Immunobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Immunobiology

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

Identification of immune biomarkers related to disease progression and treatment efficacy in human visceral leishmaniasis

Áquila S.B. Portela[a], Lourena E. Costa[a], Beatriz C.S. Salles[a], Mariana P. Lima[a], Thaís T.O. Santos[a], Fernanda F. Ramos[a], Daniela P. Lage[a], Vívian T. Martins[a], Rachel B. Caligiorne[b], Daniela R. Lessa[b], Fabiana R. Silva[b], Amanda S. Machado[b], Guilherme F. Nascimento[b], Isabela S. Gama[a], Miguel A. Chávez-Fumagalli[a], Antonio L. Teixeira[a,c], Manoel O.C. Rocha[a], Regina L. Rocha[a], Eduardo A.F. Coelho[a,*]

[a] 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, Santa Efigênia, 30130-100, Belo Horizonte, Minas Gerais, Brazil

[b] Instituto de Ensino e Pesquisa, Santa Casa de Belo Horizonte, Rua Domingos Vieira, 590, Santa Efigênia, 30150-240, Belo Horizonte, Minas Gerais, Brazil

[c] Neuropsychiatry Program, Department of Psychiatry and Behavioral Sciences, McGovern Medical School, The University of Texas Health Science Center at Houston (UTHealth), Houston, TX,USA; 1941 East Road, Houston, TX, 77041, USA

ARTICLE INFO

Keywords:
Peripheral blood mononuclear cells
Cytokines
Antibodies
Recombinant proteins
Immune response
Visceral leishmaniasis

ABSTRACT

Visceral leishmaniasis (VL) is a potentially fatal disease, in which the treatment based on chemotherapy is considered toxic. The cure of disease is associated with the life-long Th1-type immunity against the infection. The Th1-related cytokines production by peripheral blood mononuclear cells (PBMCs) seems to be crucial for host control of parasite load and clinical cure. In the current study, we used five proteins (IgE-dependent histamine-releasing factor [HRF], LiHyD, LiHyV, LiHyT and LiHyp6) recently shown to be antigenic and/or immunogenic in the canine VL, aiming to evaluate the antigen-specific antibody levels and cytokine production in PBMCs culture supernatants collected from VL patients before and after anti-VL treatment. In the results, when PBMCs were exposed to rHRF, rLiHyD and rLiHyT, higher IFN-γ and lower IL-10 levels were observed in all patients that were treated and clinically cured. Analysis of specific antibody subclasses was in line with *in vitro* cellular response, since a higher IgG2 production was found in the treated and cured patients, when compared to the IgG1 subclass levels. In addition, evaluating the diagnostic efficacy of the recombinant molecules, the rHRF, rLiHyD and rLiHyT proteins showed the best results in the serology assays to identify all VL patients, as well as these antigens were not recognized by antibodies in sera from non-infected subjects or those with leishmaniasis-related diseases. Our results corroborate the view that clinical cure of VL is associated with a sustained Th1-related response, and indicate the potential use of rHRF, rLiHyD and rLiHyT as immune biomarkers of VL treatment.

1. Introduction

Leishmaniasis is a vector-borne infection considered a neglected tropical disease. Approximately 350 million people live in endemic areas and about 14 million people are clinically affected by the disease (World Health Organization, 2016). More than 30 species of *Leishmania* have been identified of which about 20 are pathogenic to humans. The clinical manifestations depend primarily on the interaction between the parasite and the host immune response. Visceral leishmaniasis (VL) results from an infection in phagocytic cells within the reticulum-endothelial system due to metastasis of parasites and infected cells from the initial site of infection, being parasites of the *Leishmania donovani* complex in Southern Europe, Africa, Asia, and *Leishmania infantum* in Latin America as the main responsible by infection in mammalian hosts (Faleiro et al., 2014; Rodrigues et al., 2016).

The treatment of disease is based on the use of pentavalent antimonials, oral miltefosine, free or liposomal amphotericin B, and paramomycin. However, there are several concerns related with the toxicity of these compounds, duration of treatment, high cost, route of administration and/or development of drug-resistant parasites (Chávez-

* 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, 190, 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.2017.10.043
Received 6 July 2017; Accepted 19 October 2017

Fumagalli et al., 2015).

Laboratory methods to diagnose VL include parasite detection by microscopic examination of aspirates and molecular biology-based techniques for detecting the *Leishmania* kDNA. Although these methods are considered highly specific, problems related with their sensitivity have been registered (Santos et al., 2014; Carvalho et al., 2017). Serological methods are also used to diagnose VL, since high serum antibody levels are found in the active disease. Nevertheless, there are shortcomings as serology remains positive for several years after treatment (Savoia, 2015), and can be positive in endemic and *Leishmania*-related pathogens co-infection areas (Vallur et al., 2014; Salles et al., 2017), influencing the specificity of the tests.

Significant progresses have been made in the understanding of the immune mechanisms related to the resistance and susceptibility to VL (Duarte et al., 2016a; Lage et al., 2016; Martins et al., 2017). Taking into account that infected individuals after recovery become resistant to re-infection with the same parasite species, efforts have been made to develop prophylactic vaccination (Khamesipour et al., 2006; Srivastava et al., 2016). Although there is no approved human vaccine, a protective product would be possible based on the abundance of genetic and biological information available about *Leishmania*, as well as by the evidence of T cell-mediated immune mechanisms role for infection control (Srivastava et al., 2012; Duarte et al., 2016b; Santos et al., 2017).

Previous works evaluating humoral immunity have shown a variation of IgG subclasses in the human VL (Saha et al., 2005; Mukhopadhyay et al., 2012). Increased *Leishmania*-specific IgG1 and IgG3 levels have been found in active VL patients, when compared to non-infected controls (Gidwani et al., 2009; Bhattacharyya et al., 2014). On the other hand, high anti-parasite IgG2 and IgG4 levels have been found in treated and cured patients (Bhattacharyya et al., 2014; Ganguly et al., 2008). In a recent immunoproteomic study performed in *L. infantum* promastigotes and amastigotes, parasite proteins were identified by antibodies from VL dogs sera (Coelho et al., 2012). Among them, known proteins such as the *Leishmania* orthologue of the IgE-dependent histamine-releasing factor (HRF), and unknown proteins and classified as “hypothetical”, such as LiHyD, LiHyV, LiHyT and LiHyp6 were recognized and all of them showed as effective diagnostic markers for canine VL (Martins et al., 2015a; Martins et al., 2015b).

Due to the high similarity of the amino acid sequence of these proteins in distinct *Leishmania* species, as well as by presence of conserved T and B cells epitopes among them, the current study investigated these candidates in their recombinant versions as biomarkers related to the VL progression. Specifically, we evaluated protein- and parasite-specific cellular (IFN-γ and IL-10 levels) and humoral (IgG total and its IgG1 and IgG2 subclasses) responses in VL patients before and after treatment. Moreover, we investigated whether these antigens could distinguish VL patients from non-infected subjects living in endemic or non-endemic areas of disease, as well as from patients with leishmaniasis-related diseases. Our results were promising for the improvement of diagnostic and prognostic biomarkers of human VL.

2. Materials and methods

2.1. Ethics and blood samples

The present study was approved by Ethics Committee from Federal University of Minas Gerais (UFMG, Belo Horizonte, Minas Gerais, Brazil), with the protocol number CAAE–32343114.9.0000.5149. Peripheral blood samples were collected from VL patients (n = 30, including 17 males and 13 females, with ages ranging from 22 to 58 years). Infection was confirmed by PCR technique targeting *L. infantum* kDNA in aspirates from spleen and/or bone marrow of the patients. None of them had been treated prior to blood samples collection. The control group consisted from non-infected individuals living in endemic (n = 30, including 19 males and 11 females, with ages ranging from 19 to 49 years; Belo Horizonte, MG, Brazil) or non-endemic (n = 15, including 9 males and 6 females, with ages ranging from 23 to 56 years; Poços de Caldas, MG, Brazil) areas of leishmaniasis. These subjects did not present any clinical sign or symptom of disease at the moment of sample collection, also exhibiting negative results when the presence of rK39-specific antibodies was investigated by Kalazar Detect™ Test (InBios International, USA). Aiming to evaluate the cross-reactivity of the antigens, blood samples were collected from patients diagnosed with Chagas disease, paracoccidioidomycosis, leprosy or aspergillosis. Regarding Chagas disease patients (n = 30, including 22 males and 8 females, with ages ranging from 25 to 62 years), infection was confirmed by hemoculture and by Chagatest recombinant ELISA v. 3.0 kit or Chagatest hemagglutination inhibition (HAI) assay (Wiener Lab, Argentina). Samples from paracoccidioidomycosis patients (2 males and 2 females with ages ranging from 23 to 49 years) were also used. The diagnosis was performed by means of clinical examination and positive *Paracoccidioides* culture. Samples were also collected from leprosy patients (n = 20, with 12 males and 8 females, with ages ranging from 20 to 49 years), which were diagnosed by means of clinical evaluation, ML Flow rapid test and/or histopathology of biopsies from leprosy skin lesions. Sera collected from patients with aspergillosis (n = 8, including 5 males and 3 females, with ages ranging from 28 to 63 years) were also employed in the serological assays, and disease was confirmed by sample culture and detection of fungal hyphae in histopathological examination.

2.2. Parasites

Leishmania infantum (MHOM/BR/1970/BH46) strain was used. Parasites were cultured at 24 °C in complete Schneider's medium (Sigma) which was supplemented with 20% inactivated fetal bovine serum (FBS, Sigma-Aldrich), 20 mM L-glutamine, 200 U/mL penicillin and 100 μg/mL streptomycin, at pH 7.4. The soluble *Leishmania* antigenic extract (SLA) was prepared of stationary-phase promastigotes of parasites (Coelho et al., 2003).

2.3. Cloning, expression, and purification of recombinant proteins

The HRF (XP_001465979.1), LiHyD (XP_001468360.1), LiHyT (XP_001465138.1), LiHyV (XP_001462854.1) and LiHyp6 (XP_001467126.1) proteins were cloned from genomic *L. infantum* DNA. They were purified as described elsewhere to: LiHyD (Lage et al., 2016), LiHyT (Martins et al., 2015a), LiHyV (Martins et al., 2015b), LiHyp6 (Martins et al., 2015b), and HRF (Coelho et al., 2016). The rA2 protein was used as an antigen control (Zhang et al., 1996). After purification, all recombinant proteins were passed through a polymyxin-agarose column (Sigma) in order to remove any residual endotoxin content. The purity of the recombinant proteins was also checked by a one dimensional 12% SDS-PAGE.

2.4. ELISA for the serodiagnosis of human VL

For the ELISA assays, previous titration curves were performed to determine the most appropriate concentration of the antigen and antibody dilution. Flexible microtiter plates (Jet Biofil®, Belo Horizonte) were coated with the rHRF, rLiHyD, rLiHyT, rLiHyV, and rLiHyp6 proteins (0.5, 0.5, 1.0, 0.5, and 1.0 μg/mL, respectively) or with the antigen controls: rA2 and *L. infantum* SLA (1.0 μg per well, in both cases) which were all diluted in 100 μL coating buffer (50 mM carbonate buffer) pH 9.6, and incubated for 16 h at 4 °C. After, free binding sites were blocked using 250 μL of a blocking solution (5% non-fat dry milk diluted in PBS 1 x plus Tween 20 0.05%), for 1 h at 37 °C. After washing plates seven times with PBS-T (PBS 1 x plus Tween 20 0.05%), 100 μL of the individual serum samples of the different groups (1:400 diluted in PBS 1x) were added and incubated for 1 h at 37 °C. Then, plates were washed seven times with PBS-T and incubated with anti-