Vaccine 26 (2008) 5700–5711

Contents lists available at ScienceDirect

Vaccine

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

Th1-stimulatory polyproteins of soluble *Leishmania donovani* promastigotes ranging from 89.9 to 97.1 kDa offers long-lasting protection against experimental visceral leishmaniasis

Shraddha Kumari[a], Mukesh Samant[a], Pragya Misra[a], Prashant Khare[a], Brijesh Sisodia[b], Ajit K. Shasany[b], Anuradha Dube[a,*]

[a] *Division of Parasitology, Central Drug Research Institute, Lucknow, India*
[b] *Proteomics Laboratory, Genetic Resources and Biotechnology Division, Central Institute of Medicinal and Aromatic Plants, India*

ARTICLE INFO

Article history:
Received 4 July 2008
Received in revised form 5 August 2008
Accepted 11 August 2008
Available online 30 August 2008

Keywords:
Soluble *Leishmania donovani* promastigotes (SLD)
Th1-stimulatory proteins
Immunoprophylaxis
Hamsters
MALDI-TOF/MS

ABSTRACT

Our earlier studies identified a fraction (F2) of *Leishmania donovani* soluble promastigote antigen belonging to 97.4–68 kDa for its ability to stimulate Th1-type cellular responses in cured visceral leishmaniasis (VL) patients as well as in cured hamsters. A further fractionation of F2-fraction into seven subfractions (F2.1–F2.7) and re-assessment for their immunostimulatory responses revealed that out of these, only four (F2.4–F2.7) belonging to 89.9–97.1 kDa, stimulated remarkable Th1-type cellular responses either individually or in a pooled form (P4-7).

In this study these potential subfractions were further assessed for their prophylactic potential in combination with BCG against *L. donovani* challenge in hamsters. Optimum parasite inhibition (~99%) was obtained in hamsters vaccinated with pooled subfractions and they survived for 1 year. The protection was further supported by remarkable lymphoproliferative, IFN-γ and IL-12 responses along with profound delayed type hypersensitivity and increased levels of *Leishmania*-specific IgG2 antibody as observed on days 45, 90 and 120 post-challenge suggesting that a successful subunit vaccine against VL may require multiple Th1-immunostimulatory proteins. MALDI-TOF–MS/MS analysis of these subfractions further revealed that of the 19 identified immunostimulatory proteins, Elongation factor-2, p45, Heat shock protein-70/83, Aldolase, Enolase, Triosephosphate isomerase, Disulfideisomerase and Calreticulin were the major ones in these subfractions.

1. Introduction

Visceral leishmaniasis (VL), caused by the intracellular parasite *Leishmania donovani*, *L. chagasi* and *L. infantum* is characterized by defective cell-mediated immunity (CMI) and is usually fatal if not treated properly [1]. An estimated 350 million people are at risk of acquiring infection with *Leishmania* parasites worldwide with approximately 500,000 cases of VL has been reported each year. Recent epidemics of VL in Sudan and India have resulted in over 100,000 deaths [2]. With the advent of the HIV epidemics, the disease has emerged as an important opportunistic infection in AIDS patients [1]. In India, high incidence has been reported from the states of Bihar, Assam, West Bengal and eastern Uttar Pradesh where resistance and relapse are on the increase. Available chemotherapy for VL is far from satisfactory because antileishmanial drugs are costly with unpleasant side effects. The situation has further worsened with emergence of drug resistance in various regions of endemicity [1]. Vaccination would, therefore, be a better option for an effective control strategy for VL.

To control Leishmania infections in experimental and human VL, the development of an effective CMI, capable of mounting Th1-type of immune responses, play an important role [3–7]. This is derived from the fact that a large number of people living in endemic areas have self-resolving subclinical infection and the infected individuals once recovered after the treatment are immune to reinfection. This provides a rationale for designing immunoprophylactic strategies against VL [8]. So far, successful immunization with fractionated and purified antigens reported against murine VL and CL has led to very few proteins that have been taken up for preclinical/clinical evaluation [9–12]. Successful second generation vaccines with excreted factors (LiESAp) [13] or purified glycoproteins (FML) [14,15] are used in trials with efficacy and even licensed. There are only few reports in literature deal with vaccines, viz., FML, FML-QuilA Saponin, etc. against canine visceral leishmaniasis

* Corresponding author. Tel.: +91 522 212411/212418x4398; fax: +91 522 223405/223938.
E-mail addresses: anuradha_dube@hotmail.com, anuradha_dube@rediffmail.com (A. Dube).

0264-410X/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.vaccine.2008.08.021

[14,16–20]. Among the recombinant and native antigens tested in murine models, the LACK protein [21], the LPGAP peptides [22], the LeIF protein [6] and the dp72 glycoprotein of *L. donovani* [23] were protective immunogens for mice. Protection results obtained with the third generation vaccines composed of cDNA encoding leishmanial antigens cloned into an eukaryotic expression vector are still in preliminary stage [24].

Although a great number of antigens have been tested for protection against the cutaneous disease with *in vitro* cell or mouse models, no effective vaccine against human kala-azar is yet available. The reason perhaps being that the cell epitopes that were protective in the murine model did not elicit an adequate effective immune response in human [25–27]. Hence, the evaluation of potential leishmanial antigenic proteins for their ability to elicit cellular immune responses in humans as well as in experimental model was considered important [28,29] before they are further evaluated for their prophylactic potential against experimental VL. The golden hamster (*Mesocricetus auratus*) has been proven to be the most appropriate experimental model as it largely reflects the clinic-pathological features of progressive human VL, including a relentless increase in visceral parasitic burden, cachexia, hepatosplenomegaly, pancytopenia, hypergammaglobulinemia and ultimately death. Of late, it has also been used extensively for vaccination studies [28,30]. Interestingly, while infection of the hamster with *L. donovani* reproduced the features of human VL, the mechanisms of disease in the hamster differ strikingly from those obtained in mice, a suitable experimental model for early infection of VL as the infection tends to self-cure in this rodent model [31].

The main focus in vaccine development has been the identification and characterization of defined leishmanial antigens as well as the elucidation of the range and specificity of antileishmanial immune responses. Advances made in clinical proteomic technologies have further enhanced our mechanistic understanding of leishmanial pathobiology thereby defining novel vaccine targets [28,32,33]. The evaluation of such vaccine targets for their prophylactic potential will provide further lead towards the development of a candidate vaccine(s).

We have further fractionated the Th1-stimulatory fraction of 68–97.4 kDa (F2) of soluble protein of *L. donovani* promastigotes by continuous elution gel electrophoresis (Prep-Cell) on the basis of molecular weight and there of obtained seven subfractions (F2.1–F2.7) belonging to 69.0, 74.9, 78.4, 89.9, 94.9, 96.9, 97.1 kDa, respectively [86]. Out of seven subfractions only four subfractions of 89.9, 94.9, 96.9, 97.1 kDa stimulated remarkable cellular responses, i.e. lymphoproliferative, IFN-γ and IL-12 responses and suppressed IL-10 cytokine levels in cured/exposed VL patients as well as in cured *Leishmania* infected hamsters. Interestingly, all of these as pooled subfractions yielded optimum efficacy as compared to the individual ones.

In this study, these potential antigenic subfractions were assessed for their prophylactic efficacy alongwith Bacillus Calmette Guerin (BCG)—an adjuvant, against *L. donovani* infection in hamsters. Those subfractions which exhibited significant cellular responses as well as prophylactic potential were further characterized by MALDI-TOF/TOF-MS so as to ensure the protein contents as prospective vaccine targets.

2. Materials and methods

2.1. Animal and parasites

Laboratory bred male golden hamsters (*M. auratus*, 45–50 g) from the Institute's Animal House Facility were used as experimental host. They were housed in climatically controlled room and fed with standard rodent food pellet (Lipton India Ltd., Bombay) and water *ad libitum*. The usage of hamsters was approved by the Institute's Animal Ethical Committee (protocol number 24/05/Para/IAEC dated 15 September 2005).

2.2. Parasites

The *L. donovani* strain (2001) was procured from a patient admitted to the Kala-azar Medical Research Centre of the Institute of Medical Sciences, BHU, Varanasi and was cultured *in vitro* as described elsewhere [28]. Promastigotes were grown in L-15 medium at 26 °C (Sigma, USA) in 75 cm^2 culture flasks (Nunc) [34]. The strain has also been maintained in hamsters through serial passage, i.e. from amastigote to amastigote [34].

2.3. Preparation of soluble L. donovani promastigote (SLD) antigen

SLD was prepared as per method described by Gupta et al. [33,34]. Briefly, late log phase promastigotes (10^9) were harvested from 3 to 4 days of culture and washed four times in cold phosphate-buffered saline (PBS) and resuspended in PBS containing protease inhibitors cocktail (Sigma, USA) and subjected to ultrasonication and centrifugation at 40,000 × *g* for 30 min. The protein content of the supernatant was estimated [35] and stored at −70 °C.

2.4. Subfractionation of F2-fraction by continuous elution gel electrophoresis

Subfractionation of F2-fraction was done by continuous elution SDS-PAGE using Laemmli's buffer system in a 'Prep-Cell' (Model 491; BioRad, Hercules, CA) containing fraction collector [36]. Prior to this, a resolving gel concentration suitable for the whole range of proteins was determined by running a series of SDS-PAGE on mini slab gel. Accordingly, 8% resolving gel and 4% stacking gel were cast in tube. Lyophilized protein (10 mg) was loaded under standard condition [37] and electrophoresis was carried out as per protocol mentioned in manufacturer's manual, Total 120 fractions were collected and each was analyzed consequently on mini gel slabs and silver stained [38] to visualize the eluted proteins. Wide range molecular weight marker (Bangalore Genei, India) was used to identify and assess the exact molecular weights of protein bands and their number and density were assessed by software AlphaImager™2200. Fraction number 40–100 displayed the protein bands ranging between the desired molecular weight of 68–97.4 kDa. The bands with identical molecular weight were pooled in such a way so as to provide seven discrete subfractions F2.1, F2.2, F2.3, F2.4, F2.5, F2.6 and F2.7 [86]. The subfractions were further processed for SDS removal by the method of Wessel and Flügge [39]. The removal of SDS was checked by colorimetric estimation using the method of Arand et al. [40]. Protein quantification was done by Lowry's method [35].

The most potent subfractions (F2.4–F2.7) were taken both individually as well as in pooled form (referred as P4-7 subfraction) for vaccination studies. In addition, the subfractions F2.1–F2.7 were also pooled into one and used as reference antigen (F2).

2.5. Vaccination

Nine groups containing 12–15 hamsters in each were employed for the immunization with different preparations of antigenic fractions. The hamsters of Groups 1–7 were immunized intradermally on the back with each subfractions F2.4, F2.5, F2.6, F2.7, P4-7, F2-fraction and SLD (50 μg/(0.05 ml animal)) along with equal volume of BCG (0.1 mg/(0.05 ml animal)) in emulsified form. The eighth

group was given BCG only and the last ninth group which received only PBS served as control. Fifteen days later a booster dose of half of the amount of antigens along with BCG or PBS was given intradermally to all the hamsters of respective experimental groups (i.e. Groups 1–8) as mentioned above.

2.6. Infection

Twenty-one days after the booster dose, all the vaccinated and unvaccinated control groups were challenged intracardially with 10^8 late log phase promastigotes of *L. donovani*. The prophylactic efficacy of the immunogen was assessed in spleen, liver and bone marrow of three vaccinated hamsters on necropsy at different time intervals, i.e. on days 0, 45, 90, 120 post-challenge (p.c.). Peritoneal exudates cells, inguinal lymph nodes and blood were also collected at these time points to obtain cells and sera for evaluation of cellular and antibody responses [41].

The criterion of prophylactic efficacy was the assessment of parasite load as the number of amastigotes/1000 cell nuclei in each organ in comparison to the unvaccinated controls and the percentage inhibition (PI), was assessed in comparison to the unvaccinated control by following formula [28]:

$$\mathrm{PI} = \frac{\text{No. of parasite count from infected control} - \text{No. of parasite from vaccinated group}}{\text{No. of parasite count from infected control}} \times 100$$

For post-challenge survival animals from both the experimental and control groups were given proper care and were observed for their survival period which lasted for more than 12 months p.c. Survival of individual hamster was recorded and mean survival period was calculated.

2.7. Immunological assays

2.7.1. Delayed type hypersensitivity (DTH)

DTH was performed by injecting intradermally 50 μg/50 μl of SLD in PBS into one footpad and PBS alone into the other footpad of each vaccinated hamsters and unvaccinated controls. The response was evaluated 24 h later by measuring the difference in footpad swelling between each of the vaccinated and control groups of hamsters [4].

2.7.2. Lymphocyte proliferation assay (LTT)

Lymphocytes suspension (1×10^6 ml^{-1}) of vaccinated hamsters was cultured in 96-well flat bottom tissue culture plates (Nunc, Denmark).This assay was carried out as per protocol described by Garg et al. [28]. About 100 μl of predetermined concentration of mitogen-Con A (10 μg/ml, Sigma, USA) and antigen-SLD (10 μg/ml each) were added to the wells in triplicate. Wells without stimulants served as blank controls.

Cultures were incubated at 37 °C in a CO_2 incubator (5% CO_2) for 3 days in the case of Con A and for 5 days in the case of SLD. Eighteen hours prior to termination of culture, 0.5 μCi of [^{3}H] thymidine (BARC, India) was added to each well and then they were harvested on glass fibre mats (Whatman) and counted in a liquid scintillation counter. Results were expressed as stimulation index (SI) which was calculated as mean counts per minute (cpm) of stimulated culture/mean cpm of unstimulated control. SI values of more than 2.5 were considered as positive response.

2.7.3. Quantification of NO in macrophages of hamsters and cell lines

The presence of nitrite (NO_2^-) content was assessed using Griess reagent in the culture supernatants of naïve hamster peritoneal macrophages after the exposure with supernatant of stimulated lymphocyte's cultures. Briefly, isolated peritoneal macrophages [42] were suspended in culture medium and plated at 10^6 cells/well and exposed to the supernatants of above described 5-day-old antigen stimulated lymphocyte's cultures from all the study groups. The supernatants (100 μl) collected from macrophage cultures 24 h after incubation was mixed with an equal volume of Griess reagent (Sigma, USA) and left for 10 min at room temperature. The absorbance of the reaction was measured at 540 nm in an ELISA reader [43].

2.7.4. RT-PCR of mRNA cytokines and inducible NO synthase (iNOS)

RT-PCR was performed to assess the expression of various mRNA cytokines and iNOS in splenocytes of main experimental groups as well as unvaccinated control animals. Three representative hamsters from each experimental group were randomly selected to analyze the splenic cytokine profile. RNA from splenocytes of different groups of hamsters was isolated using Tri-reagent (Sigma, USA) on days 45, 90 and 120 p.c. and quantified by using Genequant (Biorad, USA). The primer sequences of cytokine and iNOS primers as described by Melby et al. [44] as mentioned in Table 1 were used to amplify their respective cDNA. HGPRT was used as a house keeping control.

One microgram of total RNA was used for the synthesis of cDNA using first strand cDNA synthesis kit (Fermentas). 0.5 μg of cDNA was amplified by PCR under the following condition: initial denaturation at 95 °C for 2 min, 40 cycles of denaturation step each at 95 °C for 30 s, annealing at 55 °C for 40 s, followed by extension step at 72 °C for 40 s. The final extension step was carried out at 72 °C for 10 min. Further, the densitometric analysis of PCR product was carried out using software AlphaImager^{TM}2200 (Alfainnotech). The same band area was taken for band intensity and was normalized to HGPRT. The mean percentage expression values were represented relative to their corresponding HGPRT values.

2.7.5. Antileishmanial antibody responses

The level of antileishmanial antibody in sera samples from hamsters of both the set of experimental groups was measured as per protocol of Voller et al. [41]. Briefly, ELISA plates (Nunc, Denmark) were coated overnight at 4 °C with 1 μg/ml SLD antigen diluted in 0.02 M phosphate buffer (pH 7.5) and were then blocked with

Table 1
Sequence of reverse and forward primers

S. no.	Primer	Primer sequence	Product size (bp)
1	HGPRT: forward; reverse	5′ATCACATTATGGCCCTCTGTG3′; 5′CTGATAAAATCTACAGTYATGG3′	125
2	iNOS: forward; reverse	5′GCAGAATGTGACCATCATGG3′; 5′CTCGAYCTGGTAGTAGTAGAA3′	198
3	IFNγ: forward; reverse	5′GGATATCTGGAGGAACTGGC3′; 5′CGACTCCTTTTCCGCTTCCT3′	309
4	IL-12: forward; reverse	5′GTACACCTGYCACAAAGGAG3′; 5′GATGTCCCTGATGAAGAAGC3′	430
5	TGFβ: forward; reverse	5′CCCTGGAYACCAACTATTGC3′; 5′ATGTTGGACARCTGCTCCAC3′	310
6	IL-4: forward; reverse	5′CATTGCATYGTTAGCRTCTC3′; 5′TTCCAGGAAGTCTTTCAGTG3′	463
7	IL-10: forward; reverse	5′ ACAATAACTGCACCCACTTC3′; 5′AGGCTTCTATGCAGTTGATG3′	432

Degenerate bases are indicated by the appropriate International Union of Pure and Applied Chemistry designation (Y = C or T, R = A or G).

1% BSA in PBS, after washing with PBS containing 0.05% Tween 20. The optimum dilution of sera for determination of IgG was found to be 1:200 and for IgG1 and IgG2 1:100 in 1% BSA–PBS at 4 °C for 1.5 h. After washing with PBS Tween the plates were incubated for 3 h with HRP-conjugated Goat anti-Hamster IgG (H + L) (1:1000) (Serotec, USA). Parallely, plates were incubated overnight with Biotin-conjugated mouse anti-Hamster IgG1 and mouse anti-Armenian and anti-Syrian hamster IgG2 (BD, Pharmingen) 2 μg/ml in PBS (100 μl/well) and incubated for 3 h according to manufactures instruction. The plates were developed using the OPD substrate (*o*-phenylene diamine dihydrochloride, Sigma) and read at 492 nm using an ELISA reader (BioTek, USA).

2.8. MALDI analysis of potent Th1-stimulatory subfractions

The potential subfractions F2.4–F2.7 were run on one-dimensional PAGE (12% resolving gel, 1.0 mm thick). Bands were separated out and were digested in-gel as per protocol described earlier [33,45]. Briefly, the coomassie blue stained bands were washed, in-gel reduced, S-alkylated and digested with trypsin (Promega, Madison, WI, USA) at 37 °C overnight. Peptides were extracted, dried in a Speed-Vac and resolubilized in 0.1% trifluoroacetic acid. Zip Tips (Millipore) were used to desalt and concentrate the peptide mixture. Peptide mass fingerprinting was performed by spotting 0.3 μl of the extracted protein digest mixed with α-cyano-4-hydroxycinnamic acid (CHCA, Sigma) on a MALDI target plate. MS and MS/MS spectrum were acquired in the positive ion mode on MALDI-TOF/TOF Mass Spectrometer, Applied Biosystems 4700 Proteomics Analyzer (Framingham, MA, USA).

The instrument was operated in the delayed extraction mode with delay time of 200 ns. Spectra were obtained by accumulation of 1000 and 4000 consecutive laser shots respectively in MS and MS/MS mode and laser intensity used were in the range of 5000–6000. Close external calibration for MS was performed with 4700 Cal Mix (Applied Biosystems, USA) a standard mixture. Peak harvesting was carried out using 4000 Series Explorer™ Software (Applied Biosystems, USA). Only baseline corrections were applied to the raw data.

Database searching for protein identifications was performed with mass spectrometry data (MS or MS/MS) using Global Proteome Server v3.5 software (Applied Biosystems, USA) equipped with MASCOT (Matrix Science) search engine. Only monoisotopic masses were used for searching against the Swiss-Prot and NCBInr databases with a minimum number of matched masses set at 4. The maximum peptide precursor tolerance was set at 40 ppm and MS/MS fragment tolerance was defined as 0.2 Da. At most one missed cleavage for tryptic peptides was allowed, and the modifications accepted were carbamidomethyl cysteines as fixed modification and methionine oxidation as variable modification. Tandem MS was performed only in the cases where identification appeared ambiguous with MALDI-TOF–MS data. The criteria used to accept identifications for peptide mass fingerprint included the probabilistic protein score-based confidence interval %, the extent of sequence coverage, the number of peptides matched and whether *Leishmania* spp. or *Trypanosoma* protein appeared as top candidates during the first search, when no restriction was applied to the species of origin. Identification criteria with MS/MS data were that peptides count should be not less than two or more and confidence interval % for the best ion score should be above 95 [33].

2.9. Statistical analysis

Results were expressed as mean ± S.D. Three sets of experiments were performed for vaccination studies and in each experiment 10–15 animals were used. The results (pooled data of three independent experiments) were analyzed by one-way ANOVA test and comparisons with control data were made with Dunnett's post-test using Graph Pad Prism software program. Comparisons were made between infected control groups and all the experimental groups. The upper level of significance was chosen as $P < 0.001$.

3. Results

3.1. Vaccination with potential subfractions in pooled form induced optimum protection against L. donovani challenge

The potential subfractions vaccinated hamsters were protected from the challenge infection of *L. donovani*, as observed by their weight gains (Fig. 1A) similar to normal hamsters kept simultaneously for the same time period, i.e. on days 45, 90 and 120 p.c. Hepatosplenomegaly, associated with challenge infection, was virtually absent in the potential subfractions vaccinated group (Fig. 1B and C). Parasite load was directly correlated with weight and size of liver and spleens among different groups: significant parasite inhibition was observed in hamsters vaccinated with F2.5, F2.6 and F2.7 subfractions, P4-7 subfractions and F2-fraction wherein the parasite load was observed to be $\leq 2 \times 10^2$. Interestingly, the prophylactic efficacy was more marked in the hamsters immunized with P4-7 subfractions. There was progressive decrease in parasite load in spleen, liver and bone marrow from $<1 \times 10^2$, a more than 96% inhibition of parasite multiplication on day 45 p.c. to a negligible level on day 120 p.c., rendering them difficult to discern, i.e. ~99% parasite inhibition was observed (Fig. 2A–C). F2 and SLD vaccinated hamsters harbored more than 2×10^2 parasites on day 45 p.c. The remaining parasite in the P4-7 pooled subfraction vaccinated group, when checked for their virulence on day 120 p.c. by putting the splenic/liver tissue and lymph nodes in *in vitro* culture, did not convert into promastigotes even after 10 days of culture.

All the vaccinated hamsters immunized with the individual subfractions F2.5–F2.7 and pooled P4-7 subfractions survived longer after the lethal challenge of *L. donovani* and remained healthy until the termination of the experiment at 12 months post-infection. While hamsters immunized with F2.4 subfraction, F2-fraction and SLD survived only till 6 months, all the hamsters which were immunized with BCG alone as well as unimmunized ones succumbed to virulent *L. donovani* challenge within 2–2.5 months.

3.2. Potential subfractions stimulate substantial DTH and mitogenic and Leishmania-specific cellular responses in immunized hamsters

To characterize the fate of cell-mediated immune response following immunization we have investigated the potent subfractions (F2.4–F2.7) induced DTH responses in hamsters challenged with *L. donovani* and the capacity of their cells to proliferate in response to mitogen and leishmania antigen-SLD on days 0, 45, 90 and 120 p.c. Fig. 3A shows that the hamsters receiving potent subfractions either individually or in pooled form displayed significantly stronger DTH response as compared with the other groups, viz., infected control and BCG vaccinated hamsters at these time points. Immunization with P4-7 induced significantly ($P < 0.001$) higher level of DTH response, i.e. eightfold increase as compared to infected control on day 45 p.c. that remained increased significantly through day 90 till day 120 p.c.

In vitro stimulation of the lymphocytes with the mitogen-Con A, showed proliferative responses with no differences between control and antigen immunized groups at pre-challenge time point (Fig. 3B and C). Following challenge, the vaccinated groups showed intact responses to Con A but, on the other hand, it

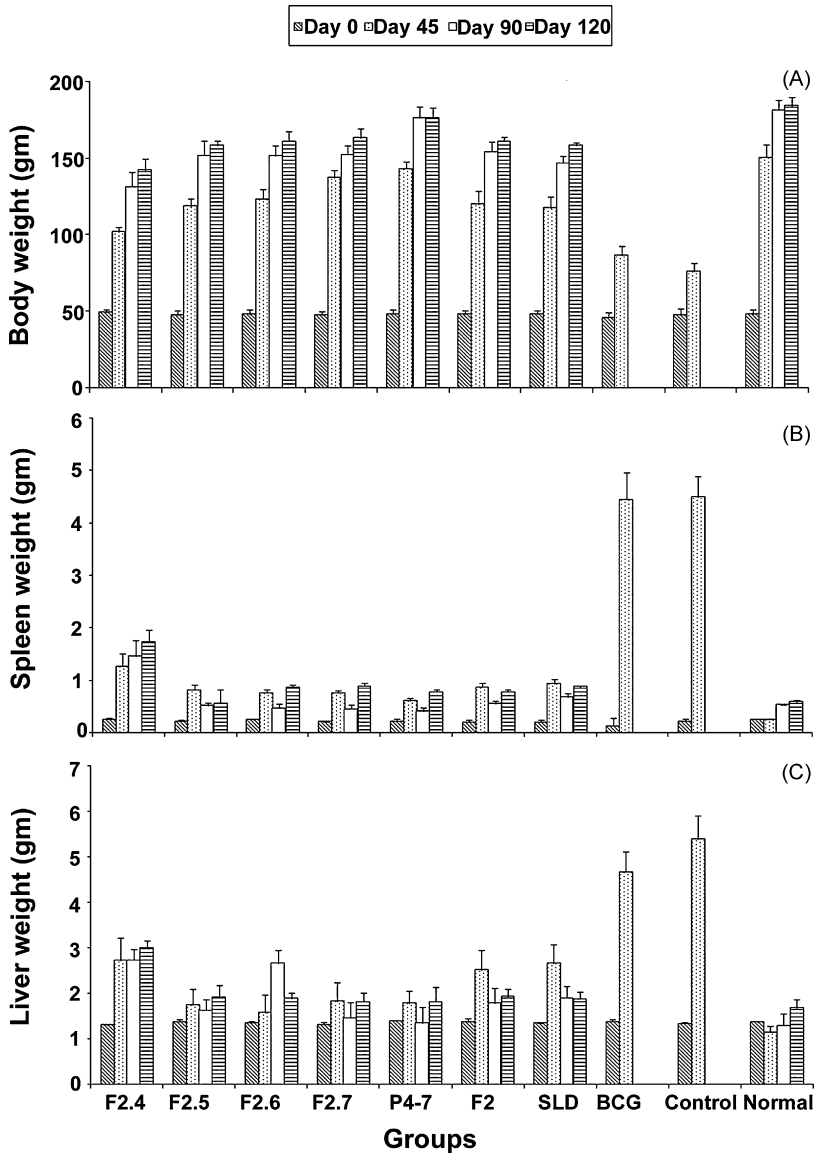

Fig. 1. Body weight (A), weight of spleen (B) and liver (C) in g on days 45, 90 and 120 p.c. of vaccinated hamsters with potent subfractions (F2.4–F2.7), pooled subfraction P4-7, F2-fraction and SLD. Each bar represents the pooled data (mean ± S.D. value) of three replicates.

was lowered in the infected control groups. In antigen-specific re-stimulation assays, performed after immunization, there was significant ($P < 0.001$) stimulatory response in the cells of hamsters vaccinated with potent subfractions F2.4–F2.7 in combination with BCG on day 45 p.c., which was ~10–20 folds higher as compared to the infected control group (Fig. 3C). Moreover, maximum lymphoproliferative response was noticed on day 45 p.c. in the cells of animals immunized with P4-7 subfraction which was ~27 folds higher and the responses increased progressively on days 90 and 120 p.c. On the other hand, there was no proliferative response in animals vaccinated with BCG alone as well as unvaccinated infected control (Fig. 3C).

Similarly, we have observed that macrophages isolated from naïve hamsters, when incubated with stimulated supernatants of lymphocytes from P4-7 subfraction vaccinated hamsters, produced significant amount of NO which was ~7 folds more than the infected controls on day 45 p.c. The NO level was further increased incredibly by days 90 and 120 p.c. In contrast, little amount of NO was produced by the cells of hamsters belonging to unvaccinated control group and BCG only (Fig. 3D).

3.3. Immunization with P4-7 subfraction elicits prominent Th1-type cytokine response in protected group of hamsters by RT-PCR

Impairment of CMI response during active VL is reflected by marked T-cell anergy specific to *Leishmania* antigens [30]. Since optimum protective efficacy was observed in P4-7 vaccinated hamsters, the expression of Th1 and Th2 cytokines was evaluated in this group only and compared with infected and normal control group of animals. A comparative RNA cytokine profile of splenocytes, analyzed on days 45, 90, and 120 p.c., showed that among the three groups of hamsters expression of IFN-γ and IL-12 transcripts was suppressed in infected group, but was significantly higher by two and three folds, respectively ($P < 0.001$) in vaccinated group (Fig. 4A). Higher expression of two to three folds of iNOS transcript was observed in P4-7 vaccinated hamsters as compared to the infected controls on 45 days p.c. (Fig. 4B). Level of expression of Th1 suppressive cytokines, TGF-β, IL-10 and IL-4 which were up-regulated in infected group, were significantly down-regulated in P4-7 vaccinated hamsters by days 45 through 120 p.c. (Fig. 4B).

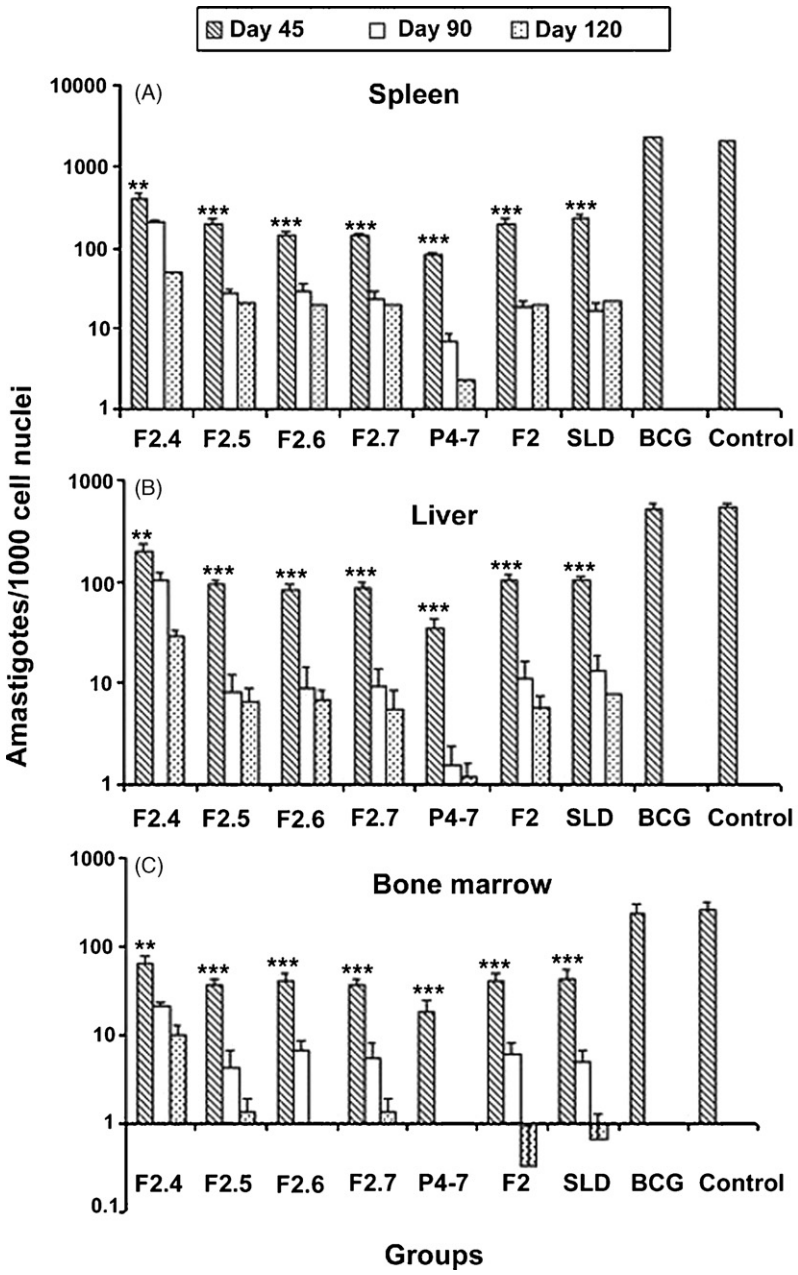

Fig. 2. Parasite burden (no. of amastigotes per 1000 cell nuclei) on days 45, 90 and 120 p.c. in (A) spleen, (B) liver and (C) bone marrow of vaccinated hamsters with potent subfractions (F2.4–F2.7), pooled subfraction P4-7, F2-fraction and SLD. Each bar represents the pooled data (mean ± S.D. value) of three replicates.

3.4. Potent subfractions cause decrease in Leishmania-specific IgG and IgG1 isotype and increase in IgG2 isotype antibody responses in vaccinated hamsters

We have assessed the leishmanial antigen-specific IgG and its isotype (IgG1 and IgG2) levels in the sera of all the groups of vaccinated hamsters through ELISA. As shown in Fig. 5C the groups of hamsters vaccinated with potential subfractions developed an effective immune response by showing substantially higher levels of IgG2 antibody, which is a measure of CMI (Fig. 5C). A highly significant difference (two folds) was found in IgG2 level between P4-7 vaccinated and infected control groups of hamsters ($P < 0.001$). In contrast, there was decreased levels of IgG and IgG1 ($P < 0.5$) in P4-7 vaccinated hamsters as compared to the infected controls (Fig. 5A and B).

3.5. Characterization of the four potent subfractions by 1-DGE and MALDI-TOF–MS

In order to assess the components of the four potent subfractions their characterization by MALDI-TOF–MS was carried out. Majority of the proteins was detected around 4–8 p*I* acidic to neutral pH range. Masses and p*I* were calculated by software; measures of the confidence of the identification on the basis of number of peptides matched and sequence coverage which was determined using MASCOT. The identified spots matched to 168 database entries. Of spots analyzed by MALDI-TOF and MS/MS, 41% were clearly identified by their homology with those of *L. major*. The proteins thus identified are listed in Table 2. The analysis revealed the similarity of protein pattern as observed with 2D gel map of F2-fraction [33]. In all, a total of 19 proteins were identified by 1-DGE and

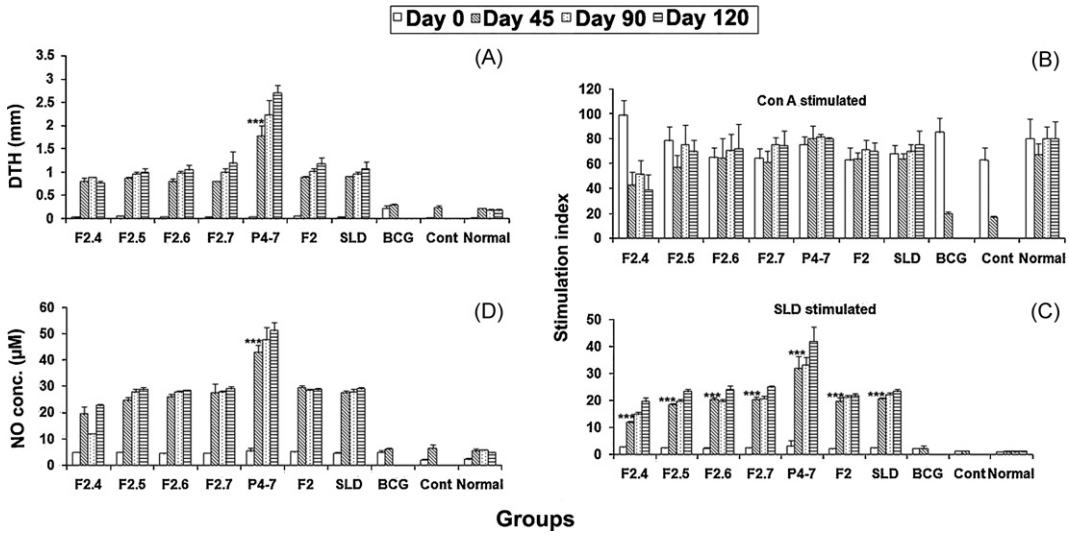

Fig. 3. Cellular responses on days 45, 90 and 120 p.c. in hamsters vaccinated with potent fraction hamsters with potent subfractions (F2.4–F2.7), pooled subfraction P4-7, F2-fraction and SLD along with BCG. Data for normal and unvaccinated infected groups have been represented as control groups, respectively. (A) DTH response (mm) to SLD in hamsters was measured as footpad swelling at 24 h, (B and C) LTT response (SI value) against Con A and SLD. SI values of more than 2.5 were considered as positive response, and (D) NO production (μg/ml). Each bar represents the pooled data (mean ± S.D. value) of three replicates.

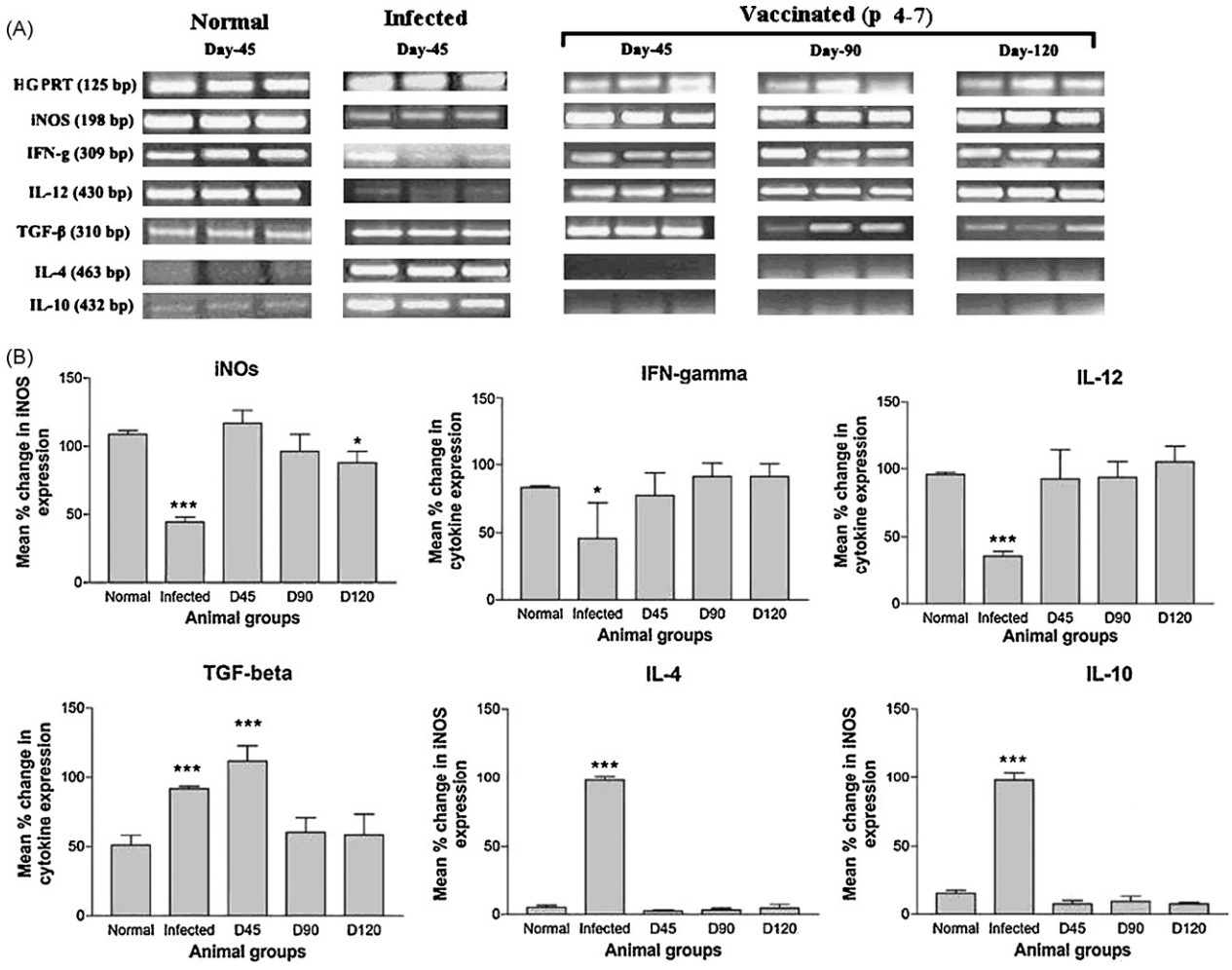

Fig. 4. Analysis of Th1 and Th2 mRNA cytokine profile in normal, infected and P4-7 vaccinated hamsters on days 45, 90 and 120 p.c. by RT-PCR. (A) Splenic iNOS and cytokine expression. Each band represents one out of three representative hamsters from each experimental group. (B) Densitometry analysis showing the relative mean % change in iNOS and cytokine mRNA expression ± S.D. in comparison to control (HGPRT). Significance values indicate the difference between various animal groups and normal group (*$P < 0.05$ and ***$P < 0.001$).

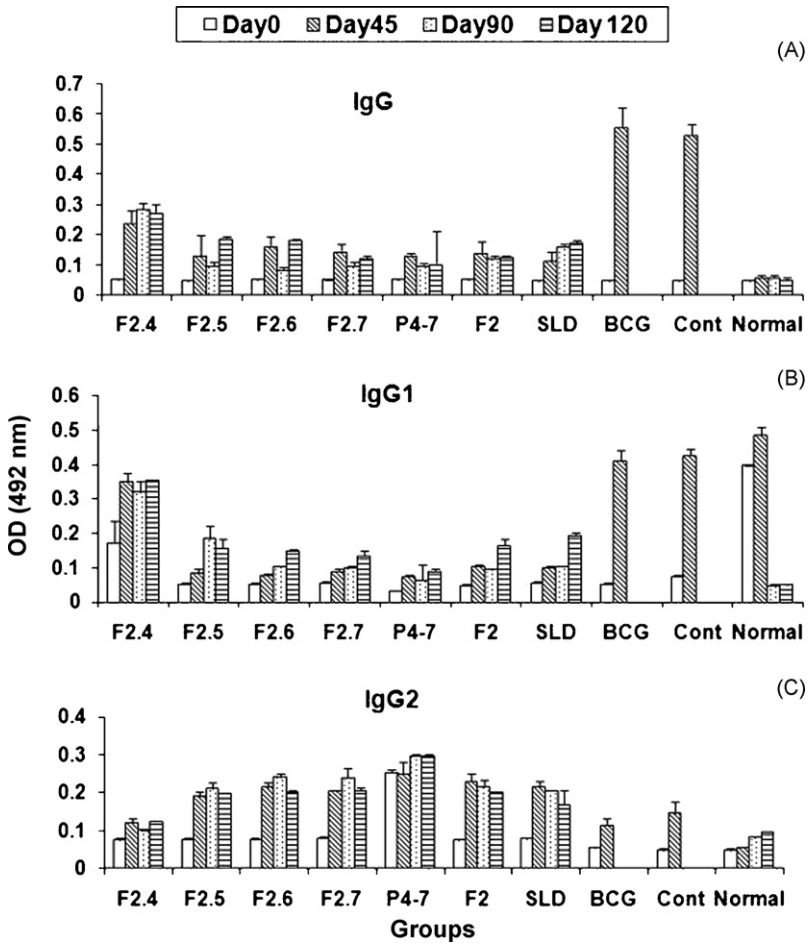

Fig. 5. Antileishmanial antibody levels (OD value) in hamsters vaccinated with potent subfractions (F2.4–F2.7), pooled subfraction P4-7, F2-fraction and SLD of *Leishmania donovani* along with BCG on days 45, 90 and 120 p.c. Data for BCG vaccinated, unvaccinated control and normal hamsters represented as positive and negative control groups, respectively (A) IgG and its isotype (B) IgG1 or (C) IgG2. Each bar represents the pooled data (mean ± S.D. value) of three replicates.

MALDI-TOF–MS in subfractions F2.4–F2.7, including five hypothetical proteins/unknowns. Among these, most of these proteins were of known immunostimulatory or immunogenic type or have been evaluated as vaccine candidates such as Elongation factor-2, p45, HSP-70, HSP-83, Fructose-1,6-Bisphosphate Aldolase (Aldolase), Enolase, Triosephosphate isomerase (TPI), Protein Disulfideisomerase and Calreticulin. Some of the other proteins including some enzymes from energy metabolism, phosphorylation pathway, amino acid metabolism pathways and from diverse metabolic route have also been reported as potential drug targets, viz., Adenosylhomocysteinase, Cofactor-independent phosphoglycerate mutase, Trypanothione reductase, and NAD-dependent deacetylase SIR2 homolog.

4. Discussion

Previous studies from this laboratory have hinted toward the prophylactic potential of F2-fraction of soluble promastigote antigen belonging to 68–97.4 kDa in experimental VL model [28]. Using the conventional screening approach four subfractions belonging to molecular weight of 89.9, 94.9, 96.9, 97.1 kDa were identified which gave considerably good cellular response, viz., lymphoproliferative as well as NO release against all the cured hamsters. However, the optimum cellular responses were observed when all the four subfractions were pooled together, i.e. P4-7. The responses of these identified subfractions in hamsters were further validated in cured patients/endemic contacts and analogous results, i.e. proliferation of lymphocytes *in vitro* and the release of very high amount of Th1-type cytokines, viz., IFN-γ and IL-12p40, were observed. Again, the excellent cellular responses were obtained when all the four subfractions were pooled together, i.e. P4-7. On the other hand, the Th2-type cytokine IL-10 was found to be suppressed in cured patients as well as in endemic contacts against all the subfractions [86].

Based on their immunostimulatory properties, the four potent individual subfractions as well as pooled one were further evaluated for their immunoprophylactic potential with BCG in hamsters. BCG had been used as an adjuvant due to its property of activating macrophages for inducing NO [46,47] and eliciting long-lasting cellular and humoral immune responses [48,49]. Although significant parasite inhibition was noticed in hamsters immunized with either of F2.5–F2.7 subfractions or F2-fraction, the efficacy was remarkable in hamsters receiving P4-7 subfractions. The parasite load in all visceral organs decreased progressively reaching a negligible level by day 120 p.c., rendering them difficult to discern demonstrating its strong vaccine potential. Moreover, post-challenge mean survival of hamsters in P4-7 subfraction vaccinated group along with F2.5–F2.7 vaccinated hamsters being more than 12 months further strengthen the evidence that combination of all the four potent subfractions are required to induce optimum prophylactic efficacy.

Most of the assays in this study were done between 45 and 120 days post-challenge as the disease progression reaches its peak by this time. Successful vaccination of humans and animals is often related to antigen induced DTH responses *in vivo* and T-cell stim-

Table 2
Immunostimulatory proteins identified in potent subfraction F2.4, F2.5, F2.6 and F2.7 of F2 MALDI-TOF–MS/MS

SFs[a]	Identified proteins[b]	Sp.[c]	Acc. no.[d]	kDa/pI[e]	Pm/Ms/Sc%[f]	FC[g]	Remarks[h]
F2.4	Cofactor-independent phosphoglycerate mutase	*Lmx*	28400787	61/5.4	10/151/20	5	DT [72]
	Trypanothione reductase	*Lmj*	7677022	53/5.8	12/117/32	5	DT [73]
	Hypothetical protein L5769.02	*Lmj*	12311865	29/6.8	10/100/25	??[i]	?[j]
	NAD-dependent deacetylase SIR2 homolog	*Lmj*	*SIR2_LEIMA	43/5.64	16/75/14	4	DT [74]
	Adenosylhomocysteinase	*Ld*	1710837	48/5.7	17/98/21	5	DT [75]
	Protein of unknown function	*Tc*	32401138	38/5.22	15/114/31	??[i]	?[j]
	Fructose-1,6-Bisphosphate Aldolase	*Lmx*	5834626	47/7.9	18/152/14	1–3	VC, DT [58,59,76]
	Elongation factor-2	*Lmj*	11244578	75/7.2	24/124/32	4	Th1 [68]
	Enolase	*Lmj*	8388689	46/5.6	21/124/38	1–4	IGP [59,63,76]
	Heatshock70-related protein1 precursor	*Lmj*	50299857	69/5.5	19/115/32	1	Th1 [65–67]
F2.5	NAD-dependent deacetylase SIR2 homolog	*Lmj*	*SIR2_LEIMA	43/5.7	12/63/28	4	DT [74]
	Adenosylhomocysteinase	*Ld*	1710837	48/5.7	10/102/23	5	DT [75]
	Protein of unknown function	*Tc*	32401138	38/5.22	15/121/36	??[i]	?[j]
	Enolase	*Lmj*	8388689	46/6.6	16/167/27	1–4	IGP [59,63,76]
	Fructose-1,6-Bisphosphate Aldolase	*Lmx*	5834626	47/7.5	16/155/38	1–3	VC, DT [58,59,76]
	dnaK-type molecular chaperone hsp70.4	*Lmj*	7441842	70/5.5	13/89/39	1	Th1 [65–67]
	Disulfideisomerase PDI	*Lmj*	25990151	52/5.2	9/100/25	1	VF, DT, VC [70]
	Elongation factor-2	*Lmj*	11244578	73/7.2	15/114/29	4	Th1 [68]
	Hypothetical protein 5769.02	*Lmj*	12311865	29/6.8	11/86/34	??[i]	?[j]
F2.6	Elongation factor-2	*Lmj*	11244578	73/7.2	15/114/29	4	Th1 [68]
	Enolase	*Lmj*	8388689	46/6.6	16/104/27	1–4	IGP [59,63,76]
	Fructose-1,6-Bisphosphate Aldolase	*Lmx*	5834626	41/7.0	16/156/32	1–3	VC, DT [58,59,76]
	Protein of unknown function	*Tc*	32401138	38/5.22	15/121/36	??[i]	?[j]
	Heatshock70-related protein1 precursor	*Lmj*	50299857	69/5.5	9/128/29	1	Th1 [65–67]
	Disulfideisomerase PDI	*Lmj*	25990151	55/5.2	9/100/25	1	VF, DT, VC [70]
	Hypothetical protein, unlikely	*Tb*	25992853	53/5.5	17/86/33	??[i]	?[j]
	p45	*Lmj*	6274526	44/6.6	15/156/37	5	T-cell st [68]
	Calreticulin	*Lmj*	5263289	33/4.7	16/87/25	1	VF, IGP [20,69]
	Triosephosphateisomerase glycosomal	*Tc*	*TPIS_TRYCR	76/6.6	15/142/38	1–3	Th1, VC [61,62]
	Heat shock protein-90	*Ld*	323030	53/5.6	24/128/34	1	Th1 [65–67]
F2.7	Calreticulin	*Lmj*	5263289	33/4.7	8/74/21	1	VF, IGP [20,69]
	Triosephosphate isomerase, glycosomal	*Tc*	*TPIS_TRYCR	76/6.6	21/212/31	1–3	Th1, VC [61,62]
	Hypothetical protein	*Lmx*	2131001	47/7.1	12/125/31	??[i]	?[j]
	Hypothetical protein L2385.08	*Lmj*	12311835	84/5.4	14/121/37	??[i]	?[j]
	Heatshock70-related	*Lmj*	50299857	69/5.5	21/137/32	1	Th1 [65–67]
	Elongation factor-2	*Lmj*	11244578	73/7.2	15/114/29	4	Th1 [68]
	Disulfideisomerase PDI	*Lmj*	25990151	52/5.2	15/110/25	1	VF, DT, VC [70]
	Enolase	*Lmj*	8388689	46/6.6	19/127/39	1–4	IGP [59,63,76]
	Fructose-1,6-Bisphosphate Aldolase	*Lmx*	5834626	45/7.9	16/102/27	1–3	VC, DT [58,59,76]
	p45	*Lmj*	6274526	41/6.6	12/126/46	5	T-cell st [68]
	Hsp83 protein	*Ldi*	362545	81/5.1	21/141/25	1	Th1 [65–67]

The protein spots indicated in this table were identified using peptide mass fingerprinting.

[a] Subfraction no.

[b] Name of the protein.

[c] Species: *Lmx*, *Leishmania mexicana*; *Lmj*, *Leishmania major*; *Li*, *Leishmania infantum*; *Ld*, *Leishmania donovani*; *Ldf*, *Leishmania donovani infantum*; *Ldc*, *Leishmania donovani chagasi*; *Tb*, *Trypanosoma brucei*; *Tbr*, *Trypanosoma brucei rhodesiense*; *Tc*, *Trypanosoma cruzi*.

[d] Accession numbers according to NCBI and *Swiss-Prot accession number.

[e] Predicted Mr and p*I*.

[f] No. of peptides matched/MOWSE score/sequence covered%.

[g] Identified proteins fell into the following major six functional categories; with some of them falling into two or more groups: 1. stress response; 2. cytoskeleton and cell membrane; 3. energy metabolism and phosphorylation; 4. cell cycle and proliferation; 5. amino acid metabolism.

[h] Remarks: VC, vaccine candidate; Th1, Th1 stimulatory; T-cell st, T-cell stimulatory proteins; VF, virulence factor; DT, drug target molecule; IGP, immunogenic protein; IDP, immunodiagnostic protein.

[i] ? Not previously described.

[j] ?? Unknowns/hypothetical function of the protein are not known.

ulation with antigen *in vitro* [4,50]. It has been further reported that a major factor that is believed to contribute to healing in leishmaniasis is the development of strong CMI responses like DTH responses, T-cell responses and NO production [28,51–53]. Notably, the hamsters vaccinated with P4-7 subfraction elicited strongest DTH reaction, LTT responses and NO production, among all the experimental groups suggesting a good correlation between CMI and resistance to infection in this model. In addition, all the hamsters vaccinated with potent subfractions challenged with *L. donovani* have a specific active T-cell response that was severely impeded in infected unvaccinated and BCG vaccinated hamsters. The generation of NO in these cultures also support the view regarding the up-regulation of inducible NO synthase (iNOS, NOS2) by Th1 cell associated cytokines and confirms that NO mediated macrophage effector mechanism is critical in the control of parasite replication in the animal model [28].

The presence of a comparable existence of Th1 and Th2 clones producing IL-12 and IFN-γ as well as IL-10 obtained from patients cured of VL encouraged us to assess whether the protective response which was utmost elicited by P4-7 vaccination in hamsters can reflect this feature of clinical findings [54–56]. Following vaccination with P4-7 subfractions in the hamster transcripts of iNOS, IL-12 and IFN-γ showed manifold increase and the synergism of IL-12 with IFN-γ might have an additional paramount effect

on leishmanicidal activities of *L. donovani* [30]. We found that significant iNOS transcript production in P4-7 vaccinated hamsters correlated proportionally with NO generation that was considerably higher with SLD stimulation. Possibly the cumulative effect of IFN-γ and IL-12 alongwith iNOS mediated the parasite killing [30,51,57,58]. The levels of Th-2 cytokines IL-4, IL-10 and TGF-β mRNA, on the other hand, were observed to be down-regulated in the vaccinated hamsters. The strong presence of IL-4, IL-10 and TGF-β in infected hamsters are reported to be the major immunosuppressive cytokines in experimental and human VL [44,58–62].

Apart from diminished cellular responses, VL is associated with the production of high level of antibody, observed prior to detection of parasite-specific T-cell response [4]. Unlike in mice, wherein IL-4 and IL-12, IFN-γ, the two cytokines that direct IgG class switching of IgG1 and IgG2a, respectively, there are no such distinct classifications of IgG in hamsters [30]. It is believed that hamster IgG2 corresponds to mouse IgG2a/IgG2b and hamster IgG1 corresponds to murine IgG1 [30]. It has been well-established that the increase of IgG and IgG1 antibodies titre is indicative of the *L. donovani* load [30]. These antibodies were quiet low in P4-7 vaccinated group which reflected the decreased parasite burden. In contrast, the level of IgG2 significantly increased only in P4-7 vaccinated animals further support the views that protection against leishmaniasis is induced by a strong T-cell response and this pattern was also seen in clinical as well as experimental VL [4,28,30,49,63–65].

To examine the molecular basis of the immune responses elicited during *Leishmania* infection, recent efforts have been focused on evaluating responses to defined parasite T-cell epitopes as vaccine targets using proteomics approaches [33]. Since MALDI-TOF/MS-MS is a very powerful analyzing tool which may pinpoint all the specific proteins contained in a fraction [32], we have done the characterization of four potent subfractions by using this tool. Interestingly, all that proteins that we have identified through 2D and MALDI analysis earlier (ref) were found to be similar to those identified through one D analysis of F2 subfractions [33]. Out of total 19 identified proteins, major immunostimulatory were Elongation factor-2, Aldolase, Enolase and HSP-70 that were present in all the four subfractions (Table 2) and detailed immunogenic information was described by Gupta et al. [33]. Protein Disulfideisomerase, p45, HSP-83, TPI and Calreticulin were absent in F2.4 subfraction. Perhaps due to this moderate protective response with an individual F2.4 subfraction was noticed. Further explanation for low success rate of subunit vaccine is perhaps due to the fact that some polypeptides may be slightly immunogenic and may provide only partial protection individually but they may be excellent in a cocktail vaccine [66–70] as has been observed with P4-7 subfraction. Some of the proteins such as Aldolase, Enolase, and TPI the glycolytic enzymes, may be considered as potential vaccine candidates, since they have been reported to be immunogenic in other organisms [71–76]. Among the other major identified immunostimulatory proteins HSPs 70 and 83/90 are the outcome of the stress response, participating in a large number of immunological pathways [33,77–81]. Elongation factors-2 and p45 are reported to induce proliferative response in cured CL patients PBMCs as well as leishmanial parasite-specific T-cell lines derived from an immune donor [33,82]. Calreticulin, a stress shock protein and identified as Th1-stimulatory, is an important multifunctional immunodominant calcium (Ca^{+2})-binding protein [33,83]. Another protein—Protein DisulfideIsomerase (LmPDI), responsible for virulence factor [84], is believed to represents a new potential component of novel immunogenic or vaccine preparations aimed at conferring immunity in humans or animals against *Leishmania* [85]. Remarkably, the study also documents completely unknown or hypothetical proteins of the parasite, which may represent potential targets for putative vaccine candidates.

Theoretically, linking the above stated antigenic proteins might increase the number of epitopes available for inducing Th1-type immune responses and protective immunity in a heterogeneous human population with diverse major histocompatibility complex types. Thus, this clearly indicate that while identification of antigens recognized by T cell is an important step in defining a protective immunogens, empirical immunization studies in vaccine models are crucial in defining a leishmanial vaccine, therefore, emphasizing that a successful subunit vaccine may require multiple immunogenic/Th1 immunostimulatory proteins that are also protective against VL. Extended studies such as cloning and expression of the best antigenic targets, as determined by their immunoprotective potential, together with their specific association and definite allocation are required to characterize these new proteins further.

Acknowledgements

We express our sincere gratitude to the Directors CDRI and CIMAP for their keen interest and for providing facilities for the experiments. Our grateful acknowledgement is due to Dr. Nikhil Kumar, for critically reviewing the manuscript and for his encouraging suggestions. We are also thankful to Dr Rajeev Singh, Scientist and Mr. Malaya Sahoo, research fellow for their kind support. Financial support to this work from DBT, New Delhi and for Senior Research Fellowship to Ms SK and Mr. MS from CSIR, New Delhi is gratefully acknowledged. This has CDRI communication no 7555.

References

[1] Handman E. Leishmaniasis: current status of vaccine development. Clin Microbiol Rev 2001;14(2):229–43.
[2] Desjeux P. Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis 2004;27(5):305–18.
[3] Murray HW, Hariprashad J, Coffman RL. Behavior of visceral *Leishmania donovani* in an experimentally induced T helper cell 2 (Th2)-associated response model. J Exp Med 1997;185(5):867–74.
[4] 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;25:6544–56.
[5] Reed SG, Scott P. T-cell and cytokine responses in leishmaniasis. Curr Opin Immunol 1993;5(4):524–31.
[6] Skeiky YA, Kennedy M, Kaufman D, Borges MM, Guderian JA, Scholler JK, et al. LeIF: a recombinant Leishmania protein that induces an IL-12-mediated Th1 cytokine profile. J Immunol 1998;161(11):6171–9.
[7] Coler RN, Skeiky YA, Bernards K, Greeson K, Carter D, Cornellison CD, et al. 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(8):4215–25.
[8] Muller I, Fruth U, Louis JA. Immunobiology of experimental leishmaniasis. Med Microbiol Immunol 1992;181(1):1–12.
[9] Modabber F. Development of vaccines against leishmaniasis. Scand J Infect Dis Suppl 1990;76:72–8.
[10] Coler RN, Goto Y, Bogatzki L, Raman V, Reed SG. Leish-111f, a recombinant polyprotein vaccine that protects against visceral leishmaniasis by the elicitation of CD4 + T cells. Infect Immun 2007.
[11] Kamil AA, Khalil EA, Musa AM, Modabber F, Mukhtar MM, Ibrahim ME, et al. Alum-precipitated autoclaved Leishmania major plus bacille Calmette-Guerrin, a candidate vaccine for visceral leishmaniasis: safety, skin-delayed type hypersensitivity response and dose finding in healthy volunteers. Trans R Soc Trop Med Hyg 2003;97(3):365–8.
[12] Khalil EA, Ayed NB, Musa AM, Ibrahim ME, Mukhtar MM, Zijlstra EE, et al. Dichotomy of protective cellular immune responses to human visceral leishmaniasis. Clin Exp Immunol 2005;140(2):349–53.
[13] Lemesre JL, Holzmuller P, Goncalves RB, Bourdoiseau G, Hugnet C, Cavaleyra M, et al. Long-lasting protection against canine visceral leishmaniasis using the LiESAp-MDP vaccine in endemic areas of France: double-blind randomised efficacy field trial. Vaccine 2007;25(21):4223–34.
[14] Borja-Cabrera GP, Correia Pontes NN, da Silva VO, Paraguai de Souza E, Santos WR, Gomes EM, et al. Long lasting protection against canine kala-azar using the FML-QuilA saponin vaccine in an endemic area of Brazil (São Gonçalo do Amarante, RN). Vaccine 2002;20(27–28):3277–84.
[15] Palatnik-de-Sousa CB. Vaccines for leishmaniasis in the fore coming 25 years. Vaccine 2008;26(14):1709–24.

[16] Santos WR, de Lima VM, de Souza EP, Bernardo RR, Palatnik M, Palatnik de Sousa CB. Saponins, IL12 and BCG adjuvant in the FML-vaccine formulation against murine visceral leishmaniasis. Vaccine 2002;21(1–2):30–43.
[17] Borja-Cabrera GP, Cruz Mendes A, Paraguai de Souza E, Hashimoto Okada LY, de ATFA, Kawasaki JK, et al. Effective immunotherapy against canine visceral leishmaniasis with the FML-vaccine. Vaccine 2004;22(17–18): 2234–43.
[18] Santos WR, Paraguai de Souza E, Palatnik M, Palatnik de Sousa CB. Vaccination of Swiss Albino mice against experimental visceral leishmaniasis with the FML antigen of *Leishmania donovani*. Vaccine 1999;17(20–21):2554–61.
[19] Palatnik de Sousa CB, Gomes EM, de Souza EP, dos Santos WR, de Macedo SR, de Medeiros LV, et al. The FML (Fucose Mannose Ligand) of *Leishmania donovani*. A new tool in diagnosis, prognosis, transfusional control and vaccination against human kala-azar. Rev Soc Bras Med Trop 1996;29(2):153–63.
[20] da Silva VO, Borja-Cabrera GP, Correia Pontes NN, de Souza EP, Luz KG, Palatnik M, et al. A phase III trial of efficacy of the FML-vaccine against canine kala-azar in an endemic area of Brazil (Sao Goncalo do Amaranto, RN). Vaccine 2000;19(9–10):1082–92.
[21] Gurunathan S, Sacks DL, Brown DR, Reiner SL, Charest H, Glaichenhaus N, et al. Vaccination with DNA encoding the immunodominant LACK parasite antigen confers protective immunity to mice infected with Leishmania major. J Exp Med 1997;186(7):1137–47.
[22] Jardim A, Tolson DL, Turco SJ, Pearson TW, Olafson RW. The *Leishmania donovani* lipophosphoglycan T lymphocyte-reactive component is a tightly associated protein complex. J Immunol 1991;147(10):3538–44.
[23] Jaffe CL, Rachamim N, Sarfstein R. Characterization of two proteins from *Leishmania donovani* and their use for vaccination against visceral leishmaniasis. J Immunol 1990;144(2):699–706.
[24] Convit J, Castellanos PL, Rondon A, Pinardi ME, Ulrich M, Castes M, et al. Immunotherapy versus chemotherapy in localised cutaneous leishmaniasis. Lancet 1987;1(8530):401–5.
[25] Russo DM, Jardim A, Carvalho EM, Sleath PR, Armitage RJ, Olafson RW, et al. Mapping human T cell epitopes in Leishmania gp63: identification of cross-reactive and species-specific epitopes. J Immunol 1993;150(10):4713.
[26] Garg R, Dube A. Animal models for vaccine studies for visceral leishmaniasis. Indian J Med Res 2006;123(3):439–54.
[27] Khamesipour A, Rafati S, Davoudi N, Maboudi F, Modabber F. Leishmaniasis vaccine candidates for development: a global overview. Indian J Med Res 2006;123(3):423–38.
[28] Garg R, Gupta SK, Tripathi P, Hajela K, Sundar S, Naik S, et al. *Leishmania donovani*: identification of stimulatory soluble antigenic proteins using cured human and hamster lymphocytes for their prophylactic potential against visceral leishmaniasis. Vaccine 2006;24(15):2900–9.
[29] Garg R, Srivastava JK, Pal A, Naik S, Dube A. Isolation of integral membrane proteins of Leishmania promastigotes and evaluation of their prophylactic potential in hamsters against experimental visceral leishmaniasis. Vaccine 2005;23(9):1189–96.
[30] Basu R, Bhaumik S, Basu JM, Naskar K, De T, Roy S. Kinetoplastid membrane protein-11 DNA vaccination induces complete protection against both pentavalent antimonial-sensitive and -resistant strains of *Leishmania donovani* that correlates with inducible nitric oxide synthase activity and IL-4 generation: evidence for mixed Th1- and Th2-like responses in visceral leishmaniasis. J Immunol 2005;174(11):7160–71.
[31] Melby PC, Yang J, Zhao W, Perez LE, Cheng J. *Leishmania donovani* p36 (LACK) DNA vaccine is highly immunogenic but not protective against experimental visceral leishmaniasis. Infect Immun 2001;69(8):4719–25.
[32] Kumari S, Kumar A, Samant M, Sundar S, Singh N, Dube A. Proteomic approaches for discovery of new targets for vaccine and therapeutics against visceral leishmaniasis. Proteomics—Clinical Application 2008;3(2):372–86.
[33] Gupta SK, Sisodia BS, Sinha S, Hajela K, Naik S, Shasany AK, et al. Proteomic approach for identification and characterization of novel immunostimulatory proteins from soluble antigens of *Leishmania donovani* promastigotes. Proteomics 2007;7(5):816–23.
[34] Dube A, Singh N, Sundar S. Refractoriness to the treatment of sodium stibogluconate in Indian kala-azar field isolates persist in in vitro and in vivo experimental models. Parasitol Res 2005;96(4):216–23.
[35] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193(1):265–75.
[36] Mehrotra J, Mittal A, Dhindsa MS, Sinha S. Fractionation of mycobacterial integral membrane proteins by continuous elution SDS-PAGE reveals the immunodominance of low molecular weight subunits for human T cells. Clin Exp Immunol 1997;109(3):446–50.
[37] Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227(5259):680–5.
[38] Wray W, Boulikas T, Wray VP, Hancock R. Silver staining of proteins in polyacrylamide gels. Anal Biochem 1981;118(1):197–203.
[39] Wessel D, Flügge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal Biochem 1984;138:141–3.
[40] Arand M, Friedberg T, Oesch F. Colorimetric quantitation of trace amounts of sodium lauryl sulfate in the presence of nucleic acids and proteins. Anal Biochem 1992;207:73–5.
[41] Voller A, Bidwell DE, Bartlett A. The enzyme linked immunosorbent assay (ELISA): a guide with abstracts of microplate application. Guernsey, CI: Filerolline Press; 1979.
[42] Garg R, Gupta SK, Tripathi P, Naik S, Sundar S, Dube A. Immunostimulatory cellular responses of cured Leishmania-infected patients and hamsters against the integral membrane proteins and non-membranous soluble proteins of a recent clinical isolate of *Leishmania donovani*. Clin Exp Immunol 2005;140(1):149–56.
[43] Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J Immunol 1988;141(7):2407–12.
[44] Melby PC, Chandrasekar B, Zhao W, Coe JE. The hamster as a model of human visceral leishmaniasis: progressive disease and impaired generation of nitric oxide in the face of a prominent Th1-like cytokine response. J Immunol 2001;166(3):1912–20.
[45] Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. Anal Chem 1996;68:850–8.
[46] MacMicking J, Xie QW, Nathan C. Nitric oxide and macrophage function. Annu Rev Immunol 1997;15:323–50.
[47] Nozaki Y, Hasegawa Y, Ichiyama S, Nakashima I, Shimokata K. Mechanism of nitric oxide-dependent killing of *Mycobacterium bovis* BCG in human alveolar macrophages. Infect Immun 1997;65:3644–7.
[48] Khalil EA, Elhassan AM, Zijlstra EE, Osman OF, Eljack IA, Ibrahim ME, et al. Safety and immunogenicity of an autoclaved Leishmania major vaccine. East Afr Med J 2000;77(9):468–70.
[49] Misra A, Dube A, Srivastava B, Sharma P, Srivastava JK, Katiyar JC, et al. Successful vaccination against *Leishmania donovani* infection in Indian langur using alum-precipitated autoclaved Leishmania major with BCG. Vaccine 2001;19(25–26):3485–92.
[50] Howard JG, Liew FY. Mechanisms of acquired immunity in leishmaniasis. Philos Trans R Soc Lond B Biol Sci 1984;307(1131):87–98.
[51] Haldar JP, Ghose S, Saha KC, Ghose AC. Cell-mediated immune response in Indian kala-azar and post-kala-azar dermal leishmaniasis. Infect Immun 1983;42(2):702–7.
[52] Ali N, Afrin F. Protection of mice against visceral leishmaniasis by immunization with promastigote antigen incorporated in liposomes. J Parasitol 1997;83(1):70–5.
[53] Afrin F, Rajesh R, Anam K, Gopinath M, Pal S, Ali N. Characterization of *Leishmania donovani* antigens encapsulated in liposomes that induce protective immunity in BALB/c mice. Infect Immun 2002;70(12):6697–706.
[54] Kemp K, Kemp M, Kharazmi A, Ismail A, Kurtzhals JA, Hviid L, et al. Leishmania-specific T cells expressing interferon-gamma (IFN-gamma) and IL-10 upon activation are expanded in individuals cured of visceral leishmaniasis. Clin Exp Immunol 1999;116(3):500–4.
[55] Kemp K. Cytokine-producing T cell subsets in human leishmaniasis. Arch Immunol Ther Exp (Warsz) 2000;48(3):173–6.
[56] Kemp M, Kurtzhals JA, Bendtzen K, Poulsen LK, Hansen MB, Koech DK, et al. *Leishmania donovani*-reactive Th1- and Th2-like T-cell clones from individuals who have recovered from visceral leishmaniasis. Infect Immun 1993;61(3):1069–73.
[57] Carvalho EM, Bacellar O, Brownell C, Regis T, Coffman RL, Reed SG. Restoration of IFN-gamma production and lymphocyte proliferation in visceral leishmaniasis. J Immunol 1994;152(12):5949–56.
[58] Gifawesen C, Farrell JP. Comparison of T-cell responses in self-limiting versus progressive visceral *Leishmania donovani* infections in golden hamsters. Infect Immun 1989;57(10):3091–6.
[59] Kenney RT, Sacks DL, Gam AA, Murray HW, Sundar S. Splenic cytokine responses in Indian kala-azar before and after treatment. J Infect Dis 1998;177(3):815–8.
[60] Melby PC, Tryon VV, Chandrasekar B, Freeman GL. Cloning of Syrian hamster (*Mesocricetus auratus*) cytokine cDNAs and analysis of cytokine mRNA expression in experimental visceral leishmaniasis. Infect Immun 1998;66(5):2135–42.
[61] Ghalib HW, Piuvezam MR, Skeiky YA, Siddig M, Hashim FA, el-Hassan AM, et al. Interleukin 10 production correlates with pathology in human *Leishmania donovani* infections. J Clin Invest 1993;92(1):324–9.
[62] Rodrigues Junior V, Da Silva JS, Campos-Neto A. Selective inability of spleen antigen presenting cells from *Leishmania donovani* infected hamsters to mediate specific T cell proliferation to parasite antigens. Parasite Immunol 1992;14(1):49–58.
[63] Bretscher PA, Ogunremi O, Menon JN. Distinct immunological states in murine cutaneous leishmaniasis by immunising with different amounts of antigen: the generation of beneficial, potentially harmful, harmful and potentially extremely harmful states. Behring Inst Mitt 1997;98:153–9.
[64] Dube A, Sharma P, Srivastava JK, Misra A, Naik S, Katiyar JC. Vaccination of langur monkeys (*Presbytis entellus*) against *Leishmania donovani* with autoclaved L. major plus BCG. Parasitology 1998;116(Pt 3):219–21.
[65] Fragaki K, Suffia I, Ferrua B, Rousseau D, Le Fichoux Y, Kubar J. Immunisation with DNA encoding *Leishmania infantum* protein papLe22 decreases the frequency of parasitemic episodes in infected hamsters. Vaccine 2001;19(13–14):1701–9.
[66] Carrion J, Folgueira C, Alonso C. Immunization strategies against visceral leishmaniosis with the nucleosomal histones of *Leishmania infantum* encoded in DNA vaccine or pulsed in dendritic cells. Vaccine 2008;26(20):2537–44.
[67] Kedzierski L, Zhu Y, Handman E. Leishmania vaccines: progress and problems. Parasitology 2006;133(Suppl):S87–112.
[68] Skeiky YA, Coler RN, Brannon M, Stromberg E, Greeson K, Crane RT, et al. Protective efficacy of a tandemly linked, multi-subunit recombinant leishmanial vaccine (Leish-111f) formulated in MPL adjuvant. Vaccine 2002;20(27–28):3292–303.

[69] Reed SG. Leishmaniasis vaccination: targeting the source of infection. J Exp Med 2001;194(3):F7–9.
[70] Coler RN, Reed SG. Second-generation vaccines against leishmaniasis. Trends Parasitol 2005;21(5):244–9.
[71] McCarthy JS, Wieseman M, Tropea J, Kaslow D, Abraham D, Lustigman S, et al. Onchocerca volvulus glycolytic enzyme fructose-1,6-bisphosphate aldolase as a target for a protective immune response in humans. Infect Immun 2002;70(2):851–8.
[72] Certa U, Ghersa P, Dobeli H, Matile H, Kocher HP, Shrivastava IK, et al. Aldolase activity of a *Plasmodium falciparum* protein with protective properties. Science 1988;240(4855):1036–8.
[73] Pal-Bhowmick I, Sadagopan K, Vora HK, Sehgal A, Sharma S, Jarori GK. Cloning, over-expression, purification and characterization of *Plasmodium falciparum* enolase. Eur J Biochem 2004;271(23–24):4845–54.
[74] Reynolds SR, Dahl CE, Harn DA. T and B epitope determination and analysis of multiple antigenic peptides for the *Schistosoma mansoni* experimental vaccine triose-phosphate isomerase. J Immunol 1994;152(1):193–200.
[75] Hooker CW, Brindley PJ. Cloning and characterisation of strain-specific transcripts encoding triosephosphate isomerase, a candidate vaccine antigen from *Schistosoma japonicum*. Mol Biochem Parasitol 1996;82(2):265–9.
[76] Mitsutake K, Miyazaki T, Tashiro T, Yamamoto Y, Kakeya H, Otsubo T, et al. Enolase antigen, mannan antigen, Cand-Tec antigen, and beta-glucan in patients with candidemia. J Clin Microbiol 1996;34(8):1918–21.
[77] Folgueira C, Carrion J, Moreno J, Saugar JM, Canavate C, Requena JM. Effects of the disruption of the HSP70-II gene on the growth, morphology, and virulence of *Leishmania infantum* promastigotes. Int Microbiol 2008;11(2):81–9.
[78] Garrido C, Schmitt E, Cande C, Vahsen N, Parcellier A, Kroemer G. HSP27 and HSP70: potentially oncogenic apoptosis inhibitors. Cell Cycle 2003;2(6):579–84.
[79] Suto R, Srivastava PK. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. Science 1995;269(5230):1585–8.
[80] de Andrade CR, Kirchhoff LV, Donelson JE, Otsu K. Recombinant Leishmania Hsp90 and Hsp70 are recognized by sera from visceral leishmaniasis patients but not Chagas' disease patients. J Clin Microbiol 1992;30(2):330–5.
[81] Rico AI, Del Real G, Soto M, Quijada L, Martinez AC, Alonso C, et al. Characterization of the immunostimulatory properties of *Leishmania infantum* HSP70 by fusion to the *Escherichia coli* maltose-binding protein in normal and nu/nu BALB/c mice. Infect Immun 1998;66(1):347–52.
[82] Probst P, Stromberg E, Ghalib HW, Mozel M, Badaro R, Reed SG, et al. Identification and characterization of T cell-stimulating antigens from Leishmania by CD4 T cell expression cloning. J Immunol 2001;166(1):498–505.
[83] Debrabant A, Lee N, Pogue GP, Dwyer DM, Nakhasi HL. Expression of calreticulin P-domain results in impairment of secretory pathway in *Leishmania donovani* and reduced parasite survival in macrophages. Int J Parasitol 2002;32(11):1423–34.
[84] Ben Achour Y, Chenik M, Louzir H, Dellagi K. Identification of a disulfide isomerase protein of Leishmania major as a putative virulence factor. Infect Immun 2002;70(7):3576–85.
[85] Kebaier C, Louzir H, Chenik M, Ben Salah A, Dellagi K. Heterogeneity of wild Leishmania major isolates in experimental murine pathogenicity and specific immune response. Infect Immun 2001;69(8):4906–15.
[86] Kumari S, Samant M, Khare P, Sundar S, Sinha S, Dube A. Induction of Th1-type cellular responses in cured/exposed *Leishmania*-infected patients and hamsters against polyproteins of soluble *Leishmania donovani* promastigotes ranging from 89.9 to 97.1 kDa. Vaccine 2008;26:4813–8.