vaccinated hosts (Coelho *et al*., 2015).

In addition, the immune stimulation induced by A4 and A8 clones can be attributed to the own phage genome. This fact can be explained by the presence of cytosine-phosphate-guanosine (CpG) motifs, which are recognized by an evolutionary conserved family of Toll-Like receptors, such as Toll-Like Receptor 9 (TLR-9), which is expressed by a number of antigen presenting cells, such as macrophages and dendritic cells, thus stimulating the production and secretion of immunostimulatory cytokines, such as IFN-γ, TNF-α, IL-1, IL-6 and IL-12, among others. These compounds were also used with adjuvant action for the induction of Th1 immune response by others (Lipford *et al*., 1997; Roman *et al*., 1997; Mohsen *et al*., 2017). The findings described here are interesting, since the absence of association of other adjuvant molecules, which can be considered inflammatory and/or induce significant humoral response in vaccinated hosts, make possible to develop a safer vaccine to protect dogs and humans against *Leishmania* infection (Costa *et al*., 2015; Aghebati-Maleki *et al*., 2016).

The outcome of *L. amazonensis* infection depends largely on host immune responses to the parasites (Wanasen *et al*., 2008). In the current study, stimulated spleen cells of A4- and A8-immunized mice showed a mounted Th1 response when a capture ELISA and flow cytometry were used in the analysis. Cytokines such as IFN-γ, IL-2 and IL-12 have described as effective at protecting against *L. amazonensis* in animal models (Sanchez *et al*., 2017; Bezerra *et al*., 2018). TNF-α has been also implicated in the activation of phagocytic cells to kill parasites, besides stimulation of B cells to produce an antileishmanial IgG2a isotype antibody (Hernández-Ruiz and Becker, 2006), while GM-CSF has been linked to macrophage activation and resistance of murine models against *L. infantum* (Santos *et al*., 2017), *L. major* (Bayih *et al*., 2017) and *L. donovani* (Saldarriaga *et al*., 2006).

Studies have indicated that $CD4^+$ T cells present the *in vivo* capacity to enhance $CD8^+$ T cell response, helping to maintain a specific immunity for a long period of time (Keene and Forman, 1982; Husmann and Bevan, 1988; Singh and Sundar, 2012). Therefore, it seems likely that an optimal antileishmanial activity can be achieved if both $CD4^+$ and $CD8^+$ parasite-specific T cell subtypes are activated by an ideal vaccine candidate (Coelho *et al*., 2015; Margaroni *et al*., 2017). In our work, mAbs were added to the *in vitro* cultures of the stimulated spleen cells, and the IFN-γ production was evaluated. In the results, the most significant reduction in the production of this cytokine was found when anti-CD8 antibodies were used, then corroborating with data presented in other studies using phage display (Costa *et al*., 2015; Ramos *et al*., 2017). On the other hand, although A4 and A8 mimotopes are composed of 7-mer residues, a decrease in the IFN-γ production was also found when the anti-CD4 antibody was added into the cultures. Although these peptides are considered short, others have also showed that small peptides can induce the activation of $CD4^+$ T cells and induce the development of a specific immune response to protect against distinct diseases (Atanackovic *et al*., 2004; Gerlach *et al*., 2005; Martins *et al*., 2015).

Sequence analysis performed in the GenBank database did not reveal any significant similarity to the amino acid sequence of our molecules with previously characterized *Leishmania* proteins, suggesting these sequences correspond to conformational epitopes of parasite proteins. In fact, bioinformatics assays showed that A4 and A8 mimotopes are present in the *Leishmania* dihydroorotate dehydrogenase protein. This molecule is a flavoenzyme that participates in the redox reaction of the *de novo*

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pyrimidine-biosynthesis pathway (Pinheiro *et al*., 2013). It has been also evaluated as therapeutic target against diseases, such as rheumatoid arthritis, cancer and parasitic diseases (Vyas and Ghate, 2011), including those caused by *Trypanosoma brucei* (Arakaki *et al*., 2008), *T. cruzi* (Inaoka *et al*., 2008; Pinheiro *et al*., 2008) and *L. major* (Cordeiro *et al*., 2012).

In our work, the recombinant dihydroorotate dehydrogenase protein and A4 and A8 synthetic peptides were not used in the stimulation of the spleen cells from vaccinated mice. As a consequence, this fact could be considered as a limitation of the study. However, we understand that the parasite- and phage-specific immune response, which was observed when SLA and each clone were used to *in vitro* stimulate the immune cells of the animals can be considered relevant, since results showed the development of a specific Th1 response, when compared with the data obtained when WTP or RP were used as stimuli. In addition, our purpose was to test the own phage clone as a vaccine, as well as to postulate its use in new experiments in other mammalian hosts; thus justifying the employ of these immunogens in their own structural conformation as candidates to protect against disease.

Leishmania proteins applied as immunogens can induce humoral and cellular responses against parasites; however, an ideal vaccine candidate should be able to induce both $CD4^+$ and $CD8^+$ T cell responses, as well as be composed of different parasite immunogenic parts in a single product, making it specific, immunogenic, easier and cheaper to produce, since the production of single recombinant proteins and synthetic peptides is expensive and laborious (Martins *et al*., 2017*a*, 2017*b*; Dias *et al*., 2018). In addition, vaccine design in the context of protect human populations against *Leishmania* infection presents problems, such as individuals displaying a different set of alleles, with potentially different binding specificities are likely to react with a different set of molecules, and alleles are expressed at different frequencies by distinct ethnicities (Poland *et al*., 2007; Oyarzun and Kobe, 2015). As a consequence, the use of few molecules such as synthetic peptides or recombinant proteins makes difficult to protect a heterogeneous population as humans. However, in the current study, the A4 and A8 phage-exposed mimotopes could be considered to composed of a formulation based on other molecules, such as a polypeptide vaccine, since these epitopes were immunogenic in human PBMCs collected from healthy subjects and treated TL patients, and showed a protection when tested in a known murine model, thus making them suitable to be considered for future studies on vaccination protocols against leishmaniasis.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182018001403.

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Conflict of interest. The authors hereby declare that they have no conflicts of interest.

Ethical standards. This study was approved by the Ethics Committee from the Federal University of Minas Gerais (UFMG; Belo Horizonte, Minas Gerais, Brazil) under the protocol number CAAE-32343114.9.0000.5149. Regarding the experiments using mice, the study was also approved by the Committee on the Ethical Handling of Research Animals of UFMG (protocol number 333/2015).

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