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A chimeric vaccine combined with adjuvant system induces immunogenicity and protection against visceral leishmaniasis in BALB/c mice

Thais Lopes Valentim Di Paschoale Ostolin [a], Mirã Rodrigues Gusmão [a], Fernando Augusto Siqueira Mathias [a,b], Jamille Mirelle de Oliveira Cardoso [a], Bruno Mendes Roatt [a,c], Rodrigo Dian de Oliveira Aguiar-Soares [a], Jeronimo Conceição Ruiz [b], Daniela de Melo Resende [b], Rory Cristiane Fortes de Brito [a], Alexandre Barbosa Reis [a,c,*]

[a] Laboratório de Imunopatologia, Núcleo de Pesquisas em Ciências Biológicas (NUPEB), Universidade Federal de Ouro Preto, Ouro Preto, Brazil
[b] Grupo Informática de Biossistemas e Genômica, Instituto René Rachou, Fiocruz Minas, Belo Horizonte, Brazil
[c] Instituto Nacional de Ciência e Tecnologia em Doenças Tropicais (INCT-DT), Salvador, Brazil

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ABSTRACT

In Brazil, canine visceral leishmaniasis is an important public health problem due to its alarming growth. The high prevalence of infected dogs reinforces the need for a vaccine for use in prophylactic vaccination campaigns. In the present study, we evaluate the immunogenicity and protection of the best dose of Chimera A selected through the screening of cytokines production important in disease. BALB/c mice were vaccinated subcutaneously with three doses and challenged intravenously with 1×10^7 *L. infantum* promastigotes. Spleen samples were collected to assess the intracellular cytokine profile production, T cell proliferation and parasite load. At first, three different doses of Chimera A (5 μg, 10 μg and 20 μg) were evaluated through the production of IFN-γ and IL-10 cytokines. Since the dose of 20 μg showed the best results, it was chosen to continue the study. Secondarily, Chimera A at dose of 20 μg was formulated with Saponin plus Monophosphoryl lipid A. Vaccination with Chimera A alone and formulated with SM adjuvant system was able to increase the percentage of the proliferation of specific T lymphocytes and stimulated a Th1 response with increased levels of IFN-γ, TNF-α and IL-2, and decreased of IL-4 and IL-10. The vaccine efficacy through real-time PCR demonstrated a reduction in the splenic parasite load in animals that received Chimera A formulated with the SM adjuvant system (92%). Additionally, we observed increased levels of nitric oxide in stimulated-culture supernatants. The Chimera A formulated with the SM adjuvant system was potentially immunogenic, being able to induce immunoprotective mechanisms and reduce parasite load. Therefore, the use of T-cell multi-epitope vaccine is promising against visceral leishmaniasis.

1. Introduction

Caused by the species *Leishmania donovani* and *L. infantum*, visceral leishmaniasis (VL) is the most serious disease among leishmaniasis since it can evolve to death in 95% of cases, if not treated. Its main characteristics are prolonged fever, weight loss, weakness, hepatosplenomegaly, hypergammaglobulinemia and pancytopenia [1,2]. VL is present in 76 countries, being endemic in 12 countries in the Americas with 52.176 reported cases in humans between 2001 and 2015 [2,3]. Brazil has the main endemic areas of the disease in the Americas [2] with increased transmission mainly in the Northeast, Southeast and Midwest [3].

Therefore, it is imperative to develop strategies that contribute to ensure the control of VL and its prevention [4]. The interruption of the disease cycle in dogs can restrict transmission to humans. Thus, developing a vaccine against canine visceral leishmaniasis (CVL) is a potential, practical and effective strategy to control the disease spread [5]. Additionally, vaccine design and development are the most economical way of controlling neglected diseases [6–8]. There are only three vaccines licensed against CVL. Among them, one approved in Brazil, Leish-Tec® (Hertape Calier, Brazil),

* Corresponding author at: Laboratório de Imunopatologia, Núcleo de Pesquisas em Ciências Biológicas, Morro do Cruzeiro, Universidade Federal de Ouro Preto, Ouro Preto, Minas Gerais, Brazil.
E-mail address: alexreis@ufop.edu.br (A.B. Reis).

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and the other two in Europe, Canileish® (Virbac, France) and Letifend® (Laboratorios Leti, Spain) [9]. It is noteworthy that the World Health Organization (WHO) encourages the development of research that investigates potential vaccines against CVL [2].

The biotechnology applied to vaccinology is a suitable tool that helps to accelerate the development of new potential vaccine candidates [10]. Reverse vaccinology is an approach that optimized data analysis and computational recognition of vaccine targets [11] by using genome sequences to identify peptides/proteins with the elevated potencial [12]. After applying *in silico* prediction techniques, vaccine candidates can be expressed as recombinant proteins and tested in appropriate *in vitro* or *in vivo* models to assess immunogenicity, memory induction, and protection [6,11]. The T cell epitopes are predicted based on the molecular events related to the processing and presentation of antigens, mainly the identification of peptides of a certain protein that will provoke the desired immune response [6,13]. However, some studies suggested that protein-based vaccines present low immunogenic potential when not formulated with adjuvants [14,15]. Regarding chimeras, Saponin adjuvant has been widely employed [16–18]. Saponins trigger a Th1 response, characterized by $CD8^+$ T cells, and Th2 by IL-4 cytokine secretion [19–21], while Monophosphoryl lipid A stimulates a polarized Th1 immune response with the production of IL-2, TNF-α and IFN-γ and expression of costimulatory molecules [21,22]. These adjuvants have also been formulated as adjuvant system [23,24]. The adjuvant system showed low reactogenicity and efficiently stimulated immune response mediated by $CD4^+$ T cells, being an appropriate candidate for use in vaccines against viruses or intracellular pathogens [23,24]. It is important to consider that the use of adjuvants in vaccine formulation reduces production costs due to improvement of vaccine efficacy and requires less antigen and number of doses [25] since adjuvants enhance the immune response to the target antigen activating innate immunity [26,27].

Brito et al. [18] mapped T-cell epitopes of known *L. infantum* proteins with immunogenic potential and then, designed Chimera A (histone H2A, Lip2a, Lip0, LACK, and CPC). According to the authors [18], Chimera A induced multifunctional T cells, $CD4^+$ and $CD8^+$ T lymphocytes proliferation and IFN-γ and TNF-α production. However, the dose–response was not evaluated, as well as the Chimera A formulated with other adjuvants than Saponin. Although previous literature showed that similar vaccines were not immunogenic, it was of our interest to assess the potential of Chimera A alone in different doses to induce the production of the main cytokines involved in the protection and susceptibility of VL. Therefore, we aimed to investigate three different doses (5 μg, 10 μg and 20 μg) of Chimera A alone regarding the cytokine production, followed by the evaluation the immunogenicity and efficacy of the best dose of Chimera A alone or formulated with the adjuvant system (Saponin and Monophosphoryl lipid A) against VL experimental.

2. Materials and methods

2.1. Ethics statement, mice and parasites

This research was approved (approval number 6977080518) by the Ethical Committee on the Use of Animals (CEUA) of Federal University of Ouro Preto (UFOP), Brazil.

Female BALB/c mice 6 to 8-week-old were sampled into four groups (6 per group) at each stage. The study was divided into two stages and all the experiments were conducted in duplicate.

Promastigotes of *L. infantum* (MCAN/BR/2008/OP46) maintained by passage in Syrian golden hamsters were cultured at 24 °C in medium *Novy-MacNeal-Nicolle/Liver Infusion Tryptose* (NNN/LIT) supplemented with 100 U/mL penicillin G and 100 mg/mL of streptomycin sulfate as described by [28]. Stationary growth parasites were used to challenge the mice and to prepare the soluble *Leishmania* antigen (SLA) [29].

2.2. Study design

2.2.1. Dose-response screening

First, the screening to choose the Chimera A dose was performed based on the production of the intracellular cytokines IFN-γ and IL-10 by $CD4^+$ and $CD8^+$ T cells (Fig. 1A) and their ratio IFN-γ/IL-10. At first, we evaluated different doses of the chimeric protein, the previously reported dose (10 μg), a lower dose (5 μg), and a higher dose (20 μg). The mice received sterile saline 0.9% (SAL), and 5 μg (ChiA-5), 10 μg (ChiA-10) or 20 μg (ChiA-20) of Chimera A. Chimera A was synthesized by GenScript as described by [18] and was dissolved in sterile saline. The mice were submitted to three subcutaneous immunizations in the dorsum, with two-week intervals between doses. Then after two weeks, the mice were challenged with 1×10^7 stationary *L. infantum* promastigotes intravenously. Animals were euthanized six-weeks post-challenge to collect the spleen.

2.2.2. Evaluation of vaccine immunogenicity and efficacy

The dose of best response selected by the screening was used to formulate the vaccine of our study. The Chimera A was employed alone and formulated with the adjuvant system, composed of two adjuvants, Saponin and Monophosphoryl lipid A. Thus, was evaluated a complete immune response vaccination-induced, production of intracellular cytokines IL-2, Th1-type cytokines (IFN-γ and TNF- α), immunoregulatory (IL-10) and Th2-type cytokine (IL-4), T lymphocytes ($CD3^+CD4^+$ and $CD3^+CD8^+$) proliferation, nitric oxide production and splenic parasite load (Fig. 2A).

Then after the choice of the best dose (20 μg), the animals were divided into another four groups (6 per group): (1) received sterile saline 0.9% (SAL); (2) adjuvant group received 30 μg of Saponin plus 12,5μg of Monophosphoryl lipid A (SM); (3) received 20 μg of Chimera A (ChiA-20); and (4) received 20 μg of Chimera A and 30 μg of Saponin plus 12,5μg of Monophosphoryl lipid A (ChiA-20 + SM). Saponin (Sigma Chemical Co., St. Louis, MO) was prepared in sterile saline, while Monophosphoryl lipid A (Avanti Polar Lipids, Inc.) was resuspended at a concentration of 2 mg/mL with a solution of BSA (*Bovine Serum Albumin*) according to the manufacturer's instructions. The chimera was dissolved in sterile saline, then added the adjuvant preparation.

2.3. Flow cytometry assessment intracellular cytokine profile production and T lymphocytes proliferation

Six-weeks post-challenge, spleen samples were collected to evaluate the immune response triggered by vaccination. Splenocytes were submitted to stimulated-culture with SLA (50 μg/ml) for 24 h to assess intracellular cytokines profile production and, for 120 h, to assess the T lymphocytes proliferation. Cells were cultured in 96-well round-bottom (Costar, USA) at 37 °C with 5% CO_2.

2.3.1. Cytokine profile production assay

Briefly, the evaluation of intracellular cytokines production by flow cytometry was performed as described by [18]. Brefeldin A (Sigma-Aldrich, USA) (10 μg/mL) was added in culture four hours before the end of the period of incubation. Some wells were stimulated with Phorbol 12-myristate 13-acetate (5 ng/mL) and Ionomycin (1 μg/mL) as a positive control. First, cells were incubated with FVS450 for 15 min, then washed. Cells were marked with antibodies against CD3 (BV650 anti-mouse, clone 145.2C11, BD Biosciences, USA), CD4 (BV605 anti-mouse, clone RM4-5, BD

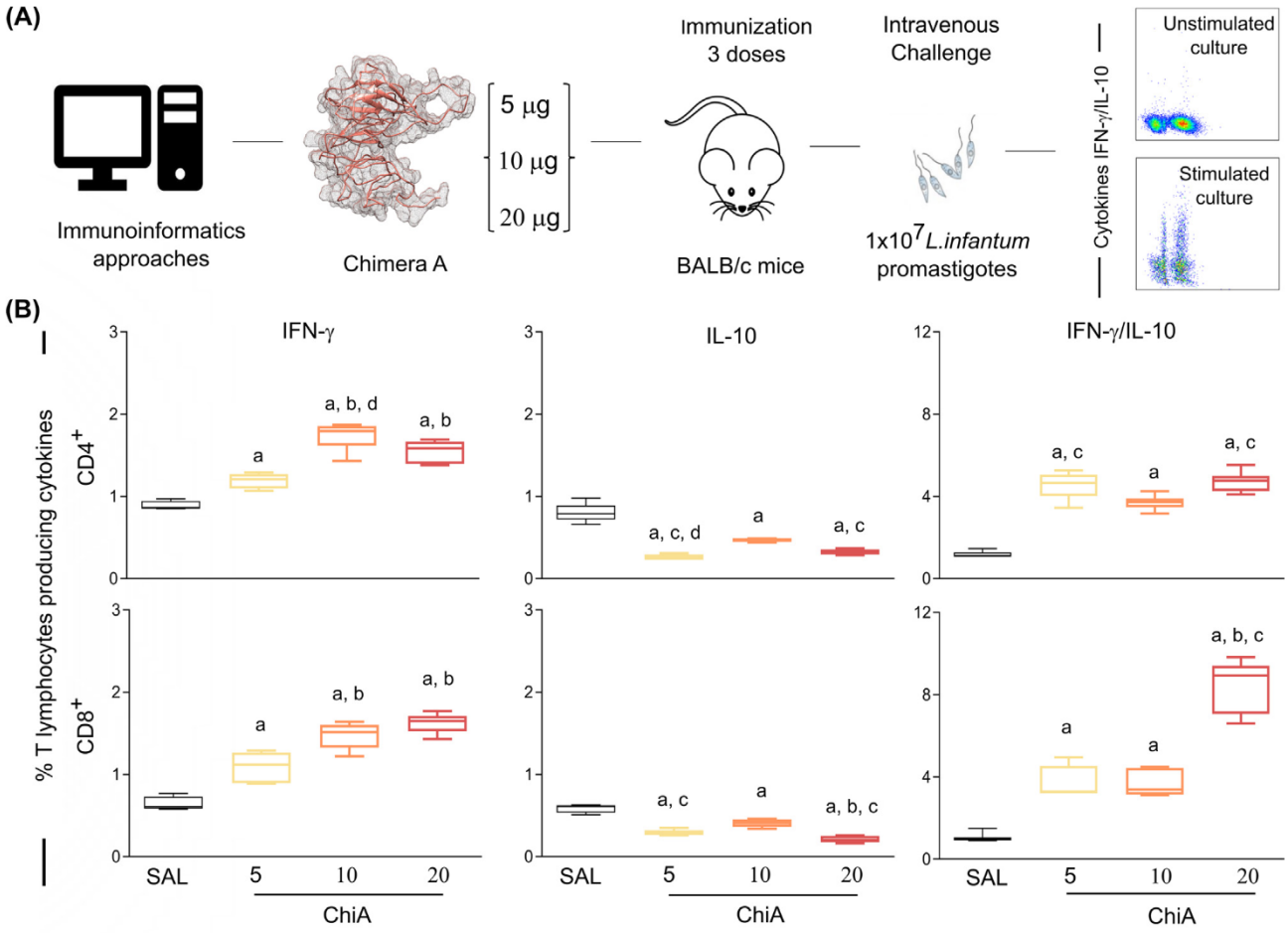

Fig. 1. Dose-response screening through the production of IFN- γ and IL-10 cytokines by T lymphocytes after vaccination with three doses of Chimera A and challenge with *L. infantum*. **(A)** Flowchart showing the study design of dose–response screening. BALB/c mice were immunized with three different doses of Chimera A (5 µg, 10 µg and 20 µg) and challenged with *L. infantum*. **(B)** Percentage of T lymphocytes producing intracellular cytokines IFN- γ and IL-10 and their ratio (IFN- γ /IL-10). Data are expressed as medians ± SD of two independent experiments. The letters "a", "b", "c" and "d" represent the significant differences with the control group SAL, ChiA-5, ChiA-10 and ChiA-20, respectively (*p-value* < 0.05).

Biosciences, USA) and CD8 (BV786 anti-mouse, clone 53–6.7, BD Biosciences, USA). Cells were fixed with FACS fixing solution (10 g/L paraformaldehyde, 10.2 g/L sodium cacodylate, and 6.63 g/L sodium chloride, pH 7.2), washed and permeabilized with PBS buffer plus 0.5% saponin and stained with antibodies against IL-2 (PE anti-mouse, clone JES6-5H4, BD Biosciences, USA), IFN-γ (AF700 anti-mouse, clone XMG1.2, BD Biosciences, USA), TNF-α (PE-Cy7 anti-mouse, clone LG.3A10, BD Biosciences, USA), IL-4 (PE anti-mouse, clone 11B11, BD Biosciences, USA) and IL-10 (PE anti-mouse, clone JES5-16E3, BD Biosciences, USA) at room temperature for 30 min.

The events (100,000) were acquired on the LSR Fortessa cytometer (BD Biosciences). Data analysis was performed using the FlowJo software. The cytokines profile was demonstrated by the percentage values of the SLA-stimulated culture. For this analysis, was conceived as a heatmap with the median of each cytokine. The production of cytokines from the SAL group was considered as basal production and discounted from the other groups.

2.3.2. Cell proliferation assay

The T cell proliferation was estimated by CFSE (*Carboxyfluorescein succinimidyl ester*) assay as described by [30]. Splenocytes were incubated with 5 µM of CFDA-SE (*Carboxyfluorescein diacetate N-succinimidyl ester*, Sigma Co.) and stimulated with SLA (50 µg/ml) for 120 h. As a positive control, some wells were stimulated with Concanavalin A mitogen (1 µg/mL). First, cells were incubated with FVS450 for 15 min, then washed. Cells were treated with PBS and an inert protein (serum albumin 5%) and stained with antibodies against CD3 (BV650 anti-mouse, clone 145.2C11, BD Biosciences, USA), CD4 (BV605 anti-mouse, clone RM4-5, BD Biosciences, USA) and CD8 (BV786 anti-mouse, clone 53–6.7, BD Biosciences, USA) at room temperature for 30 min.

The events (100,000) were acquired on the LSR Fortessa cytometer (BD Biosciences). Data analysis was performed using the FlowJo software. The T lymphocytes proliferation was demonstrated by the percentage values of the SLA-stimulated culture.

2.4. Nitric oxide production

The nitric oxide production in vaccinated mice was measured by the Griess reagent. Thus, the 120-hour stimulated-culture supernatants were collected and 50 µL dispensed in a 96-well microtiter plate. After adding 50 µL of Griess reagent, the plate was incubated at room temperature for 10 min. Absorbance was measured on an ELISA reader (BIO-RAD) at 570 nm. The results were expressed through the levels of total nitrite (µM).

2.5. Real-time PCR

In order to detect the presence of the parasites, we used the real-time PCR as previously described by [31]. The pair of primers (forward: 5′ GGG (G/T)AG GGG CGT TCT (G/C)CG 3′; reverse: 5′ (G/C)(G/C)(G/C)(A/T)CT AT(A/T) TTA CAC CAA CCC 3′), which amplify a

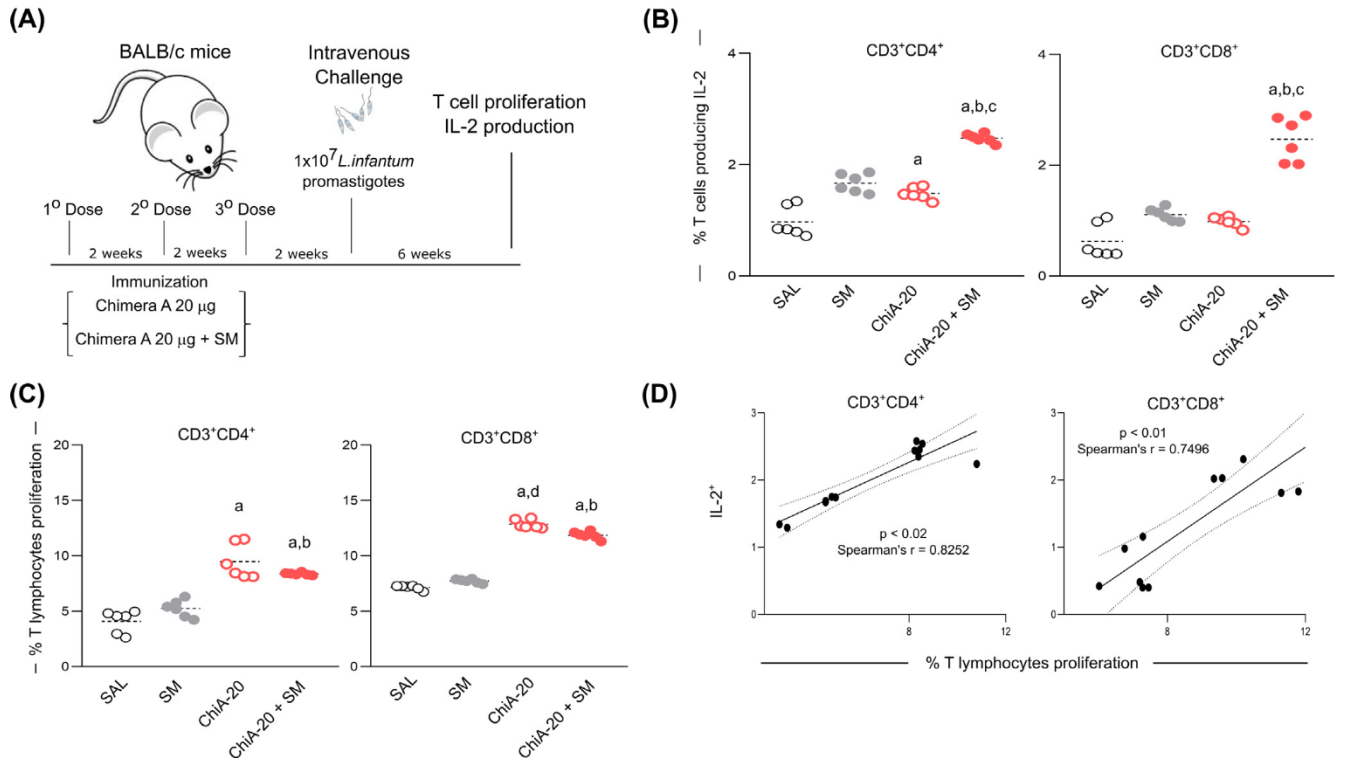

Fig. 2. Percentage of T lymphocytes proliferation and producers of IL-2 after vaccination and challenge with *L. infantum*. **(A)** Flowchart showing the study design of evaluation of T lymphocytes proliferation and intracellular IL-2 production. **(B)** Percentage of T lymphocytes ($CD3^+CD4^+$ and $CD3^+CD8^+$) proliferation after SLA stimulation in BALB/c mice splenocytes. **(C)** Percentage of T lymphocytes producing intracellular cytokine IL-2 after SLA stimulation in BALB/c mice splenocytes. Data are expressed as medians ± SD of two independent experiments. The letters "a", "b", "c" and "d" represent the significant differences with the control group SAL, adjuvant group SM, ChiA-20 and ChiA-20 + SM, respectively (*p-value* < 0.05). **(D)** Spearman's correlation test between the percentage of T lymphocytes proliferation ($CD4^+$ and $CD8^+$) and their production of the IL-2 cytokine.

120 bp fragment, were used to detect and quantify the parasite's target kinetoplastid DNA minicircle (multiple-copy gene of *L. infantum*). The constitutive gene tumor necrosis factor alpha (TNF-α) murine was used to verify DNA integrity. For amplification of the TNF-α gene, the pair of primers used was forward: 5′ TCC CTC TCA TCA GTT CTA TGG CCCA 3′ and reverse: 5′ CAGCA AGCATC-TATGCACTTAGACCCC 3′. Reactions were performed using Sybr green I (Bryt Green®, Promega, USA) fluorescence system in an ABI Prism 7500 Sequence Detection System (Applied Biosystems, USA). The quantification of the number of DNA copies of the parasite was determined by means of a linear regression using the Ct values obtained by the standard curve (efficiency 96.0%; $r^2 = 0.99$). The results were expressed by the number of copies of the parasite's DNA per milligram of spleen.

2.6. Statistical analysis

Data was analyzed using GraphPad Prism 8.0 software. The data were plotted as mean or median ± standard deviation (SD) or standard error of means (SEM) from duplicate sets of experiments. Statistical differences were determined by Kruskal-Wallis followed by Dunn's multiple comparisons test and Mann-Whitney post test. Correlation analyses were obtained by using Spearman's *r* test. The statistical significance was set at *p*-value < 0.05.

3. Results

3.1. Dose-response screening through production effector and immunoregulatory cytokines by T lymphocytes

The analysis of the effector cytokine showed an increased percentage of T lymphocytes producers of IFN-γ in all groups immunized with Chimera A when compared to the SAL group (*p*-value < 0.02) (Fig. 1B). We found increased production of IFN-γ by $CD4^+$ T cells at the dose of 10 µg when compared to the other doses evaluated (*p*-value < 0.05). For $CD8^+$ T cells, there was a significant difference between the lowest dose compared to the 10 µg and 20 µg doses (*p*-value < 0.02). In all immunized groups, we observed a decreased percentage of $CD4^+$ and $CD8^+$ T cells that produce the immunoregulatory cytokine IL-10 in comparison with the control group (*p*-value < 0.002) (Fig. 1B). Additionally, the group immunized with 20 µg of Chimera A showed reduced production of IL-10 by $CD8^+$ T cells in comparison with the groups that received the doses of 5 µg and 10 µg (*p*-value < 0.01). Regardless of the dose, the ratio between the production of intracellular cytokines (IFN-γ/IL-10) showed that Chimera A increases the production of effector cytokine, but not the immunoregulatory cytokine (Fig. 1B). This ratio was lower at the dose of 10 µg in $CD4^+$ T cells when compared to 5 µg (*p*-value < 0.05) and 20 µg (*p*-value < 0.003) doses. For $CD8^+$ T cells, the 20 µg dose had a more expressive production of IFN-γ, being different from the groups immunized with 5 µg (*p*-value < 0.01) and 10 µg (*p*-value < 0.005). Therefore, a dose of 20 µg was chosen to continue the study.

3.2. Chimera a formulated with the SM adjuvant system vaccination induce increased of T lymphocytes proliferation associated with IL-2 production

Regarding the proliferative activity of $CD4^+$ and $CD8^+$ T lymphocytes, vaccination with Chimera A alone and with the SM adjuvant system increased the percentage of proliferation after antigen-specific stimulation as compared with the SAL and adjuvant control groups (*p*-value < 0.01) (Fig. 2B). For $CD4^+$ T cells, there was

no difference for proliferation between the vaccinated groups, while for $CD8^+$ T cells there was only a small decrease in the group vaccinated with Chimera A formulated with the SM adjuvant system compare to Chimera A alone (Fig. 2B). We observed an increased percentage of $CD4^+$ T cells producing IL-2 in Chimera A compared to the SAL group (p-value < 0.005) (Fig. 2C). The IL-2 cytokine level increased for both $CD4^+$ and $CD8^+$ T cells in the group vaccinated with Chimera A formulated with the SM adjuvant system compared to the SAL, SM and Chimera A alone (p-value < 0.003) (Fig. 2C). Spearman's correlation showed a positive correlation between IL-2 production and T lymphocytes proliferation for both populations, $CD4^+$ T cells (r = 0.8252; p-value < 0.002) and $CD8^+$ T cells (r = 0.7496; p-value < 0.01) (Fig. 2D).

3.3. Vaccination led to a significant increase in IFN- γ and TNF-α and decrease the IL-4 and IL-10 produced by T lymphocytes

Chimera A alone and formulated with the SM adjuvant system demonstrated an alike profile of intracellular cytokines production in the vaccinated mice. Regarding pro-inflammatory cytokines, there was an increased percentage of T lymphocytes, $CD4^+$ and $CD8^+$, producers of IFN-γ and TNF-α in vaccinated groups (Chimera A alone and formulated with the SM adjuvant system) compared to the SAL and SM groups (p-value < 0.03) (Fig. 3A). It is important to highlight that vaccination with Chimera A formulated with the SM adjuvant system demonstrates better performance in triggering IFN- γ and TNF-α production compared to vaccination with Chimera A alone (p-value < 0.01). In addition, there was a decrease in IL-4 and IL-10 production by $CD4^+$ and $CD8^+$ T cells in the vaccinated groups compared to the SAL and the adjuvant control group (p-value < 0.02) (Fig. 3A). In summary, the use of the adjuvants system strengthened the production of pro-inflammatory cytokines and kept the production of anti-inflammatory cytokines lower than those observed in the control groups, as can be observed in the heatmap (Fig. 3B).

3.4. Chimera A formulated with the SM adjuvant system was able to induce nitric oxide production and reduce the splenic parasite load

We observed a significant increase in nitric oxide levels produced by splenocytes only in the group vaccinated with Chimera A formulated with the SM adjuvant system when compared with the SAL and SM groups (p-value < 0.001) (Fig. 4A). To evaluate the efficacy of the vaccine candidate the parasite load was quantified in the splenic compartment. No difference was found in the parasite load in the spleen of the vaccinated mice with Chimera A and the control groups. Notwithstanding, when Chimera A was formulated with the SM adjuvant system, there was a remarkably decreased parasite load in comparison with the SAL (p-value < 0.0003), adjuvant control group (p-value < 0.0005), and Chimera A alone (p-value < 0.003) (Fig. 4B).

3.5. Correlation between splenic parasite load and cytokines and nitric oxide

Correlation analyses were performed by Spearman's r test between the splenic parasite load and the percentage of T lymphocytes, $CD4^+$ and $CD8^+$, producers of the IFN-γ, TNF-α and IL-10 cytokines, as well as nitric oxide levels. We observed a positive correlation between $CD4^+$ and $CD8^+$ T cells producers of IL-10 and parasite load in the spleen of the vaccinated mice (r = 0.5425; p-value < 0.0319; r = 0.5791; p-value < 0.0259) (Table 1). However, the correlation was negative for $CD4^+IFN\text{-}\gamma^+$ (r = −0.7196; p-value < 0.0023), $CD8^+IFN\text{-}\gamma^+$ (r = −0.7105; p-value < 0.0027), $CD4^+$-$TNF\text{-}\alpha^+$ (r = −0.8047; p-value < 0.0003), and $CD8^+TNF\text{-}\alpha^+$ (r = −0.7864; p-value < 0.0008) (Table 1). We highlight the strong correlation obtained between the parasite load and $CD4^+TNF\text{-}\alpha^+$. In addition, we also found a moderate correlation with the nitric oxide levels data (r = −0.6844; p-value < 0.0044).

4. Discussion

Several efforts have been made emphasizing the characterization and testing of antigens derived from different species of *Leishmania* as potential candidates for future vaccines. However, there are still gaps in the mechanisms of protection and immune memory triggered by new vaccine and immunobiological candidates [32]. Therefore, it is important to develop more effective candidates, which can contribute not only to attenuate the dog's clinical condition, but also to interrupt the transmission cycle by providing the effective immunization of the dog and/or blocking sandfly infection.

The present study investigated the potential of Chimera A alone and formulated with an adjuvant system (Saponin plus Monophosphoryl lipid A) to induce immunogenicity in BALB/c mice against VL. Either alone or formulated with adjuvant system, vaccination with Chimera A proved to be harmless and safe for administration. Additionally, the animals did not present ulcers and/or signals of pain or behavioural changes. Our results strengthened that vaccination with Chimera A formulated with the SM adjuvant system leads to the generation of a Th1 response and proliferation of T lymphocytes against *L. infantum*.

At first, our study performed a comparative analysis of the production of IFN-γ and IL-10 cytokines induced by different doses (5 μg, 10 μg and 20 μg) of a vaccine candidate. Doses 10 and 20 μg of Chimera A showed similar behavior regarding the production of the effector cytokine. Despite the 10 μg dose presenting an increase in the production of IFN-γ, unfortunately it also increased the production of IL-10 compared to the dose of 20 μg. The IFN-γ cytokine is known for stimulates the production of nitric oxide by activated macrophages combating intracellular pathogens, promotes the differentiation of $CD4^+$ T cells in the Th1, and inhibits the development of Th2 [33]. In contrast, the cytokine IL-10 is associated with disease progression and susceptibility, acting on the modulation of the immune response by blocking the activation of the Th1 response and reducing the levels of IFN-γ and nitric oxide [34]. As known, a vaccine for leishmaniasis intends to stimulate Th1 response. When we determined the ratio for the production of intracellular cytokines by $CD4^+$ and $CD8^+$ T cells, we observed an increase in the IFN-γ production compared to IL-10. The IFN-γ/IL-10 ratio for $CD8^+$ T cells at the dose of 20 μg was substantially higher than 5 and 10 μg. Similarly, Sachdeva et al. [35] found a predominance of the Th1 response (higher values of the IFN-γ/IL-10 ratio) in BALB/c mice immunized and challenged with *L. donovani*. High values of the IFN-γ/IL-10 ratio may provide a substantial source of protection in immunized mice [36]. Therefore, the data obtained allows us to choose the Chimera A 20 μg dose to continue the study. According to [14,15], the protein-based vaccine presents low immunogenicity, which can be improved when formulated with adjuvants. To ensure enhanced immunogenicity, we evaluated the Chimera A 20 μg alone and formulated with Saponin plus Monophosphoryl lipid A.

To immunogenicity evaluation, we use flow cytometry analysis to assess T lymphocytes ($CD3^+CD4^+$ and $CD3^+CD8^+$) proliferation and the cytokine profile production after antigen-specific stimulation. The data obtained demonstrated that the Chimera A alone and formulated with the SM adjuvant system was able to increase the percentage of the proliferation of specific T lymphocytes, corroborating the findings obtained by [37,38]. The detection of responsive T lymphocytes is probably associated with a specific response to

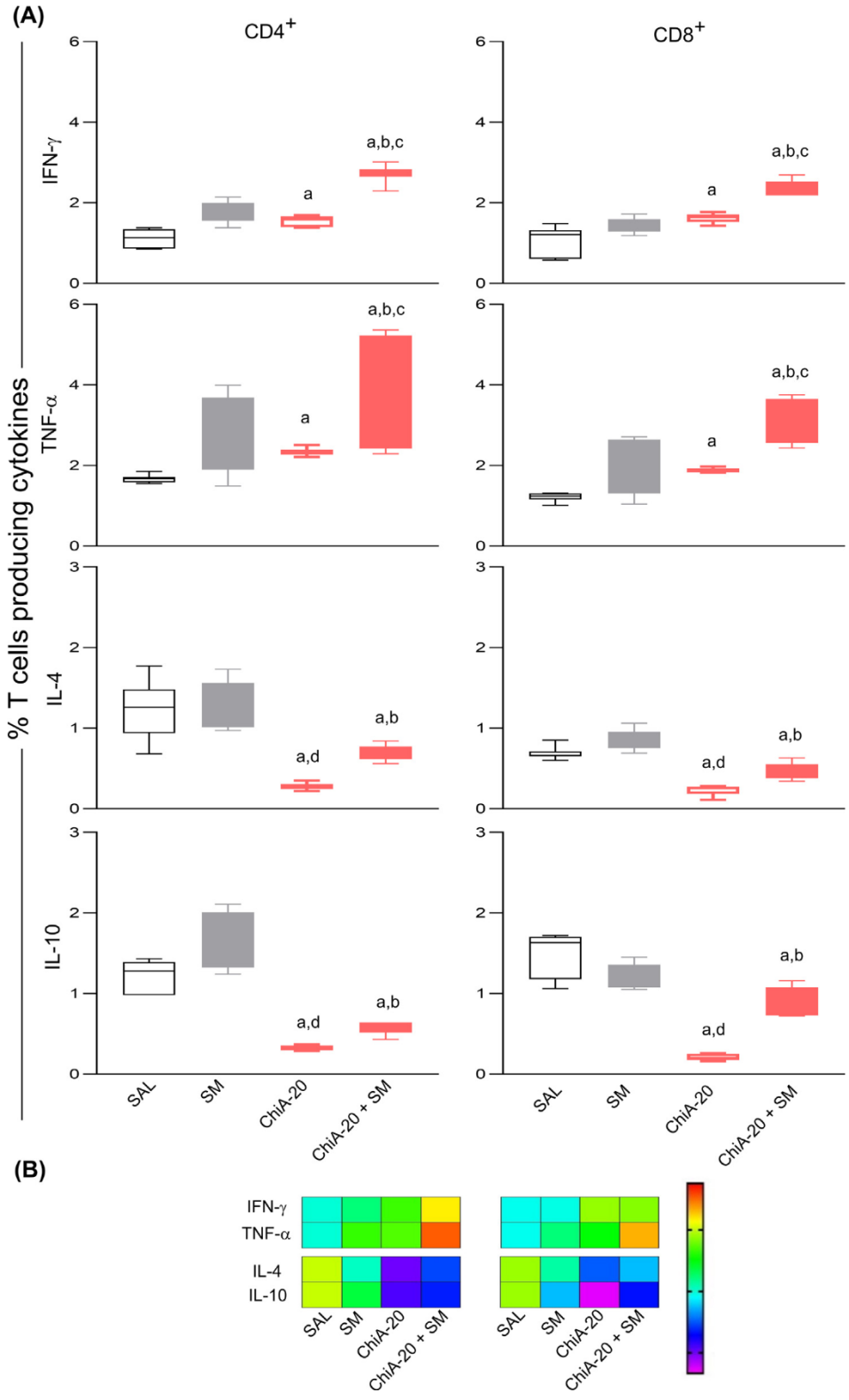

Fig. 3. Intracellular cytokine profile production induced by vaccination and challenge with *L. infantum*. **(A)** Percentage of T lymphocytes producing intracellular cytokines IFN-γ, TNF-α, IL-4 and IL-10 after SLA stimulation in BALB/c mice splenocytes. Data are expressed as medians ± SD of two independent experiments. The letters "a", "b", "c" and "d" represent the significant differences with the control group SAL, adjuvant group SM, ChiA-20 and ChiA-20 + SM, respectively (p-*value* < 0.05). **(B)** The heatmap is the rainbow type, in which the greatest production is expressed from the yellow to red, while the blue to violet tones represent a low expression of cytokines. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

experimental infection by *L. infantum*, which demonstrates the potential of Chimera A as a vaccine candidate. $CD8^+$ T response appears to be necessary, as it provides protection, as well as the presence of $CD4^+$ T cells that provides protection and control of *Leishmania* parasites due to pro-inflammatory cytokine secretion [39,40]. There was an increase in IL-2 level produced by $CD4^+$

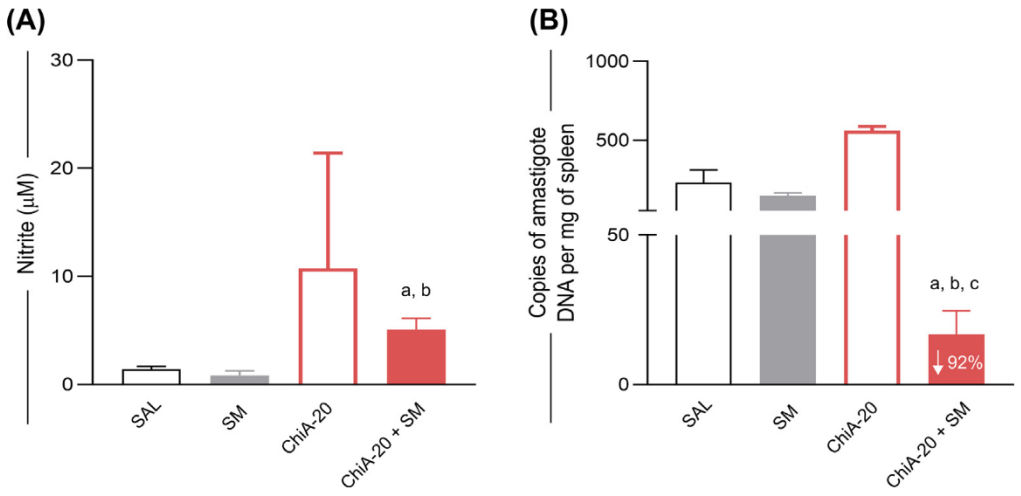

Fig. 4. Nitric oxide production and splenic parasite load after vaccination and challenge with *L. infantum*. **(A)** Nitric oxide production by splenocytes in supernatants of SLA-stimulated culture. The results were expressed through the levels of total nitrite (μM). **(B)** Splenic parasite load of vaccinated and challenged BALB/c mice. Results are expressed in amastigotes DNA copies per milligram of spleen. Data are expressed as means ± SEM of two independent experiments. The letters "a", "b", "c" and "d" represent the significant differences with the control group SAL, adjuvant group SM, ChiA-20 and ChiA-20 + SM, respectively (*p-value* < 0.05).

Table 1
Spearman's correlation analyses.

			Splenic parasite load
$CD4^+$	IFN^+	*r*	−0,7196
		p	0,0023
	TNF^+	*r*	−0,8047
		p	0,0003
	$IL\text{-}10^+$	*r*	0,5425
		p	0,0319
$CD8^+$	IFN^+	*r*	−0,7105
		p	0,0027
	TNF^+	*r*	−0,7864
		p	0,0008
	$IL\text{-}10^+$	*r*	0,5791
		p	0,0259
Nitric oxide		*r*	−0,6844
		p	0,0044

and $CD8^+$ T cells in mice vaccinated with Chimera A formulated with the SM adjuvant system. IL-2 is related to clonal expansion of $CD4^+$ and $CD8^+$ T cells and secretion of Th1 cytokines [41].

We found an increased percentage of $CD4^+$ and $CD8^+$ T cells producers of IFN-γ and TNF-α in vaccinated mice that received Chimera A formulated with the SM adjuvant system. IFN-γ and TNF-α are pro-inflammatory cytokines involved in protection against leishmaniasis that deserve to be highlighted [41]. Dikhit et al. [38] observed a significant increase in $CD4^+$ T cells producers of IFN-γ, while Martins et al. [16] found an increase of both $CD4^+$ and $CD8^+$ T cells producers of IFN-γ in vaccinated mice. Differently, Alves-silva et al. [42] showed an increase for IFN-γ and TNF-α. These findings point to a Th1 response, as expected. The literature widely addressed that effective vaccines activate innate immunity through inflammatory responses, being advantageous in providing immunogenicity [43].

After vaccination with Chimera A, we observed a decrease in IL-4 and IL-10 levels, reinforcing the establishment of an inflammatory response. The addition of the SM adjuvant system led to an increase in IL-4 and IL-10 levels compared to vaccination only with Chimera A. However, it remained lower when compared to the group inoculated with saline. Susceptibility to leishmaniasis is related to the development of a Th2 response, characterized by the production of cytokines IL-4, IL-5 and IL-13 [44]. Previous studies suggest that the production of IL-10 may be related to its role in modulating the immune system, mainly in response to a pro-inflammatory environment triggered after vaccination, acting as a feedback mechanism by increasing its secretion [44]. In addition, increased IL-10 may be necessary against an extremely polarized immune response and, hence, seeking to limit tissue damage [45,46].

The increased levels of IL-2, IFN-γ and TNF-α found in our study was also previously reported by Brito et al.[18] in BALB/c mice vaccinated with Chimera A (10 μg) associated with Saponin only. Similar findings were observed by [38,47], but using a higher dose than ours, respectively, 30 and 50 μg. Thus, even with a smaller dose, we obtained results expressive as those previously mentioned, which can be attributed to the immunogenic potential of chimera and the use of adjuvants in the formulation.

The intensity and quality of the adaptive immune response are influenced by the innate immune response, which is activated more efficiently through the action of adjuvants. The adjuvants used are TLRs agonists, which stimulate increased inflammation through cells producing IFN-γ and IL-2 and help antigen uptake and presentation by APCs [21,48,49]. The vaccination with Chimera A formulated with SM adjuvant system enhanced the cellular immune response. This finding is possibly related to adjuvants' effect that activates the immune system. Furthermore, Brito et al. [18] showed a good interaction between Chimera A and murine TLR3 and TLR4 receptors by using immunoinformatics tools.

The nitric oxide production in culture supernatant can also be considered a biomarker of immunity against *Leishmania* since their levels increase in vaccinated dogs [50]. Low nitrite production was expected in the group inoculated with saline, due to the absence of antigenic fragments during the immunization protocol. Although vaccinated mice with Chimera A alone induced nitrite production, it was not significant in comparison to the other groups, possibly due to no activation of macrophages and, hence, no reduction in the parasite burden. Instead, we observed increased nitrite production in the group vaccinated with Chimera A formulated with the SM adjuvant system, indicating possible activation of splenic macrophages. The presence of activated macrophages, which produce nitric oxide and reactive oxygen species, is related to the increase in specific $CD4^+$ T cells that produce the cytokines IFN-γ and TNF-α. Dikhit et al. [38], Lage et al. [51] and Dias et al. [17] also found similar results, which may be associated with the use of adjuvants in the formulation.

To evaluate the vaccine efficacy, we quantified the parasite load in the spleen, which is an organ largely affected by the *Leishmania* parasite. There is the establishment of a persistent infection profile in a murine model, which makes this organ useful for assessing vaccine efficacy [34,52,53]. We did not observe any reduction in parasite load in mice vaccinated with Chimera A alone. Thus, despite being immunogenic, the Chimera A alone was not able to provide protection. However, when formulated with the SM adjuvant system, the parasite load significantly decreases (92%) in comparison to the SAL group. In contrast, Martins et al. [16] had low reduction of parasite load (49.8%) in mice BALB/c vaccinated with recombinant chimeric protein formulated with Saponin. Brito et al. [18] found a reduction slightly minor than our results (82%) when evaluated this same Chimera A formulated with Saponin only. The murine model is widely useful to obtain a detailed analysis of immune response, contributing to select possible vaccine candidates and potential adjuvants [44,53,54]. Nevertheless, it is important to highlight that data obtained from murine models cannot be generalized to other hosts, which implies further investigations regarding the establishment of potential prophylactic effects in dogs and/or humans for example.

5. Conclusion

The Chimera A formulated with the SM adjuvant system was considered immunogenic and capable of inducing potential immunoprotective mechanisms against infection by *L. infantum* in BALB/c mice. The vaccination provides a proinflammatory cytokine profile with high levels of IFN-γ and TNF-α and production of nitric oxide, leading to a reduction in the splenic parasite load. The data obtained were promising and highlight the potential use of T-cell epitope-based vaccine against CVL.

Authors contributions

All the authors participated with suggestions and the development of this manuscript; T.L.V.P.D.O.; M.R.G.; F.A.S.M.; J.M.O.C.; R.C.F.D.B.; and R.D.O.A.S. performed all experiments. T.L.V.P.D.O.; R.C.F.D.B.; and B.M.R. performed the analyses of data. T.L.V.P.D.O.; M.R.G.; F.A.S.M.; J.M.O.C.; B.M.R.; R.D.O.A.S.; R.C.F.D.B. and A.B.R. participated in drafting the article and/or revising it critically for important intellectual content and created the figures and tables. R.C.F.D.B. and A.B.R. participated in the study conception, critical revision of the article, and supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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