Development of an immunogen containing CD4⁺/CD8⁺ T-cell epitopes for the prophylaxis of tegumentary leishmaniasis

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Abstract
Tegumentary leishmaniasis (TL) is a disease of high severity and incidence in Brazil, and *Leishmania braziliensis* is its main etiological agent. The inefficiency of control measures, such as high toxicity and costs of current treatments and the lack of effective immunoprophylactic strategies, makes the development of vaccines indispensable and imminent. In this light, the present work developed a gene encoding multiple T-cell (CD4⁺/CD8⁺) epitope, derived from conserved proteins found in *Leishmania* species and associated with TL, to generate a chimeric protein (rMEP/TL) and compose a vaccine formulation. For this, six T-cell epitopes were selected by immunoinformatics approaches from proteins present in the amastigote stage and associated with host-parasite interactions. The following formulations were then tested in an *L. braziliensis* murine infection model: rMEP/TL in saline or associated with MPLA-PHAD®. Our data revealed that, after immunization (three doses; 14-day intervals) and subsequent challenging, rMEP/TL and rMEP/TL + MPLA-vaccinated mice showed an increased production of key immunological biomarkers of protection, such as IgG₂a, IgG₂a/IgG₁, NO, CD4⁺, and CD8⁺ T-cells with IFN-γ and TNF-α production, associated with a reduction in CD4⁺IL-10⁺ and CD8⁺IL-10⁺ T-cells. Vaccines also induced the development of central (CD44highCD62Lhigh) and effector (CD44highCD62Llow) memory of CD4⁺ and CD8⁺ T-cells. These findings, associated with the observation of lower rates of parasite burdens in the vaccinated groups, when compared to the control groups, suggest that immunization with rMEP/TL and, preferably, associated with an adjuvant, may be considered an effective tool to prevent TL.

Key points

• Rational design approaches for vaccine development.
• Central and effector memory of CD4⁺ and CD8⁺ T-cells.
• Vaccine comprised of rMEP/TL plus MPLA as an effective tool to prevent TL.

Keywords Tegumentary leishmaniasis · *Leishmania braziliensis* · Vaccine · Immunoinformatics · T-cell epitope mapping · Chimeric protein

Introduction

Tegumentary leishmaniasis (TL) is a neglected disease with worldwide distribution and is caused by several species of parasites from the *Leishmania* genus (Lainson and Shaw 1987). TL is associated with a broad spectrum of clinical manifestations, ranging from discrete, single, or multiple skin lesions that can heal spontaneously (Grimaldi and Tesh 1993; Saravia et al. 1985), to multiple ulcerations and mucosal involvement, with a tendency to metastasize and...
relapse (Marsden 1986; Marzochi and Marzochi 1994). Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica, and Peru present the highest number of estimated cases, representing 70 to 75% of the global incidence of TL (Alvar et al. 2012; Erber et al. 2022). In Brazil alone, 16,135 cases were registered in 2019, constituting a public health problem, which is aggravated by the ineffectiveness of the control measures currently employed (SINAN 2019). Several species of *Leishmania* can cause TL, and in Brazil, *Leishmania braziliensis* is the main pathogenic agent in humans. Importantly, there is evidence that the immune response of individuals infected by different strains or isolates is variable, suggesting that the clinical and immunological characteristics developed by the infection are associated with the genotypic profile of the parasite strain (Alexander and Brombacher 2012; Vargas-Inchaustegui et al. 2004; Salay et al. 2007; Silveira et al. 2004).

During the course of the disease, infected antigen-presenting cells (APCs) migrate through the lymph nodes to secondary lymphoid organs and present the antigen to major histocompatibility complex class I (MHC I) in order to trigger a CD8+ T-cell response, and to class MHC II to trigger a CD4+ response (Gollob et al. 2014). CD4 naive T-cells are activated by cytokine IL-12, produced by APCs, and differentiate into T helper 1 (T_{H1}) cells. These cells then start to produce tumor necrosis factor (TNF)-α and interferon gamma (IFN-γ), which are cytokines responsible for activating macrophages. This will subsequently induce the production of nitric oxide (NO) and reactive oxygen species (ROS), to finally eliminate intracellular parasites and protect the host (Alexander and Brombacher 2012; Vargas-Inchaustegui et al. 2008). The T_{H1} inflammatory response observed in the simple cutaneous form of TL (TL-C) is continuously regulated by the production of IL-10, but in cases of a mucosal clinical manifestation (TL-M), there is an exacerbated and unregulated production of this response, which can lead to disfiguring lesions (Gollob et al. 2014). TL-C is characterized by the presence of CD4+ T-cell in greater quantity when compared to CD8+ T-cells, but these are balanced during the wound healing phase. In two different ways, these indicate that CD8+ T-cells stand out in terms of eliminating parasites, helping in the healing process of old lesions, and indicate that the cytotoxic immune response driven by CD8+ T-cells, when related to the expression of granzymes, directly influences the inflammatory response and lesion size, especially in cases of TL-M (Leopoldo et al. 2006; Santos Cda et al. 2013). The role of T-cell immune responses in TL pathogenesis and longer-term protective immunity is currently poorly defined; therefore, it is of utmost importance to better understand this role in order to improve therapeutic interventions and vaccine design.

In this context, the knowledge of the parasite’s infectivity mechanisms, associated with the understanding of the protective immune response elaborated by the host, makes it possible to establish rational strategies for the development of immunogens to compose vaccine formulations capable of preventing TL. In view of the serious public health problem that TL represents for Brazil, the need to advance the field is evident, seeking new alternatives with prophylactic potential and capable of inducing mass protection in endemic areas. In the past, the production of vaccines for TL was based on strategies that used protein extracts and attenuated or dead parasites. Despite potential protection, these formulations presented issues related to safety and stability to be used on a large scale (Brito et al. 2020; Mayrink et al. 1985). Our research team has previously shown in an immunoproteomics approach that two *L. braziliensis* stages, stationary-phase promastigote and amastigote-like, have different protein signatures based on the abundance of all detected targets (Duarte et al. 2015). This study has identified different antigenic proteins with the potential for the development of an immunogen comprised of multiple T-cell epitopes. This would enable the development of a new generation of vaccines based on rational design approaches. Thus, the present study proposes a gene sequence containing the fusion of T-cell epitopes from proteins previously identified in the immunoproteome of the *L. braziliensis* parasite and the use of this sequence to produce a chimeric protein. From proteins found in the extract of proteins of the amastigote stage, and using bioinformatics tools, our study identified the presence of epitopes of CD4+ and CD8+ T-cells that were used to elaborate the chimera. The major proliferative stage of *Leishmania*, amastigote, resides in the mature phagolysosomes of mammalian host cells (Kaye and Scott 2011); thus, proteins expressed at this stage have the potential to be associated with mechanisms of infectivity and the pathogenicity of the parasite, making them interesting targets for vaccines. With this strategy in mind, we developed a safe, low-cost, and easy-to-produce vaccine composition, associated with the induction of a long-lasting, protective immune response.

**Material and methods**

**Identification and selection of CD4+ and CD8+ T-cell epitopes to compose the chimeric protein**

Using sequences of proteins already identified in the immunoproteome of the parasite *L. braziliensis* (Duarte et al. 2015), immunoinformatics techniques were used to identify specific CD4+ and CD8+ T-cell epitopes and elaborate a chimeric protein to compose the vaccine formulation for TL immunoprophylaxis.

The selection of specific CD4+ T-cell epitopes was performed using the NetMHCII program, evaluating the
presence of epitopes capable of binding in more than 30% of human alleles and with specificity for the murine IAb or IAd alleles (Nielsen and Lund 2009). For the specific CD8+ T-cell epitopes, the bioinformatics program NetCTLpan was used to analyze the A2, A3, and B7 alleles present in the HLA, as well as the Db, Dd, Kb, Kd, Kk, and Ld alleles present in the murine H2 (Bakker et al. 2008; Stranzl et al. 2010).

To elaborate the chimeric protein (MEP/TL), selected epitope sequences for CD4+ and CD8+ T-cells were connected via flexible spacers (GSGSGS), to increase solubility and to prevent the interaction of “neighboring” epitopes.

Production of recombinant multi-epitope protein to compose a TL vaccine (rMEP/TL)

Gene-encoded rMEP/TL protein was synthesized by a specialized biotechnology company (GenScript company, USA). The plasmid pET-28a (+)-MEP/TL was inserted into the electrocompetent Escherichia coli BL21 Arctic Express (DE3) cells (Agilent Technologies, USA), as previously described (Menezes-Souza et al. 2015). Protein expression was induced by the addition of IPTG, and purification was performed using a chromatography column HisTrap HP affinity connected to an ÄKTAprime chromatography system (Cytiva, USA), as previously described (Garcia et al. 2021). To remove endotoxin contamination, rMEP/TL was passed through a polymyxin-agarose column (Sigma-Aldrich, St. Louis, MO, USA) to remove any residual endotoxin content (< 10 ng of LPS per 1 mg of recombinant protein, measured by the quantitative Chromogenic Limulus Amebocyte Assay QCL-1000 (BioWhittaker, MD, USA) (Lage et al. 2019; Ribeiro et al. 2019, 2020). Western blot assay which employed His-Tag antibody was carried out to confirm the correct production of the rMEP/TL, as described previously (Ribeiro et al. 2019).

Parasites and soluble L. braziliensis antigen (sLb-A)

L. braziliensis promastigotes (strain MHOM/BR/75/M2904) were cultivated and used to obtain a soluble antigen (sLb-A), as well as to produce an experimental challenge in murine model, as previously described (Dias et al. 2018; Menezes-Souza et al. 2014). The protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific™, USA) (Menezes-Souza et al. 2014).

Animals

BALB/c mice (female, 6–8 weeks old) were obtained from the Central Animal Laboratory of the Federal University of Minas Gerais (UFMG). The animals had specific pathogen-free (SPF) health status and were kept with proper handling and care conditions in the Animal Facility of the Clinical Pathology Department of COLTEC/UFMG.

Vaccination protocol and challenge with L. braziliensis parasite

Forty-eight BALB/c mice were divided into four experimental groups (female, n = 12 per group): saline (sterile saline, 0.9% NaCl, pH 7.2–7.4), MPLA (25 µg of MPLA (PHAD®)), rMEP/TL (25 µg of the rMEP/TL), and rMEP/TL + MPLA (25 µg of the rMEP/TL associated with 25 µg of MPLA (PHAD®)). Saline and MPLA were the control groups, while rMEP/TL and rMEP/TL + MPLA were the vaccinated groups. The animals were immunized subcutaneously in the dorsal region, with 100 µL of formulations. The immunization schedule was the following: three doses administered at 14-day intervals. Twenty-eight days after the third immunization, six mice per group were euthanized in order to evaluate specific immune responses. The remaining animals (six mice per group) were challenged by injecting 1 × 10⁵ of stationary L. braziliensis promastigotes, intradermally, 28 days after the last immunization (Lage et al. 2016). After 12 weeks, animals were euthanized to evaluate the vaccine protection and immune response.

Humoral response

IgG_total, IgG1, and IgG2a isotype production was evaluated in serum samples of the control and vaccinated animals. For this, sLb-A or rMEP/TL was used as an antigen (1.0 µg per well) in the plates, and samples were 1:100 diluted in PBS-T (PBS 1× plus 0.05% Tween 20), with incubation for 1 h at 37 °C. After washing plates five times, the anti-mouse IgG_total, IgG1, and IgG2a horseradish peroxidase–conjugated antibodies were added (1:5000 dilution in PBS-T), and reactions were developed by incubation with 2 µL H₂O₂, 2 mg ortho-phenylenediamine, and 10 mL citrate phosphate buffer (pH 5.0), for 30 min, and in the dark, and were stopped by the addition of 20 µL H₂SO₄ (2 N). The absorbance was read at 450 nm, using an EMax® Endpoint ELISA Absorbance Microplate Reader (EMax, Molecular Devices, USA). Absorbance values were averaged and blank-corrected.

Quantification of NO production in culture supernatant

In this study, 25 µL of supernatant from each culture was incubated with the same amount of Griess reagent (Sigma-Aldrich) for 30 min at room temperature, and the absorbance was measured at 540 nm by an EMax microplate reader. The nitric oxide concentration was
calculated by means of a standard curve using NaNO₂, as described previously (Ribeiro et al. 2020).

**Isolation of splenocytes for the analysis of intracytoplasmic cytokine production by CD4⁺ and CD8⁺ T-cells by flow cytometry analysis**

The spleen of animals from control and vaccinated groups was removed and processed to obtain splenic cell suspensions, as previously described (Brito 2018; Kaye and Scott 2011). The cells were then transferred to 96-well polyethylene U-bottom plates (Costar®) containing supplemented culture medium (20% FBS, 1% gentamicin, 1% l-glutamine, and RPMI). The splenocytes of each animal were divided into three treatments in duplicate: (i) splenocytes in RPMI medium (control), (ii) splenocytes stimulated with 25 µg/mL of sLb-A, and (iii) splenocytes stimulated with 25 µg/mL of sMEP/TL. Splenocytes were also stimulated with mitogen concanavalin A (ConA) at a concentration of 1 µg/mL (positive control). The samples were incubated for 48 h with 5% CO₂ at 37 °C. After incubation, cells were treated with 10 µg/ml of brefeldin A (Sigma) for 4 h. After, cells were blocked with anti-mouse CD16/CD32 (0.5 µg/well), harvested, washed, and treated with PBS and an inert protein (serum albumin 5%). Subsequently, T-cells were labeled using an anti-CD3 antibody (FITC, clone 145.2C11), followed by anti-CD4 (BV605, clone RM4-5) or anti-CD8 (PerCP Cy5.5, clone 53–6.7) antibodies at room temperature for 30 min. Cells were then fixed with FACS fixing solution (10 g/L paraformaldehyde, 10.2 g/L sodium cacodylate, and 6.6 g/L sodium chloride, pH 7.2), washed, and permeabilized.

Cells were acquired on a BD LSRFortessa™ Cell Analyzer, using BD FACSDiva™ software (Becton Dickinson, USA). Specific beads (CompBeads, BD) were used for cytometer compensation. For data analysis, the FlowJo® program was used. The cell population of interest was defined in plots of point distribution of forward scatter (FSC) size versus side scatter (SSC) granularity. After the automatic compensation of the cytometer, at least 200,000 events were acquired from each sample in the lymphocyte region. The analysis strategy was based on the initial identification of live cells, using the label Fixable Viability Stain 450 (FVS450, BD Biosciences) and, subsequently, T-cell analyses and the intracellular production of cytokines IFN-γ (AF700, clone XMG1.2), TNF-α (PE-Cy7, clone LG.3A10), and IL-10 (APC, clone JES5-16E3). Strategy for the analysis of intracytoplasmic cytokines in the splenocytes of vaccinated mice, by flow cytometry approach, is shown in Fig. S1.

**Analyses of memory T-cell phenotypes**

Central memory (CM) and effector memory (EM) T cells were analyzed 4 weeks after the last immunization and 12 weeks post-challenge, as described by Brito et al. (2020). Splenocytes from animals were plated at 5 x 10⁵ cells per well in duplicate in 96-well round-bottom plates. Cells were treated with the same conditions described above. After 5 days of culture, cells were then prepared for flow cytometry analysis. Samples were blocked with anti-mouse CD16/CD32 (0.5 µg/well) and stained with surface markers at room temperature using the following antibodies: anti-CD3 (FITC, clone 17A2), anti-CD4 (BV605, clone RM4-5), anti-CD8 (PerCP-Cy5.5, clone 53–6.7), anti-CD44 (PE, clone IM7), and anti-CD62L (BV510, clone MEL-14). The events were acquired (300,000 cells) on an LSR Fortessa cytometer (BD Biosciences) using FACSDiva software. For analysis, dead cells were excluded after FVS780 stain, and live cells were used for further analyses. Strategy for the analysis of memory T-cells in the splenocytes of vaccinated mice, by flow cytometry approach, is shown in Fig. S1.

**Evaluation of parasite burden by quantitative PCR**

The parasite burden in the skin lesion was evaluated using a quantitative PCR (qPCR) assay. For this, DNA was extracted from skin fragments using Wizard® Genomic DNA Purification Kit (Promega, USA) according to the manufacturer’s recommendations. The resulting DNA was resuspended in 100 µL of Milli-Q H₂O. Parasite burdens were estimated using the following primers that amplify the kDNA region of *L. braziliensis*: forward (CCTATTITACACCAAACCCCACT) and reverse (GGGTAGGGGGTCTCGGAAA). Mouse β-actin gene (forward: CAGAGCAAAGAGGTATCC; reverse: TACTGTTAAGGTGTTGTCG) was used as endogenous control to normalize (nucleated cells, single copy number) and to verify sample integrity. Standard curves were obtained from DNA extracted from 1 x 10⁸ parasites for kDNA and 1 x 10⁶ peritoneal macrophages for β-actin under the same conditions used to extract the samples of the present study (Mendonça et al. 2022). Reactions were processed and analyzed in a QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems, USA), using a 2×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 min, and then submitted to 40 cycles of 95 °C for 15 s and 60 °C for 1 min, and during each time, fluorescence data was collected. Parasite quantification for each skin sample was calculated by interpolation from the standard curve, performed in duplicate, and converted into a number of parasites per nucleated cell (multiplied by one thousand to facilitate visualization).
Heat map analysis and gene correlation network

The heat map was built using the heatmap.2 function from the gplots package, version 3.0.4 (Warnes et al. 2020), implemented in R software 3.6.1 (Team RC 2013).

Protection profile in the vaccinated groups associated with lower parasite burden was carried out using correlation network. Spearman’s rank correlation test, using R language (Team 2013), was performed to construct gene correlation networks. A correlation matrix, containing all pairwise Spearman’s test \( p \) values less than 0.05, was used to design the networks, considering genes as vertices and edges as the correlation between two specific genes (Fukushima et al. 2011). The statistical significance of each network was calculated by the number of identical networks obtained from 1000 random evaluations, using the randomGraph function. The statistical significance of each network was calculated by the number of identical networks obtained from 1000 random evaluations, using the randomGraph function of the graph library in R language, where \( p < 0.05 \) was considered significant.

Statistical analysis

The one-sample Kolmogorov-Smirnoff test was used to determine whether a variable was normally distributed. Grubbs’ test was carried out to detect outlier values in the groups. Differences between groups were analyzed by one-way ANOVA, followed by the Bonferroni’s post hoc test. The differences were considered statistically significant at \( p < 0.05 \). All the statistical analyses were performed using GraphPad Prism™ (version 8.0).

Results

From immunoinformatics analysis of the immunoproteome of the *L. braziliensis* parasite, six T-cell epitopes, with a high number of MHC class I and II alleles in human and murine, were selected to compose the chimeric protein

Six conserved proteins among the species causing TL were selected, as described in Fig. S1. The peptides identified in these proteins were selected to compose the chimeric protein as described in Fig. S1. Using the epitope analysis described herein, it was possible to identify a high amount of MHC I and MHC II alleles, from human and murine, capable of recognizing amino acid sequences present in the epitopes. Regarding CD4+ and CD8+ T cells (human + murine), 199 and 43 alleles were identified, respectively (data not shown). The gene encoding rMEP/TL has a nucleotide sequence of 879 bp and encodes a protein containing 289 amino acids and 33.8 kDa (Fig. S2). GSGSGS linkers were added to the chimera coding region, between each selected peptide, to stabilize the protein and increase the interaction with MHC. Nucleotide sequence of synthetic gene was submitted to GenBank (accession number: ON351015). Western blot assay which employed His-Tag antibody using five purification fractions of the chimeric protein confirmed the correct production of the rMEP/TL (33.8 kDa, Fig. S3).

Immunization with rMEP/TL or rMEP/TL + MPLA induces an increased production of specific antibodies (IgG-Total, IgG1, and IgG2a) and IgG2a/IgG1 ratio, in addition to an increase in nitric oxide production by splenocytes stimulated with sLb-A or rMEP/TL.

The results obtained from the evaluation of the humoral response are shown in Fig. 1A–D. In the evaluation of the production of antibodies against sLb-A and rMEP/TL, an increased production of IgG-Total (Fig. 1A), IgG1 (Fig. 1B), and IgG2a (Fig. 1C) was observed in the animals immunized with rMEP/TL or with rMEP/TL + MPLA, when compared to the saline or MPLA groups. The IgG2a/IgG1 ratio was analyzed to estimate the direction of the immune response of each experimental group. Figure 1D shows that the rMEP/TL and rMEP/TL + MPLA groups had a higher IgG2a/IgG1 ratio when compared to the control groups. Additionally, the rMEP/TL + MPLA group also showed an increase in the IgG2a/IgG1 ratio when compared to the rMEP/TL group.

The results obtained from the evaluation of NO production in the supernatant of splenocyte cultures, stimulated with sLb-A or rMEP/TL, are shown in Fig. 1E. Vaccinated animals (rMEP/TL and rMEP/TL + MPLA) showed an increased production of nitric oxide in the stimulated cultures with sLb-A or rMEP/TL when compared to saline and MPLA. Additionally, the group that received the association of the chimeric protein with the MPLA adjuvant also presented an increased NO production in relation to the rMEP/TL group when stimulated with rMEP/TL.

Immunization with rMEP/TL triggers the immune response to type 1, characterized by CD4+ and CD8+ T-cells with a high production of IFN-γ and TNF-α and a reduction in IL-10, in addition to inducing central and effector memory cells

The results of the analysis of intracytoplasmic cytokines (IFN-γ, TNF-α, and IL-10), produced by CD4+ and CD8+ T-cells, the ratio of IFN-γ/IL-10 and TNF-α/IL-10 cytokines, in addition to the generation of central or effector memory cells, are shown in Fig. 2A–J and Table S1/S2. Splenocytes were stimulated with sLb-A or rMEP/TL.

Regarding the production of cytokines after stimulation with sLb-A, it was observed that the rMEP/TL and rMEP/TL + MPLA groups showed an increase in CD4+
T-cells that produce type 1 cytokine IFN-γ in relation to the control groups (Fig. 2A). Additionally, the \( \text{rMEP/TL + MPLA} \) group also showed an increase in CD4\(^+\) T-cells producing the cytokine TNF-α, associated with a reduction in these cells producing the regulatory cytokine IL-10 when compared to the other groups (Fig. 2C and E, respectively). No differences were found for CD8\(^+\)IFN-γ\(^+\) T-cells (Fig. 2B). When analyzing CD8\(^+\) T-cells, an increase in this TNF-α production was observed in the \( \text{rMEP/TL + MPLA} \) group when compared to the other groups (Fig. 2D). A reduction in these IL-10-producing cells was also demonstrated in the vaccinated groups compared to the control groups (Fig. 2F). Evaluating the ratios of IFN-γ\(^+\)/IL-10\(^+\) cytokines produced by CD4\(^+\) T-cells, an increase was observed in the vaccinated groups compared to the saline and MPLA groups (Table S1). Regarding the ratio of CD3\(^+\)CD4\(^+\)TNF-α\(^+\)/IL-10\(^+\) T-cells, the group immunized with the association of \( \text{rMEP/TL + MPLA} \) showed an increase when compared to all other experimental groups (Table S1). The cytokine ratio data to CD8\(^+\) T-cells indicated an increase in the IFN-γ\(^+\)/IL-10\(^+\) ratio in the vaccinated groups compared to the saline group, while the increase in relation to the MPLA group was observed only in the
Fig. 2 In vitro stimulation of T-cell subpopulation with sLb-A or rMEP/TL after vaccination protocol. Splenocytes were obtained, and intracellular cytokine production (IFN-γ, TNF-α, and IL-10 cytokines) by CD4+ (A, C, and E) or CD8+ (B, D, and F) T-cells and generation of central memory (CM; CD44highCD62Lhigh; G and H) and effector memory (EM; CD44highCD62Llow; I and J) T-cells were assessed through flow cytometry. Splenocytes were unstimulated (medium) and then stimulated with sLb-A or rMEP/TL (25 µg/mL and 10 µg/mL, respectively) for 48 h at 37 °C, with 5% CO2. Graphs represented the index of intracellular cytokine production after stimulation (stimulated culture/control culture (SC/CC)). The results are presented as scattering data with overlapping bars with mean plus standard deviation. Statistical differences (p < 0.05) in comparison to the saline, MPLA, and rMEP/TL groups are shown in graphs represented by lowercase letters a, b, and c, respectively.

rMEP/TL + MPLA group (Table S1S1). However, in relation to the CD8+ T-cells, the immunized rMEP/TL group showed an increase in the TNF-α+/IL-10+ ratio when compared to the control groups, while the rMEP/
TL + MPLA group showed an increase when compared to all groups (Table S1S1). When evaluating T-cells associated with central memory, the vaccinated groups showed an increase in the CD4+CD44\text{high}CD62L\text{high} and CD8+CD44\text{high}CD62L\text{high} T-cell subpopulations in relation to the non-vaccinated groups (Fig. 2G and H, respectively). When analyzing the effector memory (CD44\text{high}CD62L\text{low}), in the subpopulations of CD4+ T-cells, an increase was observed in the MPLA, rMEP/TL, and rMEP/TL + MPLA groups compared to the saline group, while for CD8+, no significant differences were observed (Fig. 2I and J, respectively).

The results of intracytoplasmic cytokine production, after stimulation with rMEP/TL, indicated that the MPLA, rMEP/TL, and rMEP/TL + MPLA groups showed an increase in CD4\text{+}IFN-\gamma T-cells when compared to saline group, associated with an increase also present in relation to the MPLA group when the rMEP/TL + MPLA was evaluated (Fig. 2A). No differences were observed between groups for the CD4\text{+}TNF-\alpha T-cell subpopulations (Fig. 2C). Analyzing the IL-10-producing CD4\text{+} T cells, a reduction was observed in the rMEP/TL + MPLA group compared to the control groups (Fig. 2E). When evaluating CD8\text{+}IFN-\gamma T-cells, the vaccinated groups showed an increase when compared to the control groups, in addition to the association of rMEP/TL with MPLA adjuvant showing increased levels when compared to those immunized with chimera alone (Fig. 2B). No differences were observed between groups for the CD8\text{+}TNF-\alpha T-cell subpopulations (Fig. 2D). Regarding the cytokine IL-10, a reduction was observed in the rMEP/TL group when compared to the saline group, while for the rMEP/TL + MPLA group, reduced levels were found when compared to the control groups (Fig. 2F). The cytokine ratio data showed that the association of MPLA with rMEP/TL promoted an increase in all these parameters when evaluated in relation to the other groups, except for CD3\text{+}CD8\text{+}TNF-\alpha+/IL-10\text{-} T-cells, where only an increase in relation to the control groups was found (Table S2). These findings indicate a strong predominance of pro-inflammatory over regulatory cytokines for the two subpopulations of CD4+ and CD8+ T-cells (Table S2). In the group vaccinated with the isolated protein, apart from the CD4\text{+}TNF-\alpha+/IL-10\text{-} ratio, all other parameters showed an increase in relation to the control groups (Table S2). The rMEP/TL stimulation data also demonstrated increased CD4\text{+}CD44\text{high}CD62L\text{high} and CD8\text{+}CD44\text{high}CD62L\text{high} T-cell subpopulations when compared to the unvaccinated groups (Fig. 2G and H, respectively). For the rMEP/TL + MPLA group, an increase was also detected in relation to the group immunized only with the rMEP/TL for CD8\text{+}CD44\text{high}CD62L\text{high} T-cells (Fig. 2H). For CD4\text{+}CD44\text{high}CD62L\text{high} T-cell subpopulations, no differences were observed between the groups (Fig. 2I). Additionally, this study found an increase in relation to CD44\text{high}CD62L\text{low} in these subpopulations of CD8+ T-cells in the rMEP/TL + MPLA group when compared to saline and MPLA groups (Fig. 2J).

Animals vaccinated with isolated rMEP/TL or associated with MPLA, after challenged with the parasite L. braziliensis, showed an increased production of IgG\text{Total}, IgG2\text{a}, and IgG2\text{a}/IgG1, a reduced production of IgG1, and a high production of nitric oxide.

The results obtained from the evaluation of the humoral response and NO production, after challenged with the parasite L. braziliensis, are shown in Fig. S4A-E.

Initially, when analyzing IgG\text{Total} levels using sLb-A as an antigen in an ELISA, no differences were observed between the experimental groups (Fig. S4A). When evaluating the production of antibodies against rMEP/TL, a higher proportion of IgG\text{Total} production can be seen in the rMEP/TL and rMEP/TL + MPLA groups compared to the saline or MPLA group (Fig. S4A). Regarding the production of IgG1 antibodies, a reduction in the MPLA, rMEP/TL, and rMEP/TL + MPLA groups was observed in the ELISA sensitized with sLb-A when compared to saline group, while in the ELISA sensitized with rMEP/TL, there was a reduction in the levels in the immunized groups when compared to the control groups (Fig. S4B), including rMEP/TL + MPLA as compared to the rMEP/TL group. Regarding IgG2\text{a}, no differences were observed between groups in the ELISA using sLb-A (Fig. S4C). However, for rMEP/TL-sensitized ELISA, an increase in antibody levels was observed in the rMEP/TL and rMEP/TL + MPLA groups when compared to the saline and MPLA groups (Fig. S4C). In Fig. S4D, where sLb-A was used as an antigen, there was a high IgG2\text{a}/IgG1 index was increased in the immunized groups when compared to the controls and was increased in the group that associated rMEP/TL with MPLA when compared to immunization with the isolated protein (Fig. S4D).

The results obtained from the evaluation of NO production in the supernatant of splenocyte cultures, stimulated with sLb-A or rMEP/TL, showed that the vaccinated animals presented an increased production of NO when compared to the control groups (Fig. S4E). Additionally, the group that received the association of the chimeric protein with the MPLA adjuvant also showed an increase in NO production when compared to the rMEP/TL group (Fig. S4E).
After the challenge, the vaccinated animals maintained the type 1 protective immune response profile associated with an increase in CD44^{high}CD62L^{high} and CD44^{high}CD62L^{low} T-cell subpopulations

The results of the analysis of intracytoplasmic cytokines (IFN-γ, TNF-α, and IL-10), produced by CD4^+ and CD8^+ T-cells, and the ratio of IFN-γ/IL-10 and TNF-α/IL-10 cytokines, in addition to the generation of central or effector memory cells, after challenged with the parasite *L. braziliensis*, are shown in Fig. S4A–J and Table S1/S2.

The splenocytes were stimulated with sLb-A or rMEP/TL. The production of the cytokine IFN-γ by CD4^+ and CD8^+ T-cells in splenocytes after stimulation with sLb-A, post-challenge with the parasite, proved to be increased in the vaccinated groups when compared to the control groups (Fig. S5A and S5B). For the pro-inflammatory cytokine TNF-α, increased levels were observed in CD4^+ T-cells in the rMEP/TL + MPLA group when compared to the other experimental groups (Fig. S5C). Additionally, for CD8^+, this increase was also observed in the rMEP/TL + MPLA group when compared to the other groups, as was an increase in the rMEP/TL group when compared to the saline and MPLA groups (Fig. S5D). Regarding the subpopulations of CD4^+IL-10^+ T-cells, no differences were observed between the experimental groups (Fig. S5E). In the analysis of CD8^+, IL-10 data showed a low production of this cytokine in the vaccinated groups when compared to the saline group, in addition to the reduction in the rMEP/TL + MPLA group when compared to the M PLA group (Fig. S5F). Regarding the cytokine ratios (type 1/regulatory) after stimulation with rMEP/TL, an increase was observed in the M PLA group in relation to the saline group only for CD3^+CD4^+TNF-α^+/IL-10^+ and CD3^+CD8^+TNF-α^+/IL-10^+ (Table S2). Post-challenge rMEP/TL stimulation also promoted an increase in the CD3^+CD4^+IFN-γ^+/IL-10^+ T ratio in the rMEP/TL group when compared to the control groups (Table S2). However, for the CD3^+CD4^+TNF-α^+/IL-10^+ and CD3^+CD8^+TNF-α^+/IL-10^+ ratios, increased levels in the rMEP/TL group were detected only in relation to the control group (Fig. S5C). Vaccination with the two proposed formulations promoted a reduction in CD4^+IL-10^+ T-cell subpopulations when compared to the saline group, while for CD8^+IL-10^+, increased levels were detected only in the rMEP/TL + MPLA group when compared to the two control groups (Fig. S5E and S5F). Regarding the cytokine ratios (type 1/regulatory) after stimulation with rMEP/TL, an increased production of IFN-γ, for the two populations of T-cells evaluated in this study, when compared to the other groups (Fig. S5A and S5B). Evaluating the production of the pro-inflammatory cytokine TNF-α^+, an increase in this population was observed for CD4^+ T-cells in the M PLA group when compared to saline group (Fig. S5C). The rMEP/TL group showed an increase in relation to the two populations of T-cells when compared to the saline group (Fig. S5C). When analyzing the rMEP/TL + MPLA, an increase was observed for CD4^+TNF-α^+ subpopulations in relation to the other experimental groups, while for CD8^+TNF-α^+, increased levels were detected only in relation to the control groups (Fig. S5C and S5D).
in the rMEP/TL group when compared to the saline group (Fig. S5H and S5J). When evaluating the rMEP/TL + MPLA, this increase was also described in relation to the MPLA (Fig. S5H and S5J).

rMEP/TL or rMEP/TL plus MPLA adjuvant vaccine formulations were able to promote effective protection against *L. braziliensis* infection determined by lower skin parasitic burden

To investigate whether vaccination with rMEP/TL or rMEP/TL plus MPLA adjuvant formulations induces effective protection against *L. braziliensis* infection, after 12 weeks of the challenge, skin parasite burden was determined in experimental animals (Fig. 3). Parasite burden in the skin, evaluated by quantitative PCR assays, showed that rMEP/TL and rMEP/TL presented an expressive reduction in the number of parasites when compared to the saline (82.2% and 94.9%, respectively) and MPLA (80.3% and 94.4%, respectively) groups. Furthermore, a reduction in the parasite burden was also observed in the rMEP/TL + MPLA group as compared to the rMEP/TL group (71.8%).

**Protection profile in the vaccinated groups was induced by the development of CD8⁺CD44<hsup>high</sup>CD62L<sub>low</sub> and CD8⁺CD44<hsup>high</sup>CD62L<sub>low</sub> T-cells and associated with an increase of nitrite, CD4⁺IFN-γ⁺TNF-α⁺ T-cells, IgG<sub>Total</sub> and IgG<sub>2a</sub>, and lower rates of IgG<sub>1</sub>**

After vaccination protocol and challenge with *L. braziliensis* parasite, heat map analysis and biomarker networks were performed to identify the pattern of the immunological profile correlated with lower rates of parasite burden in the vaccinated groups (Fig. 4A, B).

The results demonstrated that the highest connectivity was observed for nitrite (3 positive connections with IgG<sub>Total</sub>, CD4⁺IFN-γ, and CD4⁺TNF-α and 1 negative connection with IgG<sub>1</sub>) and CD4⁺IFN-γ (3 positive connections with IgG<sub>Total</sub>, CD8⁺IFN-γ, and nitrite and 1 negative connection with IgG<sub>1</sub>). Moreover, in addition to IgG<sub>1</sub>, having negative correlations with nitrite and CD4⁺IFN-γ, a negative correlation with CD4⁺TNF-α was also observed. IgG<sub>Total</sub> also showed a positive correlation with CD8⁺IFN-γ. Two positive connections were observed for CD8⁺CD44<hsup>high</sup>CD62L<sub>high</sub> (CD8⁺CD44<hsup>high</sup>CD62L<sub>low</sub> and IgG<sub>2a</sub>). All members but IgG<sub>1</sub> demonstrated in the network showed high expression of immunological markers in vaccinated animals that presented low parasite load, as shown in the heat map analysis (Fig. 4A).

**Discussion**

The high cost to search for new antigens for the development of immunobiologics applied to the prophylaxis of infectious diseases, in addition to the long time to make the product available to the market and the high attrition risk in the development phases, has limited the interest of the industry in biotechnology in the area (Brito et al. 2020; Kalter 1994). In this sense, the identification and/or elaboration of the antigens is considered the most important step in the discovery of antigens for the development of vaccines, since the high rate of failure has been greatly attributed to an inadequate selection of targets (Brito et al. 2020). Antigens must be capable of inducing a type 1 immune response, characterized by the production of pro-inflammatory cytokines associated with a cell-type response, in addition to being able to generate central and effector memory cells to ensure prolonged protection against infection (Brito et al. 2020; Duarte et al. 2015).

In Brazil, the high incidence of TL cases is attributed to the difficulty in correct diagnosis, the high toxicity and cost of the available drugs, and the severe clinical manifestations, especially when associated with the mucosal form, in addition to the lack of an effective vaccine for the immunization.
of individuals residing in endemic areas (Palatnik-de-Sousa et al. 2008). Therefore, the present work aimed to develop new vaccine formulations for TL, capable of inducing a protective immune response profile that confers resistance to the infection by the parasite and/or attenuation or prevention of the development of severe skin lesions.

In this context, the strategy for target selection was based on the immunoproteomics data of the parasite L. braziliensis previously obtained by our research group (Duarte et al. 2015). Immunoproteomics is an important technique for elucidating new biomarkers, diagnostic tests, and vaccine candidates, thus making an important contribution to the growth and advancement of biotechnology. Among the specifications of the immunoproteomics technique, the production methodology of prophylactic vaccine candidates corresponds to the identification and preparation of specific proteins, extracted from cell extracts of the parasites, as a means of obtaining peptides destined to the exploitation of the immune response in the induction of proliferation of cells associated with type 1 response (Campos et al. 2017; Dennehy and McClean 2012; Duarte et al. 2017). Using this methodology, six proteins were selected: alpha tubulin; enolase; peroxiredoxin; heat shock protein hsp70, putative; heat shock protein 83-1; and beta tubulin. Through bioinformatics analyses, it was possible to make a comparative analysis of the similarity data with proteins from other parasites that cause TL, such as the Leishmania amazonensis species. These data showed a similarity that ranged from 69.2 to 100.0% (data not shown), thus indicating

**Fig. 4** Heat map analysis (A) and biomarker networks (B) after vaccination protocol and challenge with L. braziliensis parasite. Antibody responses in ELISA or in vitro stimulation of T-cell subpopulation were carried out using rMEP/TL. Heat map was built to define the patterns of levels of humoral response (IgG_total, IgG1, IgG2α, and IgG2a/IgG1), nitrite production (NO), intracellular cytokine production (IFN-γ, TNF-α, and IL-10 cytokines) by CD4+ or CD8+ T-cells, and generation of central memory (CM; CD44highCD62Lhigh) and effector memory (EM; CD44highCD62Llow) T-cells. Networks were built considering all significant correlations (p<0.05), with nodes representing the humoral response (IgG_total, IgG1, IgG2α, and IgG2a/IgG1), nitrite production (NO), intracellular cytokine production (IFN-γ, TNF-α, and IL-10 cytokines) by CD4+ or CD8+ T-cells, and generation of CM (CD44highCD62Lhigh) and EM (CD44highCD62Llow) T-cells. The network node neighborhood connections indicate the pattern of the immune profile in correlation with lower rates of parasite burden.
a cross-vaccination coverage for a greater number of circulating species of parasites associated with TL in Brazil. Unfortunately, at this moment, robust genomic information regarding the other six species of the subgenus Viannia is still unavailable for carrying out comparative analyses of similarity with the selected antigens (Duarte et al. 2015).

Using epitope analysis via immunoinformatics techniques, a high amount of human and murine MHC I and MHC II alleles, capable of recognizing amino acid sequences present in the six epitopes selected to compose the rMEP/TL, was identified. When evaluating the obtained results, a strong induction of the proliferation of CD4+ and CD8+ T-cells was observed, indicating that the selection was assertive in the prediction of epitopes to be applied in the murine model. These findings increase the probability of obtaining a similar return in epitope recognition when evaluating the use of this protein in future clinical trials in humans (Ribeiro et al. 2020).

The present study developed a gene encoding a rMEP/TL protein, by connecting multiple epitopes of CD4+ and CD8+ T-cells, identified among the immunoproteome proteins of the parasites of L. braziliensis and L. amazonensis (Duarte et al. 2015; Magalhaes et al. 2014). These two species were chosen following criteria on the species responsible for the largest number of cases recorded in Brazil and the availability of genomic information in the immunoinformatics database. Immunoproteomics and immunoinformatics studies, as well as experimental assays, were developed based on the etiological agents selected by the research group (Duarte et al. 2015; Magalhaes et al. 2014; Peacock et al. 2007).

For the composition of the rMEP/TL, epitope prediction analysis was developed with the aim of constituting it with a large number of MHC class I and II alleles, so that the structure was capable of inducing antigen-presenting cells (APCs). Furthermore, the addition of an adjuvant is important to delineate the ideal type of immune response (Vitoriano-Souza et al. 2019). In the present work, the selected adjuvant was MPLA due to its proven ability to induce and produce type 1 cytokines (IFN-γ and IL-12), nitric oxide, and T-cells involved in the proliferation process of Th1 cells (Margaroni et al. 2016; Nagill et al. 2015; Vitoriano-Souza et al. 2012, 2019).

The present study was carried out in two phases: the first aimed to evaluate immunogenicity, while the second aimed at the challenge of infection with the parasite L. braziliensis. In the first part, after analysis of cytokine production from the vaccination protocol, a type 1 immune response induced by rMEP/TL associated with MPLA adjuvant was obtained. This result is supported by the increase in nitric oxide, pro-inflammatory cytokines (IFN-γ and TNF-α), and CD8+ T-cells, in addition to a remarkable decrease in subpopulations of regulatory cytokine-producing lymphocytes (TCD4+IL-10+ and TCD8+IL-10+). The production of cytotoxic CD8+ T-cell expression is induced via MHC class I and through the production of cytokines that are different from CD4+ T-cells, specifically IFN-γ, IL-2, IL-4, IL-5, and IL-10. According to Hernandez-Ruiz and Becker (2006), patients with TL caused by the parasite L. braziliensis have a higher excretion profile of CD4+ than CD8+ T cells, but in inflammatory infiltrates, the presence of CD8+ is greater than in circulating blood, which is then presented as an active participant in the healing stage. The effector function of these lymphocytes contributes to the production of IFN-γ, which plays a controlling role in TL infection through the lysis of infected macrophages (Hernandez-Ruiz and Becker 2006).

As for the evaluation of humoral response, the experimental groups were evaluated in ELISAs using plates sensitized with sLb-A or rMEP/TL to determine the production of total and specific antibody subclasses. Although the rMEP/TL was elaborated based on T-cell epitopes, an increase in total antibodies and subclasses was observed. This observation is justified by the possibility of finding B-cell epitopes in the chimera amino acid chain that bind, even with a low affinity score, to B-cell receptors (BCRs) (Menezes-Souza et al. 2015). These observations, in addition to the results obtained that showed an increase in the IgG2a/IgG1 ratio in the vaccinated groups when compared to the controls, and the fact that previous studies demonstrated a direct correlation between increases in IgG2a/IgG1 with the induction of a type 1 response by T-cells indicate that the proposed vaccine compositions can induce a favorable protective response to infection by L. braziliensis, as described in other studies (Martins et al. 2017; Reis et al. 2006; Solano-Gallego et al. 2001).

Moreover, there was an increase in the production of nitric oxide, another important immunological mediator in the anti-Leishmania immune response, as seen in the evaluation of the splenocyte culture supernatant. This observation refers to the mechanism of action responsible for eliminating the parasite, which is the activation of the macrophage as a result of the type 1 immune response, promoting the production and excretion of nitric oxide (Cysne-Finkelstein et al. 2018). In the parasite-host interaction, it is important to understand the host’s immune response, since the participation of the innate immune response can be a relevant factor in the contribution of parasite control and death.

Effector memory (EM; CD44highCD62Llow) and central memory (CM; CD44highCD62Lhigh) T-cells, located in peripheral tissues, have the ability to migrate to tissues outside the lymphoid system and provide the first protective barrier during the re-infection process, while central memory T cells remain fixed in secondary lymphoid tissues (Sallusto et al. 2004). In the present study, an increase in EM and CM (CD4+ and CD8+ T-cells) was observed in the vaccinated and stimulated groups with sLb-A or rMEP/TL, when compared to the control groups. These findings corroborate with Brito et al. (2020), who evaluated the potential of a chimeric multi-epitope T-cell protein and demonstrated that the
development of central and effector memory in mouse splenocytes played an important role in decreasing the parasite load in the spleen of animals challenged with the parasite *Leishmania infantum*.

Based on the results obtained from the tests carried out in the vaccinated groups, it is understood that the formulation with the rMEP/TL protein, in association with the MPLA adjuvant, can induce a protection profile characterized mainly by the development of CD8⁻⁺CD4⁺⁺⁺CD62L⁻⁻⁻ and CD8⁻⁺CD4⁺⁺⁺CD62L⁻⁻⁻ T-cells associated to an increase of nitrite, CD4⁺⁺⁺IFN-γ⁺⁺⁺TNF-α⁺⁺⁺ T-cells, IgG₀₉₀₋₀, and IgG₂₀, and lower rates of IgG₁. These data open perspectives that this formulation could be later tested in human clinical trials for the immunophrophaxis of TL.

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**Author contribution** DM-S, TAOM, and MCD conceived and designed this study, IAF, AMRS, RCFB, BMR, VTM, DPL, FACM, and TAOM conducted the experiments. DM-S, MOCR, EAFC, and MCD contributed with the reagents or analytical tools. DM-S, TAOM, and IAF performed the result interpretation and data analysis. IAF, LRC, and DM-S wrote the manuscript. All authors read and approved the manuscript.

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**Data availability** The authors declare that (the/all other) data supporting the findings of this study are available within the article (and its supplementary information files).

**Declarations**

**Ethics approval** All procedures involving mice were approved by the Committee on Ethics of Animal Experimentation (CEUA) from the Federal University of Minas Gerais (UFMG) (protocol #216/2017). All the experiments were performed to minimize animal suffering.

**Conflict of interest** The authors declare no competing interests.

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