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Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage: http://ees.elsevier.com/apjtm

Original research http://dx.doi.org/10.1016/j.apjtm.2015.06.008

Immunogenicity and efficacy of recombinant 78 kDa antigen of *Leishmania donovani* formulated in various adjuvants against murine visceral leishmaniasis

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ARTICLE INFO

Article history:
Received 15 Apr 2015
Received in revised form 20 May 2015
Accepted 15 Jun 2015
Available online 9 July 2015

Keywords:
Visceral leishmaniasis
r78 kDa
Liposomes
MPL-A
T cell epitope

ABSTRACT

Objective: To analyze the protective efficacy of recombinant 78 kDa antigen of *Leishmania donovani* in combination with two adjuvants, that is, cationic liposomes or MPL-A against visceral leishmaniasis in BALB/c mice.

Methods: The genomic DNA of promastigotes was isolated and 583 bp of T cell epitopes of gene encoding 78 kDa was amplified using specific primers. The amplified gene was cloned into pET28c, transformed into *Escherichia coli* BL21 (DE3) and got expressed after IPTG induction. The recombinant protein was then purified using Ni-NTA and named r78. Three groups of mice were immunized with 10 μg of r78 plus MPL-A, r78 encapsulated in positively charged liposomes and control animals immunized with PBS. Two booster doses were given with the respective vaccine at an interval of 2 weeks each. Mice were challenged with 1×10^7 *Leishmania* promastigotes and sacrificed on different post infection/challenge days.

Results: Immunization with r78 along with MPL-A and liposome-encapsulated r78 brought a significant reduction in parasite load. In comparison to the infected controls, the parasite load declined by 96.2% in mice immunized with r78 plus MPL-A and 97.23% in animals immunized with liposome-encapsulated r78. The immunized animals also exhibited profound DTH response. The serum antibody responses increased from 15 to 90 days post infection/challenge. Immunized animals showed greater IgG2a levels and lesser IgG1 levels in comparison to the infected controls. The splenocytes from immunized mice were cultured, stimulated with r78 and analyzed for cytokine profile. The levels of IL-2 and IFN-γ were greater in immunized animals as compared to control mice.

Conclusions: The study proves that r78 in combination with suitable adjuvants is a potential vaccine candidate and may be instrumental in control of visceral leishmaniasis.

1. Introduction

The leishmaniases are responsible for the second-highest number of deaths due to parasitic infection globally [1]. They have an overwhelming estimated prevalence of 12 million infected humans, and cause a burden estimated at 2 357 000 disability-adjusted life years [2]. Leishmaniasis has been classified as one of the most neglected diseases, and the estimated disease burden places it second in mortality and fourth in morbidity among the tropical infections [3]. There is no satisfactory chemotherapy for cutaneous leishmaniasis. Existing chemotherapy for visceral leishmaniasis (VL) is, in general, efficient in immunocompetent patients, but is costly and not exempt from side-effects [4]. Resistant strains of *Leishmania* spp. are emerging, mostly in India [5]. In immunocompromised patients, relapses after therapy are very frequent [6]. To date, several approaches to anti-leishmanial vaccine have been tested. First generation vaccines composed of whole killed parasites have been proposed. However, most of the vaccine studies concentrate on the second generation vaccines consisting of recombinant proteins, poly-proteins or dendritic cells loaded with peptides derived from leishmanial antigens [1]. For the past 20 years, DNA cloning and characterization of genes encoding parasite proteins suitable for the development of defined vaccines have been carried out

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Peer review under responsibility of Hainan Medical University.
Foundation project: It is supported by the Indian Council of Medical Research, Department of Health Research, India for providing financial support for this study under project ref. 5/8-7(74)/2005-ECD-II.

[7]. Several native and recombinant antigens, tested in murine models, have shown different degrees of protection [1,8–11]. Recombinant Ldccys1 protein elicits protective immune responses in *Leishmania chagasi* infected BALB/c mice [12]. BALB/c mice vaccinated with *Leishmania major* (*L. major*) Ribosomal Proteins Extracts combined with CpG oligodeoxynucleotides have been reported to become resistant to disease caused by a secondary parasite challenge [13]. Recent studies have shown that subcutaneous vaccination with liposomal rGP63 partially protected susceptible BALB/c mice when challenged with *L. major* promastigotes [14].

2. Materials and methods

2.1. Parasite

Promastigotes of *Leishmania donovani* (*L. donovani*), strain MHOM/IN/80/Dd8 were grown at ambient temperature of (22 ± 1) °C in RPMI-1640 medium supplemented with 10% FCS. The *L. donovani* strain was maintained by serial sub-cultures in the same medium after every 48–72 h [15].

2.2. Animals

Inbred BALB/c mice of either gender, weighing 20–25 g, were obtained from the Central Animal House of Panjab University, Chandigarh. They were fed with water and mouse feed *ad libitum*. The ethical clearance to conduct the experiments was obtained from Institutional Animal Ethics Committee, Panjab University, Chandigarh.

2.3. Preparation of recombinant vaccine

2.3.1. Isolation of genomic DNA

Approximately 2 × 10^8 log phase promastigotes were harvested by centrifugation at 4 000 rpm for 15 min at 4 °C. The pellet was washed once with PBS (pH-7.2) and finally suspended in the same. Parasite DNA was extracted with the help of genomic DNA extraction kit as per manufacturer's protocol (Zymo Research, USA). The isolated DNA was quantitated by Nanodrop™.

2.3.2. Designing of primers and PCR amplification of gene fragment encoding 78 kDa

The T cell epitopes of gene encoding 78 kDa were identified using 'MHC Pred' online software. The region containing maximum number of T cell epitopes (583 bp) were selected for amplification (Figure 1). The following primers were designed for amplification of 583 bp sequence using IDT 'Scitools'.

Forward primer –5′ GC**CATATG**GGTGCAACAGC-TCATCAAGGACT 3′

Reverse primer -5′ GAT**GGATCC**GTTCTTCGCTTCGA-CACGTT 3′

The restriction enzymes *Nde*I and *Bam*H1 were included in the forward and reverse primer sequences respectively for cloning purpose [in pET-28c(+) vector]. PCR was carried out using commercially available kits (Chromous Biotech). Following conditions were used for the amplification of coding region of 583 bp of 78 kDa gene of *L. donovani*. Initial denaturation was performed at 94 °C for 10 min and then for 1 min. Annealing was carried out at 59 °C for one minute. Extension was carried out at 72 °C for 1 min and final extension was done at same temperature for ten minutes. PCR was carried out for thirty five cycles.

2.3.3. Electrophoresis of DNA in agarose gel

Agarose (0.8%, w/v for genomic DNA and 1%, w/v for PCR product and plasmid) was dissolved in 1 × TBE buffer by heating, when it cooled to 50 °C, ethidium bromide (0.5 μg/mL) was added and the gels were cast in the DNA electrophoresis chamber. After solidification of the agarose, DNA samples containing loading dye buffer (1×) were loaded on to the agarose gel and run in TBE (1 ×, pH 8.0) buffer. Initially, the gels were run at a constant voltage of 50 V till the dye front left the wells. The voltage was then increased to 100 V. A 2 log ladder (NEB, England) was used as standard DNA marker. The gels were

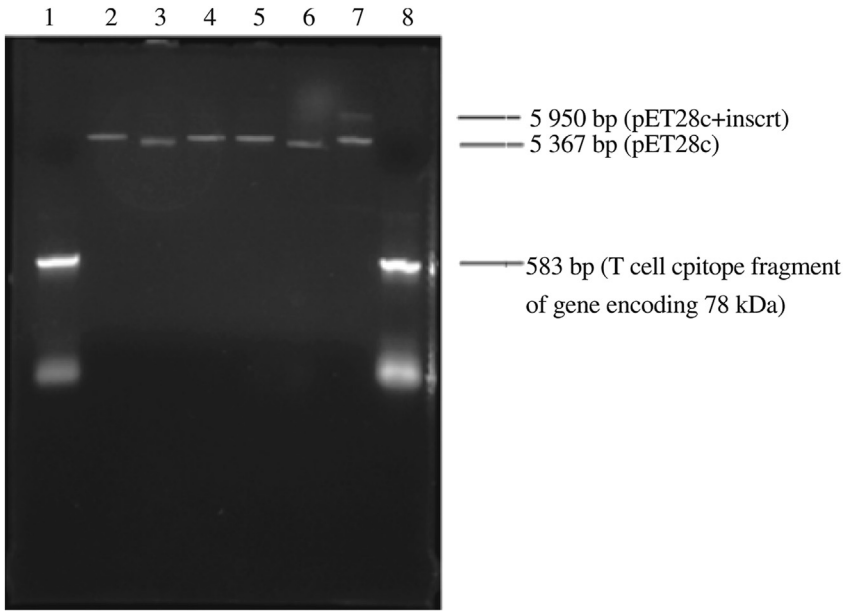

Figure 1. Agarose gel analysis of pET28c(+) vector containing 583 bp insert.
Lane 1, 8 = 583 bp fragment of gene encoding 78 kDa; Lane 2, 4, 5, 7 = pET28c vector with 583 bp insert; Lane 3, 6 = pET28c vector without insert.

visualized and results recorded on Gel Documentation Unit (Alpha Imager, India).

2.3.4. Extraction of DNA from preparatory gel

The PCR product was eluted from preparative agarose gel using gel extraction kit (Geneaid, Taiwan). The eluted DNA was then run on 1% agarose gel.

2.3.5. Enzymatic digestion and ligation of the vector and PCR products

Restriction digestions of PCR product and plasmid DNA [pET-28c(+)] were performed as per the manufacturer's instructions (MBI Fermentas, Germany). The reaction mixture was mixed gently and incubated at 37 °C. The reaction mixtures were mixed gently and incubated at 37 °C for 15 h. The double digested plasmid DNA and PCR products were electrophoresed and purified from the rest of the reaction mixtures with Gel Extraction kit (Geneaid, Taiwan). Ligation reactions were set up using the linearized pET28c and double digested PCR products. The ligation mixture was incubated at 16 °C for 16 h in ligation water bath.

2.3.6. Transformation of recombinant plasmids into Escherichia coli (E. coli)

The competent *E. coli* cells were prepared using $CaCl_2$ as described by Sambrook and Russell [16]. A loopful of *E. coli* BL21 (DE3) cells from LB agar plate were inoculated into 10 mL LB and incubated overnight at 37 °C with shaking at 180 rpm. From the overnight cultures, 1% inoculum was transferred to fresh LB broth (100 mL) and incubated at 37 °C with shaking (180 rpm) till OD_{600} of 0.5 was achieved. The cells were incubated at 4 °C for 20 min and harvested by centrifugation at 1 500 × *g* for 15 min at 4 °C in a precooled rotor. The supernatant was discarded aseptically and 10 mL of autoclaved and ice cold $CaCl_2$ (100 mM) solution was added to the cell pellets. The cells were resuspended by gentle shaking until a homogenous suspension was achieved. The cell suspension was then kept in ice for 30 min and again centrifuged (1 500 × *g*/15 min/4 °C). The supernatant was discarded aseptically and 2 mL of autoclaved $CaCl_2$ (100 mM) was added to the pellet and again resuspended gently. The cells were kept on ice overnight prior to their use. The cells were dispensed into aliquots of 200 μL each. Glycerol stocks (15%) of these cells were prepared and stored at −70 °C for subsequent use.

Transformation efficiency of the competent cells was checked by transforming them with an uncut plasmid and calculating the cfu/μg of DNA. The transformation efficiency of 1×10^8 cfu/μg DNA and above were taken as a good competent cell preparation. Transformation of *E. coli* BL21 (DE3) cells was carried out as per the instructions given by Sambrook and Russell [16].

2.3.7. Induction of recombinant 78 kDa (T cell epitope region) expression

Overnight log phase culture (1%) was transferred to LB + Km_{50} and allowed to grow to OD_{600} of 0.5–0.6 at 37 °C, 180 rpm before the expression of $(His)_6$ tagged region was induced by the addition of IPTG (final concentration of 0.1–1 mM) and incubated at temperatures ranging between 15 °C and 37 °C for 3–20 h. The induction was then analyzed by SDS-PAGE. The gels were stained for 4 h by standard coomassie brilliant blue R 250 and destained with a methanol (25%)/acetic acid (10%) solution.

2.3.8. Purification of recombinant 78 kDa (T cell epitope region) protein using Ni-NTA

For purification of the protein, IPTG induced cells from a 200 mL culture were pelleted, resuspended in lysis buffer (Tris-Cl 50 mM, pH 7.0, NaCl 300 mM, phenylmethylsulfonyl fluorid 1 mM, lysozyme 1 mM and imidazole 10 mM) and sonicated (at 20 kHz, 30 cycles of 10 s shock followed by 10 s of rest). After sonication, the cellular debris was removed by centrifugation at 14 000 × *g* for 15 min at 4 °C. The crude cell extract was loaded onto slurry of nickel-chelating agarose beads 50% (w/v) (2 mL) also equilibrated with the same buffer. The flow through was collected and the column was given washings with 5 column volumes of lysis buffer having imidazole (20 and 30 mM, respectively). Purified recombinant protein was then eluted with lysis buffer amended with imidazole (200 mM). 1 mL fractions were collected and checked for purified protein on 10% SDS-PAGE. The fractions showing purified protein were then pooled and dialyzed against Tris-Cl (20 mM, pH 7.0) to remove imidazole and NaCl. The purified recombinant protein was then named r78 (Figure 2).

2.4. In vivo immunization and challenge infection

BALB/c mice were immunized with 10 μg of purified protein r78 (region coding for T cell epitope only) along with two adjuvants namely monophosphoryl lipid A (MPL-A) (40 μg) and encapsulation in positively charged liposomes. Two booster doses with respective vaccine were given at an interval of 2 weeks each. The control animals were injected with PBS only. 15 days after the last immunization, immunized and control animals were challenged intracardially with 1×10^7 promastigotes of *L. donovani*.

2.5. Evaluation of infection

The animals were sacrificed on 15, 30, 45, 60, 75 and 90 days post infection/challenge and the course of infection was monitored by the microscopic examination of Giemsa-stained impression smears of liver. The parasite load was assessed in terms of Leishman Donovan Units by the method of Bradley and Kirkley [17].

2.6. Delayed type hypersensitivity (DTH)

All groups of mice were challenged in the right foot pad with a subcutaneous injection of leishmanin. Left foot pad was inoculated with PBS. After 48 h, the thickness of right and left foot pad was measured using a pair of Vernier calipers. Results were expressed as mean ± SD of percentage increase in the thickness of the right foot-pad as compared to the left footpad of mice [18].

2.7. ELISA

The specific serum immunoglobulin G (IgG) isotype antibody response was measured by conventional ELISA using commercially available kits (Bangalore Genei, India). 96-well ELISA plates were coated with crude antigen. Serum samples were added at twofold serial dilutions, followed by washes and addition of isotype specific HRP-conjugated secondary antibodies (rabbit anti-mouse IgG1 or IgG2a) after which the substrate and chromogen were added and absorbance was read on an ELISA plate reader at 450 nm [18].

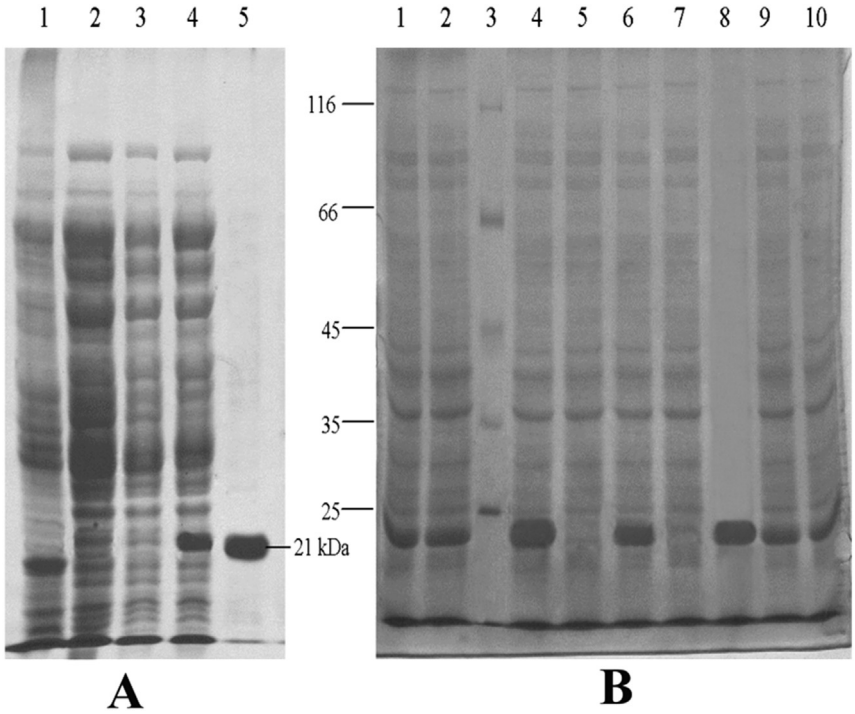

Figure 2. Purification of recombinant 78 kDa (T cell epitope region) protein using Ni-NTA.
A Lane1: Pellet of IPTG induced sonicated cells; Lane 2: Whole cell lysate of uninduced cells; Lane 3: Ni-NTA column flowthrough; Lane 4: Whole cell lysate of IPTG induced cells; Lane 5: Purified recombinant protein. B Lane 3: Standard protein molecule weight markers; Lane 1, 2, 4, 6, 9, 10: Whole cell lysate of IPTG induced cells (at different temperatures); Lane 5, 7: Whole cell lysate of uninduced cells; Lane 8: Purified recombinant protein.

2.8. Cytokine responses

The lymphocyte culture was obtained from spleens of immunized BALB/c mice. The cells were isolated and counted, and, after evaluation of their viability by trypan blue exclusion, 1×10^6 cells per well were cultured in RPMI-1640 containing 20 mM $NaHCO_3$, 10 mM HEPES, 100 U of penicillin per mL, 100 μg of streptomycin per mL, 2 mM L-glutamine, and 10% fetal calf serum (complete medium), with 50 μL β-mercaptoethanol. The spleen cells were cultured in triplicate in a final volume of 200 μL/well and stimulated with r78 antigen. The culture was incubated for 72 h at 37 °C in a humified chamber containing 5% CO_2. The IL-4, IL-10, IFN-γ and IL-2 levels in the supernatants were determined by sandwich ELISA, according to manufacturer's instructions (Diaclone, France), after sacrificing all the groups of animals [19].

2.9. Statistical analysis

All the experiments were carried out three times independently. All data comparisons were tested for significance by using Student's *t*-test; *P*-values below 0.05 were considered significant. Results were expressed as mean ± SD of one of three independent experiments.

3. Results

3.1. Parasite load

Immunization with r78 along with MPL-A (Sigma, USA) and liposome-encapsulated r78 brought a significant reduction in parasite load when compared with infected controls. In comparison to the infected controls, the parasite load declined from 47.0% to 96.2% in case of mice immunized with r78 plus MPL-A while the reduction was 49.00%–97.23% in animals immunized with liposome-encapsulated r78 (Figure 3A).

3.2. Delayed type hypersensitivity response

The animals immunized with r78 along with adjuvants exhibited profound DTH response. Significant increase in footpad thickness was observed in immunized mice as compared to control mice (Figure 3B).

3.3. Anti-leishmanial antibody response

The antibody responses increased from 15 to 90 days post infection/challenge. Immunized animals showed greater IgG2a levels and lesser IgG1 levels in comparison to the infected controls (Figure 4).

3.4. Vaccine-induced cytokine production

The splenic lymphocytes from immunized mice were cultured, stimulated with r78 and analyzed for the cytokine profile. The levels of IL-2, IFN- γ, IL-4 and IL-10 secreted by cells were measured by using cytokine assay kit according to the manufacturer's instructions (Diaclone, France). The levels of IFN- γ and IL-2 were greater in immunized animals as compared to control mice. However the concentration of IL-4 and IL-10 was lesser in immunized animals as compared to the control mice (Figures 5 and 6).

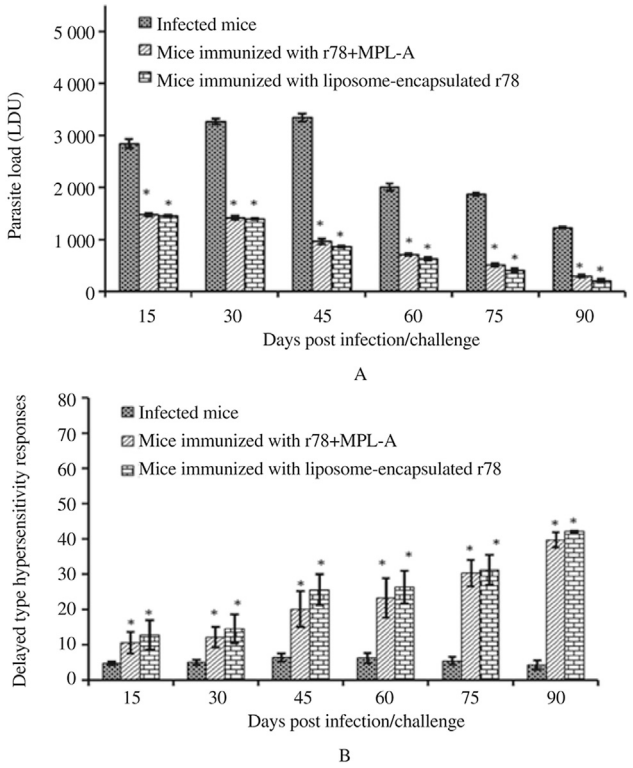

Figure 3. Leishman-Donovan units and percentage increase in footpad thickness (DTH response) of mice immunized with r78 plus different adjuvants.
A: Leishman-Donovan units; B: Percentage increase in footpad thickness. Control *vs*. r78 + MPL-A/Liposome-encapsulated r78, *$P < 0.05$.

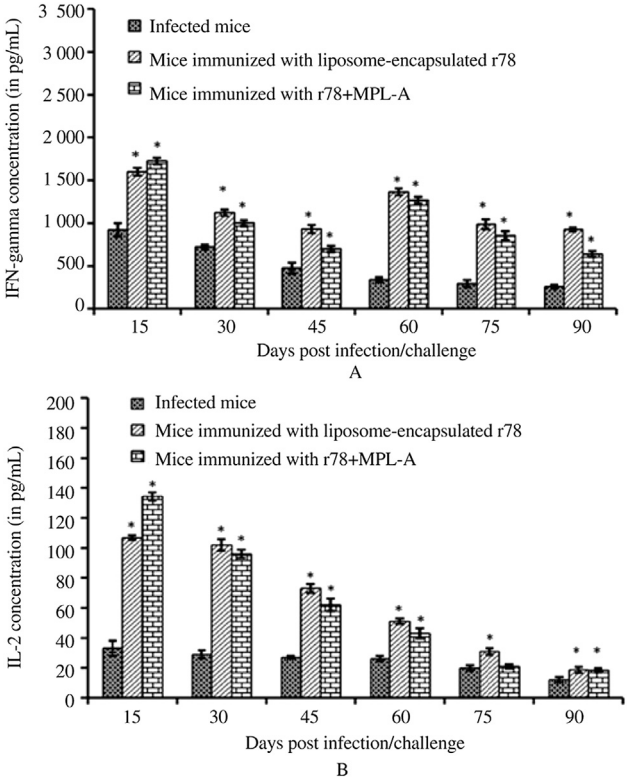

Figure 5. IFN-γ and IL-2 levels in mice immunized with r78 plus different adjuvants.
A: IFN-γ level; B: IL-2 level. Control *vs*. r78 + MPL-A/Liposome-encapsulated r78, *$P < 0.05$.

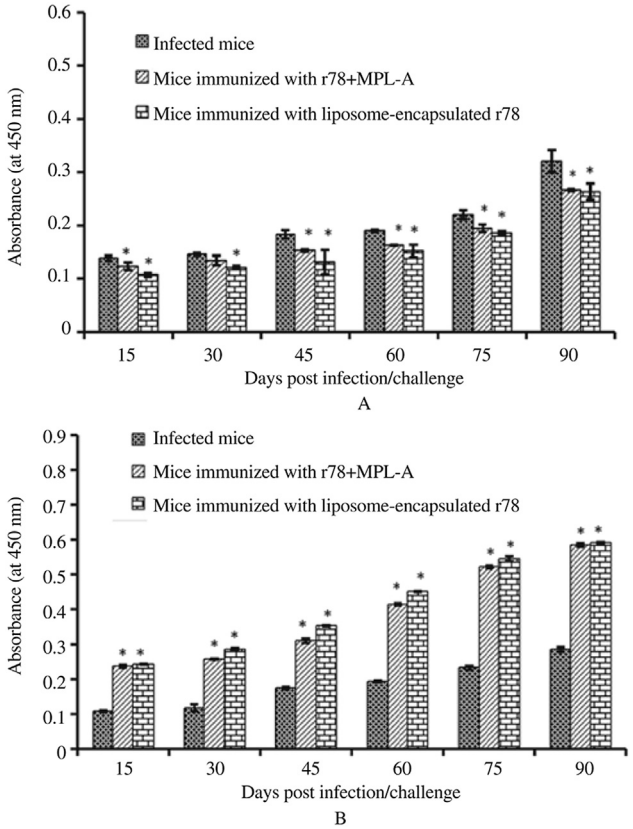

Figure 4. IgG1 levels and IgG2a levels in mice immunized with r78 plus different adjuvants.
A: IgG1 levels; B: IgG2a levels. Control *vs*. r78 + MPL-A/Liposome-encapsulated r78,*$P < 0.05$.

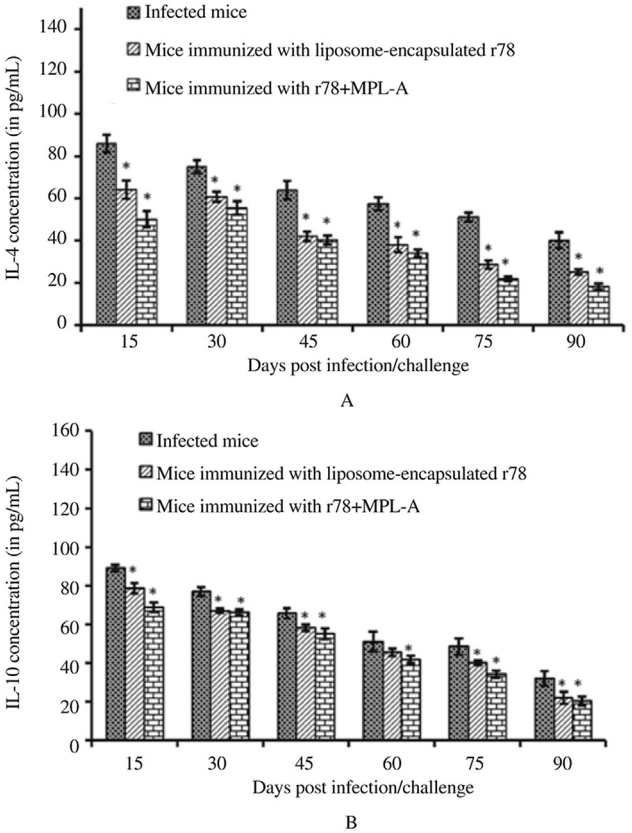

Figure 6. IL-4 and IL-10 levels in mice immunized with r78 plus different adjuvants.
A: IL-4 level; B: IL-10 level. Control *vs*. r78 + MPL-A/Liposome-encapsulated r78, *$P < 0.05$.

4. Discussion

The treatment options for leishmaniasis are limited and the increased resistance to first line drugs and toxicity of second line drugs makes the development of effective vaccine against the disease highly desirable. Though we say, on the basis of present available data, that there is no effective vaccine against any form of human leishmaniasis, yet, the palpable immunity in a person against reinfection following recovery from the disease strongly gives inkling of the feasibility of vaccination against leishmaniasis [20]. Previously, in our laboratory, we worked on the protective efficacy of 78 kDa antigen of *L. donovani* against murine visceral leishmaniasis [19]. The antigen was found to be effective against the disease though the protection afforded was not complete. Therefore, in the current study we worked on the recombinant version of the antigen in combination with two different adjuvants: MPL-A and cationic liposomes. The efficacy of the vaccine was tested in terms of parasite burden and generation of immune responses.

BALB/c mice immunized with r78 alone and in combination with MPL-A or cationic liposomes showed a significant reduction in parasite load as compared to the infected controls. Least parasite burden was observed in mice immunized with liposome-encapsulated r78. Our results are in consistence with previous studies which showed the efficacy of recombinant proteins against leishmaniasis. Animals immunized with the recombinant protein presented approximately a 100-fold reduction in parasite burden compared to controls that received PBS, BCG or *Propionibacterium acnes* alone [6]. In contrast to our study where immunization with liposome-encapsulated r78 was slightly more effective in reducing the parasite burden as compared to r78 given along with MPL-A, vaccination with liposomal rGP63 along with MPL-TDM showed almost 2-logfold, and 7–10-log-fold reduction in parasite burden compared to mice boosted with liposomal rGP63 and unvaccinated mice, respectively [21]. However, immunization with recombinant isolates of LeIF, LmSTI1 and TSA from *Leishmania braziliensis* (*L. braziliensis*) individually or in combination did not protect mice against challenge infection with *L. braziliensis* despite the induction of antibody and cell mediated responses [22].

The humoral responses to the vaccines were characterized by analyzing the distribution of IgG, IgG1 and IgG2a specific antibodies in the serum samples of immunized and control animals. ELISA results showed that immunized groups developed higher level of antibody responses than control animals. The IgG1 response is an indicator of Th2 type of immune response and IgG2a antibody response corresponds to Th1 response which is protective. The immunized animals revealed lesser IgG1 and higher IgG2a responses as compared to the infected controls. Among the immunized groups, the serum antibody levels in mice immunized with liposome-encapsulated r78 and r78 along with MPL-A were comparable.

A typical DTH reaction is characterized by activation and recruitment predominantly of T cells and macrophages at the site of intradermal injection in previously sensitized host. Peak DTH responses were observed in animals which were immunized with liposome-encapsulated r78 vaccine and these were followed by animals immunized with r78+MPL-A. The immunized mice developed a strong cell-mediated immune response and thereby resisted the challenge by parasites. The results demonstrated a positive correlation between enhanced DTH response and reduced parasite load for all groups.

The immune response (Th1 or Th2) generated by various vaccine formulations was assessed by quantifying the cytokines (IFN-γ, IL-2, IL-4 and IL-10) produced by splenocytes of vaccinated animals. Immunized animals showed greater IFN- γ and IL-2 levels as compared to the control group. Also, the levels of IL-4 and IL-10 were greater in control animals as compared to the immunized animals. High levels of IFN-γ indicate the development of a protective Th1 immune response. This IFN-γ is found to come from natural killer cells and is thought to play an important role as a component of innate immune mechanism for the activation of macrophages. Similar to our study, rLdccys1 induced a significant secretion of IFN-γ and nitric oxide (NO) [6]. Immunization of mice with recombinant LeIF stimulates the induction of Th1 type immune response with increased production of IFN-γ [23], Similarly, significantly higher levels of IFN-γ and IL-12 were observed in groups of mice receiving rGP63 in presence of liposomes and MPL-TDM during prime and boost than mice receiving liposomal rGP63 during boost, after *L. donovani* challenge infection [21]. While MPL is reported to promote IFN-γ production by Ag-specific $CD4^+$ T cells and skewing the immune response towards Th1 type [24], liposomal Ag showed protective response against leishmaniasis [25]. IL-2 plays a critical role in priming naive $CD4^+$ T cells to become IL-4 or IFN- γ producers [26] and it is a principal T cell growth factor for Th1 type of immune response. IL-10 represents the main macrophage-deactivating cytokine and plays an important regulatory role in the progression of VL [27].Our results are in consistence with those obtained from murine leishmaniasis model of both cutaneous and visceral diseases where expression of IFN-γ is associated with control of infection [28,29]. Our results are also in correspondence with the studies which showed that mice immunized with 78 kDa antigen + MPL-A significantly brought down the IL-4 and IL-10 levels in immunized mice [19]. Studies have also shown that immunization with Leish-111f + MPL-SE protected mice and hamsters against *L. infantum* infection that correlated with induction of Th1 immune response as characterized with production of IFN-γ, TNF and IL-2 with little IL-4 [30]. High levels of IL-10 in infected control animals supported the view that upregulation of this cytokine is accompanied by disease progression and depressed Th1 type of cell mediated immunity with decreased production of IFN-γ and IL-12 [31,32]. Previous studies have also confirmed that IL-10 suppresses Th1 response, which plays a protective role against active VL [33]. Moreover studies have shown that IL-10 gene deficient mice are resistant to *L. donovani* infection and produce more IFN- γ and IL-12 [34].

In light of these observations, the present study suggests that a recombinant vaccine based on r78 given along with liposomes and MPL-A as adjuvants is protective against experimental murine visceral leishmaniasis and can be further tested in higher animal models to establish its potential as a vaccine candidate against the disease.

The study proves that r78 in combination with suitable adjuvants is a potential vaccine candidate and may be instrumental in control of visceral leishmaniasis. Further studies are required to explore these vaccines in higher animal models and establish their prophylactic potential.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

The authors acknowledge the support provided by the Indian Council of Medical Research, Department of Health Research, India for providing financial support for this study under project ref. 5/8-7(74)/2005-ECD-Ⅱ.

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