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Probing the efficacy of a heterologous *Leishmania/L. Viannia braziliensis* recombinant enolase as a candidate vaccine to restrict the development of *L. infantum* in BALB/c mice

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ABSTRACT

In the present study, the *Leishmania braziliensis* enolase protein was evaluated as a vaccine candidate against visceral leishmaniasis (VL). The DNA sequence was cloned and the recombinant protein (rEnolase) was evaluated as a vaccine, associated with saponin, as an immune adjuvant. The protective efficacy of the rEnolase plus saponin combination was investigated in BALB/c mice against *Leishmania infantum* infection. The results revealed that the vaccine induced higher levels of IFN-γ, IL-12, and GM-CSF when a capture ELISA and flow cytometry were performed, as well as an antileishmanial nitrite production after using *in vitro* stimulation with rEnolase and an antigenic *Leishmania* preparation. The vaccinated animals, when compared to the control groups, showed a lower parasite burden in the liver, spleen, bone marrow, and paws' draining lymph nodes when both a limiting dilution technique and RT-PCR assay were performed. In addition, these mice showed low levels of antileishmanial IL-4, IL-10, and anti-*Leishmania* IgG1 isotype antibodies. Partial protection was associated with IFN-γ production, which was mainly mediated by $CD4^+$ T cells. In conclusion, the present study's data showed that the *L. braziliensis* enolase protein could be considered a vaccine candidate that offers heterologous protection against VL.

1. Introduction

Leishmaniasis is a vector-borne disease caused by protozoan parasites of the *Leishmania* genus and is transmitted to mammalian hosts through the bite of an infected female phlebotomine sand fly (Bates et al., 2015). The disease is endemic in 98 countries, with more than 380 million people at risk (WHO, 2016). Infections with *Leishmania donovani* or L. (*chagasi*) *infantum* species result in clinical outcomes that range from an asymptomatic infection to fatal visceral leishmaniasis (VL) (Gardinassi et al., 2016). The treatment of disease presents some problems (Kobets et al., 2012; Singh et al., 2012), which have made it necessary to develop alternative control measures, such as vaccinations (Kaye and Aebischer, 2011; Agallou et al., 2014).

The development of infective promastigote forms is an important prerequisite for the transmission of disease. The sand fly midgut microbiome is a critical factor for *Leishmania* growth and differentiation to its infective state prior to parasite transmission (Kelly et al., 2017). Although the development of a successful preventive vaccine for human leishmaniasis was still not achieved,

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control measures to curtail vector-mediated transmission in areas of endemicity are desirable. The introduction of VL transmission in non-endemic areas has motivated intensive programs using the traditional measures of infection detection in dogs, with culling of seropositive animals and insecticide spraying of households (Costa et al., 2014; Coura-Vital et al., 2014).

In addition, studies have shown that about 12% to 20% of infected humans develop the clinical disease, with this fact normally linked to the prevalence of factors including malnutrition, poverty, among others (Gardinassi et al., 2016). However, although developing an asymptomatic infection, humans can constitute a reservoir of the parasites (Kumar and Nylén, 2012; Joshi et al., 2014). In this context, to potentially reach an optimized vaccine formulation, there is an essential need to understand what types of immune responses are operating in these asymptomatic individuals. Therefore, the development of any formulation aimed to protect against local and or systemic infection that can occur in humans remains a challenge. This fact represents an important problem and reflects the complexity of parasite-host interactions on leishmaniasis, which have evolved over decades (Reed et al., 2016).

Murine models have been used to shown that the Th1 cell-mediated immunity is important to protect against *Leishmania* infection (Das and Ali, 2012; Chávez-Fumagalli et al., 2010; Ramírez et al., 2013; Costa et al., 2014). The induction of $CD4^+$ Th1 cell response against parasite antigens, based on the production of cytokines such as IFN-γ, IL-12, and GM-CSF that induce nitric oxide (NO) production by infected phagocytic cells, is necessary to control parasite replication (Green et al., 1990; Lage et al., 2015). By contrast, IL-4, IL-10, IL-13, TGF-β, among others, represent disease-promoting cytokines, leading in turn to the suppression of the Th1 response and contributing to the progression of infection (Wilson et al., 2005; Joshi and Kaur, 2014).

Enolase is an enzyme involved in glycolysis and glyconeogenesis. Besides its metabolic role, it is also related to other biological functions in distinct organisms (Pancholi, 2001). In *Leishmania*, enolase proved to be expressed on the cell surface (Quiñones et al., 2007) and to have a role in binding to the host's plasminogen, thus presenting a function in the infectivity found in macrophages (Pires et al., 2014).

An important challenge for the development of an antileishmanial vaccine is the low efficacy of the tested antigens to protect against different *Leishmania* spp., since candidates usually offer a species-specific protection (Duarte et al., 2016). In the present study, the *L. braziliensis* enolase protein, which was first identified by an immunoproteomic approach by antibodies from tegumentary leishmaniasis (TL) patients (Duarte et al., 2015), was cloned and evaluated as a cross-protective antigen, associated with saponin, against *L. infantum* infection.

2. Materials and methods

2.1. Mice and parasites

This study was approved by the Ethical Handling of Research Animals (protocol number 043/2011) from the Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil. BALB/c mice (8 weeks of age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, UFMG, and were maintained under specific pathogen-free conditions. *L. infantum* (MOM/BR/1970/BH46) and *L. braziliensis* (MHOM/BR/1975/M2904) strains were used. Parasites were cultured and soluble *Leishmania* protein extracts (SLA) were prepared according to that described in Coelho et al. (2003).

2.2. Cloning, expression, and purification of the recombinant protein

The *L. braziliensis* enolase (XP_001563419.1) sequence was obtained from the National Center for Biotechnology Information (http//www.ncbi.nlm.nih.gov). The local alignment against the available genomes of other *Leishmania spp.* was performed by BLAST. The recombinant protein was obtained after cloning the *L. braziliensis* DNA fragment containing its coding region, as described by Duarte et al. (2015). Briefly, genomic DNA was extracted by a phenol/chloroform extraction, which was used as a template. *Forward* (5′GCTAGCATGCCGATCCAGAAGGTGTA) and (5′AAGCTTTTACGCCCAGCCGGAGTA) *reverse* primers were designed according to the DNA sequence of the open reading frame (ORF) described in the *L. braziliensis* species. The PCR product was cloned into the pGEM-T *easy* vector, confirmed by sequencing and transferred to the pET28a-TEV vector using NheI and HindIII restriction enzymes, which were included in the primers (underlined). The recombinant plasmid was introduced into electrocompetent *Escherichia coli* BL21 Arctic Express (DE3) cells (Agilent Technologies, USA) by electroporation using a MicroPulser Electroporation Apparatus (Bio-Rad Laboratories, USA). The insert was sequenced using the T7 primer (Macrogen®, South Korea). For this, the MegaBACE 1000 DNA Sequencing System (GE Healthcare) was employed, in which reactions were developed using the DYEnamic™ ET Dye Terminator Kit Cycle Sequencing and runs were performed using the MegaBACE™ Long Read Matrix. The insert was analyzed by the software Sequence Analyzer version 3.0. The expression of the recombinant protein was performed adding 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG; Promega, Montreal, Canada) for 24 h at 12 °C and shaken at 200 x *g* per min. Next, cells were lysed by sonication and centrifuged at 10,000 x *g* for 30 min at 4 °C. The recombinant protein containing a tag of 6 x histidine residues was purified under non-denaturing conditions, using a 5 mL HIS-Trap column (GE Healthcare Life Science), which was attached to a fast protein liquid chromatography (FPLC) system (GE Healthcare Life Science). The column was pre-washed using 5 times its volume with phosphate buffered saline 1 x (PBS 1x) containing 30 mM imidazole, and elution was performed by adding PBS 1 x containing 500 mM imidazole. After purification, rEnolase was passed through a polymyxin-agarose column (Sigma-Aldrich) to remove the 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). To analyze the purity of the recombinant protein, rEnolase obtained at the end of the purification process (10 μg) was submitted to a 12% SDS-PAGE. Gels were stained with Coomassie blue.

2.3. Vaccination and experimental infection

Female BALB/c mice (n = 16, per group) were subcutaneously vaccinated in their left hind footpad with rEnolase (12.5 μg diluted in 20 μL PBS 1x, in each dose), added or not with saponin (*Quillaja saponaria* bark saponin, Sigma-Aldrich, 12.5 μg in each dose). As controls (n = 16, per group), animals received saline (20 μL PBS 1x, in each dose) or saponin (12.5 μg, in each dose). Three doses were administered at two-week intervals in all animals. Thirty days after the last immunization, mice (n = 8, in each group) were euthanized and their sera samples and spleen were collected to analyze the immune response. At the same time, remaining animals (n = 8, in each group) were subcutaneously infected in their right hind footpad with 1×10^7 stationary-phase promastigotes of *L. infantum* (diluted in 20 μL PBS 1x). Ten weeks after challenge, these animals were euthanized and their sera samples, spleen, liver, bone marrow (BM), and paws' draining lymph nodes (dLN) were