Immunobiology 224 (2019) 477–484

Contents lists available at ScienceDirect

Immunobiology

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

A biomarker for tegumentary and visceral leishmaniasis based on a recombinant *Leishmania* hypothetical protein

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ARTICLE INFO

Keywords:
Hypothetical proteins
Leishmaniasis
Immunogenicity
Serodiagnosis
Cytokines
Antibodies

ABSTRACT

The measures for leishmaniasis control include the precise diagnosis of disease. However, although several recombinant antigens have been tested with this biotechnological purpose, no effective product exists, which could detects patients with the active disease, as well as differentiates them from cured and treated patients. In this study, a conserved *Leishmania* hypothetical protein, which was identified in *Leishmania infantum* parasites, but evaluated to presents high homology in the amino acid sequences between distinct parasite species, was evaluated for the diagnosis of tegumentary and visceral leishmaniasis. In addition, PBMCs collected from treated and untreated mucosal leishmaniasis (ML) and visceral leishmaniasis (VL) patients, as well as in healthy subjects living in endemic region of disease, were *in vitro* stimulated, when IFN-γ, IL-4 and IL-10 levels were evaluated in the cell supernatant. Regarding the serological analyses, ELISA experiments using the recombinant protein (rLiHyL) and a human serological panel revealed high sensitivity and specificity values to detect both diseases, while control antigens showed worst results. Regarding the cellular response, results showed that rLiHyL-stimulated cells produced higher IFN-γ and lower IL-4 and IL-10 levels in the supernatants. Also, the anti-protein antibody production was evaluated in these patients, and data showed higher IgG2 and lower IgG1 levels found in the treated patients and healthy controls, demonstrating the stimulation of a Th1-type response induced by the rLiHyL protein. In conclusion, this hypothetical protein can be considered as antigenic in TL and VL, as well as a vaccine candidate to be tested in future studies to protect against disease.

1. Introduction

Leishmaniases are neglected diseases considered as important public health problem in the world. This disease complex threatens about 380 million people in regions, such as Africa, Asia and Americas; with about two million people affected leading to their morbidity and/or mortality (WHO, 2016). The main clinical manifestations of disease are comprised between tegumentary (TL) and visceral (VL) leishmaniasis, which present a spectrum of diseases commonly referred to their clinical and pathologic features. Although TL is not a fatal disease, it can manifests as cutaneous leishmaniasis (CL), which may heal spontaneously; mucosal leishmaniasis (ML), which causes hyperergic ulcerative lesions and can progresses in the absence of apparent cellular response; and diffuse cutaneous leishmaniasis (DCL), a anergic clinic

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https://doi.org/10.1016/j.imbio.2019.05.008
Received 13 April 2019; Received in revised form 8 May 2019; Accepted 28 May 2019
Available online 30 May 2019

form able to causes morbidity in the patients (Burza et al., 2018). On the other hand, VL can cause systemic clinic symptoms in the patients, where hepatomegaly and splenomegaly are found; leading to their death, if active and left untreated (Singh et al., 2016).

The treatment against leishmaniasis is usually based on parenteral administration of pentavalent antimonials; however, the toxicity in the liver, spleen and kidneys are problems known (Chávez-Fumagalli et al., 2015; Tamiru et al., 2016). Amphotericin B-containing liposomal formulations present higher efficacy and lower toxicity; however, they are expensive mainly to under-developing countries (Lucero et al., 2015; Sereno et al., 2019). As a consequence, a precise diagnosis of disease could allow to a faster treatment, which could shorten the period of exposition to the chemotherapeutics and cause lower toxicity to the patients by lower exposition for drugs (Uliana et al., 2018; Ribeiro et al., 2018).

Currently, there is no gold standard test to diagnose leishmaniasis, and a combination between clinical evaluation and laboratorial methods is needed to obtain precise results. Regarding to the TL, where cutaneous and/or mucosal lesions are present, the microscopic examination of Giemsa-stained biopsy smears followed by histopathological analysis and/or immunohystochemical exams performed in lesions fragments' triturates are performed. Alternatively, the polymerase chain reaction (PCR) can be employed to detect parasite DNA (Duarte et al., 2015). Regarding to the VL, the clinical evaluation and demonstration of amastigote forms in splenic aspirates are performed; however, variable sensitivity and/or specificity, besides the invasive collection of the samples are problems known (Salles et al., 2017). As a consequence, immunological methods such as these using anti-leishmanial serology could be improved, since the detection of anti-parasite antibodies in patients sera present advantages in relation to the other methods, such as simplicity, lower cost, and the fact that the collection of the samples be considered minimally invasive, when compared to the collect from lesion and mucosal fragments (TL) or organ aspirates (VL) (Srividya et al., 2012).

The paradigm of Th1/Th2 response, each one characterized by presence of specific cytokines secreted by T cells in infected patients has been discussed regarding to the different mammalian hosts (Lockard et al., 2019). Predominantly, it is accept that the protective response is characterized by the production of pro-inflammatory cytokines, such as IFN-γ, IL-2, IL-12, GM-CSF, among others, as well as higher specific IgG2 production; which activate infected cells to kill parasites (Rodrigues et al., 2016). However, the susceptibility to infection is discussed, since the presence of polarized Th2 response with the production of cytokines, such as IL-4, IL-10, TGF-β, among others; and the development of a weak Th1 response have been related with the development of disease (Kumar and Samant, 2016; Maspi et al., 2016). In this context, antigens able to induce the specific Th1 response in immunized hosts could be considered as potential candidates to be used to protect against *Leishmania* infection (Lage et al., 2016; Dias et al., 2018; Joshi et al., 2019).

Leishmaniasis can cause morbidity and/or mortality in the patients, if not promptly diagnosed and reated (Marlais et al., 2018). As a consequence, the precise identification of infected subjects is important for controlling the spread of disease (Passero et al., 2018). However, diagnostic methods have showed variable sensitivity and specificity, since false-negative and/or false-positive results have been found when different antigens and/or methodologies have been employed in studies (Shanks et al., 2015; Lima et al., 2017). In this context, new antigens have been tested to diagnose the disease, although distinct sensitivity and specificity values are still found. One of the greatest challenges is based on the discrepancy from results obtained when similar serological assays are evaluated in different regions (Duarte et al., 2015; Hazra and Patra, 2018). The variation in the results can be due to the type of antigen, method, parasite strain and/or immune state of the patients. Also, assays can be negative in asymptomatics, as well as positive in patients that completed their treatment, did not allowing differentiate between treated and current infections (Zhang et al., 2018; Dhom-Lemos et al., 2019).

As a consequence, the present study evaluated a conserved *Leishmania* hypothetical protein, which was recently identified in *L. infantum* protein extracts by antibodies from VL patients' sera, when an immunoproteomic approach was performed (unpublished results). This protein, namely LiHyL (XP_003886492.1) showed high conservation degree in the amino acid sequence between different *Leishmania* species, being thus evaluated for the serodiagnosis of human TL and VL. Due to the absence of defined immunogenic antigens present in human vaccine to protects against *Leishmania* infection, the immunogenicity induced by LiHyL protein was evaluated after immune stimulation of peripheral blood mononuclear cells (PBMCs) collected from ML or VL patients, as well as from healthy subjects living in endemic region of disease; when the levels of IFN-γ, IL-4 and IL-10 cytokines were measured in the cellular supernatant. In addition, and to accomplish the evolution of the treatment regarding to humoral response, the specific IgG total, IgG1 and IgG2 response was also evaluated before and after treatment.

2. Materials and methods

2.1. Ethics statement and blood samples to evaluate the cellular response

The study was approved by the Ethics Committee on Human Research from Federal University of Minas Gerais (UFMG; protocol CAAE–32,343,114.9.0000.5149). A written informed consent was obtained from all subjects, who received an individual copy of the study policy. Blood samples were collected from ML patients (n = 8; including 3 males and 5 females, with ages ranging from 25 to 55 years) living in endemic region of disease (Belo Horizonte, Minas Gerais, Brazil). The diagnosis was confirmed by clinical exam, as well as by Giemsa-stained smears from mucosal fragments and PCR technique to identify *L. braziliensis* kDNA. Samples were also collected from VL patients (n = 8; including 4 males and 4 females, with ages ranging from 28 to 52 years), which were diagnosed by clinical exam and by PCR technique to identify *L. infantum* kDNA. Both patient´s classes were submitted to the same therapeutic regimen using the pharmaceutics, and they did not suffered from any infections or had pre-existing disease. Blood samples were also collected of healthy individuals (n = 8; including 6 males and 2 females, with ages ranging from 24 to 42 years) living in endemic region of disease (Belo Horizonte). These subjects showed no signs of leishmaniasis and showed negative serological results by using the Kalazar Detect™ kit.

2.2. Sera samples for the serological assays

Sera samples were collected from CL (n = 25; including 16 males and 9 females with ages ranging from 25 to 59 years), ML (n = 25; including 19 males and 6 females with ages ranging from 31 to 62 years) and VL (n = 25, including 17 males and 8 females with ages ranging from 23 to 55 years) patients, which were obtained from an endemic region of disease (Belo Horizonte). All patients were diagnosed as previously described. Samples were also collected from healthy individuals (n = 25, including 20 males and 5 females with ages ranging from 24 to 53 years) living in endemic region of disease, which did not showed clinical signs and presented negative serological result. Sera collected from Chagas Disease (n = 20, including 12 males and 8 females with ages ranging from 28 to 47 years), paracoccidioidomycosis (n = 10, 6 males and 4 females with ages ranging from 26 to 51 years), leprosy (n = 10, with 5 males and 5 females, with ages ranging from 35 to 63 years), and aspergillosis (n = 10, including 6 males and 4 females, with ages ranging from 26 to 47 years) patients were also used in the serological assays.

2.3. Cloning, expression and purification of the rLiHyL protein

The LiHyL (XP_003886492.1) gene was cloned from *L. infantum* kDNA by using the following primers: 5´-GCTGCTAGCATGCGTGAGG CTATCTGC -3´ (*forward*) and 5´- TCATGGATCCACACCGAGTTCGTGA TGT -3´ (*reverse*), and the restriction enzymes *NheI* and *BamHI*. The DNA fragment was excised from gel, purified and linked into a pGEM®-T vector system (Promega, USA). The recombinant plasmid (LiHyL-pGEM®-T) was transformed in *E. coli* XL1-Blue cells, and the obtained material was digested and ligated into the pET28a-TEV vector, and thus transformed again. The process was confirmed by colony PCR and using a MegaBace 1000 automatic sequencer (Amersham Biosciences, USA). For the purification of the recombinant protein, bacteria were induced with IPTG (0.5 mM) for 4 h at 37 °C, shaking at 200 x *g* per min. Then, they were ruptured by six cycles of ultrasonication with cycles of 30 s each (38 MHz). Cellular debris were removed by centrifugation, and rLiHyL was purified onto HisTrap HP affinity column connected to an AKTA system. The eluted fractions containing the recombinant protein (~32.3 kDa) were concentrated in Amicon® ultra15 centrifugal filters 10,000 NMWL (Millipore, Germany), and purified on a Superdex™ 200 gel-filtration column (GE Healthcare Life Sciences, USA). The recombinant A2 (rA2) protein was purified according described (Zhang et al., 1996). After purification, the recombinant proteins were passed through a polymyxin-agarose column (Sigma), aiming to remove residual endotoxin content (< 10 ng of LPS per 1 mg of recombinant protein, which was measured by the Quantitative Chromogenic Limulus Amebocyte Assay QCL-1000, BioWhittaker, MD, USA).

2.4. Parasites

Leishmania infantum (MHOM/BR/1970/BH46) and *L. braziliensis* (MHOM/BR/1975/M2903) were used. The stationary promastigotes were grown in complete Schneider's medium (Sigma-Aldrich, USA), which was composed by Schneider´s medium added with 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U/mL penicillin, and 100 µg/mL streptomycin at pH 7.4, 24 °C. To prepare the *Leishmania* antigenic extract (SLA), stationary promastigotes (10^9 cells) were washed three times in cold sterile phosphate-buffered saline (PBS 1x), and submitted to five cycles of freezing and thawing. Then, the suspension was centrifuged at 10,000 x *g* for 15 min at 4 °C, and aliquots containing SLA were collected and stored at −80 °C until use (Coelho et al., 2003). Protein concentration was estimated by the Bradford method (Bradford, 1976).

2.5. ELISA experiments

Titration curves were previously performed to determine the most appropriate antigen concentration and antibody dilution. After, microtiter immunoassay plates (Jetbiofil®, Belo Horizonte) were coated with the rLiHyL, rA2 or *L. braziliensis* and *L. infantum* SLA (1.0, 1.0, 2.0, and 1.0 µg per well, respectively), which were diluted in 100 µL of coating buffer (50 mM carbonate buffer, pH 9.6) for 16 h at 4 °C. Next, free binding sites were blocked using 200 µL of PBS 1x plus Tween 20 0.05% (PBS-T) containing 5% casein, for 1 h at 37 °C. After washing the plates five times with PBS-T, they were incubated with 100 µL of human sera (1:200 diluted in PBS-T) for 1 h at 37 °C. Plates were washed five times and incubated with anti-human IgG horseradish-peroxidase conjugated antibody (1 1:10,000 diluted in PBS-T) for 1 h at 37 °C. Then, they were washed and reactions were developed by incubation with 2 µL H_2O_2, 2 mg ortho-phenylenediamine and 10 mL citrate-phosphate buffer, at pH 5.0, for 30 min in the dark. Reactions were stopped by adding 25 µL 2 N H_2SO_4, and the optical density (OD) values were read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nm.

2.6. Evaluation of the IgG total, IgG1 and IgG2 production

Sera samples collected from ML and VL patients (n = 8 in both cases), which donated blood to obtain PBMC cultures, were used to evaluate the protein and parasite-specific antibody production (Lima et al., 2018). For this, ELISA microplates (JetBiofil®, Belo Horizonte) were coated with the rLiHyL, *L. braziliensis* or *L. infantum* SLA (1.0, 2.0, and 1.0 µg per well, respectively), diluted in coating buffer pH 9.6 and incubated for 16 h at 4 °C. The technical protocol was similar to this described above (subitem 2.6). The anti-human IgG total, IgG1 and IgG2-conjugated antibodies were used 1:10,000 in all cases, diluted also in PBS-T, for 1 h at 37 °C.

2.7. PBMC culture and cytokine production

Blood samples collected from untreated or treated ML and VL patients (n = 8 in both cases), as well as from healthy subjects living in region endemic of disease (n = 8), were used to obtain PBMCs, according described (Dias et al., 2018). Briefly, cells were purified by density centrifugation through Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), and cultured in RPMI 1640 medium, together with 20% FBS, 2 mM L-glutamine, 200 U/mL penicillin, 100 µg/mL streptomycin, 50 µM 2-mercaptoethanol, 1 mM sodium pyruvate, and 1× non-essential amino acid. PBMCs (10^6 cells) were plated in 48-well flat-bottomed tissue culture plates (Costar, Cambridge, MA, USA), and incubated alone (medium) or stimulated with rLiHyL or SLA (10 and 25 µg/mL, respectively), for 5 days at 37 °C in 5% CO_2. Cellular supernatants were collected and IFN-γ, IL-4 (ML patients and healthy subjects) and IL-10 (VL patients and healthy subjects) levels were measured in the supernatants by capture ELISA (Human IFN-γ, IL-4, and IL-10 ELISA Sets, BD Biosciences, USA), according to manufacturer's instructions. Results were interpolated from a standard curve using recombinant cytokines (expressed in pg/mL).

2.8. Statistical analysis

Results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed by GraphPad Prism™ (version 6.0 for Windows). The Receiver Operating Characteristic (ROC) curves were constructed to obtain the sensitivity (Se), specificity (Sp), area under curve (AUC), and Youden index. The unpaired Student *t*-test was used and significant differences were considered with $P < 0.05$.

3. Results

3.1. Serological evaluation of the antigens to detect human leishmaniasis

Initially, rLiHyL was tested for the serodiagnosis of leishmaniasis. ELISA experiments were conducted using this recombinant protein, as well as rA2 and *Leishmania* antigenic preparations (SLA), which were used as antigen controls. When serological assays were performed for the diagnosis of VL (Fig. 1), results showed that rLiHyL-specific IgG antibodies were present in higher levels in all VL patients' sera, but not in those from healthy subjects living in endemic region of disease (Fig. 1A). The rA2 protein showed worst results to discriminate between positive and negative samples (Fig. 1B), as well as when *L. infantum* SLA was used as an antigen, where DO values between VL patients and controls were near (Fig. 1C). ROC curves were constructed with the results obtained in the ELISA assays, in order to obtain the sensitivity, specificity, and AUC values (Fig. 1D).

When serological assays were performed to diagnose TL (Fig. 2), results showed also that rLiHyL-specific IgG antibody levels were higher in TL patients, but not in those from healthy subjects living in endemic region of disease (Fig. 2A). On the other hand, when rA2 and *L. braziliensis* SLA were used as antigens, worst results to discriminate between positive and negative samples were obtained (Fig. 2B and C,

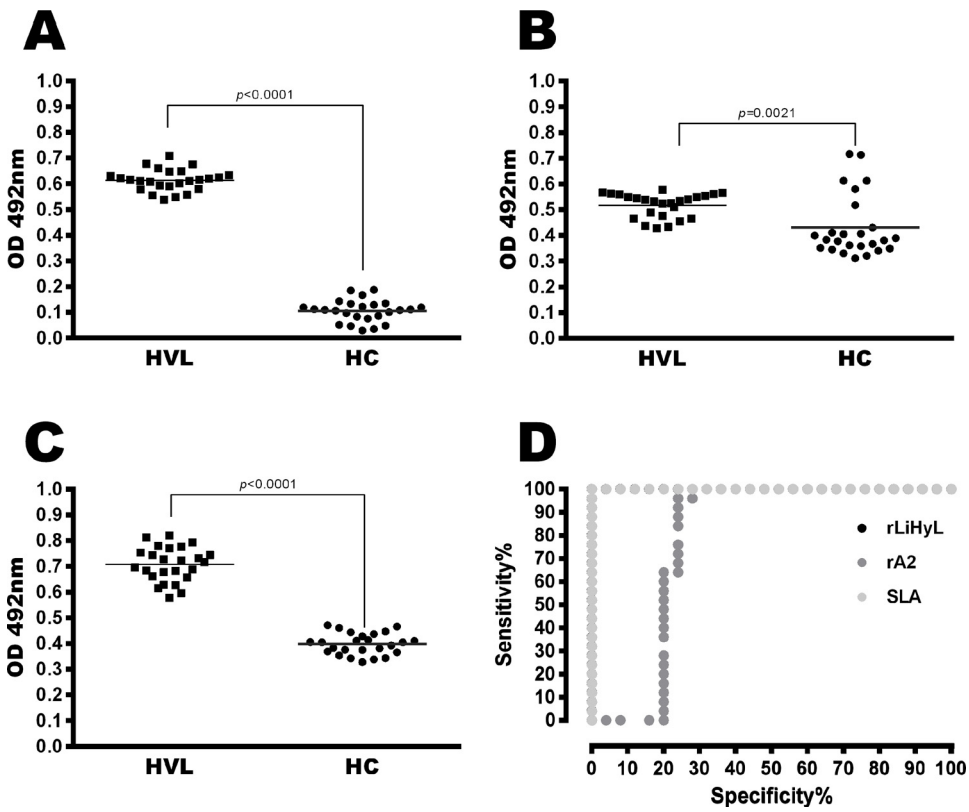

Fig. 1. ELISA assays using the recombinant antigens against sera from visceral leishmaniasis patients and controls. ELISA experiments were performed with sera samples from visceral leishmaniasis (HVL, n = 25) patients, as well as from healthy individuals living in endemic region of disease (HC, n = 25). The individual optical density (OD) values for each serum sample against the antigens: rLiHyL (A), rA2 (B), and L. *infantum* SLA (C) are shown. The mean of each group is indicated, as well as significant differences between HVL and HC groups. Receiver Operating Characteristic (ROC) curves were constructed with the results obtained in the ELISA assays, in order to obtain the sensitivity, specificity, and AUC values (D).

respectively). ROC curves were also constructed, in order to obtain the sensitivity, specificity, and AUC values (Fig. 2D). The cross-reactivity of the diagnostic antigens was also evaluated and results are shown. The cut-off values were calculated (Fig. 2E), being possible to observe that anti-rLiHyL IgG antibody levels were lower regarding the cut-off values for both VL and ML, while when rA2 and SLA were used; cross-reactive samples showed higher reaction with these antigens in the serological assays.

Results obtained for each antigen in terms of sensitivity, specificity, AUC, and Youden index were calculated, and are shown (Table 1). The rLiHyL protein presented sensitivity and specificity values of 100% for both VL and ML, besides of an AUC value of 1.0. When cross-reactive sera were evaluated, rLiHyL did not shown cross-reactivity with sera from Chagas Disease, leprosy, aspergillosis or paracoccidioidomycosis patients; while rA2 and SLA presented lower specificity. The percentage of false-positive results was calculated, and results are shown (Table 2). While rLiHyL did not present any false-positive result, rA2 and SLA antigens showed false-positive results for several tested cross-reactive sera.

3.2. Cytokine production obtained before and after treatment

The cytokine profile was evaluated in the cell supernatant from stimulated PBMC cultures (Fig. 3). Results showed that rLiHyL-stimulated cells, which were collected from treated VL patients and controls (healthy subjects), produced higher IFN-γ and lower IL-10 levels, in comparison to the values found using rA2 or SLA were used as stimuli (Fig. 3A). When PBMCs from ML patients were *in vitro* stimulated, higher IFN-γ and lower IL-4 levels were found in the rLiHyL-stimulated cell supernatant, when both treated patients and controls were evaluated (Fig. 3B).

3.3. Antibody response obtained before and after treatment of mucosal and visceral leishmaniasis

The humoral response based on the IgG total, IgG1 and IgG2 antibodies production was evaluated in VL and ML patients, when sera samples were collected before and six months after treatment. Results showed a significant decrease in the anti-rLiHyL IgG total levels, when the evaluation was performed after treatment. Both VL and ML patients showed also higher IgG2 and lower IgG1 isotype levels after treatment (Fig. 4). On the other hand, when rA2 and SLA were used as antigens, similar IgG total levels were obtained before and after treatment, as well as when the specific IgG1 and IgG2 production was evaluated. More importantly, higher ratios between rLiHyL-specific IgG2 and IgG1 levels were found in treated patients, in comparison to the results obtained using rA2 or SLA as antigens.

4. Discussion

Leishmania proteins have been tested to stimulate PBMCs from leishmaniasis patients, aiming to evaluate the specific immune response generated in the cellular supernatant, which could suggest new immunogens to be evaluated in vaccine studies (Ramos et al., 2017; Dias et al., 2018; Carvalho et al., 2019). Also, the humoral response developed in these patients could indicate the specific immune profile for these candidates (Portela et al., 2018). In this light, in the present work, we have tested a conserved *Leishmania* hypothetical protein, which was identified in an immunoproteomic study as expressed in *L. infantum* protein extracts by antibodies from VL patients' sera (unpublished results), as antigenic and immunogenic on leishmaniasis. The recombinant protein was evaluated as antigenic in ELISA experiments to diagnose TL and VL, due to the high conservation degree between distinct *Leishmania* spp., and as immunogenic to evaluate the cytokine production in PBMCs collected from treated and untreated ML and VL patients, as well as from healthy subjects living in endemic area of disease, which were stimulated with it. Results showed that our

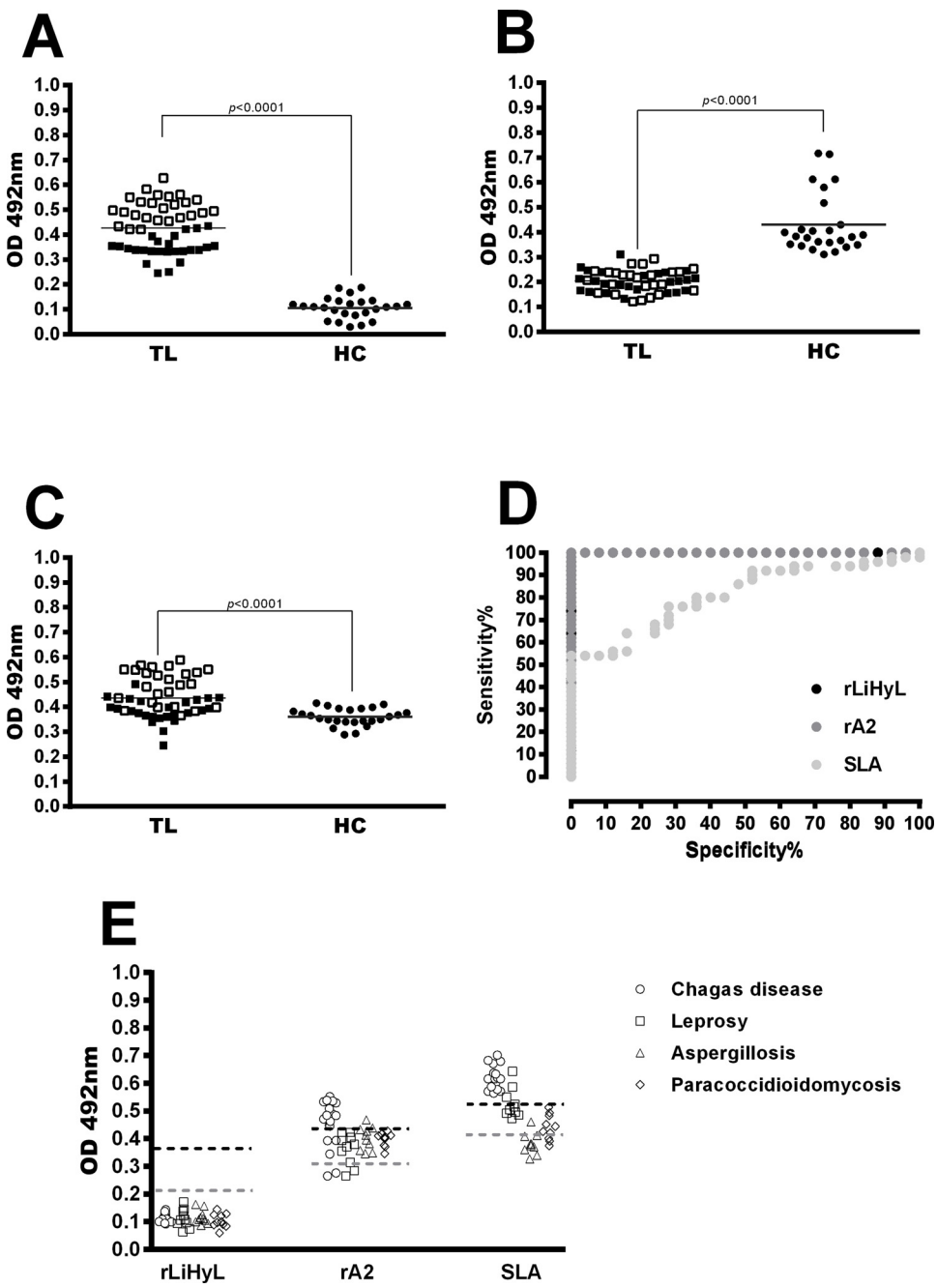

Fig. 2. Evaluation of the recombinant antigens against sera from tegumentary leishmaniasis patients and controls. Serological assays were performed using sera from tegumentary leishmaniasis (TL) patients, which were composed by samples from cutaneous (black square, n = 25) and mucosal (white square, n = 25) leishmaniasis patients, as well as from healthy individuals living in endemic region of disease (HC, black circle, n = 25). The individual optical density (OD) values for each serum sample against the antigens: rLiHyL (A), rA2 (B), and L. *braziliensis* SLA (C) are shown. The mean of each group is indicated, as well as significant differences between TL and HC groups. Receiver Operating Characteristic (ROC) curves were constructed with the results obtained in the ELISA assays, in order to obtain the sensitivity, specificity, and AUC values (D). The cross-reactivity was also evaluated, by using sera samples from Chagas Disease (n = 20), paracoccidioidomycosis (n = 10), leprosy (n = 10), and aspergillosis (n = 10) patients, which were reacted against rLiHyL, rA2, and L. *infantum* SLA. Cut-off values were calculated by ROC curves to determine the unspecific reactions against visceral (black dotted line) and tegumentary (grey dotted line) leishmaniasis patients' sera (E).

recombinant antigen was highly effective to differentiate leishmaniasis patients from healthy subjects and patients presenting cross-reactive diseases, as well as it induced a Th1-type immune response in PBMC from treated leishmaniasis patients, thus allowing infer about an immunogenic role developed by this protein on human leishmaniasis.

Distinct works evaluating the cellular response generated by candidates to protect against leishmaniasis have showed that higher IFN-γ and lower IL-4 and IL-10 levels are necessary to protect against infection by parasites in mammalian hosts, such as mice (Hojatizade et al., 2018) and dogs (Abbehusen et al., 2018). IL-4 is a cytokine related with

Table 1
Diagnostic performance of the antigens for the diagnosis of human leishmaniasis. The human serological panel was used in ELISA experiments against rLiHyL, rA2 and L. *infantum* or L. *braziliensis* SLA. Results obtained were used to calculate sensitivity (Se; 95% CI), specificity (Sp; 95% CI), confidence interval (95%CI), area under curve (AUC), and Youden index (*J*).

	Healthy control *versus* Visceral leishmaniasis								Healthy control *versus* Tegumentary leishmaniasis							
Antigen	AUC	*p*-value	Cut-off	Se	95%CI	Sp	95%CI	*J*	AUC	*p*-value	Cut-off	Se	95%CI	Sp	95%CI	*J*
rLiHyL	1.00	< 0.0001	> 0.363	100	86.28-100	100	86.28-100	1.00	1.00	< 0.0001	> 0.216	100	92.89-100	100	86.28-100	1.00
rA2	0.78	0.0006	> 0.431	96.00	79.65-99.90	76.00	54.87-90,64	0.72	1.00	< 0.0001	< 0.311	100	92.89-100	100	86.28-100	1.00
SLA	1.00	< 0.0001	> 0.525	100	86.28-100	100	86.28-100	1.00	0.81	< 0.0001	> 0.416	54.00	39.32-68.19	100	86.28-100	0.54

Table 2
Diagnostic performance of the antigens regarding to the cross-reactive samples. Serological assays were performed using rLiHyL, rA2 and L. *infantum* or L. *braziliensis* SLA against sera samples derived from Chagas Disease, paracoccidioidomycosis, leprosy, and aspergillosis patients. ROC curves were constructed and the percentage of false-positive results was determined for each antigen tested.

Disease	Antigens					
	rLiHyL		rA2		SLA	
	VL	TL	VL	TL	VL	TL
Chagas Disease	0	0	75.0	90.0	100	20.0
Leprosy	0	0	0	80.0	0	10.0
Aspergillosis	0	0	20.0	100	0	20.0
Paracoccidioidomycosis	0	0	0	100	0	70.0

the susceptibility to infection, mainly in parasite species able to cause TL (Jafari et al., 2018), while IL-10 is a molecule more related with the development of active VL (Pirdel et al., 2014). Here, we showed that rLiHyL induced significantly higher IFN-γ levels in the PBMC cultures, when cells from both treated ML and VL patients and healthy subjects were stimulated. In addition, lower rLiHyL-specific IL-4 and IL-10 levels were found in the cell supernatant of these groups. In addition, the evaluation of humoral response showed that higher anti-rLiHyL IgG2 levels were found in treated ML and VL patients, when compared to the IgG1 levels. As a consequence, we can infer about an immunogenic role developed by our protein, since it stimulated the development of a Th1-type cellular response, which was similar to this observed when other immunogenic proteins were tested against *Leishmania* (Lima et al., 2017; Dias et al., 2018; Joshi et al., 2019); thus demonstrating the feasibility to evaluate this antigen as protective candidate against infection by parasite in mammalian hosts. Also, the polarized Th1 response specific to rLiHyL protein found in immune cells from healthy subjects living in an endemic area of disease can be considered relevant, since these individuals could be considered resistant against parasites, suggesting a previous exposure of this population to natural infection by *Leishmania* (Tripathi et al., 2006). Otherwise, our molecule could be also considered as a potential immunogen, since it induced higher IFN-γ production and higher IgG2 levels in this population, then corroborating with the immune profile of resistance against disease (Botana et al., 2018; Lima et al., 2018; Portela et al., 2018).

Leishmaniasis presents high rates of morbidity and mortality in the patients, if not promptly diagnosed and treated. In this context, rapid, sensitive and specific diagnostic tests capable of detect precisely the disease should be worked (Dea-Ayuela et al., 2018). Distinct recombinant antigens have been tested for the serodiagnosis of TL and VL, although problems related with the variable sensitivity and/or specificity of the tests have been reported (Borja et al., 2018; Salles et al., 2019; Santos et al., 2019). Between these antigens, kinesin-derived molecules have showed progress in the serological assays for canine and human VL, although they have failed to detect cases where the presence of low antileishmanial antibody levels are found, as well as when sera samples from treated patients are evaluated (Abass et al., 2013; Sarkari et al., 2018). In this respect, the follow-up of antileishmanial serology after treatment should be also considered as a clinical cure criteria, and this immunological parameter should be adequately worked with the proposed diagnostic antigens (Lima et al., 2017; Portela et al., 2018). In our study, rLiHyL presented a significant decrease in the specific antibody levels after treatment of both ML and VL patients, with similar DO values to those obtained in non-infected subjects; thus demonstrating the feasibility to evaluate this recombinant antigen as a serological marker related with the treatment and clinical cure of the patients.

A variety of serological tests has been tested for diagnosis of leishmaniasis, including ELISA, direct agglutination test, indirect fluorescence antibody test, and immunochromatographic tests (Sarkari et al., 2018). The detection of parasite-specific antibodies is a promising alternative, by the fact that these techniques are simple and rapid; in contrast to parasitological diagnosis that require invasive sampling, trained professionals and sophisticated equipment (Osorio et al., 2018; Sundar and Singh, 2018). Here, when rLiHyL was used in ELISA

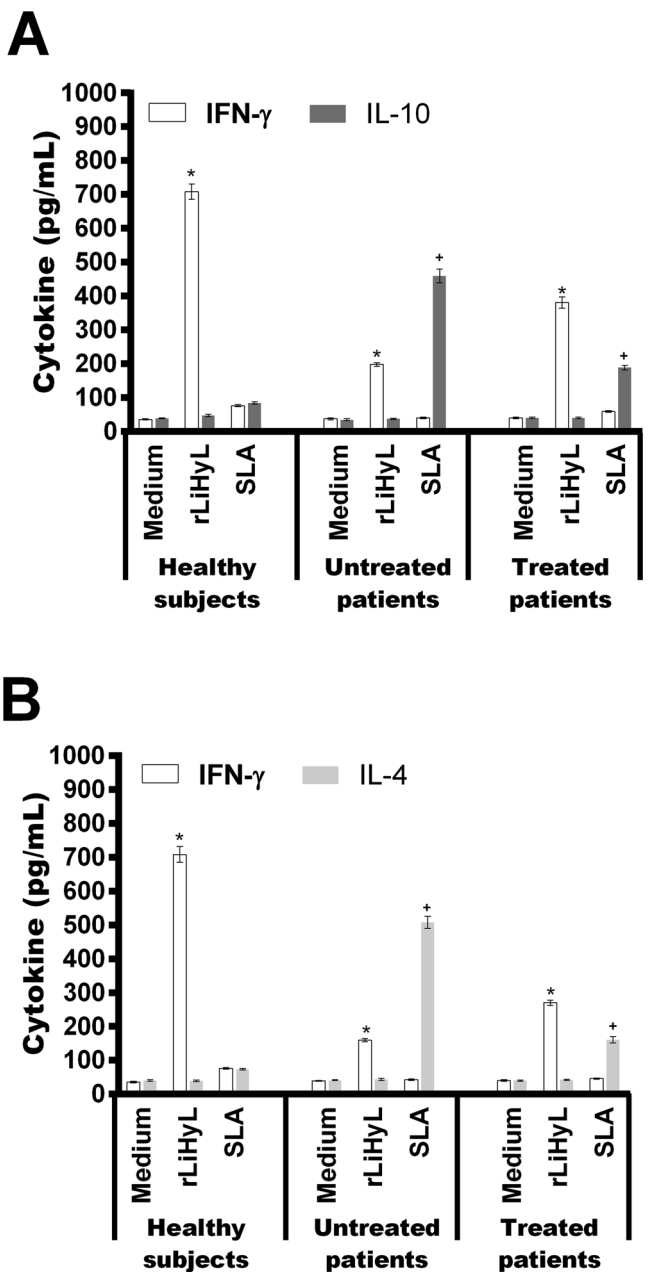

Fig. 3. Antigen-specific cellular response obtained before and after treatment. PBMCs were purified from blood samples collected from visceral or mucosal leishmaniasis patients (n = 8 in both cases), which were obtained before and six months after treatment, as well as from healthy subjects living in endemic region of disease (n = 8). Cells (10^6) were unstimulated (medium) or stimulated with rLiHyL or SLA (10 and 50 μg/mL, respectively) for 5 days at 37 °C in 5% CO_2. Then, IFN-γ, IL-4 and IL-10 levels were measured in the culture supernatant by capture ELISA. White and grey bars indicate the mean ± standard deviation of the groups, before and after treatment, respectively, for visceral (A) and mucosal (B) leishmaniasis patients. (*) indicates statistically significant difference in relation to the unstimulated (medium) and SLA-stimulated cultures $P < 0.0001$). (+) indicates statistically significant difference in relation to the unstimulated (medium) and rLiHyL-stimulated cultures $P < 0.0001$).

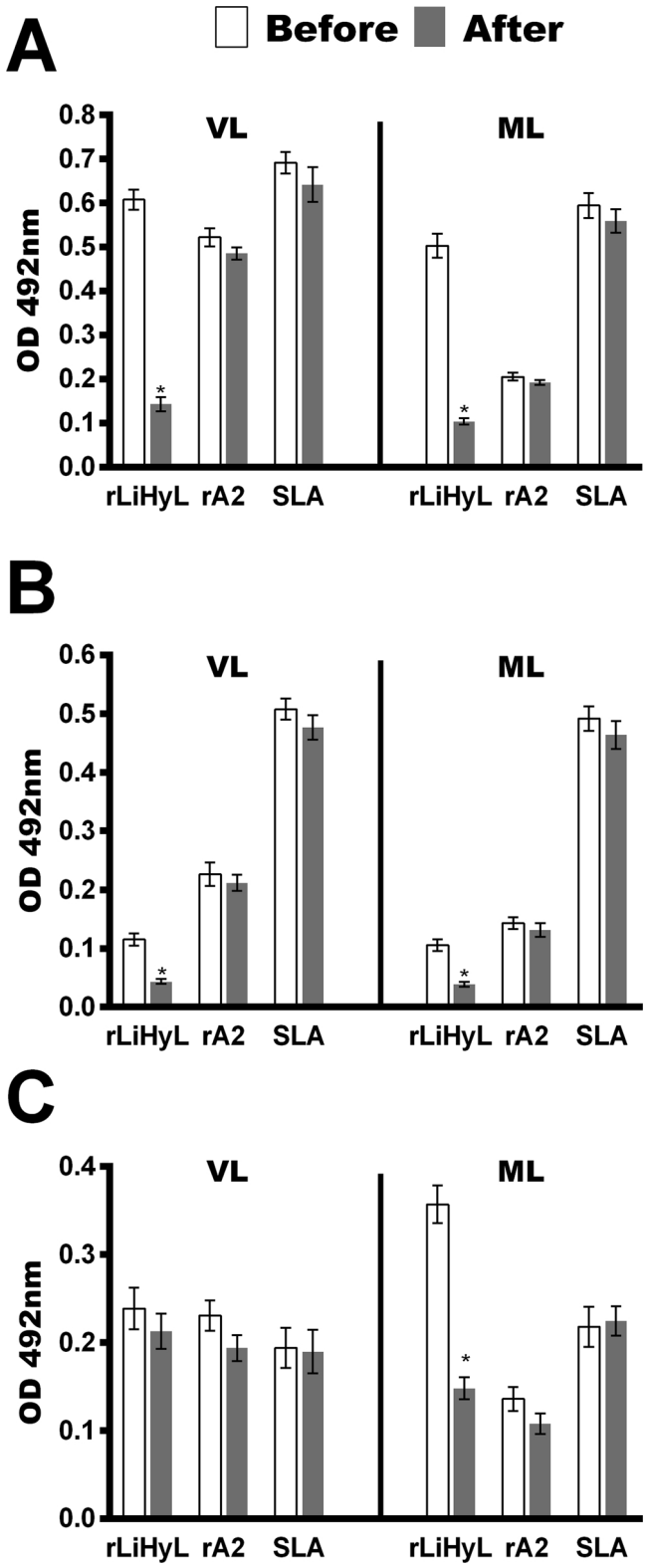

Fig. 4. IgG total, IgG1 and IgG2 levels specific to the antigens obtained before and after treatment. ELISA assays were performed using sera samples from visceral (n = 8) or mucosal (n = 8) leishmaniasis patients; which were collected before (white bars) or six months after (black bars) treatment, against the antigens: rLiHyL, rA2, and SLA. The individual optical density (OD) values for IgG total (A), IgG1 (B), and IgG2 (C) levels are shown. Bars indicate the mean ± standard deviation of the groups. (*) indicates statistically significant difference in relation to the results obtained before treatment ($P < 0.0001$).

experiments against a large serological panel composed by distinct cross-reactive samples, the protein was highly effective in react with antibodies from sera from both leishmaniasis patient´s classes, presenting high sensitivity and specificity to identify positive, but not negative or cross-reactive sera; thus demonstrating the possibility to use this antigen in future studies for a precise and safe serodiagnosis of disease.

As limitation of this study, the immune response was evaluated once after treatment, and other endpoints should be considered to validate the use of our recombinant antigen as biomarker of progression and cure of TL and VL. Also, rLiHyL could be incorporated in other diagnostic systems, such as those using immunochromatographic strips. However, this study demonstrated that rLiHyL is able to induce a Th1-type response in PBMCs from treated ML and VL patients and from healthy subjects living in endemic area of leishmaniasis, highlighting its application as a vaccine candidate against disease. In addition, rLiHyL showed also satisfactory diagnostic performance, thus suggesting an antigenic role and possible application for the serodiagnosis of disease.

Conflict of interest

The authors declare no commercial or financial conflict of interest.

Acknowledgments

The authors would like thank to CAPES, CNPq, and FAPEMIG for the scholarships. This work was supported by grants from CNPq (APQ-408408/2016-2 and APQ-408675/2018-7).

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