Investigation of the antigenicity and protective efficacy of Leishmania promastigote membrane antigens in search of potential diagnostic and vaccine candidates against visceral leishmaniasis

Sarfaraz Ahmad Ejazi
CSIR-Indian Institute of Chemical Biology
Smriti Ghosh
CSIR-Indian Institute of Chemical Biology
Anirban Bhattacharyya
CSIR-Indian Institute of Chemical Biology
Mohd Kamran
CSIR-Indian Institute of Chemical Biology
Sonali Das
CSIR-Indian Institute of Chemical Biology
Sudipta Bhowmick
CSIR-Indian Institute of Chemical Biology
Mehebubar Rahaman
School of Tropical Medicine
Rama Prosad Goswami
School of Tropical Medicine, Kolkata
Nahid Ali (nali@iicb.res.in)
Indian Institute of Chemical Biology CSIR

Research

Keywords: Biochemistry, Parasitology, Leishmaniasis, Immunology, Diagnosis, Vaccination, Th1/Th2, Cytokines,

Posted Date: March 2nd, 2020

DOI: https://doi.org/10.21203/rs.2.19783/v2

Version of Record: A version of this preprint was published at Parasites & Vectors on May 30th, 2020. See the published version at https://doi.org/10.1186/s13071-020-04138-7.

Abstract

Background Visceral leishmaniasis (VL), a parasitic disease causes serious medical consequences if treatment is delayed. Despite a decline in the number of VL cases in the Indian Subcontinent, commencement of the disease in newer areas continues to be a major concern. Although serological diagnosis mainly by immunochromatographic tests has been found to be effective, test for cure in different phases of treatment is still desired. Even though good prophylactic response has been obtained in murine models by a number of vaccine candidates, few have been proposed for human use. Methods In this study, nine antigenic components (31, 34, 36, 45, 51, 63, 72, 91 and 97 kDa) of Leishmania promastigote membrane antigens, LAg, were electroeluted and evaluated through ELISA to diagnose and distinguish active VL from one month cured and six month past infection. Further, to investigate the immunogenicity of electroeluted proteins, humans PBMCs of cured VL patients were stimulated with 31, 34, 51, 63, 72, and 91 kDa proteins. Results We found that 34 and 51 kDa fractions show 100% sensitivity and specificity with healthy controls and other diseases. After six months post treatment antibodies to 72 and 91 kDa antigens show a significant decline to almost normal levels. This suggests that 34 and 51 kDa are efficient in diagnosis whereas 72 and 91 kDa may be used to monitor treatment outcome. In another study, 51 and 63 kDa proteins demonstrated maximum ability for up-regulate IFN-g and IL-12 with minimum induction of IL-10 and TGF-β. The results indicating that 51 and 63 kDa proteins could be strong candidates for human immunization against VL. In contrast, 34 and 91 kDa demonstrated a reverse profile and may not be a good vaccine candidate. Conclusions The preliminary data obtained in this study proposes the potential of some of the antigens in Leishmania diagnosis and for test of cure. Additionally, some antigens demonstrated good immunoprophylactic cytokine production through T cell mediated immune response suggesting future vaccine candidates for VL. However, further studies are necessary to explore these antigens in diagnosis and to access long-term immune response.

Introduction

Despite reduction in the number of visceral leishmaniasis (VL) cases in previous endemic areas, many regions still show presence of disease burden and also spread in newer areas has been reported. Early diagnosis with complete treatment of cases together with the development of vaccine should be considered as an important solution [1, 2, 3]. Clinical diagnosis of VL depends on the demonstration of parasites in spleen or bone marrow aspirate, a complicated procedure to perform, but still considered as the gold standard in routine diagnosis [4]. High levels of anti-leishmanial antibodies are developed during the acute disease which is maintained up to several years and can be detected by various serological tests such as ELISA, IFAT and direct agglutination test (DAT). Development of rk39 antigen-based strip test has brought a major advancement in VL diagnosis for practice in field settings. Nevertheless, the poor sensitivity of the rK39 in East Africa and suboptimal in Brazil necessitate the scope of research for better diagnostic tools [5]. Failure to differentiate between active and past infection is another limitation with rk39 test. Since a single antigen is not effective for all endemic areas newer antigens have been searched and evaluated for the serodiagnosis of VL. Recombinant leishmanial antigens such as rKLO8,

rKE16 and rK28 are few of them [6, 7, 8, 9]. We in our previous studies have reported the diagnostic value of antigen, LAg, through various immunological techniques like ELISA, immunoblot, and dipstick test [5, 10, 11]. Moreover, anti-leishmanial antibodies in the sera of active and cured VL patients against several proteins of LAg in immunoblot have shown variable reactivity [12].

L. donovani infections in humans do not always result in disease manifestations. In VL endemic areas self-resolving infection has also been observed by developing parasite-specific antibodies and/or T cell response [13, 14]. Furthermore, patients who have recovered from kala-azar are usually immune to reinfection, which suggests that vaccination against leishmaniasis should be feasible [15, 16]. Studies from animal models have shown that protection against *Leishmania* can be achieved using parasite-specific proteins, DNA or genetically attenuated parasites [17, 18]. Advances in our understanding of *Leishmania*–host interactions, *Leishmania* pathogenesis, protective immunity and the availability of the complete *L. donovani* genome sequence, could take this a step further. Reports from earlier studies in our laboratory have demonstrated the immunogenicity of *L. donovani* promastigote membrane antigens, LAg, either free or in liposomal preparation [19, 20]. Besides inducing very good protection in murine model, it could induce remarkable lymphoproliferation and protective cytokines (IFN-g and IL-12) production in successfully treated kala-azar patients [21]. Similar results were also observed with soluble leishmanial antigens, SLA, partially purified from leishmanial membrane antigens, which when entrapped in cationic liposomes conferred almost complete protection as a prophylactic or therapeutic vaccine against *L. donovani* in BALB/c mice [22]. These indicate that, some of these peptides are more immunogenic than the others in experimental mice models and could be interesting to investigate their immunogenicity against human.

Screening of the most immunodominant antigens of formerly tested purified antigens in response to the human immune system is an important task. On the other hand, differentiation between active and past infection is one of the major challenges for serodiagnosis of VL. Moreover, antigens used in recent years are mostly recombinants which evade post translational modification unlike purified antigens [23]. Therefore, in this paper, we have separated leishmanial antigens, LAg and SLA, and evaluated different eluted fractions, 31, 34, 36, 45, 51, 63, 72, 91, and 97 kDa proteins of LAg in their native state to differentiate active VL from healthy controls and cured individuals through ELISA. We have also characterized 31, 34, 51, 63, 72 and 91 kDa polypeptides of LAg for immune stimulatory efficacy of PBMCs of cured VL patients through cytokine analysis as potential vaccine candidates.

Methods

Sample collection

Serum samples used in this study were collected from School of Tropical Medicine (STM), Kolkata. Twenty three VL patients were enrolled for the longitudinal study having single dose liposomal amphotericin B therapy (10mg/kg). Blood samples were collected before the treatment started, active VL cases (AVL), one month after the treatment cured VL cases (CVL), and about six months post treatment

follow-ups (FU). Sera also collected from 23 other symptomatically similar diseases comprised of four samples each from malaria, tuberculosis, pneumonia, typhoid and viral fever and one sample from liver abscess, systemic lupus erythematosus and pancreatitis. Sample collection continued with 23 healthy people too as controls from Indian Institute of Chemical Biology (IICB), Kolkata.

Parasite culture and purification of leishmanial antigens

L. donovani strain AG83 (ATCC© PRA-413™) of the parasite was regularly maintained in hamsters. Amastigotes were isolated from sacrificed hamsters and allowed to transform into promastigotes in culture medium (M199) with necessary supplements at 22^0C. Promastigotes were subcultured through fresh medium passages and 3rd to 5th passage cultures were harvested and centrifuged to get cell pellets. Cell pellets were then washed in PBS and stored at -20^0C until use.

Leishmania promastigote antigen, LAg, was purified from the cell pellet. In a typical experiment cells were suspended in 5mM Tris-HCl having pH 7.4 and vortexed for 12 minutes (2 min for 6 times) to get the parasite membrane leaky. Parasites were then centrifuged to collect the ghost membrane pellet (2310 g, 10 min, 4^0C) which was then resuspended in the same buffer and subjected to ultrasonication (30 sec for 6 times). The suspension was centrifuged again to obtain the antigens in the supernatant (5190 g, 30 min, 4^0C). The concentration of LAg was estimated by Lowry's methods and stored at -80^0C for further use. Soluble leishmanial antigen, SLA, was also purified from *Leishmania* promastigote culture similar to LAg with some modifications. Cell pellet was suspended in 1mM EDTA, 5 µg leupeptin, 1mM iodoacetamide and 1mM phenylmethylsulfonyl fluoride in 5mM Tris-HCl buffer, pH 7.4. Suspension was vortexed, centrifugation and sonicated as mentioned for LAg followed by solubilisation in (1% w/v) octyl-β-D-glucopyranoside at 4^0C for overnight. Next day solubilised suspension was centrifuged at 100,000g for 1h. The supernatant collected contains SLA which was dialyzed and finally stored at -80^0C after concentration estimation by Lowry's method.

SDS-PAGE and electroelution

Proteins were first denatured by the reducing agent, βME, and resolved in 10% SDS-PAGE. Different molecular weight polypeptides were separated from LAg (10 µg/lane) and SLA (5 µg/lane). The pattern of polypeptides of proteins LAg and SLA were visualized by Coomassie blue. Rf values for the molecular weights of the respective proteins were determined by the automated Image Lab software in comparison to the standards. For electroelution, 31, 34, 36, 45, 51, 63, 72, 91, and 97 kDa protein bands were excised from the LAg gel and subjected to electroelution as per the protocol (BioRad, Model-422). Subsequently, each protein was dialyzed against PBS. Electroeluted proteins after quantification were resolved on SDS-PAGE for reconfirmation of their molecular weights.

Indirect ELISA for antibody detection

Indirect ELISA to capture antibodies was performed on 96-well flat bottom plates (Nunc Maxisorp, Denmark). In brief, wells were coated with 1 µg/well concentration of purified and electroeluted proteins

with phosphate buffer (100 µl/well) and incubated overnight in 37^0C. Next day, antigen coated wells were blocked with 1% BSA in PBS (200 µl/well) for 2h at 37^0C. Subsequently, serum samples (1:2000) followed by peroxide conjugated antihuman IgG were applied to the wells in PBS buffer (100 µl/well) and incubated for 1h at 37^0C. Plates were washed at each step with PBS and Tween 20 to remove any non specific binding. Finally wells were incubated in the substrate, o-phenylenediamine dihydrochloride (OPD), and H_2O_2 in phosphate-citrate buffer (50 µl/well). Biological reaction was stopped with sulfuric acid and optical density values were acquired by using ELISA plate reader (RS232C, Thermo Scientific, USA) at 492 nm wavelength.

Stimulation of PBMCs and cytokines analysis

Peripheral blood mononuclear cells (PBMCs) from heparinized blood samples of one month cured VL patients and healthy individuals were isolated by density gradient using Histopaque-1077 (Sigma-Aldrich, USA) and finally resuspended in Medium RPMI 1640 with serum supplements and antibiotics. PBMCs obtained from each individual were cultured in triplicate (1X 10^6 cells/well) with and without antigen stimulation. Stimulation of cured VL and healthy PBMCs with LAg (12.5 µg/ml) and cured VL PBMCs with electroeluted antigens (1.5 µg/ml) 31, 34, 51, 63, 72, and 91 kDa were performed at 37^0C in CO_2 incubator. Supernatant from the cultures were collected after 96 h and stored at -20^0C for cytokine analysis. Level of cytokines, IFN-g, IL-12, IL-10 and TGF-β along with IFN-g/ IL-10 and IFN-g/ TGF-β were measured through ELISA according to the manufacturers' instructions (BD OptEIA ELISA kit, BD Biosciences). Briefly, capture antibodies specific to the cytokines were coated in the wells in phosphate buffer overnight at 4^0C. Subsequently, wells were blocked and incubated with culture supernatants for 1h. Cytokine specific detection antibodies were used in the wells followed by TMB substrate. Optical density values were obtained in the ELISA reader at 450 nm.

Mass spectrometry

Proteins bands of molecular masses, 34 and 45 kDa were stained with Coomassie blue and excised from 10% SDS-PAGE of LAg. The proteins imbibed in gel bands were digested through in-gel tryptic digestion kit according to the protocol provided by the manufactures (ThermoFisher Scientific). Subsequently, co-crystallization of the digested peptides with matrix was done and subjected to MALDI-TOF/TOF for MS/MS spectra (Applied Biosystems). Data obtained from mass spectrometry were identified through MASCOT search engine.

Statistics

Statistical studies were conducted with Graph Pad Prism software version V. Two-tailed student *t* test were performed for paired and unpaired samples in indirect ELISA. P values less than 0.05 was considered significant with 95% confidence intervals. Cut off values were determined by the Receiver-Operator curve (ROC) curve where 100% sensitivity was obtained. Difference in the cytokine production was determined by the two-tailed unpaired student's *t* test.

Results

Immunoreactivity of LAg and SLA with Leishmania infected human serum samples.

Leishmania promastigote membrane antigen (LAg) and soluble leishmanial antigen (SLA) were isolated from *L. donovani* promastigote culture and subjected to 10% polyacrylamide gel electrophoresis. LAg and SLA were resolved in their components of different molecular masses through SDS PAGE and share many immunogenic proteins. LAg contains approximately 25-30 polypeptides whereas SLA is a mixture of about ten dominant polypeptides (Fig. 1).

One of the most challenging objectives for *Leishmania*-specific antibody-based serodiagnosis is to differentiate an active VL patient from the past infection. In this regard reactivity of LAg and SLA with paired serum samples of 13 VL patients before treatment, one month cured and six months follow-up cases were evaluated through ELISA. We observed that there was comparatively higher antibody titer in the active VL sera against LAg in comparison to other study groups. However, cured VL patients just after the treatment show a significant decrease in the antibody titer but 84.16% positivity in ELISA (Fig. 2A). Follow-up patient sera after six months of treatment showed a remarkable decrease in the antibody titer with no positive reactivity against LAg and the mean was below the cut off line. No cross reaction of LAg was found with 13 other diseases and 13 healthy controls tested. Similar study was also performed with SLA. Both active and one month cured VL patients showed 100% positivity with SLA (Fig. 2B). However, 7.69% of the follow-up patients were still positive with SLA. Healthy controls and other diseases, however, did not show cross reactivity with SLA. These results reveal that LAg and SLA have strong serodiagnostic potential for VL. Both LAg and SLA could not satisfactorily differentiate active VL from past infections following one month post therapy. However, after six months of infection, antibody titers significantly decreased below the cut off and become 100% negative for LAg. Therefore in the next step, we isolated different components of LAg for immunoreactive studies of individual antigens.

Electroelution of LAg antigens.

To access the immunoreactive potential of different protein fractions of LAg nine polypeptides of LAg, 31, 34, 36, 45, 51, 63, 72, 91, and 97 kDa were eluted out electrophoretically from Coomassie stained gels and subjected to SDS-PAGE (Fig. 3). To differentiate active disease from cured VL and follow-up patients ELISA were carried out with all the nine electroeluted antigens.

Reactivity of electroeluted proteins in ELISA.

Nine electroeluted antigens were evaluated for their ability to distinguish active disease from other diseases and healthy controls as well as from the cured VL and follow-up samples. Cut off values were set for each eluted antigen where antibody titer above the cut off line was considered as positive and below as negative in ELISA. Cut offs for VL positivity were obtained from ROC curve where maximum sensitivity and specificity were achieved in comparison with healthy controls. Out of the nine antigens, 100% sensitivity with active VL sera was observed with all electroeluted antigens. The specificity of the

antigens was calculated based on the cross reactivity of the antigens with healthy control and other diseases. With healthy control samples tested, the mean optical density values for all the electroeluted antigens were below the cut off line. 31, 34, 36, 51, and 97 kDa proteins found to be 100% specific whereas antigens 63 and 91 kDa showed more than 90% specificity. The reactivity of eluted antigens with other diseases showed 100% specificity with 34 and 51 kDa antigens followed by 91 and 97 kDa with 95.75% specificity. Therefore, for the purpose of diagnosis antigens 34 and 51 kDa proteins we found to be best to distinguish active VL from healthy controls and other diseases. The percent sensitivity and specificity of all the electroeluted antigens are listed in Table 1.

The study to differentiate active VL from cured and past infections using electroeluted antigens demonstrated considerable differences among the patients' groups (Fig. 4). There was a statistically considerable decline in the serum IgG levels against all the antigens in the one month cured patient sera compared to their respective active disease sera. However, except 63, 72 and 91kDa none of the antigens showed mean optical density values below its cut off. Recognition of electroeluted antigens with sera after one month cure ranged from 8.69-100% which is still positive and not ideal for the differentiation of the diseased state from cure. Follow-up patients who recovered from VL six months post treatment showed a significant decline in the IgG levels against 31, 34, 51, 63, 72, and 91kDa antigens and their levels were comparable to the healthy control sera the mean lying below the cut off. Amongst all the antigens, 72 and 91 kDa showed the most promising results with 100% negative reactivity in follow-up patients.

Evaluation of electroeluted proteins for in vitro cytokine analysis with PBMCs of cured VL patients.

Immunity to leishmaniasis is known to depend on protective cellular responses against the parasites. In this regard LAg-induced cytokine production from the culture supernatants from PBMCs of cured VL individuals were evaluated who are known to present positive cell mediated immunity along with PBMCs from healthy individuals as control. In order to assess the immunogenicity of eluted proteins, 31, 34, 51, 63, 72 and 91 kDa were used to stimulate the PBMCs of cured VL patients for the production of protective cytokines IFN-g and IL-12, and anti-inflammatory cytokines, IL-10 and TGF-β, which promote disease progression. IFN-g is the most dominant Th1 cytokine required to control *Leishmania* infection. Analysis of cytokines revealed variable levels of IFN-g production when PBMCs were stimulated with electroeluted antigens whereas unstimulated cultures produced negligible IFN-g (Fig. 5A). Prominent levels of IFN-g were produced by the LAg stimulated PBMCs of VL treated and healthy individuals as compared to unstimulated PBMCs. Significantly high levels of IFN-g were produced by 51 and 63 kDa antigens followed by 31 and 72 kDa antigens when compared with unstimulated cultures. In comparison 34 and 91 kDa antigen produced low levels of IFN-g. IL-12 is another protective cytokine produced by the macrophages and in turn, activates the T cells to produce IFN-g and accelerates the leishmanicidal function. Healthy and treated PBMCs exhibited significant IL-12 induction against LAg (Fig. 5B). Similar to the LAg stimulated cultures, significantly elevated levels of IL-12 were produced by 51and 63 kDa antigens followed by 31 and 72 kDa. 34 and 91 kDa antigens produced comparatively lower levels of IL-12.

IL-10 and TGF-β are two immunosuppressive cytokines that promote parasite survival and help in disease progression in VL infection. Our study demonstrated that LAg stimulated PBMCs from VL treated and healthy subjects produced moderate levels of IL-10. Among electroeluted antigens, 34 and 91 kDa antigens showed maximum levels of IL-10. In contrast, comparatively low levels of IL-10 were produced by 31, 51, 63, and 72 kDa electroeluted antigens (Fig. 5C). Low levels of TGF-β were produced by the healthy PBMCs than the cured PBMCs against LAg whereas, 31, 51, 63 and 72 kDa antigens produced median levels of TGF-β by the treated PBMCs (Fig. 5D). Again, 34 and 91 kDa antigens produced highest levels of TGF-β similar to that produced by LAg stimulated cultures (Fig. 5D). Ratios of IFN-g and IL-10 as well as IFN-g and TGF-β were plotted in Figure 5E and 5F.

Identification of electroeluted proteins

Out of nine fractions of LAg evaluated in this study, except 34 and 45 kDa all proteins were identified in our earlier reports (Table 2). Therefore, we identified the remaining two proteins through MALDI-TOF mass spectrometry and matched with *Leishmania* sequence. The 34 kDa protein was identified as *Leishmania* analogue of the receptors of activated C kinase (LACK) whereas 45 kDa protein was β tubulin (Supplementary Fig. 1).

Discussion

Visceral leishmaniasis is one of the foremost fatal infectious diseases for which no effective vaccine is currently available. Moreover, there is scope for finding better and effective tests for its diagnosis to access treatment response. In this study we have isolated and evaluated several *Leishmania* promastigote membrane antigens which can be used as probable candidates for diagnosis as well as for vaccine development.

Estimating parasites microscopically from tissue aspirates is considered the gold standard for diagnosis but bears risk of internal bleeding, pain, and dependence on a trained person is the major hurdle. VL is also characterized by the production of *Leishmania* specific antibodies during disease. Hence in the last two decades, several serological methods have been evaluated largely using recombinant antigens with varying degrees of sensitivity and specificity. However, defined antigens do not go for post translational modifications such as glycosylation. Therefore, these antigens lack carbohydrate and lipid moieties as present in the native state of the antigen [23]. Thus antigens in their native state were electroeluted to validate their diagnostic properties. Additionally, most of the serological tests are for the diagnosis of active VL conditions. Therefore, to assess treatment efficacy remains an important challenge for VL management and elimination. In the present study, two purified leishmanial antigens, LAg and SLA, together with nine electroleluted proteins of LAg were evaluated for serodiagnosis of VL as well as to differentiate active VL from past infections. LAg has been earlier reported to demonstrate strong potential for the serodiagnosis of VL and PKDL by detecting antigen-specific antibodies in serum and urine samples [10, 24]. SLA has also been reported in vaccine-mediated protection in murine VL [22, 25]. However, there is no study to investigate the different components of these purified antigens separately

for diagnosis and investigating their reactivity during different treatment phases to access their immunogenicity, Herein we have observed that there is a significant decline in the *Leishmania*-specific antibody titers in the patients' immediate after completion of treatment for both LAg and SLA, However, they were still positive at the set cut offs. Sera collected after six months of treatment demonstrated a sharp decline in antibody titers against both LAg and SLA. Antibody levels for SLA remained above the cut off line in some patients after six months follow-up in contrast to LAg where at this time point antibodies decreased to the level below the cut off. Therefore, absolute differentiation between active VL sera from past infections is possible with LAg. In search of more defined immunoreactive antigens of LAg, nine antigens, 31, 34, 36, 45, 51, 63, 72, 91, and 97 were electroeluted to study their reactivity individually. We found that few electroeluted antigens introduced improvement over total antigen LAg and some of which hold strong potential for serodiagnosis of VL. Antigens 31, 34, 36, 51, and 97 kDa were found to be 100% sensitive to detect active VL cases with 100% specificity against healthy controls. Antigens, 34 and 51 kDa differentiate active VL and other diseases with 100% specificity. On the basis of the overall sensitivity and specificity of the antigens these two antigens could be prospective candidates for future serodiagnosis of VL.

In kala-azar therapy, initial cure is determined just after completion of treatment and a definite cure is determined six months post treatment. Therefore, it is more important to distinguish the active disease from past infections of VL after at least six months of therapy. Very few studies have been undertaken to distinguish active VL from past infections of VL using serum samples. Antibody levels in sera were found to fall in response to rK26 and rK18 antigens after six months of treatment [26]. Studies of Indian and Sudanese *L. donovani* patient sera with crude antigen showed a significant decrease in IgG1 antibody levels after six months of cure [27]. A recent study with rK39 antigen demonstrated a greater decline in IgG1 isotypes in follow-up patients than IgG [28]. Therefore to distinguish active disease from past infections of VL an antigen with high sensitivity for active VL may or may not be a good candidate for use in a test of cure. In our attempt, we were to some extent successful in differentiating active disease from past infections of VL using the electroeluted antigens. Interestingly, serum samples after six months of cure did not show reactivity with 72 and 91 kDa antigens. The other antigens which also hold strong potential to distinguish active VL from past infections of VL were 34 and 63 kDa and all these antigens had high reactivity with diseased VL sera and low reactivity with follow-up patient sera.

Most of the antigens electroeluted in this study have been identified through MALDI ms/ms in the earlier reports. The role of these parasitic antigens in disease progression has been ascertained in mice models from several previous studies. 31 kDa protein was identified as ATP synthase a chain of *Leishmania* [29]. ATP synthase complex subunits are functionally associated with the membrane and thus can be potential targets for drugs, diagnostic probes or vaccine components against *Leishmania*. Protein with molecular weight of 34 kDa was identified in this study through mass spectrometry and recognized as *Leishmania* analogue of the receptors of activated C kinase (LACK). LACK has been reported to induce strong parasite-specific immune response and protects experimental mice against *L. donovani* infection [30]. 36 kDa antigen was identified as elongation factor 1- α (EF1-α), a translation factor induces cellular proliferative response and imparting long term immunity [31]. 45 kDa antigen was identified in the current

study and appeared as beta-tubulin. 51 kDa was also identified as beta-tubulin, which exhibits high levels of protective cytokines and reduced infection in mice [29]. 63 kDa protein is a membrane-anchored glycoprotein that corresponds to the infective stage and virulence [32]. Another protein 72 kDa belongs to a set of highly evolutionary conserved heat shock proteins. They behave as chaperones, and induced Th1-type of cellular responses in cured patients and hamsters and hamsters [33, 34]. The 91 kDa polypeptide showed homology with b-tubulin but did not show immunogenicity and protective immunity as compared to the other tubulins in the experimental *L. donovani* vaccine study [29]. The 97 kDa protein was recently reported as nucleoporins 93 (NUP93) that presents in both promastigotes and amastigotes forms and studied as immunoprophylactic agent [35].

Identification of key antigenic targets of the protective human immune response against VL is central to the development of an efficacious vaccine against *Leishmania.* Cell-mediated immune responses are key determinants of the natural course of infection of *Leishmania*. Although human VL is known to elicit mixed Th1 and Th2 response, protective immunity is achieved when the cell-mediated Th1 response is predominant. Thus antigens that predominantly stimulate Th1 response are considered as potential protective antigens [36]. IL-12 driven IFN-g dominated Th1 response is associated with resistance to infection against *Leishmania* as it has a direct effect on the macrophage microbicidal response and other effector killing mechanisms [37]. Conversely, antigens that predominantly stimulate a Th2 response from these cells have been regarded as of lesser interest as vaccine candidates because they are likely to be associated with pathology [38]. IL-10 and TGF-b are the major immunosuppressive cytokines in VL and they can modulate macrophages and T-cell functions and promote multiplication of *Leishmania* parasites as evident in patients with VL [21].

Studies have evaluated purified and recombinant proteins as well as whole parasite lysate for potential immunostimulatory activity in experimental models. Hence, it is important to evaluate leishmanial antigens for cellular immune responses in humans. Our earlier report reveals that entrapment of LAg in the cationic liposomes conferred significant levels of protection against infection in hamsters and BALB/c mice [39]. A few immunodominant parasite antigens from LAg, 31, 51, 63, 72, and 91 kDa antigens elicited protective immune responses in experimental model [29]. Since PBMCs derived from VL treated patients were exposed to *Leishmania* infection they showed increase in protective cytokine response against LAg in the present study. However, PBMCs derived from healthy individuals also showed similar results suggesting the role of LAg antigens in prophylaxis. We characterized the immune response through cytokine analysis of six electroeluted antigens of LAg, 31, 34, 51, 63, 72 and 91 kDa that were recognized by sera of active and cured VL patients. Although the six antigens were able to stimulate PBMCs from cured VL individuals, the response was quite variable. 51 and 63 kDa antigens induced maximum elevation of protective Th1 cytokines IFN-g and IL-12, similar to that of LAg, followed by 31 and 72 kDa antigens that produced comparatively lower levels of IFN-g and IL-12. 34 and 91 kDa antigens, however, did not show significant production of IFN-g and IL-12. Lower levels of immunosuppressive cytokines IL-10 and TGF-b were secreted by 31, 51, 63 and 72 kDa antigens. In contrast, 34 and 91 kDa antigens produced significantly higher levels of the suppressive cytokines, comparable to LAg. Therefore, these two antigens could be the reason for high IL-10 and TGF-b

production from LAg. Moreover, the ratio of IFN-g/IL-10 and IFN-g/ TGF-b of treated patients also demonstrated the protective immune profile against LAg and 51 and 63 kDa among electroeluted antigens.

From this study, 51 and 63 kDa antigens may be considered to be the most potent vaccine candidates based on the cytokine analysis. 31 and 72 kDa were found to be less immunogenic and 34 and 91 kDa antigens did not show any potential to be considered as a vaccine candidate. Although the diagnostic and immunoprophylactic responses of antigens were clearly described in these preliminary experiments, the number of samples used were not many. This we consider as a limitation of our work and will try to overcome this in the next phase of the study.

Conclusions

We report here the evaluation of electroeluted *Leishmania* membrane proteins some of which showed high sensitivity for active VL cases, as well as ability to differentiate from past VL infection. Thus they can be used as probable diagnostic candidates as well as for test of cure. Moreover, some of the electroeluted antigens stimulate significant protective cytokines IFN-g and IL-12 and low levels of disease progressive cytokines IL-10 and TGF-β from human PBMCs collected after cure of VL. Therefore, they may be considered as potential subunit vaccine candidates against VL.

Abbreviations

VL: Visceral leishmaniasis

PKDL: Post kala-azar dermal leishmaniasis

HIV: Human Immunodeficiency Virus

LAg: *Leishmania* promastigote membrane antigens

SLA: Soluble leishmanial antigen

ELISA: Enzyme Linked Immuno Sorbent Assay

DAT: Direct agglutination test

IFAT: Immunofluorescence antibody test

MALDI-TOF: Matrix Assisted Laser Desorption/Ionization-Time Of Flight

SDS-PAGE: Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

EHC: Endemic Healthy Control

NEHC: Non Endemic Healthy Control

OD: Other Diseases

HC: Healthy Control

AVL: Active visceral leishmaniasis

CVL:Cured visceral leishmaniasis

FU: Follow-up patients

Declaration

Ethics approval and consent to participate

Approval for sample collection and research work was obtained from Institutional Ethical Committee, CSIR-Indian Institute of Chemical Biology (IICB) and School of Tropical Medicine (STM), Kolkata. The study was conducted according to the guidelines and suggestions set by the committees. Patients involved in this study were informed before sample collection and written consent was obtained.

Consent for publication

Not Applicable

Availability of data materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare that they have no competing interests.

Funding

This work has, in part, received funding from UK Research and Innovation via the Global Challenges Research Fund under grant agreement ‘A Global Network for Neglected Tropical Diseases’ grant number MR/P027989/1, Sir J. C. Bose Fellowship, India and Council of Scientific and Industrial Research, India.

Authors’ contributions

SAE, SG and NA conceived and designed this study. SAE and SG carried out the experiments and wrote the manuscript. AB, MK and SD provided experimental support, analysed results and reviewed the manuscript. SB conducted the MALDI experiment for 34 kDa protein. MR, and RPG contributed in clinical sample collection, conducted confirmatory tests and provided ethical clearance from the hospitals.

Acknowledgments

Not Applicable

References

1. Burza S, Croft SL, Boelaert M. Leishmaniasis. Lancet. 2018;392 10151:951-70; doi: S0140-6736(18)31204-2 [pii] 10.1016/S0140-6736(18)31204-2. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=30126638.
2. Lindoso JAL, Moreira CHV, Cunha MA, Queiroz IT. Visceral leishmaniasis and HIV coinfection: current perspectives. HIV AIDS (Auckl). 2018;10:193-201; doi: 10.2147/HIV.S143929 hiv-10-193 [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=30410407.
3. Singh OP, Gedda MR, Mudavath SL, Srivastava ON, Sundar S. Envisioning the innovations in nanomedicine to combat visceral leishmaniasis: for future theranostic application. Nanomedicine (Lond). 2019;14 14:1911-27; doi: 10.2217/nnm-2018-0448. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=31313971.
4. Ejazi SA, Ali N. Developments in diagnosis and treatment of visceral leishmaniasis during the last decade and future prospects. Expert Rev Anti Infect Ther. 2013;11 1:79-98; doi: 10.1586/eri.12.148. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23428104.
5. Ejazi SA, Bhattacharya P, Bakhteyar MA, Mumtaz AA, Pandey K, Das VN, et al. Noninvasive Diagnosis of Visceral Leishmaniasis: Development and Evaluation of Two Urine-Based Immunoassays for Detection of Leishmania donovani Infection in India. PLoS Negl Trop Dis. 2016;10 10:e0005035; doi: 10.1371/journal.pntd.0005035 PNTD-D-16-00788 [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=27741241.
6. Sivakumar R, Dey A, Sharma P, Singh S. Expression and characterization of a recombinant kinesin antigen from an old Indian strain (DD8) of Leishmania donovani and comparing it with a commercially available antigen from a newly isolated (KE16) strain of L. donovani. Infect Genet Evol. 2008;8 3:313-22; doi: S1567-1348(08)00018-X [pii] 10.1016/j.meegid.2008.02.004. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18374635.
7. Abass E, Bollig N, Reinhard K, Camara B, Mansour D, Visekruna A, et al. rKLO8, a novel Leishmania donovani - derived recombinant immunodominant protein for sensitive detection of visceral leishmaniasis in Sudan. PLoS Negl Trop Dis. 2013;7 7:e2322; doi: 10.1371/journal.pntd.0002322

PNTD-D-13-00113 [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23875052.

8. Pattabhi S, Whittle J, Mohamath R, El-Safi S, Moulton GG, Guderian JA, et al. Design, development and evaluation of rK28-based point-of-care tests for improving rapid diagnosis of visceral leishmaniasis. PLoS Negl Trop Dis. 2010;4 9; doi: 10.1371/journal.pntd.0000822. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20856856.
9. Bhargava P, Singh R. Developments in diagnosis and antileishmanial drugs. Interdiscip Perspect Infect Dis. 2012;2012:626838; doi: 10.1155/2012/626838. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23118748.
10. Saha S, Goswami R, Pramanik N, Guha SK, Saha B, Rahman M, et al. Easy test for visceral Leishmaniasis and post-Kala-azar Dermal Leishmaniasis. Emerg Infect Dis. 2011;17 7:1304-6; doi: 10.3201/eid1707.100801. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21762596.
11. Ejazi SA, Ghosh S, Saha S, Choudhury ST, Bhattacharyya A, Chatterjee M, et al. A multicentric evaluation of dipstick test for serodiagnosis of visceral leishmaniasis in India, Nepal, Sri Lanka, Brazil, Ethiopia and Spain. Sci Rep. 2019;9 1:9932; doi: 10.1038/s41598-019-46283-9 10.1038/s41598-019-46283-9 [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=31289323.
12. Ravindran R, Anam K, Bairagi BC, Saha B, Pramanik N, Guha SK, et al. Characterization of immunoglobulin G and its subclass response to Indian kala-azar infection before and after chemotherapy. Infect Immun. 2004;72 2:863-70; doi: 10.1128/iai.72.2.863-870.2004. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14742530.
13. Duthie MS, Raman VS, Piazza FM, Reed SG. The development and clinical evaluation of second-generation leishmaniasis vaccines. Vaccine. 2012;30 2:134-41; doi: S0264-410X(11)01759-2 [pii] 10.1016/j.vaccine.2011.11.005. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22085553.
14. van Griensven J, Diro E. Visceral Leishmaniasis: Recent Advances in Diagnostics and Treatment Regimens. Infect Dis Clin North Am. 2019;33 1:79-99; doi: S0891-5520(18)30092-8 [pii]10.1016/j.idc.2018.10.005. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=30712769.
15. De Brito RCF, Cardoso JMO, Reis LES, Vieira JF, Mathias FAS, Roatt BM, et al. Peptide Vaccines for Leishmaniasis. Front Immunol. 2018;9:1043; doi: 10.3389/fimmu.2018.01043. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=29868006.

16. Olliaro PL, Shamsuzzaman TA, Marasini B, Dhariwal AC, Be-Nazir A, Mondal D, et al. Investments in Research and Surveillance Are Needed to Go Beyond Elimination and Stop Transmission of Leishmania in the Indian Subcontinent. PLoS Negl Trop Dis. 2017;11 1:e0005190; doi: 10.1371/journal.pntd.0005190PNTD-D-16-01049 [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=28125596.
17. Moafi M, Rezvan H, Sherkat R, Taleban R. Leishmania Vaccines Entered in Clinical Trials: A Review of Literature. Int J Prev Med. 2019;10:95; doi: 10.4103/ijpvm.IJPVM_116_18 IJPVM-10-95 [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=31360342.
18. Bi K, Chen Y, Zhao S, Kuang Y, John Wu CH. Current Visceral Leishmaniasis Research: A Research Review to Inspire Future Study. Biomed Res Int. 2018;2018:9872095; doi: 10.1155/2018/9872095. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=30105272.
19. Mazumdar T, Anam K, Ali N. A mixed Th1/Th2 response elicited by a liposomal formulation of Leishmania vaccine instructs Th1 responses and resistance to Leishmania donovani in susceptible BALB/c mice. Vaccine. 2004;22 9-10:1162-71; doi: 10.1016/j.vaccine.2003.09.030 S0264410X03007059 [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15003644.
20. Saha S, Mazumdar T, Anam K, Ravindran R, Bairagi B, Saha B, et al. Leishmania promastigote membrane antigen-based enzyme-linked immunosorbent assay and immunoblotting for differential diagnosis of Indian post-kala-azar dermal leishmaniasis. J Clin Microbiol. 2005;43 3:1269-77; doi: 43/3/1269 [pii] 10.1128/JCM.43.3.1269-1277.2005. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15750095.
21. Saha S, Mondal S, Ravindran R, Bhowmick S, Modak D, Mallick S, et al. IL-10- and TGF-beta-mediated susceptibility in kala-azar and post-kala-azar dermal leishmaniasis: the significance of amphotericin B in the control of Leishmania donovani infection in India. J Immunol. 2007;179 8:5592-603; doi: 179/8/5592 [pii] 10.4049/jimmunol.179.8.5592. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17911647.
22. Bhowmick S, Ravindran R, Ali N. Leishmanial antigens in liposomes promote protective immunity and provide immunotherapy against visceral leishmaniasis via polarized Th1 response. Vaccine. 2007;25 35:6544-56; doi: S0264-410X(07)00616-0 [pii] 10.1016/j.vaccine.2007.05.042. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17655984.
23. Kuhne V, Rezaei Z, Pitzinger P, Buscher P. Systematic review on antigens for serodiagnosis of visceral leishmaniasis, with a focus on East Africa. PLoS Negl Trop Dis. 2019;13 8:e0007658; doi: 10.1371/journal.pntd.0007658 PNTD-D-19-00230 [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=31415564.

24. Ejazi SA, Bhattacharyya A, Choudhury ST, Ghosh S, Sabur A, Pandey K, et al. Immunoproteomic Identification and Characterization of Leishmania Membrane Proteins as Non-Invasive Diagnostic Candidates for Clinical Visceral Leishmaniasis. Sci Rep. 2018;8 1:12110; doi: 10.1038/s41598-018-30546-y 10.1038/s41598-018-30546-y [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=30108316.
25. Bhowmick S, Ravindran R, Ali N. gp63 in stable cationic liposomes confers sustained vaccine immunity to susceptible BALB/c mice infected with Leishmania donovani. Infect Immun. 2008;76 3:1003-15; doi: IAI.00611-07 [pii] 10.1128/IAI.00611-07. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18195029.
26. Vallur AC, Tutterrow YL, Mohamath R, Pattabhi S, Hailu A, Abdoun AO, et al. Development and comparative evaluation of two antigen detection tests for Visceral Leishmaniasis. BMC Infect Dis. 2015;15:384; doi: 10.1186/s12879-015-1125-3 10.1186/s12879-015-1125-3 [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=26395447.
27. Bhattacharyya T, Ayandeh A, Falconar AK, Sundar S, El-Safi S, Gripenberg MA, et al. IgG1 as a potential biomarker of post-chemotherapeutic relapse in visceral leishmaniasis, and adaptation to a rapid diagnostic test. PLoS Negl Trop Dis. 2014;8 10:e3273; doi: 10.1371/journal.pntd.0003273 PNTD-D-14-01084 [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=25340782.
28. Mollett G, Bremer Hinckel BC, Bhattacharyya T, Marlais T, Singh OP, Mertens P, et al. Detection of Immunoglobulin G1 Against rK39 Improves Monitoring of Treatment Outcomes in Visceral Leishmaniasis. Clin Infect Dis. 2019;69 7:1130-5; doi: 5239677 [pii] 10.1093/cid/ciy1062. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=30541022.
29. Bhowmick S, Ali N. Identification of novel Leishmania donovani antigens that help define correlates of vaccine-mediated protection in visceral leishmaniasis. PLoS One. 2009;4 6:e5820; doi: 10.1371/journal.pone.0005820. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19503834.
30. De Oliveira Gomes DC, Schwedersky RP, Barbosa De-Melo LD, Da Silva Costa Souza BL, De Matos Guedes HL, Lopes UG, et al. Peripheral expression of LACK-mRNA induced by intranasal vaccination with PCI-NEO-LACK defines the protection duration against murine visceral leishmaniasis. Parasitology. 2012;139 12:1562-9; doi: S0031182012000868 [pii] 10.1017/S0031182012000868. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23036534.
31. Sabur A, Bhowmick S, Chhajer R, Ejazi SA, Didwania N, Asad M, et al. Liposomal Elongation Factor-1alpha Triggers Effector CD4 and CD8 T Cells for Induction of Long-Lasting Protective Immunity against Visceral Leishmaniasis. Front Immunol. 2018;9:18; doi: 10.3389/fimmu.2018.00018.

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=29441060.

32. Kaur T, Sobti RC, Kaur S. Cocktail of gp63 and Hsp70 induces protection against Leishmania donovani in BALB/c mice. Parasite Immunol. 2011;33 2:95-103; doi: 10.1111/j.1365-3024.2010.01253.x. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21226722.
33. Rasouli M, Karimi MH, Kalani M, Ebrahimnezhad S, Namayandeh M, Moravej A. Immunostimulatory effects of Leishmania infantum HSP70 recombinant protein on dendritic cells in vitro and in vivo. Immunotherapy. 2014;6 5:577-85; doi: 10.2217/imt.14.28. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=24896626.
34. Jaiswal AK, Khare P, Joshi S, Kushawaha PK, Sundar S, Dube A. Th1 stimulatory proteins of Leishmania donovani: comparative cellular and protective responses of rTriose phosphate isomerase, rProtein disulfide isomerase and rElongation factor-2 in combination with rHSP70 against visceral leishmaniasis. PLoS One. 2014;9 9:e108556; doi: 10.1371/journal.pone.0108556 PONE-D-14-08545 [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=25268700.
35. Singh MK, Jamal F, Dubey AK, Shivam P, Kumari S, Pushpanjali, et al. Visceral leishmaniasis: A novel nuclear envelope protein 'nucleoporins-93 (NUP-93)' from Leishmania donovani prompts macrophage signaling for T-cell activation towards host protective immune response. Cytokine. 2019;113:200-15; doi: S1043-4666(18)30291-6 [pii] 10.1016/j.cyto.2018.07.005. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=30001865.
36. Gillespie PM, Beaumier CM, Strych U, Hayward T, Hotez PJ, Bottazzi ME. Status of vaccine research and development of vaccines for leishmaniasis. Vaccine. 2016;34 26:2992-5; doi: S0264-410X(16)00289-9 [pii] 10.1016/j.vaccine.2015.12.071. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=26973063.
37. Rodrigues V, Cordeiro-da-Silva A, Laforge M, Silvestre R, Estaquier J. Regulation of immunity during visceral Leishmania infection. Parasit Vectors. 2016;9:118; doi: 10.1186/s13071-016-1412-x 10.1186/s13071-016-1412-x [pii]. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=26932389.
38. Jain K, Jain NK. Vaccines for visceral leishmaniasis: A review. J Immunol Methods. 2015;422:1-12; doi: S0022-1759(15)00093-9 [pii] 10.1016/j.jim.2015.03.017. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=25858230.
39. Bhowmick S, Mazumdar T, Sinha R, Ali N. Comparison of liposome based antigen delivery systems for protection against Leishmania donovani. J Control Release. 2010;141 2:199-207; doi: S0168-

3659(09)00658-0 [pii]10.1016/j.jconrel.2009.09.018. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19818373

Tables

Table 1. Results of the sensitivity and specificity of electroeluted antigens with percent recognitions.

Antigens (kDa)	Sensitivity of active VL patients (%)	Sensitivity of cured VL patients (%)	Sensitivity of 6 month follow-up patients (%)	Specificity with other diseases (%)	Specificity with healthy samples (%)
LAg	100(13/13)	84.16(11/13)	0(0/13)	100(13/13)	100(13/13)
SLA	100(13/13)	100(13/13)	7.69(1/13)	100(13/13)	100(13/13)
31	100(23/23)	60(14/23)	26.08(6/23)	39.1(9/23)	100(23/23)
34	100(23/23)	73.91(17/23)	13.04(3/23)	100(23/23)	100(23/23)
36	100(23/23)	69.56(16/23)	78.26(18/23)	40(8/23)	100(23/23)
45	100(23/23)	100(23/23)	60.86(14/23)	30.43(7/23)	60.86(14/23)
51	100(23/23)	91.30(21/23)	30.43(7/23)	100(23/23)	100(23/23)
63	100(23/23)	47.82 (11/23)	4.34(1/23)	91.30(21/23)	95.65(22/23)
72	100(23/23)	39.1(9/23)	0(0/23)	65.21(15/23)	73.91(17/23)
91	100(23/23)	8.69(2/23)	0(0/23)	95.75(22/23)	91.30(21/23)
97	100(23/23)	78.23(18/23)	86.95(20/23)	95.75(22/23)	100(23/23)

Table 1. The table contains percent recognition of purified antigens, LAg and SLA and the elctroeluted antigens of 31, 34, 36, 45, 51, 63, 72, 91, and 97 kDa with the sera of VL patients before the treatment started, after one month cured and after six month follow-up along with other diseases and healthy individuals.

Table 2. List of electroeluted fractions of LAg and corresponding proteins.

Table 2. Electroeluted proteins, 31, 34, 36, 45, 51, 63, 72, 91, and 97 kDa of LAg are listed here with their identified protein names obtained through MALDI assay from previous studies. 34 and 45 kDa proteins were identified in the current study.

Mass (kDa)	Protein name	References
31	ATP synthase α chain	[29]
34	*Leishmania* analogue of the receptors of activated C kinase (LACK)	Current study
36	Elongation factor 1α	[31]
45	β tubulin	Current study
51	Elongation factor 1α	[29]
63	Glycoprotein	[24]
72	Heat shock 70-related protein 1 mitochondrial precursor (HSP 70)	[29]
91	β tubulin	[29]
97	Not yet indentified from LAg/ May be nucleoporins-93 (NUP-93)	[35]

Figures

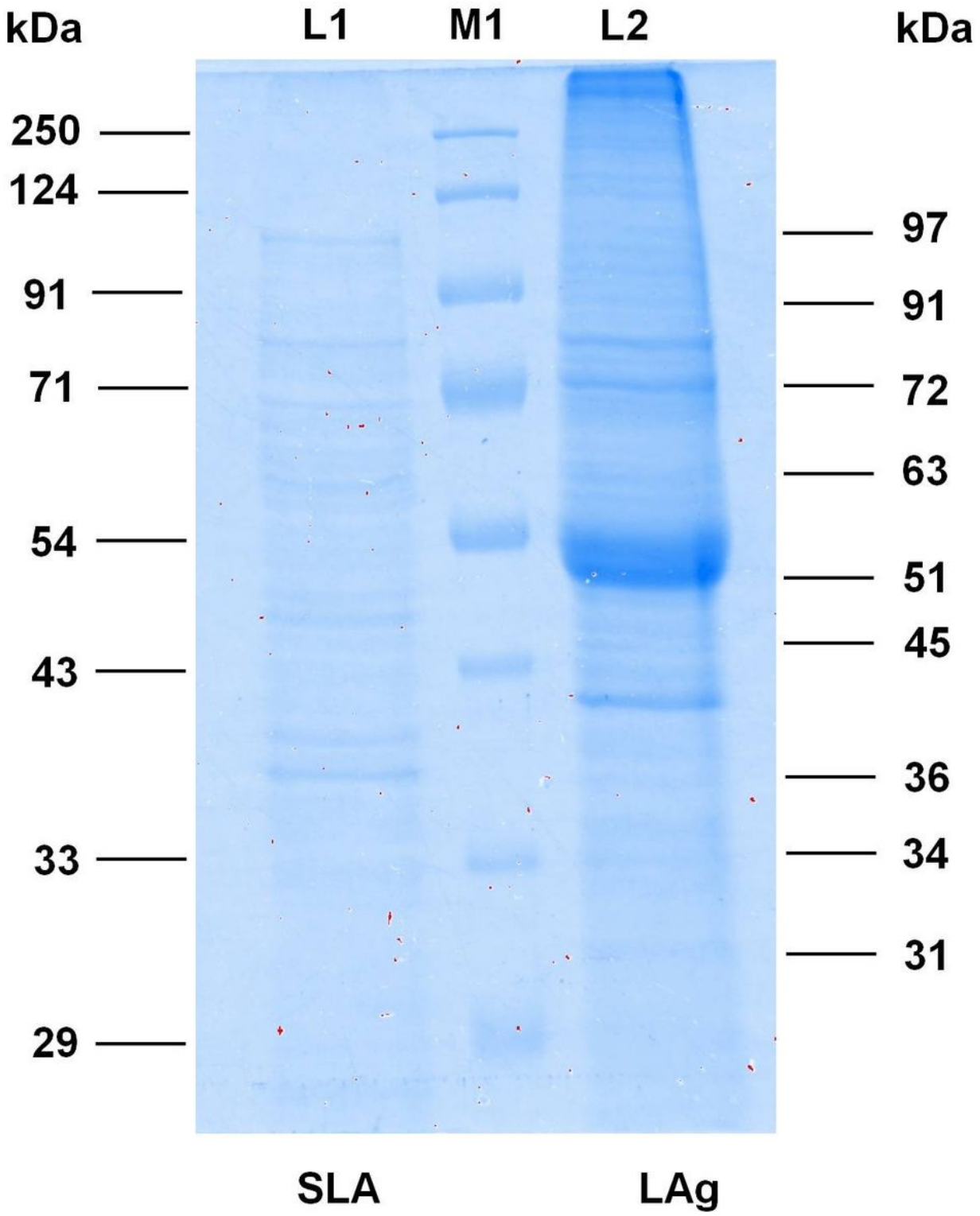

Figure 1

SDS-PAGE of purified antigens. Purified antigens, SLA and LAg were separated in SDS-PAGE and stained with Coomassie to observe the individual proteins of each antigen. Lane M1 is protein molecular weight markers. Lane L1 and L2 correspond to SLA and LAg, respectively.

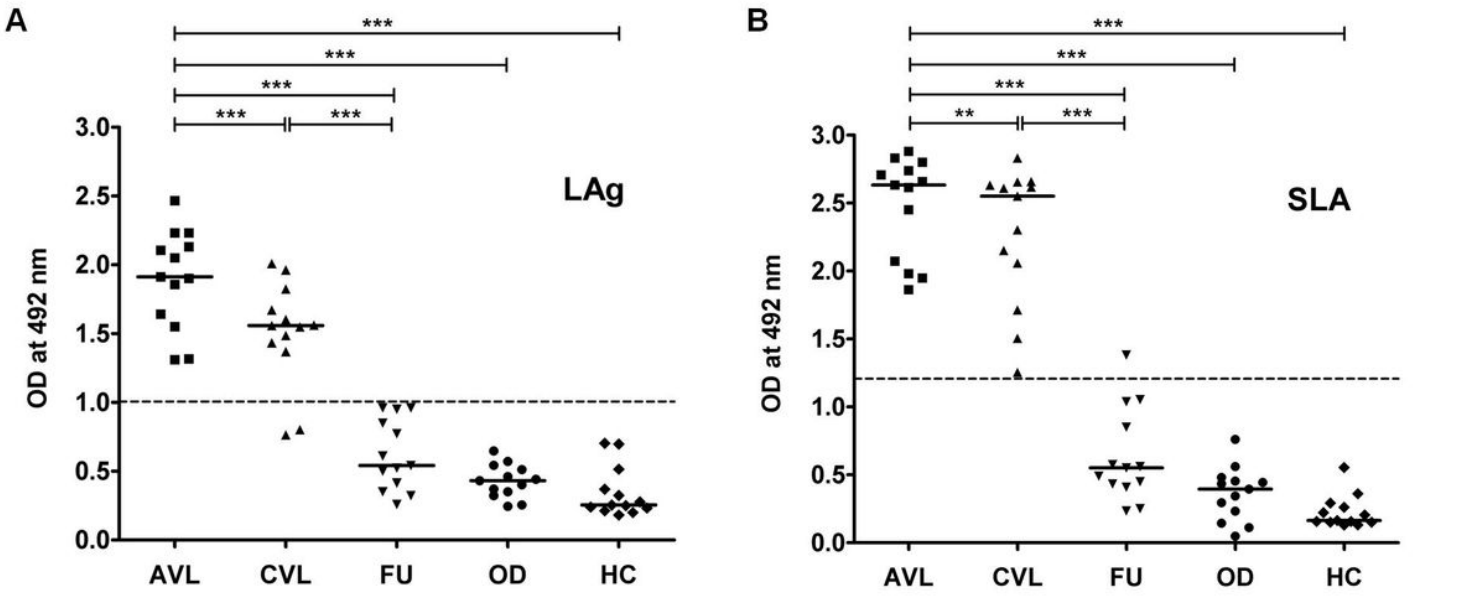

Figure 2

Indirect ELISA using purified antigens LAg and SLA. Optical density values were obtained in ELISA for detection of serum antibodies against antigens (A) LAg and (B) SLA. Paired serum samples (n=13) from confirmed VL cases before treatment started (AVL), cured VL one month after treatment (CVL), and six month follow-ups (FU) were investigated against purified leishmanial antigens, LAg and SLA. Serum samples from 13 healthy individuals (HC) and 13 from other diseases were also tested. The cut off values were selected from the ROC curve where 100% sensitivity and specificity achieved. Each point represents an average of triplicate values obtained from a single sample.

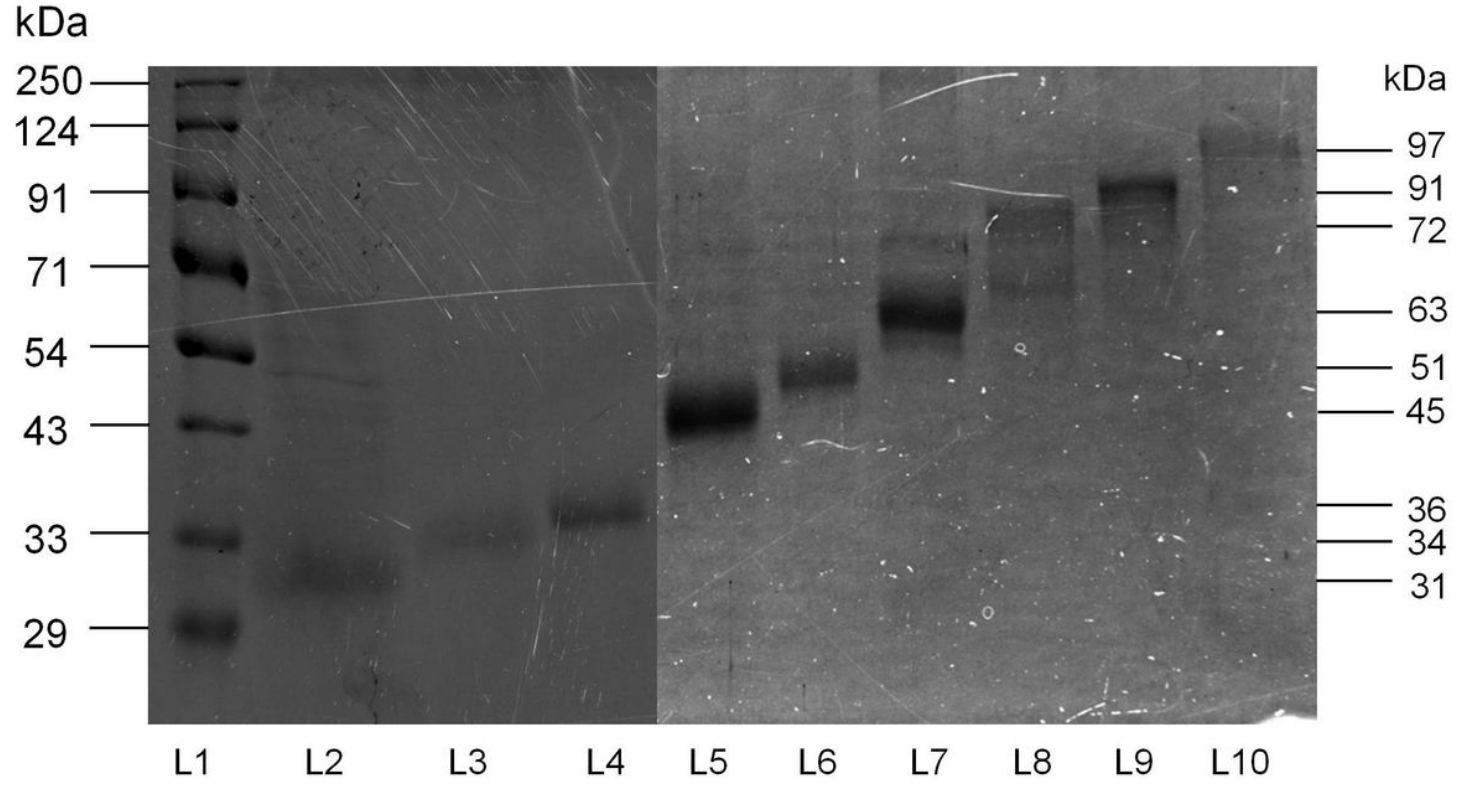

Figure 3

SDS-PAGE of electroeluted proteins. Electroeluted proteins of 31, 34, 36, 45, 51, 63, 72, 91, and 97 kDa were run on 10% SDS-PAGE. L1-marker, L2- 31 kDa, L3- 34 kDa, L4-36 kDa, L5- 45 kDa, L6- 51 kDa, L7- 63 kDa, L8- 72 kDa, L9- 91 kDa, and L10- 97 kDa. The figure depicted here was obtained from two separate gels.

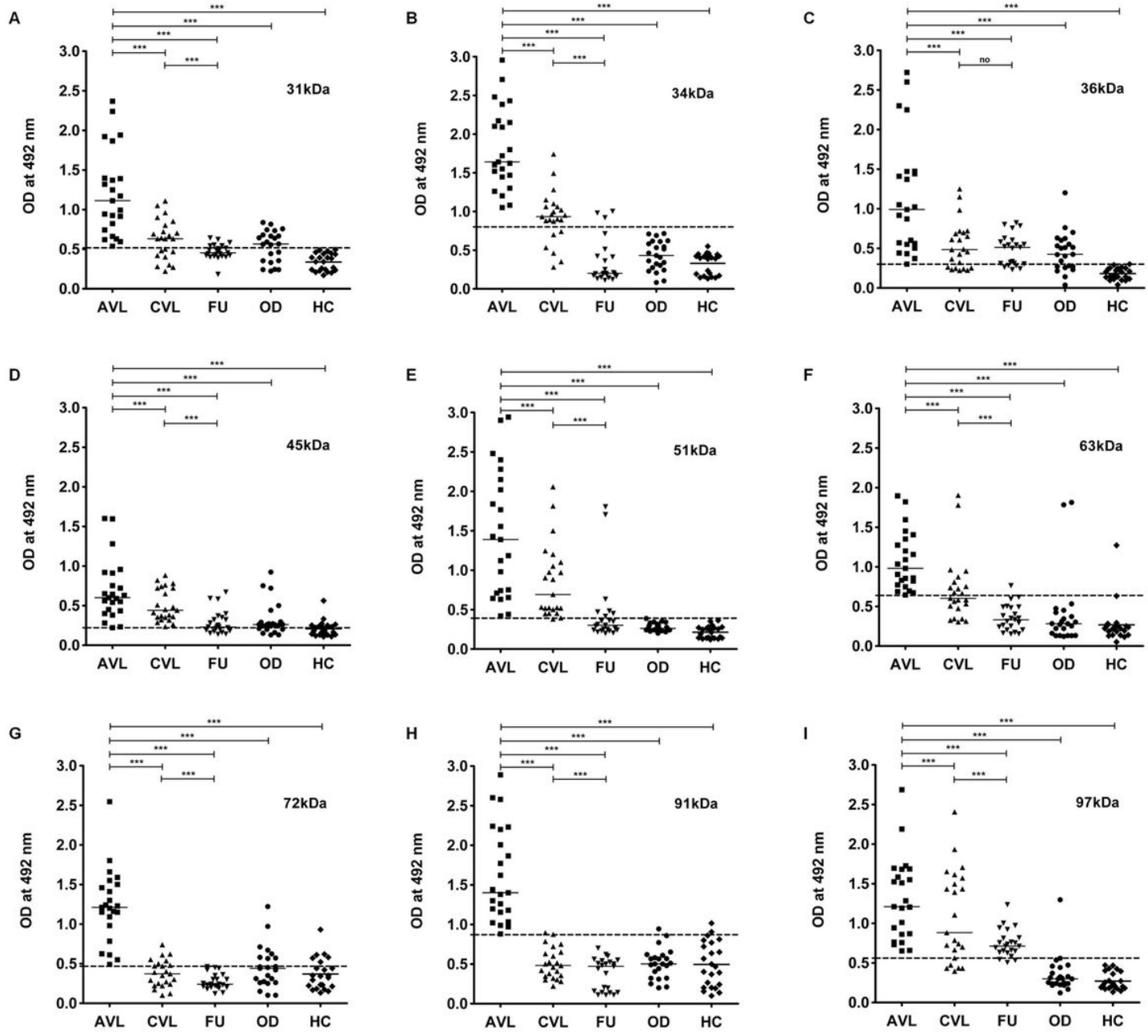

Figure 4

Indirect ELISA using electroeluted antigens. IgG reactivity of eluted antigens (A) 31, (B) 34, (C) 36, (D) 45, (E) 51, (F) 63, (G) 72, (H) 91, and (I) 97 kDa fractions with serum samples from 23 VL patients at three different time points that is before treatment started, active VL (AVL), after one month cured VL (CVL), and after six months follow-up cases (FU). Study also included sera from other symptomatically similar

diseases (OD), n= 23 and healthy controls (HC), n = 23. Horizontal line denotes the mean value of each group. Dotted line denotes the cut off value selected from the ROC curve where 100% sensitivity and specificity achieved. Each point represents an average of triplicate values obtained from a single sample.

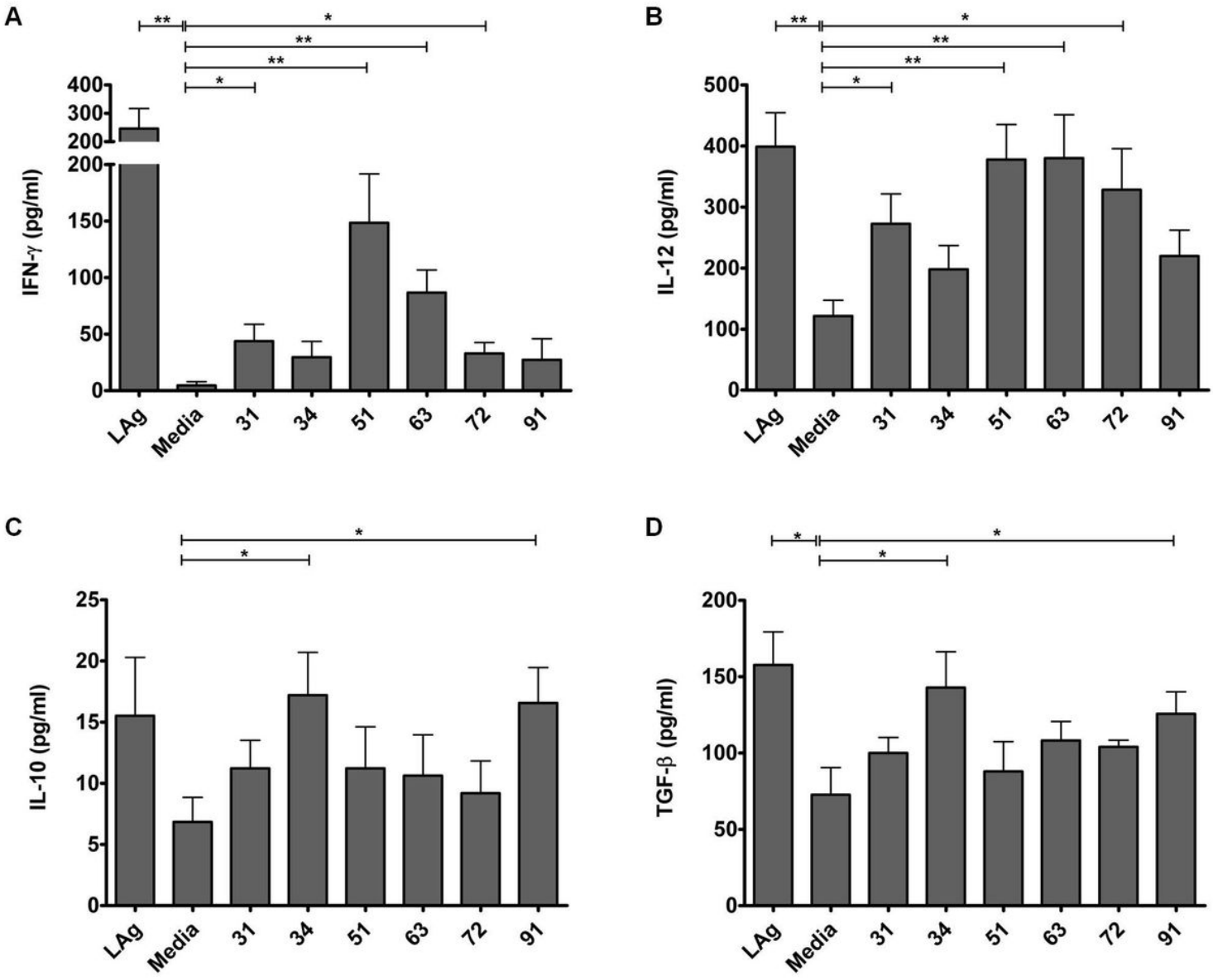

Figure 5

Sandwitch ELISA for cytokine assay. In vitro cytokine production was shown by PBMCs of cured VL individuals in cytokine ELISA upon stimulation with electroeluted 31, 34, 51, 63, 72 and 91 kDa proteins for 96 hrs, (n = 6). Cytokines were measured by ELISA for (A) IFN-□ production, (B) IL-12 production, (C) IL-10 production, and (D) TGF-β production. Unpaired student t tests were used to analyse the data.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementary.pdf