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APPLIED GENETICS AND MOLECULAR BIOTECHNOLOGY

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 (*r*MEP/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: *r*MEP/TL in saline or associated with MPLA-PHAD®. Our data revealed that, after immunization (three doses; 14-day intervals) and subsequent challenging, *r*MEP/TL and *r*MEP/TL + MPLA-vaccinated mice showed an increased production of key immunological biomarkers of protection, such as IgG_{2a}, IgG_{2a}/IgG_1, 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 (CD44^{high}CD62L^{high}) and effector (CD44^{high}CD62L^{low}) 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 *r*MEP/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

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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 (Grimaldi and Tesh 1993; Indiani de Oliveira 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 (*r*MEP/TL)

Gene-encoded *r*MEP/TL protein was synthetized by a specialized biotechnology company (GenScript company, USA). The plasmid *p*ET-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, *r*MEP/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 *r*MEP/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®)), *r*MEP/TL (25 µg of the *r*MEP/TL), and *r*MEP/TL + MPLA (25 µg of the *r*MEP/TL associated with 25 µg of MPLA (PHAD®)). Saline and MPLA were the control groups, while *r*MEP/TL and *r*MEP/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 1×10^7 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}, IgG_1, and IgG_{2a} isotype production was evaluated in serum samples of the control and vaccinated animals. For this, sLb-A or *r*MEP/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}, IgG_1, and IgG_{2a} horseradish peroxidase–conjugated antibodies were added (1:5000 dilution in PBS-T), and reactions were developed by incubation with 2 µL H_2O_2, 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 H_2SO_4 (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 $NaNO_2$, 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 polystyrene 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 *r*MEP/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% CO_2 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 5×10^5 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 H_2O. Parasite burdens were estimated using the following primers that amplify the kDNA region of *L. braziliensis*: *forward* (CCTATTTTACACCAACCCCCAGT) and *reverse* (GGGTAGGGGCGTTCTGCGAAA). Mouse β-actin gene (*forward*: CAGAGCAAGAGAGGTATCC; *reverse*: TCA TTGTAGAAGGTGTGGTGC) 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 1×10^8 parasites for kDNA and 1×10^8 peritoneal macrophages for β-actin under the same conditions used to extract the samples of the present study (Mendonca 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 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 *r*MEP/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 *r*MEP/TL (33.8 KDa, Fig. S3).

Immunization with rMEP/TL or rMEP/TL + MPLA induces an increased production of specific antibodies (IgG_{Total}, IgG_1, and IgG_{2a}) and IgG_{2a}/IgG_1 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 *r*MEP/TL, an increased production of IgG_{Total} (Fig. 1A), IgG_1 (Fig. 1B), and IgG_{2a} (Fig. 1C) was observed in the animals immunized with *r*MEP/TL or with *r*MEP/TL + MPLA, when compared to the saline or MPLA groups. The IgG_{2a}/IgG_1 ratio was analyzed to estimate the direction of the immune response of each experimental group. Figure 1D shows that the *r*MEP/TL and *r*MEP/TL + MPLA groups had a higher IgG_{2a}/IgG_1 ratio when compared to the control groups. Additionally, the *r*MEP/TL + MPLA group also showed an increase in the IgG_{2a}/IgG_1 ratio when compared to the *r*MEP/TL group.

The results obtained from the evaluation of NO production in the supernatant of splenocyte cultures, stimulated with sLb-A or *r*MEP/TL, are shown in Fig. 1E. Vaccinated animals (*r*MEP/TL and *r*MEP/TL + MPLA) showed an increased production of nitric oxide in the stimulated cultures with sLb-A or *r*MEP/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 *r*MEP/TL group when stimulated with *r*MEP/TL.

Immunization with *r*MEP/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 *r*MEP/TL.

Regarding the production of cytokines after stimulation with sLb-A, it was observed that the *r*MEP/TL and *r*MEP/TL + MPLA groups showed an increase in $CD4^+$

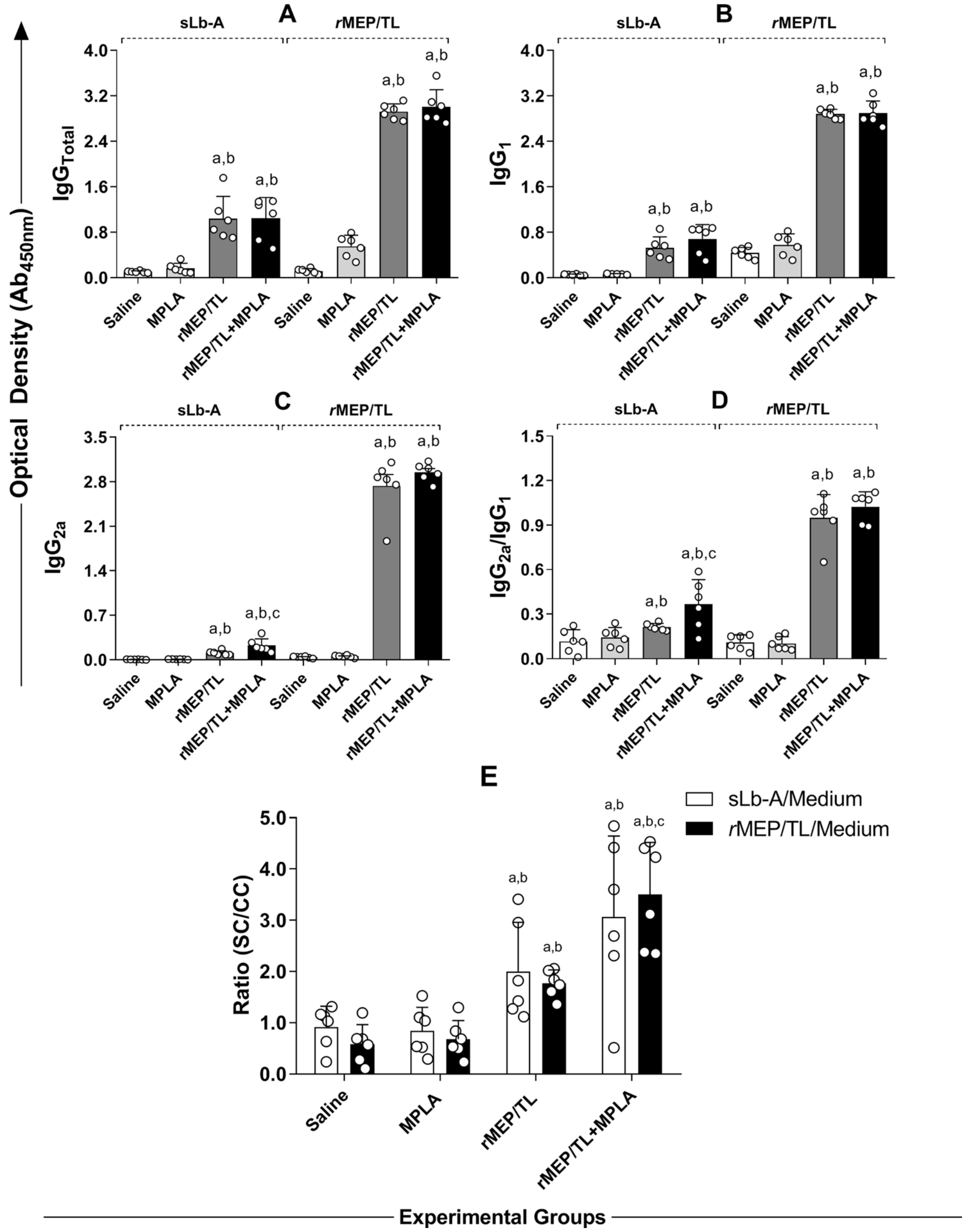

Fig. 1 Humoral response and nitrite production after vaccination protocol. IgG_{Total} (**A**), IgG_1 (**B**), IgG_{2a} (**C**), and IgG_{2a}/IgG_1 (**D**) antibody responses in ELISA-employed sLb-A or *r*MEP/TL. Nitrite production levels (**E**) in the culture supernatants obtained from splenocytes unstimulated (medium) and stimulated with sLb-A or *r*MEP/TL (25 µg/mL and 10 µg/mL, respectively) for 48 h at 37 °C, with 5% CO_2. Graphs represented the index of nitrite production [stimulated culture/control culture (SC/CC)]. The results (**A**–**E**) 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 *r*MEP/TL groups are shown in graphs represented by lowercase letters *a*, *b*, and *c*, respectively

T-cells that produce type 1 cytokine IFN-γ in relation to the control groups (Fig. 2A). Additionally, the *r*MEP/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 $CD8^+IFN\text{-}\gamma^+$ T-cells (Fig. 2B). When analyzing $CD8^+$ T-cells, an increase in this TNF-α production was observed in the *r*MEP/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 $IFN\text{-}\gamma^+/IL\text{-}10^+$ cytokines produced by $CD4^+$ Tcells, an increase was observed in the vaccinated groups compared to the saline and MPLA groups (Table S1). Regarding the ratio of $CD3^+CD4^+TNF\text{-}\alpha^+/IL\text{-}10^+$ T-cells, the group immunized with the association of *r*MEP/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 $IFN\text{-}\gamma^+/IL\text{-}10^+$ ratio in the vaccinated groups compared to the saline group, while the increase in relation to the MPLA group was observed only in the

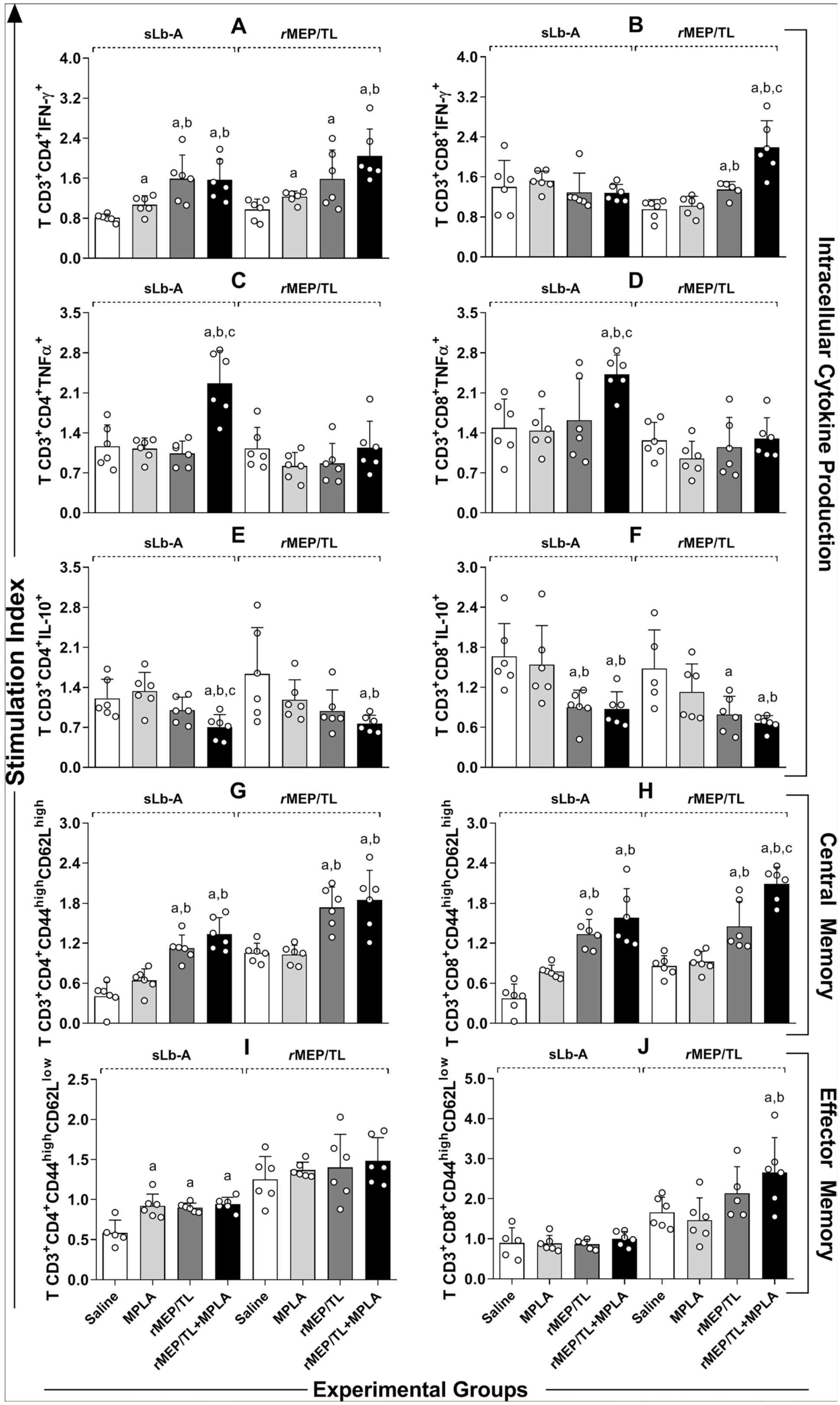

Fig. 2 In vitro stimulation of T-cell subpopulation with sLb-A or *r*MEP/TL after vaccination protocol. Splenocytes were obtained, and intracellular cytokine production (IFN-γ, TNF-α, and IL-10 cytokines) by $CD4^+$ (**A**, **C**, and **E**) or $CD8^+$ (**B**, **D**, and **F**) T-cells and generation of central memory (CM; $CD44^{high}CD62L^{high}$; **G** and **H**) and effector memory (EM; $CD44^{high}CD62L.^{low}$; **I** and **J**) T-cells were assessed through flow cytometry. Splenocytes were unstimulated (medium) and then stimulated with sLb-A or *r*MEP/TL (25 µg/mL and 10 µg/mL, respectively) for 48 h at 37 °C, with 5% CO_2. 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 *r*MEP/TL groups are shown in graphs represented by lowercase letters *a*, *b*, and *c*, respectively

*r*MEP/TL + MPLA group (Table S1S1). However, in relation to the $CD8^+$ T-cells, the immunized *r*MEP/TL group showed an increase in the TNF-α^+/IL-10^+ ratio when compared to the control groups, while the *r*MEP/

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 $CD4^{+}CD44^{high}CD62L^{high}$ and $CD8^{+}CD44^{high}CD62L^{high}$ T-cell subpopulations in relation to the non-vaccinated groups (Fig. 2G and H, respectively). When analyzing the effector memory ($CD44^{high}CD62L^{low}$), in the subpopulations of $CD4^{+}$ T-cells, an increase was observed in the MPLA, *r*MEP/TL, and *r*MEP/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 *r*MEP/TL, indicated that the MPLA, *r*MEP/TL, and *r*MEP/TL + MPLA groups showed an increase in $CD4^{+}$IFN-γ^{+} T-cells when compared to saline group, associated with an increase also present in relation to the MPLA group when the *r*MEP/TL + MPLA was evaluated (Fig. 2A). No differences were observed between groups for the $CD4^{+}$TNF-α^{+} T-cell subpopulations (Fig. 2C). Analyzing the IL-10-producing $CD4^{+}$ T cells, a reduction was observed in the *r*MEP/TL + MPLA group when compared to the control groups (Fig. 2E). When evaluating $CD8^{+}$IFN-γ^{+} T-cells, the vaccinated groups showed an increase when compared to the control groups, in addition to the association of *r*MEP/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 $CD8^{+}$TNF-α^{+} T-cell subpopulations (Fig. 2D). Regarding the cytokine IL-10, a reduction was observed in the *r*MEP/TL group when compared to the saline group, while for the *r*MEP/TL + MPLAP group, reduced levels were found when compared to the control groups (Fig. 2F). The cytokine ratio data showed that the association of MPLA with *r*MEP/TL promoted an increase in all these parameters when evaluated in relation to the other groups, except for $CD3^{+}CD8^{+}$TNF-α^{+}/IL-10^{+} 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 $CD4^{+}$TNF-α^{+}/IL-10^{+} ratio, all other parameters showed an increase in relation to the control groups (Table S2). The *r*MEP/TL stimulation data also demonstrated increased $CD4^{+}CD44^{high}CD62L^{high}$ and $CD8^{+}CD44^{high}CD62L^{high}$ T-cell subpopulations when compared to the unvaccinated groups (Fig. 2G and H, respectively). For the *r*MEP/TL + MPLA group, an increase was also detected in relation to the group immunized only with the *r*MEP/TL for $CD8^{+}CD44^{high}CD62L^{high}$ T-cells (Fig. 2H). For $CD4^{+}CD44^{high}CD62L^{high}$ T-cell subpopulations, no differences were observed between the groups (Fig. 2I). Additionally, this study found an increase in relation to $CD44^{high}CD62L^{low}$ in these subpopulations of $CD8^{+}$ T-cells in the *r*MEP/TL + MPLA group when compared to saline and MPLA groups (Fig. 2J).

Animals vaccinated with isolated *r*MEP/TL or associated with MPLA, after challenged with the parasite *L. braziliensis*, showed an increased production of IgG_{Total}, IgG_{2a}, and IgG_{2a}/IgG_{1}, a reduced production of IgG_{1} 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_{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 *r*MEP/TL, a higher proportion of IgG_{Total} production can be seen in the *r*MEP/TL and *r*MEP/TL + MPLA groups when compared to the saline or MPLA group (Fig. S4A). Regarding the production of IgG_{1} antibodies, a reduction in the MPLA, *r*MEP/TL, and *r*MEP/TL + MPLA groups was observed in the ELISA sensitized with sLb-A when compared to saline group, while in the ELISA sensitized with *r*MEP/TL, there was a reduction in the levels in the immunized groups when compared to the control groups (Fig. S4B), including *r*MEP/TL + MPLA as compared to the *r*MEP/TL group. Regarding IgG_{2a}, no differences were observed between groups in the ELISA using sLb-A (Fig. S4C). However, for *r*MEP/TL-sensitized ELISA, an increase in antibody levels was observed in the *r*MEP/TL and *r*MEP/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 no difference between the groups for the IgG_{2a}/IgG_{1} ratio. In the assays using *r*MEP/TL as an antigen, the IgG_{2a}/IgG_{1} index was increased in the immunized groups when compared to the controls and was increased in the group that associated *r*MEP/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 *r*MEP/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 *r*MEP/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 *r*MEP/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 *r*MEP/TL + MPLA group when compared to the other experimental groups (Fig. S5C). Additionally, for $CD8^+$, this increase was also observed in the *r*MEP/TL + MPLA group when compared to the other groups, as was an increase in the *r*MEP/TL group when compared to the saline and MPLA groups (Fig. S5D). Regarding the subpopulations of $CD4^+IL\text{-}10^+$ 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 *r*MEP/TL + MPLA group when compared to the MPLA group (Fig. S5F). Regarding the cytokine ratios (type 1/regulatory), the data showed that vaccination promoted an increase in the $CD3^+CD4^+IFN\text{-}\gamma^+/IL\text{-}10^+$ ratio when compared to the control groups (Table S1). The analysis of $CD3^+CD4^+TNF\text{-}\alpha^+/IL\text{-}10^+$ T-cell subpopulations revealed an increase in the *r*MEP/TL + MPLA group in relation to the other groups (Table S1). In the $CD3^+CD8^+IFN\text{-}\gamma^+/IL\text{-}10^+$ subpopulations, an increase was observed in the vaccinated groups in relation to the saline group, in addition to an increase in the *r*MEP/TL + MPLA group as compared to the MPLA group (Table S1). Vaccinated groups showed an increased $CD3^+CD8^+TNF\text{-}\alpha^+/IL\text{-}10^+$ T-cell subpopulation when compared to saline and MPLA groups (Table S1). Data on the generation of cells associated with CM indicated an increase in the $CD4^+CD44^{high}CD62L^{high}$ population in the *r*MEP/TL and *r*MEP/TL + MPLA groups when compared to controls (Fig. S5G). CM associated with $CD8^+$ T-cells showed an increase in these populations in the *r*MEP/TL + MPLA group when compared to the other experimental groups (Fig. S5H). Regarding the EM $CD4^+$ T-cells (Fig. S5I), the MPLA and the other vaccinated groups showed an increase in relation to the saline group. When analyzing the EM $CD8^+$ subpopulations, the *r*MEP/TL vaccinated group showed increased levels only in relation to the saline group, while for the *r*MEP/TL + MPLA group, an increase was observed as compared to the other experimental groups (Fig. S5J).

From the stimulation of splenocytes with *r*MEP/TL, post-challenge, it was observed that the *r*MEP/TL and *r*MEP/TL + MPLA groups showed 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 $TNF\text{-}\alpha^+$, an increase in this population was observed for $CD4^+$ T-cells in the MPLA group when compared to saline group (Fig. S5C). The *r*MEP/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 *r*MEP/TL + MPLA, an increase was observed for $CD4^+TNF\text{-}\alpha^+$ subpopulations in relation to the other experimental groups, while for $CD8^+$ $TNF\text{-}\alpha^+$, increased levels were detected only in relation to the control groups (Fig. S5C and S5D). Vaccination with the two proposed formulations promoted a reduction in $CD4^+IL\text{-}10^+$ T-cell subpopulations when compared to the saline group, while for $CD8^+IL\text{-}10^+$, increased levels were detected only in the *r*MEP/TL + MPLA group when compared to the two control groups (Fig. S5E and S5F). Regarding the cytokine ratios (type 1/regulatory) after stimulation with *r*MEP/TL, an increase was observed in the MPLA group in relation to saline group only for $CD3^+CD4^+TNF\text{-}\alpha^+/IL\text{-}10^+$ and $CD3^+CD8^+TNF\text{-}\alpha^+/IL\text{-}10^+$ (Table S2). Post-challenge *r*MEP/TL stimulation also promoted an increase in the $CD3^+CD4^+IFN\text{-}\gamma^+/IL\text{-}10^+$ T ratio in the *r*MEP/TL group when compared to the control groups (Table S2). However, for the $CD3^+CD4^+TNF\text{-}\alpha^+/IL\text{-}10^+$ and $CD3^+CD8^+TNF\text{-}\alpha^+/IL\text{-}10^+$ ratios, increased levels in the *r*MEP/TL group were detected only in relation to the saline group (Table S2). Interestingly, the *r*MEP/TL + MPLA group showed an increase for all cytokine ratios (type 1/regulatory) in subpopulations of $CD4^+$ and $CD8^+$ T-cells, after stimulation with *r*MEP/TL, when compared to the control groups (Table S2). Additionally, evaluating the $CD3^+CD4^+TNF\text{-}\alpha^+/IL\text{-}10^+$ ratio, an increase was also observed in the *r*MEP/TL + MPLA group when compared to the group immunized with the chimeric protein alone (Table S2). Post-challenge *r*MEP/TL stimulation data showed an increase in $CD4^+CD44^{high}CD62L^{low}$ and $CD8^+CD44^{high}CD62L^{low}$ T-cell subpopulations

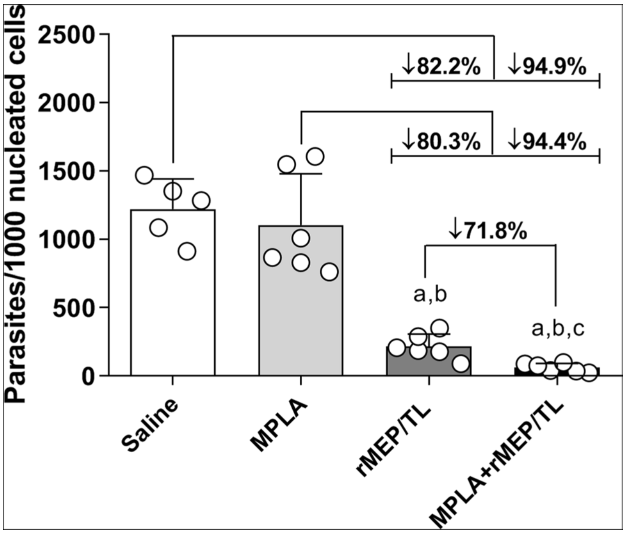

Fig. 3 Parasitic burden in the skin of vaccinated animals. Detection of parasite burden by *q*PCR carried out after infection challenge with *L. braziliensis*. Results were converted into a number of parasites (in log) per nucleated cell (multiplied by 1000 to facilitate visualization). The results are presented as scattering data with overlapping bars with mean plus standard deviation. The percentage of reduction of the parasitic load in the vaccinated groups (*r*MEP/TL and *r*MEP/TL + MPLA) in relation to the controls (saline and MPLA) is shown by the symbol "↓%." Statistical differences ($p < 0.05$) of the parasite burden compared to the saline, MPLA, and *r*MEP/TL groups are shown in graphs represented by lowercase letters *a*, *b*, and *c*, respectively

in the *r*MEP/TL group when compared to the saline group (Fig. S5H and S5J). When evaluating the *r*MEP/TL + MPLA, this increase was also described in relation to the MPLA (Fig. S5H and S5J).

*r*MEP/TL or *r*MEP/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 *r*MEP/TL or *r*MEP/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 *r*MEP/TL and *r*MEP/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 *r*MEP/TL + MPLA group as compared to the *r*MEP/TL group (↓71.8%).

Protection profile in the vaccinated groups was induced by the development of $CD8^+CD44^{high}CD62L^{high}$ and $CD8^+CD44^{high}CD62L^{low}$ T-cells and associated with an increase of nitrite, $CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T-cells, IgG_{Total}, and IgG_{2a}, and lower rates of IgG_1

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_{Total}, $CD4^+IFN\text{-}\gamma$, and $CD4^+TNF\text{-}\alpha^+$ and 1 negative connection with IgG_1) and $CD4^+IFN\text{-}\gamma$ (3 positive connections with IgG_{Total}, $CD8^+IFN\text{-}\gamma$, and nitrite and 1 negative connection with IgG_1). Moreover, in addition to IgG_1 having negative correlations with nitrite and $CD4^+IFN\text{-}\gamma$, a negative correlation with $CD4^+TNF\text{-}\alpha^+$ was also observed. IgG_{Total} also showed a positive correlation with $CD8^+IFN\text{-}\gamma$. Two positive connections were observed for $CD8^+CD44^{high}CD62L^{high}$ ($CD8^+CD44^{high}CD62L^{low}$ and IgG_{2a}). All members but IgG_1 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 immunobiologicals 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 antigen 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

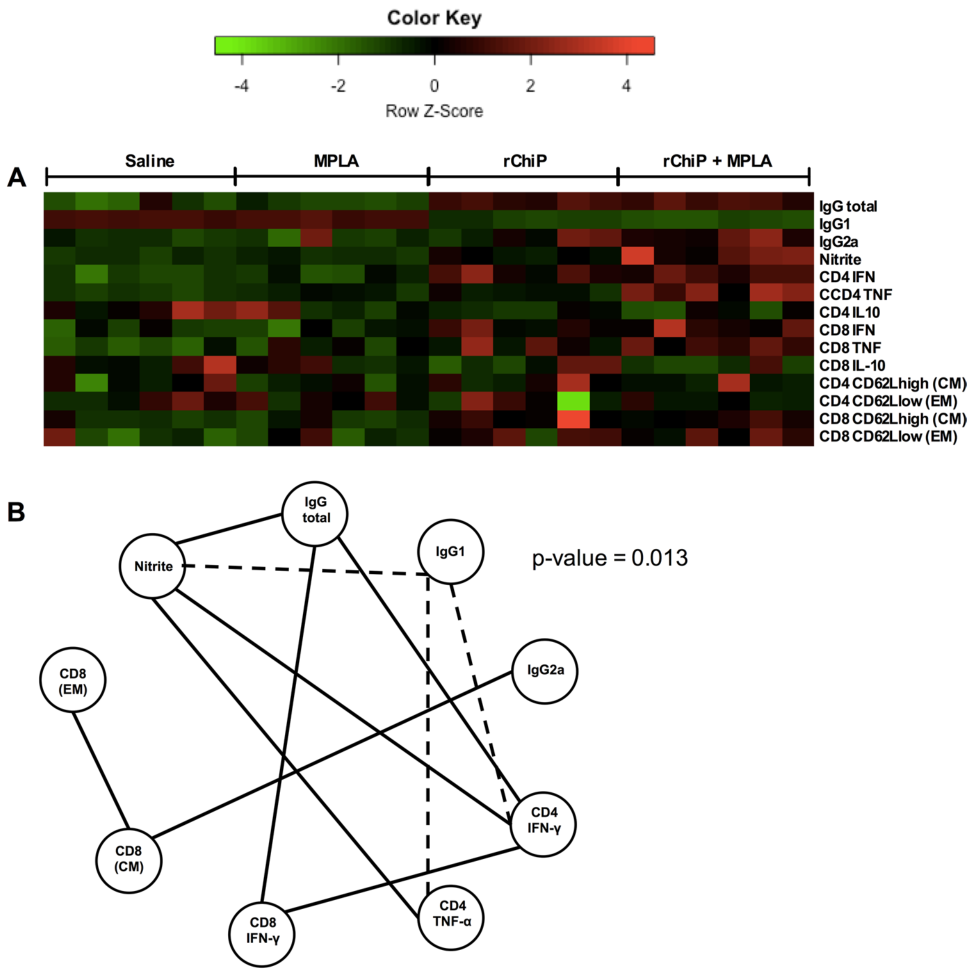

Fig. 4 Heat map analysis (**A**) and biomarker networks (**B**) after vaccination protocol and challenge with *L. braziliensis* parasite. Antibody responses in ELISA or in vitro stimulation of T-cell subpopulation were carried out using *r*MEP/TL. Heat map was built to define the patterns of levels of humoral response (IgG_{Total}, IgG_1, IgG_{2a}, and IgG_{2a}/IgG_1), 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; $CD44^{high}CD62L^{high}$) and effector memory (EM; $CD44^{high}CD62L^{low}$) T-cells. Networks were built considering all significant correlations ($p < 0.05$), with nodes representing the humoral response (IgG_{Total}, IgG_1, IgG_{2a}, and IgG_{2a}/IgG_1), nitrite production (NO), intracellular cytokine production (IFN-γ, TNF-α, and IL-10 cytokines) by $CD4^+$ or $CD8^+$ T-cells, and generation of CM ($CD44^{high}CD62L^{high}$) and EM ($CD44^{high}CD62L.^{low}$) T-cells. The network node neighborhood connections indicate the pattern of the immune profile in correlation with lower rates of parasite burden

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 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 *r*MEP/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 *r*MEP/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 *r*MEP/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 T_H1 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 *r*MEP/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 ($TCD4^+IL\text{-}10^+$ and $TCD8^+IL\text{-}10^+$). 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 *r*MEP/TL to determine the production of total and specific antibody subclasses. Although the *r*MEP/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 IgG_{2a}/IgG_1 ratio in the vaccinated groups when compared to the controls, and the fact that previous studies demonstrated a direct correlation between increases in IgG_{2a}/IgG_1 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; $CD44^{high}CD62L^{low}$) and central memory (CM; $CD44^{high}CD62L^{high}$) 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 *r*MEP/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 *r*MEP/TL protein, in association with the MPLA adjuvant, can induce a protection profile characterized mainly by the development of $CD8^+CD44^{high}CD62L^{high}$ and $CD8^+CD44^{high}CD62L^{low}$ T-cells associated to an increase of nitrite, $CD4^+IFN\text{-}\gamma^+TNF\text{-}\alpha^+$ T-cells, IgG_{Total}, and IgG_{2a}, and lower rates of IgG_1. These data open perspectives that this formulation could be later tested in human clinical trials for the immunoprophylaxis 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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