Immunobiology 222 (2017) 251–260

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

A vaccine composed of a hypothetical protein and the eukaryotic initiation factor 5a from *Leishmania braziliensis* cross-protection against *Leishmania amazonensis* infection

Mariana Costa Duarte[a,b], Daniela Pagliara Lage[b], Vívian Tamietti Martins[b], Lourena Emanuele Costa[b], Ana Maria Ravena Severino Carvalho[a], Fernanda Ludolf[b], Thaís Teodoro de Oliveira Santos[b], Danniele Luciana Vale[a], Bruno Mendes Roatt[a], Daniel Menezes-Souza[a,b], Ana Paula Fernandes[c], Carlos Alberto Pereira Tavares[d], Dr. Eduardo Antonio Ferraz Coelho (Prof.)[a,b,*]

[a] *Departamento de Patologia Clínica, COLTEC, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901, Pampulha, Belo Horizonte, Minas Gerais, Brazil*
[b] *Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Av. Prof. Alfredo Balena, 190, 30130-100, Belo Horizonte, Minas Gerais, Brazil*
[c] *Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901, Pampulha, Belo Horizonte, Minas Gerais, Brazil*
[d] *Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Av. Antônio Carlos, 6627, 31270-901, Pampulha, Belo Horizonte, Minas Gerais, Brazil*

ARTICLE INFO

Article history:
Received 23 June 2016
Received in revised form
23 September 2016
Accepted 26 September 2016
Available online 28 September 2016

Keywords:
Hypothetical proteins
Eukaryotic initiation factor 5a
Cross-protection
Vaccine
Tegumentary leishmaniasis
Th1 immunity

ABSTRACT

In the present study, two proteins cloned from *Leishmania braziliensis* species, a hypothetical protein (LbHyp) and the eukaryotic initiation factor 5a (EiF5a), were evaluated to protect BALB/c mice against *L. amazonensis* infection. The animals were immunized with the antigens, either separately or in combination, using saponin as an immune adjuvant in both cases. Spleen cells from vaccinated and later infected mice produced significantly higher levels of protein and parasite-specific IFN-γ, IL-12, and GM-CSF, in addition to low levels of IL-4 and IL-10. Evaluating the parasite load by means of a limiting dilution technique and quantitative Real-Time PCR, vaccinated animals presented significant reductions in the parasite load in both infected tissues and organs, as well as lower footpad swelling, when compared to the control (saline and saponin) groups. The best results regarding the protection of the animals were achieved when the combined vaccine was administered into the animals. Protection was associated with an IFN-γ production against parasite antigens, which was mediated by both $CD4^+$ and $CD8^+$ T cells and correlated with antileishmanial nitrite production. In conclusion, data from the present study show that this polyprotein vaccine, which combines two *L. braziliensis* proteins, can induce protection against *L. amazonensis* infection.

1. Introduction

Leishmaniasis is a disease caused by species of the kinetoplastid parasite *Leishmania* spp. and transmitted by hematophagous sandflies. The disease represents a major, although grossly underestimated, health problem: over 350 million people are at risk, with a worldwide prevalence of more than 12 million cases. The clinical presentation is dependent upon both parasite species and the host's immune response (Alvar et al., 2012). The murine model of *Leishmania* infection is considered suitable for studying leishmaniasis, which, despite its incidence of 1.5 to 2.0 million new cases per year, is still considered as a neglected disease (WHO, 2010). Studies developed using such experimental models are essential to understanding this disease in mammalian hosts, such as dogs and humans, due to similarities in the physiology and advantages provided by the infection profile within each mouse strain (Pereira

* Corresponding author at: Laboratório de Pesquisa do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, Faculdade de Medicina, Universidade Federal de Minas Gerais, Av. Prof. Alfredo Balena, 130, 30130-100, Belo Horizonte, Minas Gerais, Brazil.
E-mail address: eduardoferrazcoelho@yahoo.com.br (E.A.F. Coelho).

http://dx.doi.org/10.1016/j.imbio.2016.09.015
0171-2985/

and Alves, 2008). Among the *Leishmania* species known to cause disease in humans, in the Americas, *Leishmania amazonensis* is an important etiological agent of tegumentary leishmaniasis (TL), presenting a wide spectrum of the disease's clinical manifestations (Barral et al., 1991). Recent findings also indicate that this parasite species is increasing its geographic distribution in Brazil into new transmission areas, also leading to unusual clinical manifestations, such as visceral leishmaniasis (Azeredo-Coutinho et al., 2007; Pereira and Alves, 2008).

The course of cutaneous infection caused by *L. amazonensis* differs among inbred mouse strains. BALB/c mice are susceptible to infection and develop chronic lesions when infected with this parasite species (Calabrese and Costa, 1992). Likewise, C57BL/10 mice also develop persistent lesions with significant parasite loads in infected tissues (Afonso and Scott, 1993). In contrast, other lineages, such as C3H, C57BL/6, and CBA, are less susceptible to *L. amazonensis* infection and develop small lesions, although a persistent parasite load is commonly found in these infected hosts (Barral et al., 1983; Soong et al., 1997; Jones et al., 2002). Th1 cell immunity is important for protection against *Leishmania* infection (Afonso and Scott, 1993; Coelho et al., 2003; Das and Ali, 2012; Ramírez et al., 2013). The induction of a parasite-specific $CD4^+$ Th1 cell response is crucial to controlling the infection, where cytokines, such as IFN-γ and IL-12, induce the production of nitric oxide (NO) and other toxic compounds, which are responsible for killing internalized parasites in infected phagocytic cells (Green et al., 1990; Costa et al., 2014). In addition, $CD8^+$ T cells are also linked to protection against infection, and studies have shown that these cells also play an important role in controlling primary infection by inducing the Th1 response through an IFN-γ-dependent mechanism (Kharazmi et al., 1999; Stäger et al., 2000; Costa et al., 2015).

By contrast, cytokines, such as IL-4, IL-10, IL-13, and TGF-β, represent disease promoting molecules, contributing to the suppression of the Th1 immune response and inhibiting the activation of infected phagocytes (Stäger et al., 2003; Wilson et al., 2005; Joshi and Kaur, 2014). Despite the importance of T cell-mediated immunity in defining the outcome of *L. amazonensis* infection; other components of the adaptive immune system also demonstrate an influence in the disease outcome. For example, circulating antibodies play an important role in keeping the infection in the body, since genetically modified mice that did not present circulating antibodies were refractory to infection against this parasite species (Kima et al., 2000). One possible explanation could be based on the fact that IgG-opsonized parasites activate the production of IL-10 by infected macrophages, and this cytokine has been directly related with the progression of the disease caused by *L. amazonensis* (Yang et al., 2007).

Advances in the development of vaccines against TL have been based on molecularly defined antigens to offer species-specific protection (Julia et al., 1996; Ramírez et al., 2013; Martins et al., 2015; Lage et al., 2016), although an effective vaccine still does not exist. However, vaccine candidates combining distinct proteins leading to the development of polyprotein vaccines could help to produce a more robust prophylactic product by sharing different protective antigens and could allow for the induction of a more effective protection against different *Leishmania* species (Beaumier et al., 2013; Saljoughian et al., 2013; Zahedifard et al., 2014). In this context, in the present study, two antigenic *L. braziliensis* proteins – the eukaryotic initiation factor 5a protein and a hypothetical protein – which were identified in the promastigote and amastigote stages of this parasite species by antibodies in the sera of TL patients (Duarte et al., 2015), were combined in a polyprotein vaccine and used to protect BALB/c mice against a cross-infection caused by *L. amazonensis*. Results showed that this composition induced an effective protection in the infected animals, which was based on the development of an anti-parasite Th1 immune response, as well as on significant reductions in footpad swellings and in the parasite load in the infected tissues and different evaluated organs, leading to the possibility of developing a new pan-*Leishmania* vaccine.

2. Material and methods

2.1. Cloning, expression, and purification of LbHyp and EiF5a proteins

The hypothetical protein (LbrM30.3350) and eukaryotic initiation factor 5a (LbrM25.0580) codifying genes were cloned using *L. braziliensis* DNA, as described by Duarte et al. (2015). After purification, the recombinant proteins were placed on a polymyxin-agarose column (Sigma-Aldrich) in an attempt to remove the residual endotoxin content (less than 10 ng of lipopolysaccharide per 1 mg of protein, measured by the Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000, BioWhittaker, USA).

2.2. Mice, parasite, and immunization protocol

BALB/c mice (female, 8 weeks old) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), and were kept under specific pathogen-free conditions. Experiments were approved by the Committee on the Ethical Handling of Research Animals from UFMG, under protocol number 333/2015. *L. amazonensis* (IFLA/BR/1967/PH-8) and *L. braziliensis* (MHOM/BR/1975/M2904) strains were used. Parasites were grown at 24 °C in Schneider's medium (Sigma, St. Louis, MO, USA), which was supplemented with 20% heat-inactivated fetal bovine serum (FBS, Sigma), 20 mM L-glutamine, 100 U/mL penicillin, and 50 μg/mL streptomycin, at a pH of 7.4. The soluble *Leishmania* antigenic extracts were prepared from stationary promastigotes of the parasites (2×10^8 cells), as described by Coelho et al. (2003). For the immunization protocol, mice (n = 16 per group) were subcutaneously vaccinated in their left hind footpad with 25 μg of rLbHyp or rEiF5a proteins, or with the combined vaccine (12.5 μg of each protein), all associated with 25 μg of saponin (*Quillaja saponaria* bark saponin, Sigma). Additional animals were immunized with only 25 μg of saponin or received saline. Three doses were administered at two-week intervals.

2.3. Infection and parasite burden

Thirty days after the last immunization, half of the animals per group (n = 8) were euthanized and serum samples and spleens were collected to evaluate the immunogenicity. At the same time, the remaining animals (n = 8 per group) were infected subcutaneously in the right hind footpad with 10^6 stationary-phase promastigotes of *L. amazonensis*. The course of the infection was monitored at weekly intervals by measuring footpad thickness with a metric caliper and expressed as the increased thickness of the infected footpad compared to the uninfected footpad. Ten weeks post-challenge, these animals were euthanized and their serum samples, lesion fragments, spleen, liver, and infected paws' draining lymph nodes (dLN) were collected for parasitological and immunological analysis. To evaluate the parasite load, infected tissues and organs were submitted to a limiting dilution technique and quantitative Real-Time PCR (*q*PCR). When the limiting dilution assay was performed, infected tissues and organs were homogenized using a glass tissue grinder in sterile PBS 1×, and cell debris were removed by centrifugation at 150×*g*. Next, cells were concentrated by centrifugation at 2000×*g*, and the pellet was resuspended in 1 mL of complete Schneider's medium. Two hundred and twenty microliters were plated onto 96-well flat-bottom microtiter plates (Nunc,