Identification of *L. infantum chagasi* proteins in VL patients' urine: a promising antigen discovery approach of vaccine candidates

Suely S. Kashino[1], **Claudia Abeijon**[2], **Lizeng Qin**[1], **Kelly A. Kanunfre**[3], **Flávia S. Kubrusly**[4], **Fernando O. Silva**[5], **Dorcas L. Costa**[5], **Dioclécio Campos Jr.**[6], **Carlos H.N. Costa**[5], **Isaias Raw**[4], and **Antonio Campos-Neto**[1,2,*]

[1]The Forsyth Institute, Cambridge, MA

[2]DetectoGen Inc., Grafton, MA

[3]Institute of Tropical Medicine, University of São Paulo, São Paulo, SP, Brazil

[4]BioIndustrial Division, Butantan Institute/Foundation, São Paulo, SP, Brazil

[5]Laboratory of Leishmaniasis, Natan Portela Institute of Tropical Medicine, Federal University of Piauí, Teresina, PI, Brazil

[6]Department of Pediatrics, University of Brasilia, Brasília, DF, Brazil

Abstract

Visceral leishmaniasis (VL) is a serious lethal parasitic disease caused by *Leishmania donovani* in Asia and by *Leishmania infantum chagasi* in Southern Europe and South America. VL is endemic in 47 countries with an annual incidence estimated to be 500,000 cases. This high incidence is due in part to the lack of an efficacious vaccine. Here, we introduce an innovative approach to directly identify parasite vaccine candidate antigens that are abundantly produced *in vivo* in humans with VL. We combined RP-HPLC and mass spectrometry and categorized three *L. infantum chagasi* proteins, presumably produced in spleen, liver, and bone marrow lesions and excreted in the patients' urine. Specifically, these proteins were the following: *Li-isd1* (XP_001467866.1), *Li-txn1* (XP_001466642.1), and *Li-ntf2* (XP_001463738.1). Initial vaccine validation studies were performed with the r*Li-ntf2* protein produced in *E. coli* mixed with the adjuvant *Bp*MPLA-SE. This formulation stimulated potent Th1 response in BALB/c mice. Compared to control animals, mice immunized with *Li-ntf2* + *Bp*MPLASE had a marked parasite burden reduction in spleens at 40 days post-challenge with virulent *L. infantum chagasi*. These results strongly support the proposed antigen discovery strategy of vaccine candidates to kala-azar and opens novel possibilities for vaccine development to other serious infectious diseases.

Keywords

Leishmania infantum chagasi; vaccine; *ntf2*; kala-azar; visceral leishmaniasis; urine

[*]Corresponding author. Mailing address: Global Infectious Disease Research Center, The Forsyth Institute, 245 First Street, Cambridge, MA 02142. Phone: (617) 892-8393. Fax: (617) 892-8610. acampos@forsyth.org.

INTRODUCTION

Visceral leishmaniasis (VL) or kala-azar is a worldwide protozoal vector borne disease, endemic in 47 countries with approximately 200 million people at risk of infection and with an annual incidence estimated to be 500,000 cases (http://www.doctorswithoutborders.org/publications/reports/2010/MSF-Kala-Azar-Fact-Sheet.pdf). In addition to being human disease, VL is a zoonotic infection as well. Foxes and domestic dogs are major vertebrate reservoirs of the parasite. Canine VL (CVL) is widely distributed in Latin America and southern Europe (1, 2). In the USA the potential for CVL to be a significant problem has been recently highlighted (3–5).

Acquired resistance to leishmaniasis is mediated by T cells. T cell deficient mice rapidly succumb after infection with most species of *Leishmania* and adoptive transfer of normal T cells confers resistance to the animals. Moreover, patients with AIDS are highly susceptible to VL either as a result of concurrent infection or as a reactivation of older sub-clinical infection (6). Among the T cells CD4+ (Th1) are crucial for resistance and CD8+ T cells seem to participate more in the memory events of the immune response involved in parasite elimination (6–11).

Although little success has been achieved in vaccine development to VL, vaccine candidates have been tested in mice and dogs and proven to induce some level of protection (12–20). In contrast, vaccination against cutaneous leishmaniasis (CL) has been practiced for centuries. Deliberate inoculation of virulent organisms from the pus of an active lesion was an ancient practice, a process known as leishmanization, has proven to be efficacious and is still used in some countries, notably Uzbekistan (21). Vaccination using crude antigen preparation obtained from promastigote forms of various species of *Leishmania* (cutaneous and visceral complexes) have been tested in human clinical trials in both the Old and New World. The results vary from 0–75% efficacy against CL and only modest protection against VL (22–25). Although none of these vaccine approaches is ideal, their results do support the proposal that induction of protection against leishmaniasis (CL and VL) is feasible and can be achieved with either a viable or a sub-unit vaccine. Indeed, a recombinant protein vaccine for CL, developed by our group that induces excellent protection in the mouse and monkey models of CL (21, 26–28) is currently in human clinical trials (29).

Here, we describe an innovative approach for the direct identification of VL vaccine candidate molecules that are produced *in vivo* during disease and that are present in bodily fluids of patients with VL. This approach led to the identification of several polypeptides of *L. infantum chagasi,* one of which has been extensively studied and is reported here. Importantly, this antigen *Li-ntf*-2 (NCBI accession XP_001463738.1), tested as a vaccine candidate formulated with the adjuvant *Bp*MPLA-SE, induced marked parasite burden reduction in a mouse model of VL.

MATERIAL AND METHODS

Human samples

A total of seven urine samples were evaluated in this study. These samples were collected from seven patients diagnosed with VL based on the following criteria: a clinical course consistent with VL (e.g., fever, anemia, hepatosplenomegaly), and confirmatory laboratory findings (identification of *Leishmania* in bone marrow aspirates). Two patients were from the University Hospital, University of Brasília Medical School (Brasília, DF, Brazil) and five patients were from the Natan Portela Institute of Tropical Diseases, Federal University of Piauí, Teresina, PI, Brazil. Urine donation protocol was approved by the Investigational

Review Boards and Ethics Committees of both University hospitals. Urines were frozen immediately after collection and sent frozen to The Forsyth Institute, Cambridge, MA.

Mass spectroscopy analysis

Individual samples (15 ml) were concentrated using Centricon P3 (3kDa cutoff filters) to ~200–300μl. Urine samples were then submitted to SDS-PAGE followed by Coomassie staining. Bands were excised from the gel and submitted for mass spectroscopy analysis at the Taplin Mass Spectrometry Facility, Harvard Medical School, Boston, MA. Gel bands were then trypsin-digested into peptides. Peptides were analyzed by nano-scale liquid chromatography coupled to a tandem mass spectrometer. Eluted peptides first had their molecular masses measured, then were fragmented, and finally the fragment masses were measured. The specific fragmentation pattern was computer-searched against predicted tryptic peptides from all known proteins from genome sequencing projects of human and *Leishmania*. The power of the technique is in its redundancy. Because many peptides are generated from the initial gel band, multiple matches to the protein of interest are detected. In this way, the protein identity is completely unambiguous. Peptide score cutoff values were chosen at Xcorr of 1.8 for singly charged ions, 2.5 for doubly charged ions, and 3.0 for triply charged ions, along with deltaCN values of 0.1, and RSP values of 1. The cross correlation values chosen for each peptide assure a high confidence match for the different charge states, while the deltaCN cutoff insures the uniqueness of the peptide hit. The RSP value of 1 insured that the peptide matched the top hit in the preliminary scoring and that the peptide fragment file only matched to one protein hit.

Animals

Female BALB/c mice and LVG Golden Syrian hamsters were purchased from Charles River Laboratories (Wilmington, MA) and kept under specific pathogen-free conditions. Animals were used at 12 weeks of age. All experiments were carried out under the guidelines of the Institutional Animal Care and Use Committee at the Forsyth Institute.

Parasite

The *L. infantum chagasi* strain used in these studies was kindly supplied by Dr. Mary E. Wilson (University of Iowa, Iowa City, IA) and was maintained *in vivo* in hamsters. Metacyclic promastigotes of the parasite were used for challenge infections. For challenge infection of BALB/c mice parasites were isolated from the spleen of hamster and cultured in Schneider's medium (Invitrogen, Carlsbad, CA) supplemented with 20% FBS (Hyclone, Thermo Scientific, Rockford, IL) and 2mM L-glutamine (Gibco-Invitogen, Carlsbad, CA) for 7–10 days at 26°C.

Cloning of *Leishmania infantum chagasi* gene, protein expression and purification

Oligonucleotide PCR primers were designed to amplify the full-length open reading frame of the target gene from genomic DNA of *L. infantum chagasi*. The forward primer contained an *Nde* I restriction site at the ATG initiation codon followed by sequences derived from the gene. The reverse primer included a *Bam* HI restriction site followed by a stop codon and sequences from the gene. The resultant PCR product was digested with restriction enzymes and subcloned into pET-14b expression vector, which was similarly digested for directional cloning. Alternatively, the *Li-ntf-2* DNA sequence was codon optimized for expression in *E. coli* containing the same restriction enzyme sites (*Nde* I and *Bam* HI). The DNA fragment was synthesized (Blue Heron, Bothel, WA) and the synthetic gene was sub-cloned into pET-14b as well. A ligated pET-14b vector was subsequently used to transform *E. coli* BL21(DE3)pLysS host cells (Novagen, Madison, WI) for expression. Recombinant protein was obtained and purified from 100ml of IPTG-induced batch cultures by affinity

chromatography using QIAexpress Ni-NTA agarose matrix (QIAGEN, Chatsworth, CA) as described (30, 31). The yields of recombinant protein were 10–20mg per liter of induced bacterial culture, and purity was assessed by SDS-PAGE followed by Coomassie blue staining. Endotoxin level present in the in the purified recombinant protein preparations was measured by the Limulus Amebocyte Lysate assay (Lonza, Walkersville, MD) and shown to be 2.89 EU/ml.

Western Blot

Purified recombinant proteins (100 ng) and whole antigen extract from *Leishmania infantum chagasi* were fractionated by SDS-PAGE (4–20% gradient gel) and transferred to polyvinylidene fluoride membrane (PVDF, Millipore, Medford, MA). Crude *Leishmania chagasi* lysates were prepared from amastigotes freshly recovered from spleen of infected hamster, or after promastigote parasites were cultured for 7–10 days in complete Schneider's medium at 26°C (promastigote lysate preparation). The blots were blocked overnight at 4°C with Tris-buffered saline with 0.1% Tween 20 (TBS-T) containing 1% bovine serum albumin (BSA) and subsequently probed with antigen specific rabbit antisera and pre-immune rabbit. After several rinses with TBS-T, goat anti-rabbit IgG labeled with horseradish peroxidase (ThermoScientific Pierce, Rockford, IL) was added. After additional washings, bound conjugates were detected using ECL enhanced chemiluminescence system (Amersham/GE Healthcare, Piscataway, NJ) and proteins were visualized by autoradiography (Kodak BioMax, Rochester, NY).

Immunization regimen and challenge

Groups of 10 mice were injected subcutaneously as follow: control mice were injected with saline plus adjuvant only (20μg/ml), and immunized mice were injected with recombinant protein (5μg/mouse) in presence of 20μg/ml of the adjuvant *Bp*MPLA-SE (Institute Butantan, São Paulo, Brazil). *Bp*MPLA-SE is an oil-in water emulsion containing monophosphoryl lipid A (MPLA) derived from *Bordetella pertussis*. The *B. pertussis* MPLA-SE is a stable oil in water emulsion that contains squalene (the oil phase) and the surfactant Tween 80. A detailed description of this adjuvant formulation and its biological and immunological properties has been described elsewhere (32, 33). Mice in each group were vaccinated three times with same doses of inoculums following three weeks intervals. Ten days after the last immunization three mice from each group were euthanized to carry out T cell immunogenicity assays, and four weeks after immunization remainder mice were challenged intravenously with 10×10^6 *L. infantum chagasi* promastigotes to assess vaccination efficacy.

In vitro cytokine response assay

Ten days after the last boost spleens were excised and splenocytes harvested using 70μm cell strainers. After centrifugation over Histopaque (Sigma, St. Louis, MO) and washings, mononuclear cell suspensions were prepared in RPMI supplemented with 10% FBS (Hyclone), 100μg/ml streptomycin, 100U/ml penicillin, 25mM HEPES, 2mM L-glutamine, 0.05mM 2-ME (all Sigma). Cell viability was estimated with Trypan Blue 0.4% (Sigma). 2×10^5 cells were added to the wells of a 96-well flat-bottomed culture microplate (Costar, Lowell, MA). Cells were stimulated for 72h with 5 μg/ml of recombinant protein. Supernatants were collected and frozen until use. Cells cultured in presence of ConA (5μg/ml) or complete medium alone were included as controls. Cytokine (IFN-γ and IL-4) concentration in the supernatants was measured using specific sandwich ELISA kit (R&D Systems, Minneapolis, MN).

Flow cytometry and intracellular cytokine staining assay

Ten days after the last boost mice were sacrificed, spleens were excised and splenocytes were obtained as described above. Cells from both immunized and control mice were counted and adjusted to 2×10^7 cells/ml in RPMI 1640 medium (Gibco-Invitrogen) supplemented with 10 % FBS, 25 mM HEPES, 2 mM L-glutamine, 20 U of penicillin/ml, 20 μg of streptomycin/ml. Two million cells (100 μl) from individual mice were incubated with medium or recombinant protein (2 μg/ml) plus Golgi Stop (2 μl/ml) on a 96-cell culture plate. As positive control, splenocytes were incubated with PMA (2 μg/ml), ionomycin (10 μg/ml) and Golgi Stop. The cells were incubated at 37°C for 6h and transferred to 4°C overnight. The following day, cells were washed with 2% FBS in PBS followed by incubation with monoclonal antibodies specific for cell surface molecules for an additional 30 min. Permeabilization was performed for one hour with Cytofix/Cytoperm solution (BD Biosciences). Cells were washed with 1X Perm/Wash buffer (BD Biosciences) and then stained with anti-cytokine mAb (anti-IFN-γ or anti IL-4). After an additional washing with 1X Perm/Wash buffer, the cells were fixed in 2% formaldehyde-PBS. Samples were collected on FACSAria III instrument (BD Biosciences, San José, CA) and analyzed using FlowJo software (Tree Star). The following antibodies from BD Biosciences, San José, CA were used: anti-CD3e-FITC (145–2C11); anti-IFN-γ-APC (XMG1.2); anti-IL-4-PE (BVD4-1D11); and anti-CD4-APC-Cy7 (GK1.5).

Parasite quantification

The load of *Leishmania* in the spleen was estimated by limiting dilution assay. Forty days after challenge with *L. infantum chagasi* promastigotes animals were euthanized and spleens were removed and homogenized in complete Schneider's medium. Homogenates were 10-fold serially diluted in 12 replicates for each dilution in 96-well flat-bottomed microplates (one plate per spleen) and incubated for 7–10 days at 26°C. Number of viable parasites was calculated from the highest dilution in which parasites could be found under phase contrast light microscopy in any of the 12 replicates (34).

Antibody ELISA

IgG1 and IgG2a antibodies raised against *L. infantum chagasi* recombinant protein were titrated by standard ELISA protocol. High binding 96-well microplates (Costar) were coated with purified recombinant protein (2μg/ml) prepared in 0.2M sodium carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Wells were washed with PBS containing 0.05% Tween 20 (PBS-T) and blocked with 1% BSA in PBS-T for 2 hours. Serum samples were added at 2-fold serial dilutions in PBS-T containing 0.1% BSA and plates were incubated for 1 hour at RT. After another washing step, biotinylated rat anti-mouse IgG1 or IgG2a mAb (2μg/ml, BD Biosciences) was added and incubated for 1 hour. Streptavidin-HRP conjugate (BD Biosciences) was used at 1/2000 followed by addition of ready-to-use substrate solution containing tetramethylbenzidine (KPL, Gaithersburg, MD). Color development was stopped using 1N HCl. Optical density data were recorded at A_{450}.

Statistical analysis

Data are presented as mean and SEM. Statistical significance was determined by the Student t test for comparison of two groups. Values of $\rho < 0.05$ were considered statistically significant.

RESULTS

Antigen discovery of <u>L. infantum chagasi</u> proteins in urine of VL patients

The general protocol to analyze the human urine samples from VL patients is illustrated in Fig. 1. Urines were initially collected from seven patients with active, culture-confirmed VL. These patients did not have any clinical signs or symptoms or laboratory findings compatible with renal or urinary tract abnormalities. None of the patients were under anti-leishmaniasis therapy at the time of urine collection. Individual urine samples were analyzed by mass spectrometry and generated approximately 400 peptide sequences. As expected, most sequences of the identified peptides had identical sequence homologies with that of human proteins. Importantly, eight peptide sequences that had no known homologies with human proteins had identical sequence homologies with the deduced sequences of three different *L. infantum chagasi* proteins. Figure 2 depicts the discovered proteins and highlights the peptides found in the patients' urines. Importantly, these *L. infantum chagasi* peptides were identified in multiple urine samples, thus strongly validating these findings. Overall, these findings suggest that these proteins are abundant antigens of *L. infantum chagasi* produced during the disease and broadly present in human bodily fluids.

Both *L. infantum chagasi* iron superoxide dismutase 1 and *L. infantum chagasi* tryparedoxin 1 have been previously described and their immunological properties studied in some details (35–37). In contrast, little is known about the biology of the putative *L. infantum* nuclear transport factor 2 (*Li-ntf2*) (38) and no reports were found about the immunological significance of the molecule. Therefore, the present study concentrates on the evaluation of *Li-ntf2* as a vaccine candidate for VL.

The open reading frame of the full-length gene coding *Li-ntf2* was amplified by PCR and sub-cloned into the *E. coli* pET-14b expression vector. This cloning strategy resulted in no expression of protein. However, using a synthetic gene with optimized sequence for *E. coli* followed by sub-cloning in pET-14b resulted in excellent over-expression of the recombinant protein, which was then purified using the Ni-NTA agarose resin.

In order to validate the *E. coli*-expressed and purified protein (Fig. 3A) as true *L. infantum chagasi* protein, a crude lysate of the parasite and the recombinant protein were immunoblotted and then probed with specific polyclonal rabbit anti-recombinant *Li-ntf2* antisera (Fig. 3B). The antiserum clearly recognized a protein band in the crude parasite lysate at similar molecular weight as the recombinant protein. Therefore, the results support that the recombinant *Li-ntf2* is a replica of the native protein produced by *L. infantum chagasi*. A second band of MW ~45 kDa was also recognized by the antiserum. However, the nature of this band is not known. It is possible that the *Li-ntf2* aggregates with itself, or is part of a larger protein complex, or a building block of a larger protein originated by post-translational modification.

Immunogenicity studies in mice

Because CD4+ T cells of the Th1 phenotype are crucial mediators of resistance to VL, we chose to immunize mice with a formulation containing the purified recombinant *Li-ntf2* plus the adjuvant monophosphoryl lipid A (MPLA) from *Bordetella pertussi*s emulsified with squalene (Butantan Institute, São Paulo, SP, Brazil). An MPL based adjuvant was chosen because this type of formulation has been shown to be safe and to preferentially stimulate Th1 responses (26, 29, 32, 33). Squalene is added to condition a stable oil-in-water emulsion (*Bp*MPLA-SE) to further increase the stimulation of the immune system. Mice were immunized three times with 5µg of *Li-ntf2* alone or mixed with 20µg of *Bp*MPLA-SE. As control, another group of mice was immunized with saline + *Bp*MPLA-SE. The choice of 20µg of *BP*MPLA-SE was based on initial dose response studies that indicated that this

concentration, compared to 2.5μg, 5.0μg, and 10μg, induced the most robust immune response (not shown). Ten days after the last immunization mice were bled (for serologic studies) and sacrificed. Spleens were removed and splenic cell suspensions were prepared and stimulated for 6 hours with either medium (control) or purified recombinant *Li-ntf2*. Cells were harvested and cytokine responses (IFN-γ and IL-4) were measured by flow cytometry for intracellular cytokine staining (ICS). Culture supernatants from cells stimulated for 72 hours were used to measure total cytokine response by conventional capture ELISA.

Figure 4 shows the serological titration of the IgG1 and IgG2a specific antibody response to the recombinant *Li-ntf2*. High titers of both IgG1 and IgG2a were generated after immunization with *Li-ntf2 + Bp*MPLA-SE. In contrast, animals immunized with the protein alone (mixed with saline) produced low titers of IgG1 and no detectable levels of IgG2a. Because class switch to IgG2a is dependent of production of IFN-γ, this result is consistent with a stimulation of Th1 response by the antigen/adjuvant formulation.

The antigen stimulated cytokine response of cells obtained from animals immunized with *Li-ntf2 + Bp*MPLA-SE as well as with saline + *Bp*MPLA-SE was initially tested in the culture supernatant of spleen cell cultures stimulated with *Li-ntf2*. Results are illustrated in Fig. 5. In the cell cultures obtained from antigen immunized mice and stimulated *in vitro* with *Li-ntf2* the Th1 cytokine IFN-γ was readily detected in the culture supernatants. This cytokine was not detected in the culture supernatants of cells stimulated with medium alone nor in the cultures from spleen cells obtained from mice immunized with saline + *Bp*MPLA-SE and stimulated *in vitro* with recombinant *Li-ntf2.* Moreover, no IL-4 could be detected in the supernatants of antigen-stimulated cultures. However, both IFN-γ and IL-4 were readily detected in the culture supernatants of cultures stimulated with the mitogen ConA thus confirming the potential of the cells to produce both cytokines. These results confirm the antigen specificity of the responses obtained from cells of mice immunized with recombinant *Li-ntf2* plus *Bp*MPLA-SE, i.e., the trace amount of LPS (<2,9 EU/ml) present in the antigen preparation is not responsible for the antigen induction of cytokine production by cells from immunized *Li-ntf2*-immunized mice. Finally, no IL-4 could be detected in any of the culture supernatants (not shown).

The confirmation that the IFN-γ detected in the culture supernatants was produced by CD4+ T cells was obtained by intracellular cytokine staining (ICS). The results are illustrated in Fig. 6. As can be seen, the results obtained using ICS clearly point to CD4+ T cells as an important source of IFN-γ produced upon stimulation with recombinant *Li-ntf2*. In the cell cultures obtained from antigen immunized mice and stimulated *in vitro* with *Li-ntf2* the Th1 cytokine IFN-γ was readily detected (Fig. 6A, upper panel). In contrast no IL-4 producing cells could be detected (Fig. 6B, lower panel). As expected, no IFN-γ or **IL-4** producing cells were detected in cells obtained from mice immunized with saline + *Bp*MPLA-SE and re-stimulated *in vitro* with *Li-ntf2*. However, CD4+ T cells producing both IFN-γ and IL-4 were clearly detected in cultures stimulated with the polyclonal activators PMA + ionomicin (Fig. 6C). Together these results confirm that the immunization protocol using the adjuvant *Bp*MPLA-SE induces a preferential Th1 response to the *L. infantum chagasi* vaccine candidate *Li-ntf2*.

Protection studies in mice

Although mice is not considered an ideal model of VL these animals have been used and accepted for initial evaluation and selection of vaccine candidate antigens for future validation in large animal models (e.g., dogs). Two groups of BALB/c mice were immunized three times (three weeks interval) with either 5μg of the recombinant *Li-ntf2* mixed with 20μg of the adjuvant *Bp*MPLA-SE or with saline mixed with 20μg of *Bp*MPLA-

SE. For these experiments, a group of animals immunized with *Li-ntf2* alone was not included because the immunogenicity studies indicated that this formulation stimulated little or undetectable immune response. Thirty days after the last immunization mice were challenged i.v. with 10×10^6 promastigotes (stationary phase of growth) freshly derived from amastigotes isolated from the spleen of *L. infantum chagasi*-infected hamster. To determine protection, animals were sacrificed 40 days later and spleen homogenates were prepared in a serial dilution manner and incubated in Schneider's medium for limiting dilution quantification. Parasite enumeration was performed one week later and results are expressed in Fig. 7. As can be seen immunization with recombinant *Li-ntf2* plus *Bp*MPLA-SE caused significant reduction in the parasite burden ($1.3 \log^{10}$) compared to animals immunized with saline + *Bp*MPLA-SE. Therefore, these results support the hypothesis that abundant *L. infantum chagasi* antigens present in bodily fluids of humans during disease can be potential target vaccine candidates.

DISCUSSION

Although much effort has been placed on the development of anti-VL human vaccine little or no success has been achieved. Two vaccines to canine visceral leishmaniasis (CVL) are commercially available in Brazil but these products have not been approved for human use due to low protection efficacy in dogs as well as complications with standard operating procedures for the manufacture of one of them, which is a glycoprotein fraction purified from *L. donovani* promastigotes (39, 40).

The barriers to achieve better success in vaccine development to VL are not entirely understood. However, most of the antigen discovery approaches tested so far have used antibodies and in some cases PBMC from patients with VL to identify parasite molecules of interest. Although these approaches have been validated for other diseases, including CL, we believe that they are not ideal for VL because VL patients have both massive hypergammaglobulinemia due to polyclonal activation of B cells and severe immunossupression of T cell responses. Consequently, these readouts do not necessarily reflect host resistance to *L. donovani/infantum chagasi*.

In this work we address this critical barrier of antigen discovery. Our target vaccine candidates are parasite molecules that are abundantly produced *in vivo* in human patients with active VL. The hypothesis of this proposal is that microbial antigens found *in vivo* in the bodily fluids of the host are potentially effective vaccine candidate molecules because they represent microbial molecules actively and continuously produced and released during disease, therefore by definition, good targets for vaccine development. Moreover, our former studies in tuberculosis confirm this hypothesis (31).

Thus, we combined RP-HPLC and mass spectrometry (MS) and categorized three distinct *L. infantum chagasi* proteins present in the urine of seven VL patients. The rationale to use urine as source of the pathogen's antigens was based on the premise that *L. infantum chagasi* proteins or their breakdown products (peptides) produced *in vivo*, e.g., in spleen/ liver/bone marrow lesions, would reach the host's blood circulation and subsequently would be excreted in the patient's urine. The three *L. infantum chagasi* proteins identified in the urines of seven patients with VL were: *L. infantum chagasi* iron superoxide dismutase, *L. infantum chagasi* tryparedoxin, and *L. infantum chagasi* nuclear transport factor 2 (*Li-ntf2*). The MS results were unambiguously conclusive that the peptides identified in the patients' urines were indeed derived from the parasite's proteins. This assertion is strongly supported by the facts that the inferred amino acid sequences of the derived peptides had very high XCorr (>3.0) and several different peptides found in the patients' urines spanned at different sequence positions of the corresponding peptide donor proteins.

The concept of detecting microbial molecules in human bodily fluids of infected individuals particularly for diagnosis purposes has strong precedent. For example, molecules from numerous viruses (e.g., hepatitis A and B), bacteria (e.g., *Streptococcus pneumonia, Streptococcus pyogenes, Legionella pneumophila*), and parasites (e.g., *Entamoeba histolytica*) have long been described in various human samples (e.g., blood, mucous secretions, and feces) of patients suffering from the diseases caused by these microorganisms (41–46). Interestingly, some of these molecules have been successfully used as vaccines in humans (e.g., for hepatitis A and B). Regarding leishmaniasis, *L. donovani* polysaccharide antigens have also been described to be present in the urine of VL patients and have been used in an antigen detection assay for the diagnosis of this disease (47–52). However, because of the polysaccharide nature of these molecules they are, by definition, not rational antigens to be used as vaccine candidates to VL. Resistance to VL is T cell mediated and it is well known that polysaccharide antigens, in contrast to proteins, by in large do not induce T cell responses. Therefore, the proteins described in the present studies can be of great interest as vaccine candidates to VL.

The gene coding for the identified *L. infantum chagasi* proteins are also present, in various other members of the *Trypanosomatidae* family including *L. donovani, L. major, L. brasiliensis, L. amazonensis,* African trypanosomes, and *Trypanosoma cruzi*. However, at this point we have no information if these molecules are either abundantly produced *in vivo* or are present in bodily fluids of patients with the diseases caused by these parasites. Future investigation will be necessary to verify these possibilities.

The present studies were concentrated on the putative *Li-ntf2* because little is known about the biological and immunological properties of this leishmanial molecule. Cloning of the gene and production and purification of the recombinant molecule was easily achieved. Approximately 20mg of purified protein was obtained per liter of induced *E. coli* culture broth. Importantly, Western blot analyzes using specific rabbit antiserum raised against the purified recombinant *Li-ntf2* clearly showed that this molecule is constitutively produced by *L. infantum chagasi*. Two clear bands present in the parasite's whole antigen preparation could be detected by the antiserum. One band that migrates at approximately 14 kDa position coincides with the predicted MW of the native *Li-ntf2* (13.89 kDa). A second band migrates at ~45 kDa MW position. As expected, the recombinant *Li-ntf2* migrates slightly slower than the native molecule, which is due to its slightly higher MW due to the presence of the 6 His tag. It is interesting to note that this band reacts stronger with the specific anti-*Li-ntf2* antiserum than the band that matches the predicted MW of the native molecule. At this point, we have no experimental evidences about the nature of this molecule. One possibility is that the native molecule is either a homo or heteropolymer of *Li-ntf2*. However, further investigation will need to be carried out to support this possibility.

To verify the protection potential of *Li-ntf2* we chose to formulate this antigen with a monophosphoryl lipid A (MPL) adjuvant. This choice was based in our former and successful experience with this type of adjuvant in vaccine development to cutaneous leishmaniasis (27, 53–55). MPL has proven to modulate the immune system to a predominant Th1 response (31, 56, 57), which is essential to control leishmaniasis and several other infectious processes. The MPL used in the present studies (*Bp*MPLA-SE) is the monophosphoryl lipid A (MPLA) derived from *Bordetella pertussis* lipopolysaccharide containing squalene and one surfactant that facilitates the formation of a stable oil-in-water emulsion. This adjuvant has proven to induce potent immune response to influenza virus (including H5N1) vaccine candidates (32, 33). In the present studies we confirmed that this adjuvant formulated with recombinant *Li-ntf2* induces in BALB/c mice a potent Th1 response to this protein.

This conclusion was reached based on several evidences. First, high titer of specific antibody response of both IgG1 and IgG2a isotypes was generated against *Li-ntf2*. This pattern of high level of IgG2a production is generally accepted as reliable surrogate of Th1 response (58). Because IL-4 is known for many years to promote immunoglobulin class switching to IgG1, an antibody response that included this isotype of immunoglobulin was believed in the past to be a surrogate of the Th2 response. However, later evidences demonstrated that IgG1 antibodies are divided in two distinct sub-families of molecules (59). One that is dependent on IL-4 (Th2 associated) and another that is dependent on IL-12 and IFN-γ (Th1 associated). Therefore, the generation of high titers of IgG1 antibody can only be interpreted as a surrogate of a polarized Th2 response in the absence of IgG2a antibody. In contrast, because the class switch to IgG2a is solely dependent on IFN-γ, high titers IgG2a antibody, whether or not associated with IgG1 antibody has been generally accepted as a good surrogate of a typical Th1 response. Second, *in vitro* antigen stimulation of cells obtained from mice immunized with *Li-ntf2* + *Bp*MPLA-SE clearly indicated that IFN-γ was readily produced by CD4+ T cells. In contrast IL-4 producing CD4+ T cells could not be detected. Together, these findings clearly point to a typical Th1 phenotype of immune response induced by the antigen/adjuvant formulation.

The protection experiments in the mouse model were carried out using an identical immunization protocol used for the immunogenicity studies. Although the mice were challenged i.v. using a high inoculum (10×10^6 promastigote forms of the parasite at stationary phase of growth) of *L. infantum chagasi* freshly derived from amastigote forms isolated from infected hamsters, excellent protection was achieved. Over 1.3 log reduction in parasite burden was observed in vaccinated animals compared to controls. This level of protection is comparable and even superior to most VL vaccine reported to date (14–17, 60). The reasons to explain the difficulty to elicit a more complete protection in this animal model are not known. One possibility to explain this limitation is that most regimens of immunization do not necessarily generate CD8+ T cell responses to parasite antigens, and these cells seem to also participate in immunity/memory mechanisms of protection against *Leishmania* (11). Indeed, this possibility has been evaluated in more details in infectious caused by other pathogens such as *Mycobacterium tuberculosis* and *Plasmodium*. Interestingly, using heterologous prime/boost protocols of immunizations that generate both CD4+ and CD8+ T cell responses to the vaccine candidate, better protection has been achieved than with immunizations that used conventional boosting strategies (61, 62). In a typical protocol, animals are primed with a vaccine candidate using an antigenic formulation that induces preferential CD4+ T cells (e.g., protein plus adjuvant) and are boosted using a formulation that stimulates preferentially CD8+ T cells (e.g., genetic immunizations using virus or plasmid DNA carriers of the vaccine candidate). Experiments designed to evaluate the efficacy of this strategy to improve the protection induced by *Li-ntf2* are in progress.

Although further protection experiments using different protocols of immunization, larger animal samples, different animals models such as hamster and dogs need to be performed before the formulation *Li-ntf2* + *Bp*MPLA-SE can be tested in clinical trials. It is important to emphasize that the findings reported in this work strongly support the proposed antigen discovery strategy of vaccine candidates to VL. Moreover, and equally important, this simple but innovative approach of antigen discovery opens novel and broader possibilities for vaccine development to other serious infectious diseases like tuberculosis, trypanosomiasis, malaria, and AIDS.

Acknowledgments

REFERENCES

1. Belazzoug S. Leishmaniasis in Mediterranean countries. Vet Parasitol. 1992; 44:15–19. [PubMed: 1441186]

2. Dujardin JC, Campino L, Canavate C, Dedet JP, Gradoni L, Soteriadou K, Mazeris A, Ozbel Y, Boelaert M. Spread of vector-borne diseases and neglect of Leishmaniasis, Europe. Emerg Infect Dis. 2008; 14:1013–1018. [PubMed: 18598618]

3. Boggiatto PM, Gibson-Corley KN, Metz K, Gallup JM, Hostetter JM, Mullin K, Petersen CA. Transplacental transmission of *Leishmania infantum* as a means for continued disease incidence in North America. PLoS Negl Trop Dis. 2011; 5:e1019. [PubMed: 21532741]

4. Eddlestone SM. Visceral leishmaniasis in a dog from Maryland. J Am Vet Med Assoc. 2000; 217:1686–1688. 1659. [PubMed: 11110461]

5. Gaskin AA, Schantz P, Jackson J, Birkenheuer A, Tomlinson L, Gramiccia M, Levy M, Steurer F, Kollmar E, Hegarty BC, Ahn A, Breitschwerdt EB. Visceral leishmaniasis in a New York foxhound kennel. J Vet Intern Med. 2002; 16:34–44. [PubMed: 11822802]

6. Montalban C, Martinez-Fernandez R, Calleja JL, Garcia-Diaz JD, Rubio R, Dronda F, Moreno S, Yebra M, Barros C, Cobo J. Visceral leishmaniasis (kala-azar) as an opportunistic infection in patients infected with the human immunodeficiency virus in Spain. Rev Infect Dis. 1989; 11:655–660. [PubMed: 2672242]

7. Locksley RM, Heinzel FP, Holaday BJ, Mutha SS, Reiner SL, Sadick MD. Induction of Th1 and Th2 CD4+ subsets during murine *Leishmania major* infection. Res Immunol. 1991; 142:28–32. [PubMed: 1829257]

8. Melby PC. Recent developments in leishmaniasis. Curr Opin Infect Dis. 2002; 15:485–490. [PubMed: 12686880]

9. Muller I, Pedrazzini T, Farrell JP, Louis J. T-cell responses and immunity to experimental infection with *Leishmania major*. Annu Rev Immunol. 1989; 7:561–578. [PubMed: 2653376]

10. Reiner SL, Locksley RM. The regulation of immunity to *Leishmania major*. Annu Rev Immunol. 1995; 13:151–177. [PubMed: 7612219]

11. Stager S, Alexander J, Kirby AC, Botto M, Rooijen NV, Smith DF, Brombacher F, Kaye PM. Natural antibodies and complement are endogenous adjuvants for vaccine-induced CD8+ T-cell responses. Nat Med. 2003; 9:1287–1292. [PubMed: 14502281]

12. Palatnik-de-Sousa CB, Silva-Antunes I, Morgado AA, Menz I, Palatnik M, Lavor C. Decrease of the incidence of human and canine visceral leishmaniasis after dog vaccination with Leishmune in Brazilian endemic areas. Vaccine. 2009; 27:3505–3512. [PubMed: 19464528]

13. Fernandes AP, Costa MM, Coelho EA, Michalick MS, de FE, Melo MN, Luiz TW, Resende DM, Hermont V, Abrantes CF, Gazzinelli RT. Protective immunity against challenge with *Leishmania (Leishmania) chagasi* in beagle dogs vaccinated with recombinant A2 protein. Vaccine. 2008; 26:5888–5895. [PubMed: 18786587]

14. Ghosh A, Zhang WW, Matlashewski G. Immunization with A2 protein results in a mixed Th1/Th2 and a humoral response which protects mice against *Leishmania donovani* infections. Vaccine. 2001; 20:59–66. [PubMed: 11567746]

15. Goto Y, Bhatia A, Raman VS, Liang H, Mohamath R, Picone AF, Vidal SE, Vedvick TS, Howard RF, Reed SG. KSAC, the first defined polyprotein vaccine candidate for visceral leishmaniasis. Clin Vaccine Immunol. 2011; 18:1118–1124. [PubMed: 21632891]

16. Rafati S, Nakhaee A, Taheri T, Taslimi Y, Darabi H, Eravani D, Sanos S, Kaye P, Taghikhani M, Jamshidi S, Rad MA. Protective vaccination against experimental canine visceral leishmaniasis using a combination of DNA and protein immunization with cysteine proteinases type I and II of L. infantum. Vaccine. 2005; 23:3716–3725. [PubMed: 15882533]

17. Dondji B, Perez-Jimenez E, Goldsmith-Pestana K, Esteban M, Mahon-Pratt D. Heterologous prime-boost vaccination with the LACK antigen protects against murine visceral leishmaniasis. Infect Immun. 2005; 73:5286–5289. [PubMed: 16041057]

18. Bertholet S, Goto Y, Carter L, Bhatia A, Howard RF, Carter D, Coler RN, Vedvick TS, Reed SG. Optimized subunit vaccine protects against experimental leishmaniasis. Vaccine. 2009; 27:7036–7045. [PubMed: 19786136]

19. Goto Y, Bogatzki LY, Bertholet S, Coler RN, Reed SG. Protective immunization against visceral leishmaniasis using *Leishmania* sterol 24-c-methyltransferase formulated in adjuvant. Vaccine. 2007; 25:7450–7458. [PubMed: 17804125]

20. Chakravarty J, Kumar S, Trivedi S, Rai VK, Singh A, Ashman JA, Laughlin EM, Coler RN, Kahn SJ, Beckmann AM, Cowgill KD, Reed SG, Sundar S, Piazza FM. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine for use in the prevention of visceral leishmaniasis. Vaccine. 2011; 29:3531–3537. [PubMed: 21414377]

21. Modabber, F.; Reed, S.; Campos-Neto, A. Vaccines against *Leishmania*. In: Levine, Myron M.; Kaper, James B.; Rappuoli, Rino; Liu, Margaret A.; Good, Michael F., editors. New Generation Vaccines. 3rd. ed.. New York, Basel: Mar Dekker, Inc.; 2004. p. 903-914.

22. Genaro O, de Toledo VP, da Costa CA, Hermeto MV, Afonso LC, Mayrink W. Vaccine for prophylaxis and immunotherapy, Brazil. Clin Dermatol. 1996; 14:503–512. [PubMed: 8889328]

23. Gunders AE, Naggan L, Michaeli D. Follow-up study of a vaccination programme against cutaneous leishmaniasis. I. Vaccination with a 5 year-old human strain of *L. tropica* from the Negev. Trans R Soc Trop Med Hyg. 1972; 66:235–238. [PubMed: 5048789]

24. Khalil EA, El Hassan AM, Zijlstra EE, Mukhtar MM, Ghalib HW, Musa B, Ibrahim ME, Kamil AA, Elsheikh M, Babiker A, Modabber F. Autoclaved *Leishmania major* vaccine for prevention of visceral leishmaniasis: a randomised, double-blind, BCG-controlled trial in Sudan. Lancet. 2000; 356:1565–1569. [PubMed: 11075771]

25. Sharifi I, FeKri AR, Aflatonian MR, Khamesipour A, Nadim A, Mousavi MR, Momeni AZ, Dowlati Y, Godal T, Zicker F, Smith PG, Modabber F. Randomised vaccine trial of single dose of killed *Leishmania major* plus BCG against anthroponotic cutaneous leishmaniasis in Bam, Iran. Lancet. 1998; 351:1540–1543. [PubMed: 10326536]

26. Campos-Neto A, Porrozzi R, Greeson K, Coler RN, Webb JR, Skeiky YA, Reed SG, Grimaldi G Jr. Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. Infect Immun. 2001; 69:4103–4108. [PubMed: 11349082]

27. Campos-Neto, A. Anti-leishmania vaccine. In: Farrell, Jay P., editor. Leishmania. Vol. vol. 4. Kluwer Academic Publishers; 2002. p. 169-190.

28. Kenney RT, Sacks DL, Sypek JP, Vilela L, Gam AA, Evans-Davis K. Protective immunity using recombinant human IL-12 and alum as adjuvants in a primate model of cutaneous leishmaniasis. J Immunol. 1999; 163:4481–4488. [PubMed: 10510390]

29. Nascimento E, Fernandes DF, Vieira EP, Campos-Neto A, Ashman JA, Alves FP, Coler RN, Bogatzki LY, Kahn SJ, Beckmann AM, Pine SO, Cowgill KD, Reed SG, Piazza FM. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+MPL-SE vaccine when used in combination with meglumine antimoniate for the treatment of cutaneous leishmaniasis. Vaccine. 2010; 28:6581–6587. [PubMed: 20688040]

30. Kashino SS, Pollock N, Napolitano DR, Rodrigues V Jr, Campos-Neto A. Identification and characterization of *Mycobacterium tuberculosis* antigens in urine of patients with active pulmonary tuberculosis: an innovative and alternative approach of antigen discovery of useful microbial molecules. Clin Exp Immunol. 2008; 153:56–62. [PubMed: 18460016]

31. Mukherjee S, Kashino SS, Zhang Y, Daifalla N, Rodrigues V Jr, Reed SG, Campos-Neto A. Cloning of the gene encoding a protective *Mycobacterium tuberculosis* secreted protein detected in vivo during the initial phases of the infectious process. J Immunol. 2005; 175:5298–5305. [PubMed: 16210635]

32. Miyaki C, Quintilio W, Miyaji EN, Botosso VF, Kubrusly FS, Santos FL, Iourtov D, Higashi HG, Raw I. Production of H5N1 (NIBRG-14) inactivated whole virus and split virion influenza vaccines and analysis of immunogenicity in mice using different adjuvant formulations. Vaccine. 2010; 28:2505–2509. [PubMed: 20123051]

33. Quintilio W, Kubrusly FS, Iourtov D, Miyaki C, Sakauchi MA, Lucio F, Dias SC, Takata CS, Miyaji EN, Higashi HG, Leite LC, Raw I. *Bordetella pertussis* monophosphoryl lipid A as adjuvant for inactivated split virion influenza vaccine in mice. Vaccine. 2009; 27:4219–4224. [PubMed: 19393709]

34. Titus RG, Marchand M, Boon T, Louis JA. A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. Parasite Immunol. 1985; 7:545–555. [PubMed: 3877902]

35. Cabral SM, Silvestre RL, Santarem NM, Tavares JC, Silva AF, Cordeiroda-Silva A. A *Leishmania infantum* cytosolic tryparedoxin activates B cells to secrete interleukin-10 and specific immunoglobulin. Immunology. 2008; 123:555–565. [PubMed: 18028371]

36. Marin C, Longoni SS, Urbano J, Minaya G, Mateo H, de Diego JA, Rosales MJ, Perez-Cordon G, Romero D, Sanchez-Moreno M. Enzyme-linked immunosorbent assay for superoxide dismutase-excreted antigen in diagnosis of sylvatic and Andean cutaneous leishmaniasis of Peru. Am J Trop Med Hyg. 2009; 80:55–60. [PubMed: 19141840]

37. Ismail SO, Skeiky YA, Bhatia A, Omara-Opyene LA, Gedamu L. Molecular cloning, characterization, and expression in *Escherichia coli* of iron superoxide dismutase cDNA from *Leishmania donovani* chagasi. Infect Immun. 1994; 62:657–664. [PubMed: 8300222]

38. Casanova M, Portales P, Blaineau C, Crobu L, Bastien P, Pages M. Inhibition of active nuclear transport is an intrinsic trigger of programmed cell death in trypanosomatids. Cell Death Differ. 2008; 15:1910–1920. [PubMed: 19011643]

39. Palatnik-de-Sousa CB, Barbosa AF, Oliveira SM, Nico D, Bernardo RR, Santos WR, Rodrigues MM, Soares I, Borja-Cabrera GP. FML vaccine against canine visceral leishmaniasis: from second-generation to synthetic vaccine. Expert Rev Vaccines. 2008; 7:833–851. [PubMed: 18665780]

40. Saraiva EM, de Figueiredo BA, Santos FN, Borja-Cabrera GP, Nico D, Souza LO, de OM-A, de Souza EP, Fampa P, Parra LE, Menz I, Dias JG Jr, de Oliveira SM, Palatnik-de-Sousa CB. The FML-vaccine (Leishmune) against canine visceral leishmaniasis: a transmission blocking vaccine. Vaccine. 2006; 24:2423–2431. [PubMed: 16386824]

41. Schaffner A, Michel-Harder C, Yeginsoy S. Detection of capsular polysaccharide in serum for the diagnosis of pneumococcal pneumonia: clinical and experimental evaluation. J Infect Dis. 1991; 163:1094–1102. [PubMed: 1826918]

42. Socan M, Marinic-Fiser N, Kese D. Comparison of serologic tests with urinary antigen detection for diagnosis of legionnaires' disease in patients with community-acquired pneumonia. Clin Microbiol Infect. 1999; 5:201–204. [PubMed: 11856250]

43. Helbig JH, Uldum SA, Bernander S, Luck PC, Wewalka G, Abraham B, Gaia V, Harrison TG. Clinical utility of urinary antigen detection for diagnosis of community-acquired, travel-associated, and nosocomial legionnaires' disease. J Clin Microbiol. 2003; 41:838–840. [PubMed: 12574296]

44. Grundy MS. Preliminary observations using a multi-layer ELISA method for the detection of *Entamoeba histolytica* trophozoite antigens in stool samples. Trans R Soc Trop Med Hyg. 1982; 76:396–400. [PubMed: 6287685]

45. Pillai S, Mohimen A. A solid-phase sandwich radioimmunoassay for *Entamoeba histolytica* proteins and the detection of circulating antigens in amoebiasis. Gastroenterology. 1982; 83:1210–1216. [PubMed: 6290308]

46. Karki BM, Parija SC. Co-agglutination test for the detection of circulating antigen in amebic liver abscess. Am J Trop Med Hyg. 1999; 60:498–501. [PubMed: 10466984]

47. Attar ZJ, Chance ML, El-Safi S, Carney J, Azazy A, El-Hadi M, Dourado C, Hommel M. Latex agglutination test for the detection of urinary antigens in visceral leishmaniasis. Acta Trop. 2001; 78:11–16. [PubMed: 11164745]

48. Diro E, Techane Y, Tefera T, Assefa Y, Kebede T, Genetu A, Kebede Y, Tesfaye A, Ergicho B, Gebre-Yohannes A, Mengistu G, Engers H, Aseffa A, Desjeux P, Boelaert M, Hailu A. Field evaluation of FD-DAT, rK39 dipstick and KATEX (urine latex agglutination) for diagnosis of visceral leishmaniasis in northwest Ethiopia. Trans R Soc Trop Med Hyg. 2007; 101:908–914. [PubMed: 17624385]

49. Riera C, Fisa R, Lopez P, Ribera E, Carrio J, Falco V, Molina I, Gallego M, Portus M. Evaluation of a latex agglutination test (KAtex) for detection of *Leishmania* antigen in urine of patients with HIV-*Leishmania* coinfection: value in diagnosis and post-treatment follow-up. Eur J Clin Microbiol Infect Dis. 2004; 23:899–904. [PubMed: 15599651]

50. Salam MA, Khan MG, Mondal D. Urine antigen detection by latex agglutination test for diagnosis and assessment of initial cure of visceral leishmaniasis. Trans R Soc Trop Med Hyg. 2011; 105:269–272. [PubMed: 21353275]

51. Sarkari B, Chance M, Hommel M. Antigenuria in visceral leishmaniasis: detection and partial characterisation of a carbohydrate antigen. Acta Trop. 2002; 82:339–348. [PubMed: 12039673]

52. Sundar S, Agrawal S, Pai K, Chance M, Hommel M. Detection of leishmanial antigen in the urine of patients with visceral leishmaniasis by a latex agglutination test. Am J Trop Med Hyg. 2005; 73:269–271. [PubMed: 16103587]

53. Coler RN, Skeiky YA, Bernards K, Greeson K, Carter D, Cornellison CD, Modabber F, Campos-Neto A, Reed SG. Immunization with a Polyprotein Vaccine Consisting of the T-Cell Antigens Thiol-Specific Antioxidant, *Leishmania major* Stress-Inducible Protein 1, and Leishmania Elongation Initiation Factor Protects against Leishmaniasis. Infect Immun. 2002; 70:4215–4225. [PubMed: 12117930]

54. Reed SG, Coler RN, Campos-Neto A. Development of a leishmaniasis vaccine: the importance of MPL. Expert Rev Vaccines. 2003; 2:239–252. [PubMed: 12899575]

55. Skeiky YA, Coler RN, Brannon M, Stromberg E, Greeson K, Crane RT, Webb JR, Campos-Neto A, Reed SG. Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. Vaccine. 2002; 20:3292–3303. [PubMed: 12213399]

56. Fujiwara RT, Vale AM, Franca da Silva JC, Da Costa RT, Quetz JS, Martins Filho OA, Reis AB, Correa OR, hado-Coelho GL, Bueno LL, Bethony JM, Frank G, Nascimento E, Genaro O, Mayrink W, Reed S, Campos-Neto A. Immunogenicity in dogs of three recombinant antigens (TSA, LeIF and LmSTI1) potential vaccine candidates for canine visceral leishmaniasis. Vet Res. 2005; 36:827–838. [PubMed: 16120256]

57. Miret J, Nascimento E, Sampaio W, Franca JC, Fujiwara RT, Vale A, Dias ES, Vieira E, Da Costa RT, Mayrink W, Campos NA, Reed S. Evaluation of an immunochemotherapeutic protocol constituted of N-methyl meglumine antimoniate (Glucantime) and the recombinant Leish-110f + MPL-SE vaccine to treat canine visceral leishmaniasis. Vaccine. 2008; 26:1585–1594. [PubMed: 18328956]

58. Stevens TL, Bossie A, Sanders VM, Fernandez-Botran R, Coffman RL, Mosmann TR, Vitetta ES. Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. Nature. 1988; 334:255–258. [PubMed: 2456466]

59. Faquim-Mauro EL, Coffman RL, Abrahamsohn IA, Macedo MS. Cutting edge: mouse IgG1 antibodies comprise two functionally distinct types that are differentially regulated by IL-4 and IL-12. J Immunol. 1999; 163:3572–3576. [PubMed: 10490948]

60. Stager S, Smith DF, Kaye PM. Immunization with a recombinant stage-regulated surface protein from *Leishmania donovani* induces protection against visceral leishmaniasis. J Immunol. 2000; 165:7064–7071. [PubMed: 11120835]

61. Rollier CS, Reyes-Sandoval A, Cottingham MG, Ewer K, Hill AV. Viral vectors as vaccine platforms: deployment in sight. Curr Opin Immunol. 2011; 23:377–382. [PubMed: 21514130]

62. Draper SJ, Biswas S, Spencer AJ, Remarque EJ, Capone S, Naddeo M, Dicks MD, Faber BW, de Cassan SC, Folgori A, Nicosia A, Gilbert SC, Hill AV. Enhancing blood-stage malaria subunit vaccine immunogenicity in rhesus macaques by combining adenovirus, poxvirus, and protein-in-adjuvant vaccines. J Immunol. 2010; 185:7583–7595. [PubMed: 21098232]

VL Patient

↓

Urine

↓

Sequence peptides by mass spectrometry

↓

BLAST sequences against *L. infantum chagasi* and human protein databases using Sequest software

↓

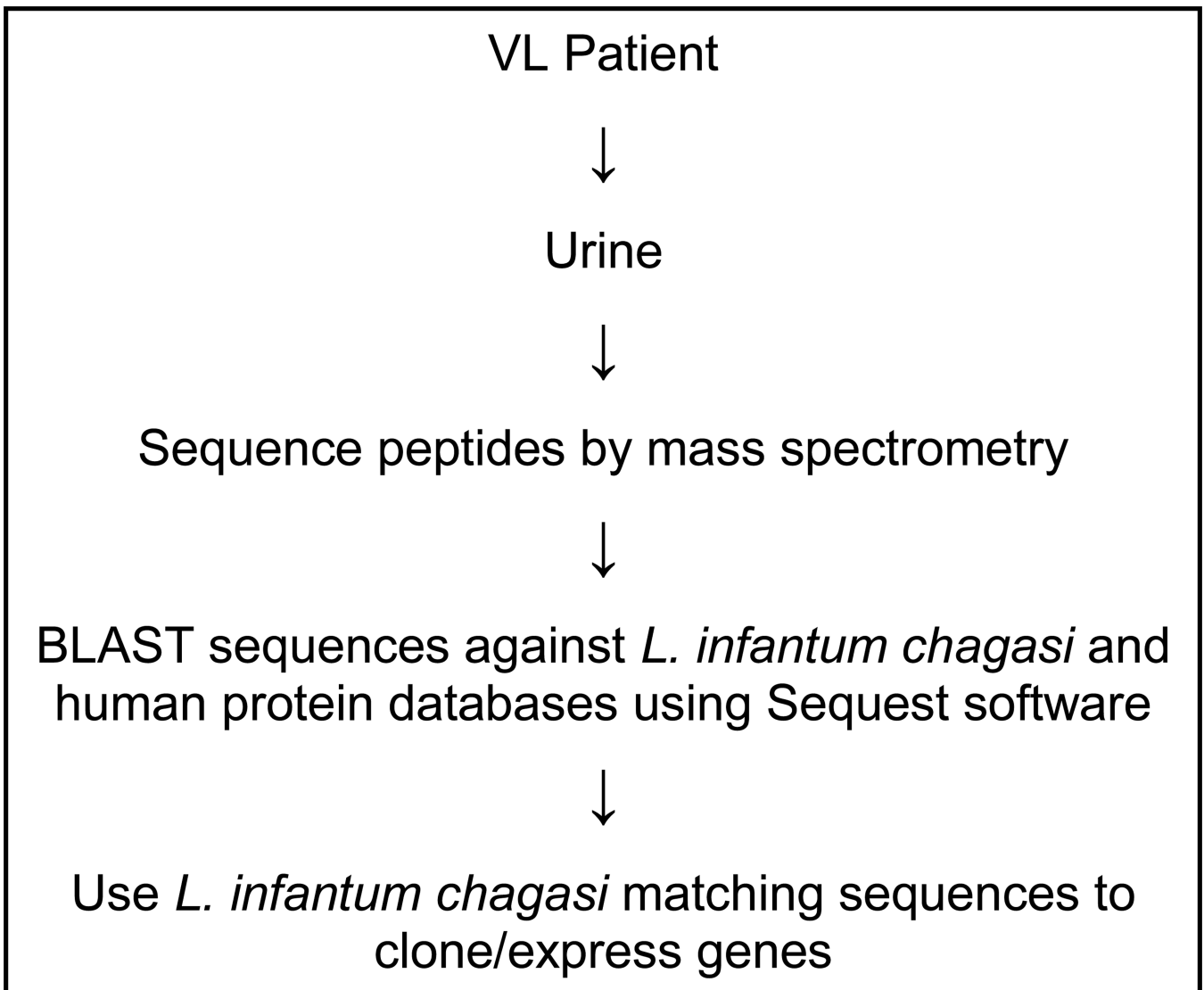Use *L. infantum chagasi* matching sequences to clone/express genes

Figure 1. Schematic representation of antigen discovery strategy of *L. infantum chagasi* proteins produced *in vivo* and excreted in urine of patients with active VL

Protein 1: *Leishmania infantum* iron superoxide dismutase (NCBI accession: XP_001467866.1; Gene ID EuPathDB: LinJ.32.1910)

MPFAVQPLPYPHDALASKGMSKEQVTFHHEKHHKGYAVK**LNAAAESNSGLASK**SL
VDIIKSEKGPAFNCAAQIFNHDFFWRCLSPR**GGGEPSGPLASAIVDSFGSFASFK**KE
FTDAANGHFGSGWAWLVKDKSSGKLKVLQTHDAGCPLTEPNLVPILTCDIWEHAYY
IDYRNDRASYVNAFWNMVDWDFASSQL

## Protein 2: *Leishmania infantum* tryparedoxin (NCBI accession: XP_001466642.1; Gene ID EuPathDB: LinJ.29.1250)

MSGVSKHLGDVLKLQK**QNDMVDMSSLSGK**TVFLYFSASWCPPCRGFTPKLVEFYE
KHHNSKNFEIILASWDEEEEDFNGYYSK**MPWLSIPFEK**RNVVEALTK**QYKVESIPTLI
GLNADTGDTVTTR**ARHALTQDPEGEQFPWRDE

Protein 3: *Leishmania infantum* nuclear transport factor 2 (NCBI accession XP_001463738.1; Gene ID EuPathDB: LinJ.10.0900)

MSFEDVGVGFVQHYYNFFANQRDQLAGIYRPNTLLTWQK**EQVQGVDAIMAR
FANLGFTEAAFK**QDSIDCQPSMSGGVIVIVNGEVKLRGEDHSLKFNDFFHLAQDNG
QWYVSNQVFNLVGGGGQ

Figure 2. *L. infantum chagasi* **peptide sequences discovered in urine of VL patients**
Peptide sequences and positioning within the peptide donor protein sequences are
highlighted in red/bold/underline. Note that several different peptides spanning at different
sequence positions of the donor proteins were identified, which unambiguously define them
as truly *L. infantum chagasi* peptides/proteins.
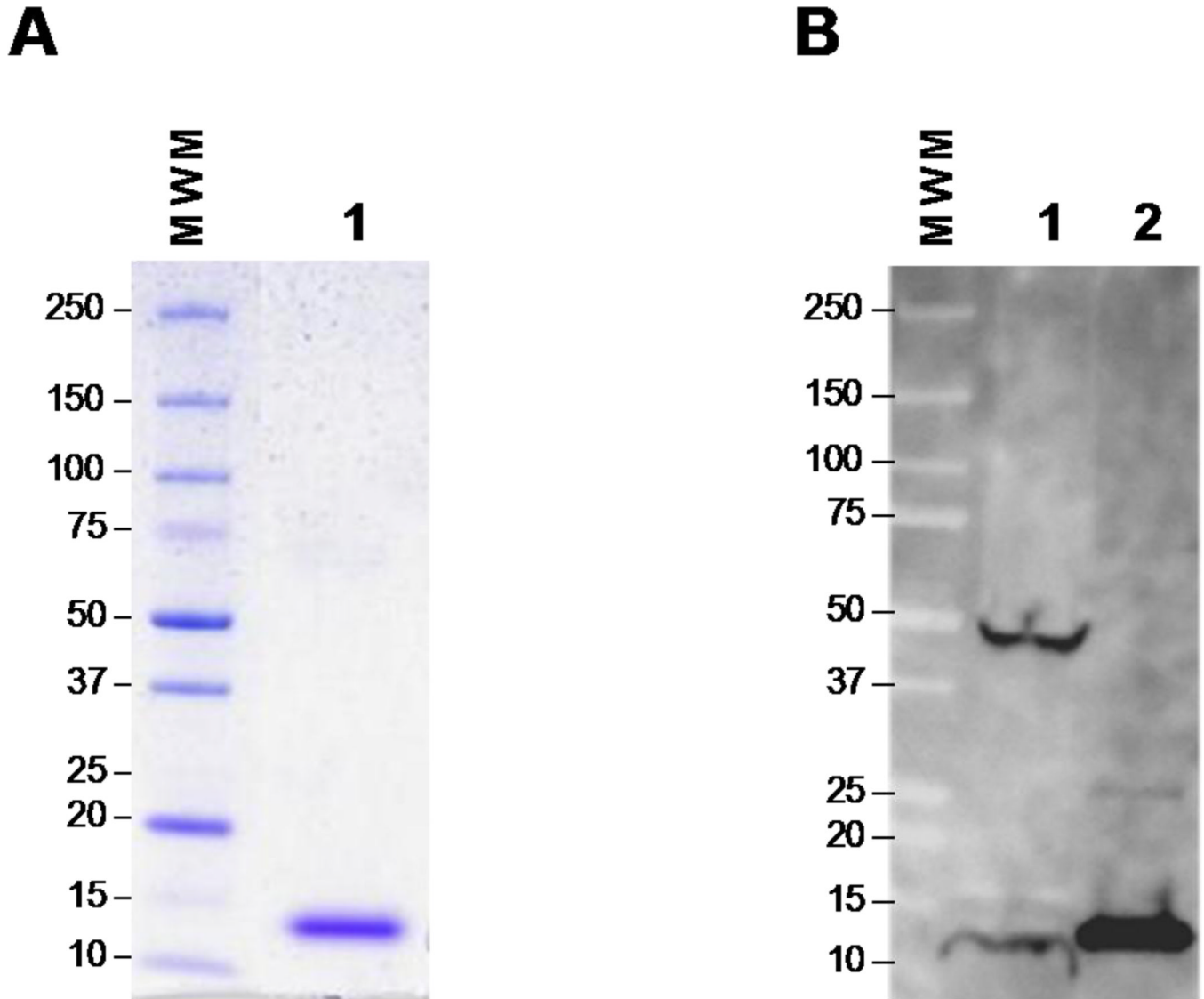
Figure 3. Characterization of purified recombinant *Li-ntf2*
Purity of the protein was evaluated by SDS-PAGE (4–20% gradient polyacrylamide gel) and
Coomassie blue staining (A). Characterization was done by Western blot analysis (B) using
a crude antigenic preparations of *L. infantum* promastigotes (lane 1) and purified
recombinant *Li-ntf2* (lane 2). Proteins were identified using specific rabbit anti-recombinant
Li-ntf2 antiserum. Reactivity was detected with goat anti-rabbit IgG labeled with
horseradish peroxidase followed by autoradiography. Numbers on the left side indicate the
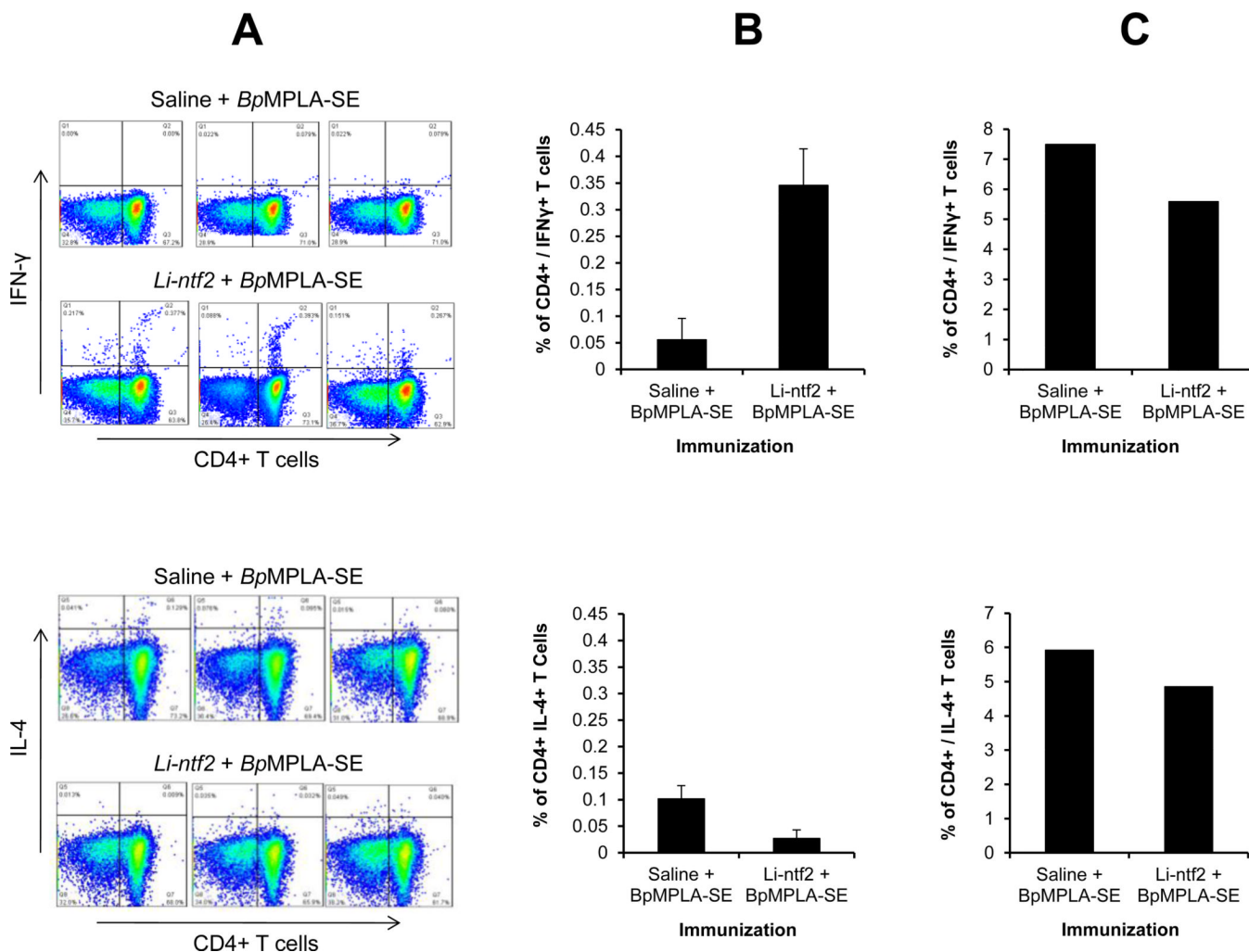molecular weights of the markers.
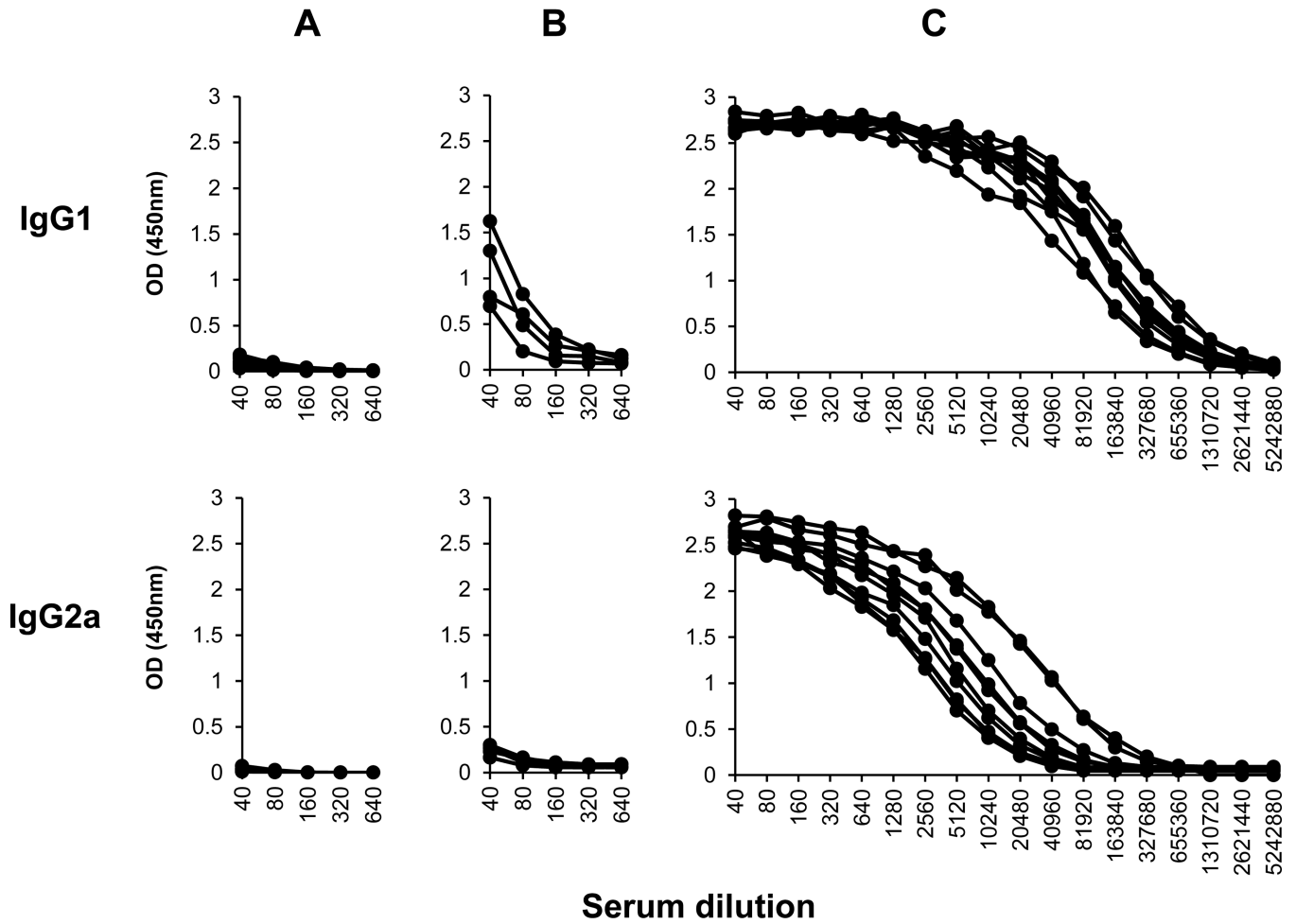
Figure 4. Isotype specific antibody response of mice immunized with purified recombinant *Li-ntf2* formulated with the adjuvant MPLA-SE

Ten mice were immunized three times (three weeks apart) **with saline plus the adjuvant *Bp*MPLA-SE (A), or with the *Li-ntf2* in saline (B), or with *Li-ntf2* mixed with *Bp*MPLA-SE (C).** Animals were bled 10 days after the last immunization and antibody responses of IgG1 and IgG2a isotypes were measured by ELISA using biotinylated isotype rat mAbs specific for mouse IgG isotypes. The curves represent the responses of each individual mouse.
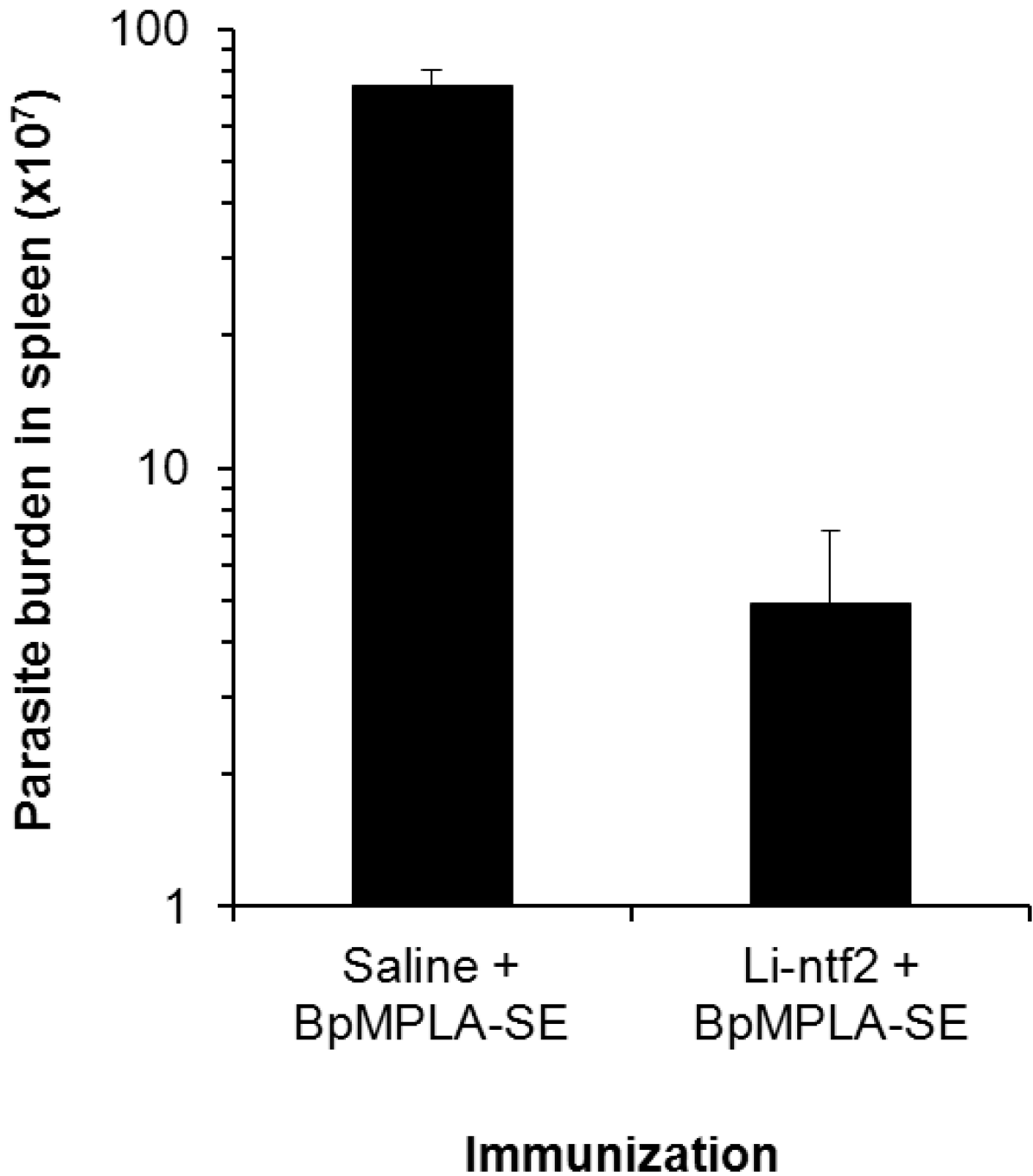
A

B
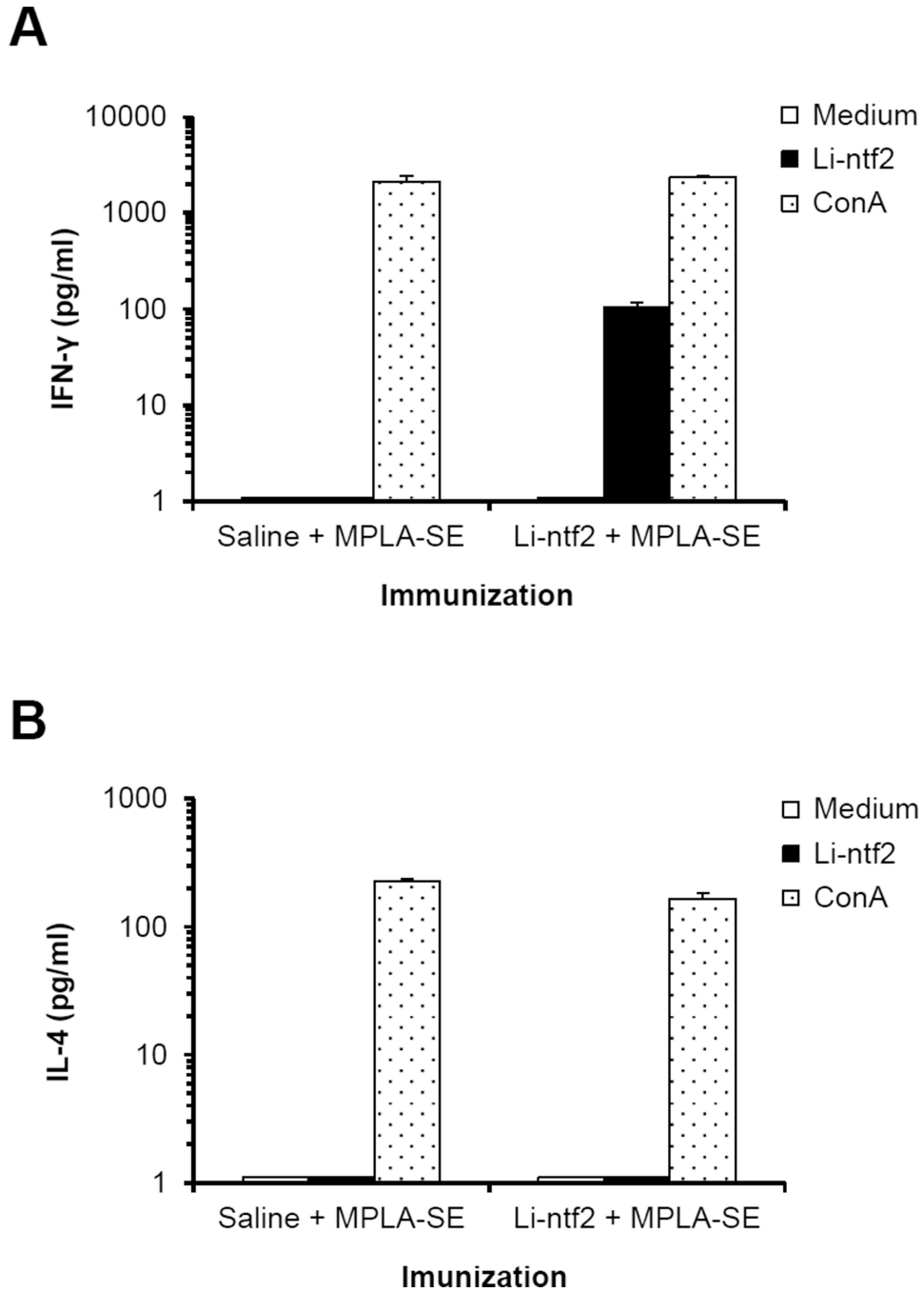
Figure 5. Cytokine response of spleen cells from mice immunized with purified recombinant *Li-ntf2* formulated with the adjuvant *Bp*MPLA-SE
BALB/c mice (three) were immunized as described in Fig. 4. Spleen cells were harvested 10 days after the last immunization either with saline + *Bp*MPLA-SE or purified recombinant *Lintf2* + *Bp*MPLA-SE. Cells were cultured for 72h in the presence of medium only, or with 5μg/ml of the recombinant protein, or with 5μg/ml of ConA;. Culture supernatants were collected and assayed for the presence of IFN-γ and IL-4 by sandwich ELISA.

Figure 6. Phenotype of CD4+ T cell response of mice immunized with purified recombinant *Li-ntf2* formulated with the adjuvant *Bp*MPLA-SE

Three mice were immunized as described in Fig. 4. Spleen cells were harvested 10 days after the last immunization either with saline + *Bp*MPLA-SE or purified recombinant *Li-ntf2* + *Bp*MPLA-SE. Production of IFN-γ (Upper panel) and IL-4 (Lower panel) by CD4+ T cells was determined by intracellular cytokine staining and analyzed by flow cytometry. (A), Dot plots represent the responses of individual mouse in each group after stimulation with *Li-ntf2*; (B) Percentage double positive CD4+ / IFN-γ+ T cells (upper panel) or CD4+ / IL-4+ T cells (lower panel) stimulated with the *Li-ntf2* (average of responses of cells from three mice per group); and (C) Percentage double positive CD4+ / IFN-γ+ T cells (upper panel) or CD4+ / IL-4+ T cells (lower panel) stimulated with the polyclonal activators PMA + Ionomicin (average of responses of cells from three mice per group). Note the abundance of CD4+ / IFN-γ+ double positive cells and the scarce presence of CD4+ / IL4+ double positive cells in cultures obtained from mice immunized with *Li-ntf2* + *Bp*MPLA-SE and re-stimulated *in vitro* with *Li-ntf2*. Cells from both groups of immunized mice produced abundant quantities of both IFN-γ and IL-4 when stimulated with PMA + Ionomicin. Little or no cytokine producing cells were observed in the cell cultures from animals immunized with saline + *Bp*MPLA-SE and equally re-stimulated *in vitro* with the antigen. Similarly, no cytokine producing cells were detected in any culture stimulated with medium only (not shown).

Figure 7. Vaccination of mice against VL with purified recombinant *Li-ntf2* formulated with the adjuvant *Bp*MPLA-SE

BALB/c mice (7 animals per group) were immunized three times as described in Figure 4. As control, mice (n=7) were immunized with saline plus *Bp*MPLA-SE. One month after the last immunization the animals were challenged i.v. with 10×10^6 promastigote forms (stationary phase of growth) of *L. infantum chagasi*. Parasite burden in the animals' spleens was determined 40 days later by limiting dilution assay. Bars represent SEM of the results given by 7 mice per group. Student t test was used to compare the average of parasite burden observed for animals immunized with *Li-nft2 + Bp*MPLA-SE with that of animals immunized with Saline + *Bp*MPLA-SE. ρ value was <0.005.