Infection, Genetics and Evolution 70 (2019) 27–35

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

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

Research paper

# Protection induced by *Leishmania Major* antigens and the imiquimod adjuvant encapsulated on liposomes in experimental cutaneous leishmaniasis

Ahmad Mehravaran[a,b,*], Maryam Rezaei Nasab[b], Hadi Mirahmadi[a,b], Iraj Sharifi[c], Ebrahim Alijani[d], Amin Reza Nikpoor[e], Javad Akhtari[f]

[a] Infectious Diseases and Tropical Medicine Research Center, Resistant Tuberculosis institute, Zahedan University of Medical Sciences, Zahedan, Iran
[b] Department of Parasitology and Mycology, Faculty of Medicine, Zahedan University of Medical Sciences, Zahedan, Iran
[c] Leishmaniasis Research Center, Kerman University of Medical Sciences, Kerman, Iran
[d] Clinical Immunology Research Center, Zahedan University of Medical Sciences, Zahedan, Iran
[e] Immunogenetic and Cell Culture Department, Immunology Research Center, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[f] Immunogenetics Research Center, Department of Medical Nanotechnology, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

ARTICLE INFO

*Keywords:*
Liposome
Imiquimod
Immune response
*Leishmania major*

ABSTRACT

There is a need for new, effective, and less expensive and toxic treatment for Leishmaniasis. It seems that the use of a suitable adjuvant and a delivery system is effective in inducing immune reactions for protection. Liposomes can be applied as immunoadjuvants to trigger immune reactions to different antigens. The adjuvant effects of imiquimod using DSPC liposomes containing SLA (soluble *Leishmania* antigens) were studied on the type and intensity of the produced immune reaction to the challenge *of Leishmania major* in BALB/c mice. Liposomes were produced by the lipid film procedure. BALB/C mice were immunized subcutaneously, three times at 2-week intervals and with various formulations. Lesion development and the parasite burden in the spleens and feet after the challenge with *Leishmania major*, Th1 cytokine (IFN-γ), and the IgG isotype titration were assessed to evaluate the induced immune reaction and the protection level.

The group of mice immunized with Liposome DSPC + Imiquimod + SLA revealed less severe footpad swelling, being significantly different ($P < .05$) from other groups. A higher level of IgG2a and IFN-γ secretion was observed in the mice immunized with Liposome DSPC + Imiquimod + SLA than the control group. These observations imply that the DSPC liposome containing imiquimod induces the Th1 immune response that is protective against the challenge of *Leishmania major*.

## 1. Introduction

Leishmaniasis is a protozoan infectious disease that is caused by some species of *Leishmania* and is transmitted by the infected sand fly bites. Based on the parasite species and the immune reaction of the host, the disease outcomes range from asymptomatic infections to clinical forms (WHO, 2016). Symptoms are manifested more prevalently as single self-healing cutaneous lesions, uncontrolled parasite replications, the generation of the non-healing cutaneous, mucosal, or even visceral illness, and chronic metastatic dissemination in the skin. The manifestations are multifactorial and depend on complicated interactions among the host, the parasite, as well as environmental factors, such as the genetic background, *Leishmania* species, and the immunological status of the host (Hartley et al., 2014).

Research has revealed that most available medications have major challenges and limitations, including fast renal expulsion or a short half-life in the blood circulation system, toxicity, and high costs. In most cases, complications hamper the treatment significantly (Heidari-Kharaji et al., 2016). No certain vaccine has so far been found against leishmaniasis. Vaccination using controlled infection with the viable parasites, or "leishmanization" practiced in the West Asian countries has been stopped because of undesired complications (Greenblatt, 1980; Nadim et al., 1983).

In addition, the use of live vaccines creates numerous problems, including the development of many cases of uncontrolled skin damage, psoriasis exacerbation, and other skin diseases. Nevertheless, despite creative endeavors made in the past, a few first-generation vaccines of whole killed *Leishmania* alone or mixed with BCG reached phase III

* Corresponding author at: Department of Parasitology and Mycology, Zahedan University of Medical Sciences, Zahedan, P.o Box: 9816913396, Iran.
*E-mail address:* ahmadmehravaran55@gmail.com (A. Mehravaran).

https://doi.org/10.1016/j.meegid.2019.01.005
Received 20 October 2018; Received in revised form 22 December 2018; Accepted 7 January 2019
Available online 07 February 2019
1567-1348/ 

clinical treatments (Badiee et al., 2007; Coler et al., 2002; Raman et al., 2012). Thus, a definitive stage in the vaccine development attempts requires an enhanced perception of the functional heterogeneity of T-cell cytokine reactions. Naive CD4 + T cells may turn into a functional proper subset called Th1 or Th2 cells after activation. Thus, a critical phase in the development of the vaccine needs the enhanced perception of the functional heterogeneity of T-cell cytokine reactions (Copland et al., 2005; Darrah et al., 2007).

The control of leishmaniasis is regulated by the cell-mediated immune response, since the parasites evade the humoral immune response by residing in the phagolysosomes of macrophages. The proliferation of Th1 (T helper) cells induces immunization through leishmanicidal cytokines, including IL-12, IFN-γ and TNF-α, yet the Th2 cell induces immunization through IL-4, IL-5, IL-10, and IL-13, with the transforming growth factor beta generation being related to susceptibility to the disease (Coler et al., 2002; Darrah et al., 2007; Rhee et al., 2002; Uzonna et al., 2001).

One of the reasons for the limited effectiveness of first-generation vaccines is the lack of a convenient adjuvant. Using a proper adjuvant is crucial in inducing the immune reaction required for protection (Noazin et al., 2008; Soto et al., 2009). Imiquimod (R837) and the more powerful analogues, i.e. R-848 and S-28463, are from a family of imidazoquinolines, with imiquimod having an immune response modifier with a potent indirect antiviral activity. The antiviral activity of imiquimod was first shown in guinea pigs infected with the herpes simplex virus. Imiquimod is now an approved treatment for external genital warts caused by the human papillomavirus infection(Jurk et al., 2002).

The innate immune response can be activated by various pathogen-associated molecular patterns via Toll-like receptors (TLRs), with this playing an important role in directing the acquired immune response (Iwasaki and Medzhitov, 2004). From among the 11 mammalian TLRs, TLRs 3, 7, 8, and 9 are present in the endosomes of cells and detect the nucleic acids of intracellular DNA and RNA pathogens(Takeda et al., 2003; Wickelgren, 2006).

Through its immunomodulatory activity, imiquimod exerts some effects on various cells involved in the immune system and induces the release of a number of cytokines, including interferon (IFN-γ), the tumor necrosis factor (TNF)-α, interleukin (IL)-1b, IL-6, and IL-8 (Iwasake and Medzhitov, 2004; Jurk et al., 2002).

Using a gene array approach, consistent with the ability of imiquimod to activate the killing of intracellular *Leishmania* amastigotes, it was demonstrated that the imiquimod's related compound S-28463 induced gene expression associated with macrophage activation and inflammatory responses, including NF-κB, IL-1, iNOS, and MIP-1 (Buates and Matlashewski, 2001). Later on, it was discovered that imiquimod resembled structurally the single stranded RNA capable of activating macrophages through stimulating the TLR7 pathway (Hemmi et al., 2002).

Monocytes and macrophages are the principal target cells for imiquimod (Iwasake and Medzhitov, 2004; Jurk et al., 2002), since imiquimod has been shown to modulate the activity of monocytes and macrophages, resulting in antiviral effects; it has also been proved as a safe and effective medication against cutaneous lesions caused by viral infections. The combination of delivery systems and immunopotentiating adjuvants is a useful approach to the rational design of vaccines (Moon et al., 2011; Nordly et al., 2011).

Despite efforts that now span more than a century, a licensed vaccine for use in human is not yet available. It seems that the limited efficacy of experimental vaccines is due to lack of appropriate adjuvant and/or delivery system(Badiee et al., 2013; Khamesipour et al., 2006). Hence, the use of particulate delivery systems as the carriers of antigens or immunostimulatory adjuvants is a valuable strategy for the effective delivery to the antigen-presenting cells (APCs) to enhance vaccine efficacy. Particle-based delivery systems, such as liposomes have the potential for the successful delivery of antigens, because of their biodegradability, biocompatibility, and non-immunologic size and surface charge, which can then be further improved through the incorporation of additional antigenic or immustimulatory adjuvant components to or onto the particle carrier system (Iwasake and Medzhitov, 2004; Jurk et al., 2002).

So far, apart from liposomes, various nanocarriers, such as micelles, polymersomes, archaeosomes, and ISCOMs have been employed to deliver protein antigens to professional APCs (Badiee et al., 2008; Joshi et al., 2012; Kataoka et al., 2012; Krishnan et al., 2003; Mazumder et al., 2011; Mehravaran et al., 2016; Mehravaran et al., 2015a; Mehravaran et al., 2015b; Smith et al., 2013). Utilizing liposomes as a transport method to deliver antigens was first conducted some years ago. Liposomes are artificial closed vesicles made of concentric lipid bilayers, which are isolated by aqueous domains and used as delivery systems for proteins, peptides, drugs, and DNA and may also be applied as immunoadjuvants to stimulate immune reactions in different antigens (Alving, 1995; O'Hagan and Singh, 2003). In this study, distearoyl phosphatidylcholine (DSPC) was examined because of its great transition point and cholesterol, which makes the liposomes turn into steady liposomes that inhibit easy clearance from blood. Numerous studies have shown that liposomes act as immunological adjuvants for a wide range of involved bacterial and viral antigens associated with human and veterinary immunization. The major feature of particulate adjuvants and transfer systems is the efficient antigen delivery for improved uptake by antigen exhibiting cells (APCs), especially dendritic cells (DCs). These adjuvants are mainly taken up by classical APCs, such as macrophages and DCs (Badiee et al., 2009; Davis and Gregoriadis, 1987; Patere et al., 2017; Tafaghodi et al., 2016).

In the current research, the adjuvant effects of imiquimod were studied using DSPC liposomes containing SLA (soluble *Leishmania* antigens) on the type and intensity of the produced immune reaction to the challenge of *Leishmania major* in BALB/c mice.

## 2. Materials and methods

### 2.1. Animals, ethics statement

Studies were performed with Female 6–8 week old BALB/c mice. The mice were holed in the Animal Research Center of Zahedan University of Medical Sciences and fed with tap water and standard laboratory pellet chow. Animals were kept at 21 °C in a colony room at 12/12 h light/dark cycle with free access to food and water. The protocol was confirmed by the Institutional Ethical Committee and Research Advisory Committee of Zahedan University of Medical Sciences (Education Office dated March 31, 2010; proposal code, 88527), on the basis of the Specific National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry Of Health and Medicinal Education (MOHME) of Iran.

### 2.2. Parasites, imiquimod, and soluble leishmania antigen (SLA)

*L. major* strain (MRHO/IR/75/ER) employed in this treatment was utilized in leishmanization and to prepare experimental *Leishmania* vaccine and leishmanin test in Iran(Alimohammadian et al., 1993; Khamesipour et al., 2006). Imiquimod (R837) was provided from Invivogen Company. The preparation of SLA was done applying the protocol developed with some modifications. The parasites were harvested at stationary phase and rinsed three times using HEPES buffer (10 mM + sucrose 10%, pH 7.4) (Scott et al., 1987). Then, promastigotes was set to 1.2 × 109 per mL in buffer having enzyme inhibitor cocktail, 50-μl/ml (Sigma, St. Louis, MO, USA). The parasites were then lysed by freeze-thaw procedure accompanied by probe sonication in an ice bath. The supernatant of the centrifuged lysate parasites was gathered, dialyzed against HS buffer solution, and sterilized by passage through a 0.22 μm membrane and kept at −70 °C. The concentration of protein of the SLA was indicated applying BCA (bicinchoninic acid) protein assay kit (Thermo Scientific, USA)(Bainor et al., 2011).

### 2.3. Liposome preparation and characterization

Liposomes were provided applying lipid film procedure. The lipid phase made of DSPC (1,2-distearoyl-*sn*-glycero-3-phosphocholine) (20 mM; Avanti polar lipids, USA) and cholesterol (10 mM; Avanti polar lipids, USA) (2:1 M ratio) were dissolved in a sterile tube in chloroform. The solvent was removed using rotary evaporation (Hettich, Germany), depositing a thin lipid film on the wall of tube. The lipid film was then freeze–dried (TAITEC, Japan) across the night to remove the solvent totally. The lipid film was hydrated and dispersed in sterile buffer (HEPES buffer 10 mM pH 7.4) with SLA (2 mg/ml). The multilamellar vesicles (MLVs) were converted to unilamellar vesicles by bath sonicator (Bandelin, Germany) at 45 °C for 15 min under argon. The liposome dispersion was extruded 13 times by 400 nm polycarbonate membranes (Avestin, Canada). The zeta potential and particle size of liposome preparations were obtained applying Dynamic Light Scattering Instrument (Nano-ZS, Malvern, UK). Particle sizes were reported as poly dispersity index (PDI) and the mean ± standard deviation (n = 3). The zeta potentials were determined on the same machine by the zeta potential mode as the means ± zeta deviation (n = 3) (O'Hagan and Singh, 2003).

### 2.4. Characterization of the prepared formulations

Analysis of SLA and liposomal SLA analytical SDS-PAGE was done to qualitatively calculate the SLA concentration encapsulated in the liposomal SLA (Lip-SLA). The discontinuous system was made of running and stacking gel of 1 mm thickness (12.5% and 4.78% *w/v* acrylamide, respectively). The electrophoresis buffer was 25 mM Tris, 192 mM glycine, and 0.1% SDS at pH 8.3. Electrophoresis was done for 45 min at 140 V constant voltages. The same SLA amount (2.5 or 5_g) was loaded to every well for various formulations. After electrophoresis, the gels were stained for protein detection with silver (Torchilin, 2005).

### 2.5. Immunization of BALB/c mice

BALB/c mice, 10 mice in every group, were immunized subcutaneously (SC) three times at a 3-week interval in the footpad (RF) with one the after formulations: HEPES buffer, SLA, Lip DSPC, Lip + imiquimod, Lip + Imiquimod + SLA, in final volume of 50 µl.

### 2.6. Challenge with L. major promastigotes

The control and immunized group of mice; two weeks following the last injection of booster, $1 \times 10^6$ late stationary phase L. *major* promastigotes in 50 µl volume inoculated SC into the right footpad. In each mouse lesion progression was followed by weekly measurement of the thickness of the footpad using a digital caliper (Mitutoyo Measuring Instruments, Japan). Grading of lesion size was done by subtracting the thickness of uninfected contralateral footpad from that of the infected one.

### 2.7. Quantitative parasite burden after challenge

The viable parasite, spleens, and footpad from mice vaccinated with L. *major* were harvested in every treatment group. The number of viable L. *major* parasites in the mice footpad and spleen was obtained by limitation of dilution assay procedure as previously defined (Taswell, 1981; Titus et al., 1985). The mice were killed at week 6 after-challenge. The feet were removed aseptically and homogenized in RPMI 1640 supplemented with 10% *v/v* heat inactivated FCS (Eurobio, Scandinavie), 2 mM glutamine, 100 units of penicillin per ml, and 100 µg/ml of streptomycin sulfate (RPMI-FCS). The homogenate was diluted in eight serial 10-fold dilutions with the media and put in every well of flat-bottom 96-well microtiter plates (Nunc, Denmark), having solid layer of rabbit blood agar in tetraplicate; they were incubated for 7–10 days at 25 ± 1 °C. The negative and positive wells (absence and presence of motile parasite, respectively) were identified by an invert microscope (CETI, UK). The number of viable parasite per spleen was determined using GraphPad Prism software, a statistical technique for limitation of the dilution assay (Taswell, 1981).

### 2.8. Antibody isotype assay

The levels of antigen-specific serum IgG subclasses were measured by a standard enzyme linked immunosorbent assay (ELISA) procedure. Samples of blood were obtained from mice at week 2 prior to and at week 6 after challenge. The sera were separated and stored at −20 °C. The evaluation of anti-SLA IgG total, IgG1, and IgG2a was carried out to identify bound antibodies (Badiee et al., 2007). Microtiter plates (Nunc, Denmark) were covered with 50 µl of SLA (10 µg/ml) in PBS buffer (0.01 M, pH 7.3) and serial dilutions of serum at 4 °C overnight. The plates were treated with HRP-rabbit anti-mouse IgG isotype based on the instructions of the manufacturer (Invitrogen Inc., USA). Optical density (OD) was indicated at 450 nm by 630 nm as the criterion wavelength.

### 2.9. ELISpot

ELISpot assay was done for identification of gamma interferon (IFN-γ) and interleukin-4(IL-4), applying mouse ELISpot kits from U-cytech (Utrecht, the Netherlands). Three mice from every group at week 2 after the last injection of booster (before challenge) were killed, and their splenocytes were separated and restimulated in vitro via either SLA as a recalled antigen or mitogen Concanavalin A (Con A) as a positive control. ELISpot plates were covered with anti-IFN-γ or anti-IL-4 antibodies and incubated overnight at 4 °C. The splenocytes (5 × 105 cells/well) were cultured in triplicate in 200 µl with merely DMEM (as background responses), medium having Con A (as positive controls), or medium with 10 µg/ml of SLA in the pre-coated plates. Spot counting was performed by Kodak 1D software (Version 3.5, Eastman Kodak, Rochester, New York) after incubation (37 °C, 5% CO2) for 24 h (for IFN-γ assay) or 48 h (for IL-4 assay). The average number of spots ± SD in triplicate wells was obtained and stated as spot-forming units (SFU) per 105 splenocytes.

### 2.10. Flow cytometry

Determine cellular uptake of formulations, spleenocytes were separated 2 weeks after the last booster and stained for intracellular cytokine IFN-γ (anti-IFN-γ–FITC) and IL-4 (anti-IL-4-FITC) based on BD protocols Cytofix/Cytoperm™ plus Fixation/Permeabilization Kit. Splenocytes ($10^6$ cells/ml) in medium with GolgiPlug™ (1 µl/ml) were excited with PMA/ionomycin cocktail (2 µl/ml) for 4 h at 37 °C. $10^5$ splenocytes were added into tubes of flow cytometry after stimulation, and rinsed twice with stain buffer (2% FCS in PBS). Splenocytes were stained with1 µl anti CD4-PE-cy5 antibody and 1 µl anti-CD8a-PE-cy5 antibody in isolated tubes at 4 °C for 30 min. The cells were rinsed with stain buffer and fixed by solution og Cytofix/Cytoperm™. The fixed cells were rinsed twice with Perm/Wash™ buffer and then stained with 1 µl anti-IFN-γ- FITC antibody at 4 °C for 30 min. CD4 cells were also stained with 1 µl anti-IL-4-PE antibody. The cells were washed with Perm/Wash™ buffer and suspended in 300 µl stain buffer for flow cytometric analysis Calibur (BD Biosciences, USA).

### 2.11. Statistical analysis

The Statistical difference among groups were recorded and analyzed in GraphPad Prism software. One-way ANOVA statistical test assessed the significance of the variations between different groups. Tukey–Kramer multiple comparisons were done as a post-test to evaluate the means in different mice groups. $P < .05$ was assumed as

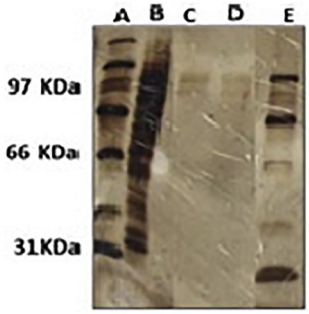


**Fig. 1.** SDS-PAGE analysis; Lane A: Low-range protein standard (Sigma, USA); Lane B: SLA; Lane C: Empty Liposome; Lane D: Empty Liposome + Imiquimod; Lane E: Lip + Imiquimod + SLA.

statistically meaningful.

## 3. Results

### 3.1. Liposome characterization

Lip + SLA + Imiquimod, Lip + Imiquimod, and Empty liposome in this research were homogenous vesicles with, respectively, average diameters of 215 ± 11 nm (PDI: 0.3 ± 0.01), 195 ± 8 nm (PDI: 0.321 ± 0.01) and 174 ± 12 nm (PDI: 0.218 ± 0.01). The zeta potentials were, respectively, −6.5 ± 2 and − 1.84 ± 3 and 8.83 ± 1 mV, (n = 3). The SLA entrapment in Liposome was estimated 64.6 ± 6.2% (n = 3). The SLA concentration in prepared formulations was set to 50 μg per 50 μl just prior to injection. Liposomal SLA and SLA characterization was done by SDS-PAGE electrophoresis (Fig. 1). SLA SDS-PAGE analysis demonstrated different protein bands with several ranges. The liposomal SLA analysis showed almost each band alike free SLA, revealing that proteins with SLA were entrapped into the formulation after the preparation of liposomes.

### 3.2. Challenge results

Swelling of footpad in BALB/c mice was controlled by weekly evaluation (Fig. 2). The size of lesion was developed at a fast rate in controls receiving SLA or buffer in comparison with the group of mice immunized with Lip DSPC, Lip + Imiquimod, and Lip + Imiquimod + SLA after challenge. At the end of the research, the lowest lesions sizes with significant variation ($P < .05$) was observed in mice immunized Lip + Imiquimod + SLA with other mice.

### 3.3. Parasite burden in footpad after challenge

The viable L. *major* number was determined in the infected footpad of various mice groups at week 6 post inoculation (Fig. 3A). Mice immunized with Lip + Imiquimod + SLA revealed the least parasite burden in comparison to others, but there was no meaningful variation in the parasites' number in all vaccinated groups in comparison to the control.

### 3.4. Parasite burden in spleen

At week 7 post challenge, the viable L. *major* parasites number was estimated in the spleen of various mice groups (Fig. 3B). Mice immunized with Lip + Imiquimod + SLA revealed the least live parasites compared with other groups. However, no meaningful difference was seen in the splenic parasite count in all vaccinated groups in comparison to the control.

### 3.5. Antibody response

To indicate the type of immune reaction generated, the anti-SLA IgG

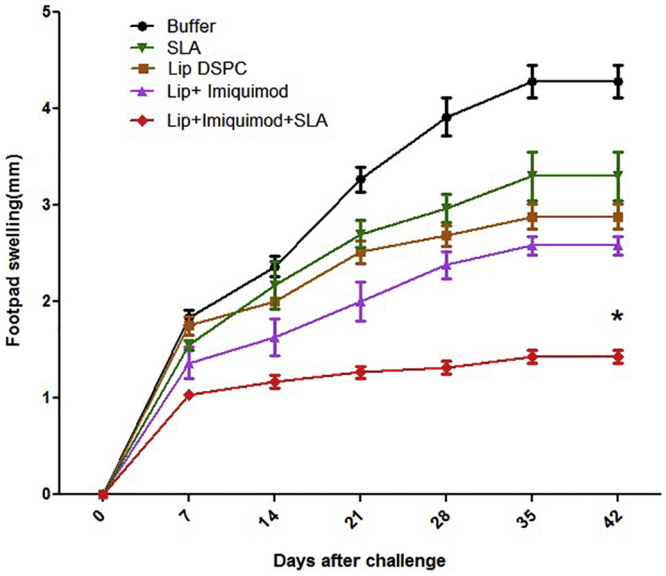


**Fig. 2.** Footpad swelling in BALB/c mice immunized SC, three times in 3-week intervals, with SLA, Lip DSPC, Lip + Imiquimod, Lip + Imiquimod + SLA or buffer alone. The footpad thickness of each mouse was measured on both footpads for 42 days. Each point represents the average increase in footpad thickness ± SEM (n = 7). *$P < .05$ when the mice immunized with Lip + Imiquimod + SLA are compared with the mice that received HEPES buffer.

antibodies specific IgG1, IgG2a, and IgG subclasses were titrated prior to (Fig. 4A–C) and after (Fig. 5A–C) the challenge. Before challenge as shown in Fig. 4A–C, there was a considerable ($P < .0001$) variation in the levels of IgG1in Lip DSPC + Imiquimod and Lip DSPC + Imiquimod + SLA compared with the control group (1/200 serum dilutions) (Fig. 4A). The greatest IgG2a level was observed in the mice sera immunized with Lip DSPC + Imiquimod + SLA, Lip DSPC + Imiquimod and Lip DSPC that were notably ($P < .0001$) higher than the control receiving HEPES buffer(Fig. 4B). Also there was a considerable ($P < .0001$) variation IgG Abs in the mice sera immunized with Lip DSPC, Lip DSPC + Imiquimod, Lip DSPC + Imiquimod + SLA in comparison to the control receiving HEPES buffer (Fig. 4C). Following challenge with L. *major* promastigotes, a critical variation was seen in the IgG1, IgG2a, and IgG Abs levels in the mice sera immunized with different formulations in comparison to the control receiving HEPES buffer (Fig. 5A–C).

Interestingly, the mice sera immunized with Lip DSPC + Imiquimod + SLA, Lip DSPC + Imiquimod and Lip DSPC produced considerably ($P < .0001$) the greatest IgG2a and IgG1 antibodies titer in comparison with those that received HEPES buffer (1/200, 1/2000, 1/20000 serum dilutions) (Fig. 5A, B). Also, the IgG level in the mice sera immunized with all formulations was considerably ($P < .0001$) more than the HEPES buffer group (1/200 serum dilution) (Fig. 5C).

### 3.6. ELISpot results

To determine the level of INF-γ and IL-4 and evaluate the effectiveness of formulations in induction of cellular immune reaction, various liposomal constructs were calculated by ELISpot assay. The ELISpot assays revealed that splenocytes separated from the mice immunized with SLA ($P < .01$), Lip DSPC, Lip + Imiquimod, and Lip + Imiquimod + SLA secreted notably ($P < .0001$) higher IFN-γ compared to the mice immunized with HEPES buffer (Fig. 6). In contrast, IL-4 production was identifiable in spleen after stimulation of antigen for all mice groups, amounts of IL-4 were significantly different in Lip + Imiquimod and Lip + Imiquimod + SLA formulations compared with the mice receiving HEPES buffer (Fig. 6).

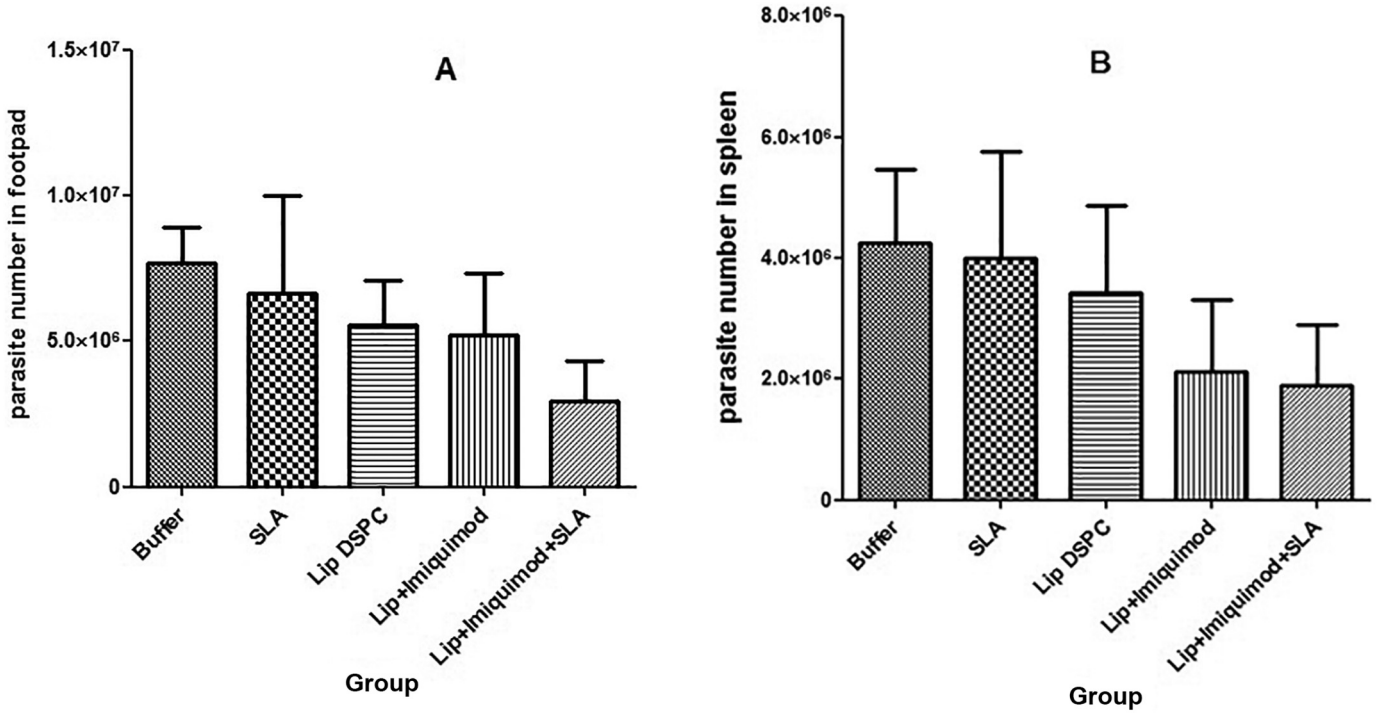


**Fig. 3.** Footpad parasite burden (3A) and spleen parasite burden (3B) in BALB/c mice. Mice immunized SC, three times in 3-week intervals with SLA, Lip DSPC, Lip + Imiquimod, Lip + Imiquimod + SLA or buffer alone after challenge with L. *major* promastigotes. A limiting dilution analysis was performed after challenge on the cells isolated from the spleen and foot of individual mice and cultured in tetra plicate in serial 8-fold dilutions. The wells were assessed microscopically for *Leishmania major* growth, and the number of viable parasite per spleen was determined by GraphPad Prism5 software. The bar represents the average score ± SEM (n = 3).

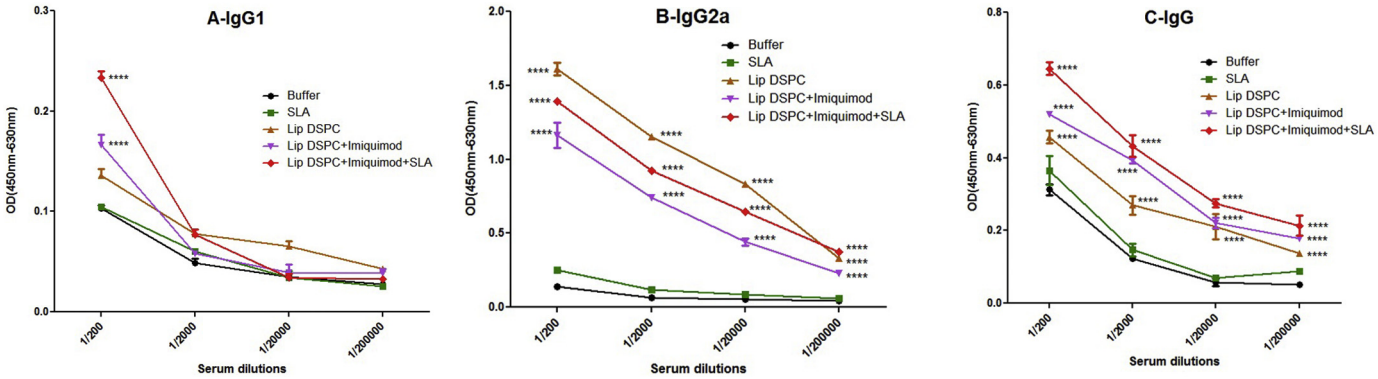


**Fig. 4.** The levels of anti-SLA specific IgG1 (A), IgG2a (B), and IgG (C) antibodies based on mean absorbance in sera of BALB/c mice before challenge. Mice immunized SC, three times in 3-week intervals, with SLA, Lip DSPC, Lip DSPC + Imiquimod, Lip DSPC + Imiquimod + SLA or buffer alone. Blood samples were collected from the mice 2 weeks after the last booster. The assays were performed using ELISA method in triplicate at 200, 2000, 20,000, or 200,000-fold dilution for each serum sample. Values are represented as mean ± SD. * indicates significant difference between the groups, ****, $P < .0001$.

### 3.7. Flow cytometry results

For assessing the antigen-specific T cell reactions, splenocytes were separated in various mice groups after vaccination. Extracellular staining was applied for surface markers of CD8 and CD4. Intracellular cytokine staining was applied for IL-4 and IFN-γ cytokines accompanied by flow cytometry analyses. CD4 and CD8 markers show the IFN-γ and IL4 frequency generating cells, respectively, in Th1 and Th2 population. Fig. 7 shows that CD8 + /IFN-γ cells frequency in the mice immunized with Lip + Imiquimod and Lip + Imiquimod + SLA were significantly ($P < .01$, $P < .001$) more than other mice, respectively. Moreover, flow cytometric also revealed IL-4 generation in cells CD4, implying that T cell-dependent humoral immunity; Lip + Imiquimod + SLA formulation was less than other mice groups ($P < .05$).

## 4. Discussion

Leishmaniasis is an overlooked tropical disease associated with immunosuppression and poverty. The high toxicity of accessible medications (amphotericin B, miltefosine, and paromomycin), HIV co-infection, and resistant parasites pose a worldwide threat in terms of leishmaniasis (Evans and Kedzierski, 2011). In spite of advancements in terms of purification and delivery, *Leishmania* genomics and proteomics (Myler and Fasel, 2008), modern biotechnology for antigen expression, and the great accessibility of murine models in the field of experimental immunology, *Leishmania* vaccinology still suffers from several impediments that limit developments towards the production of efficient and universal vaccines (Gradoni, 2015; Myler and Fasel, 2008).

The production the *Leishmania* vaccine is a challenging task, mainly hampered by the insufficient knowledge of parasite pathogenesis and the immune complexity reactions required for protection (Mutiso et al.,

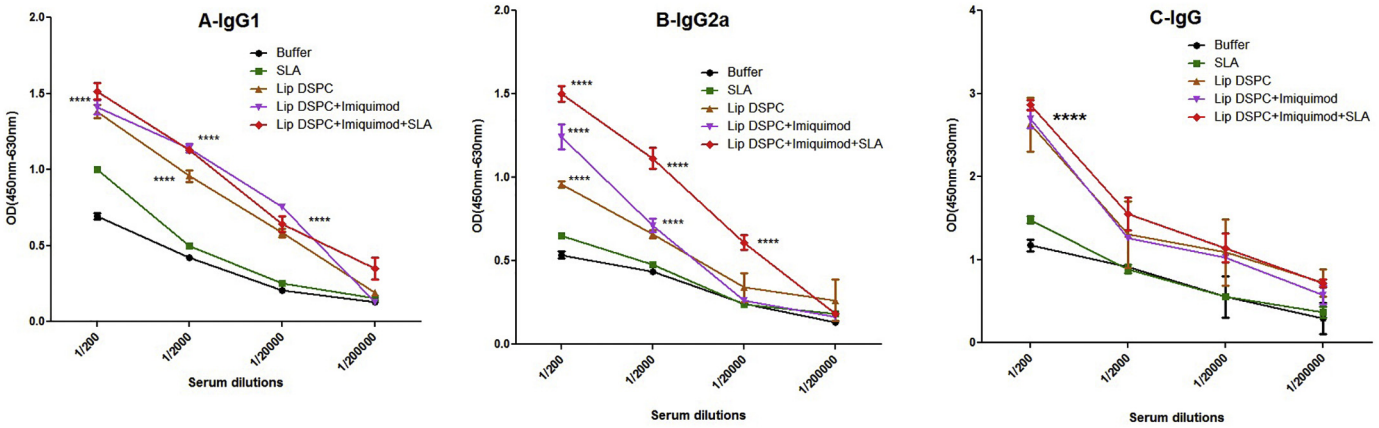


**Fig. 5.** Levels of anti-SLA specific IgG1 (A), IgG2a (B), and IgG (C) in sera of BALB/c mice; Mice immunized SC, three times in 3-week intervals, with, SLA, Lip DSPC, Lip DSPC + Imiquimod, Lip DSPC +Imiquimod + SLA, or buffer alone; Blood samples were collected from the mice 2 weeks after the last booster and 8 weeks after challenge. The anti-SLA IgG1, IgG2a, and IgG levels were assessed using ELISA method. The assays were performed in triplicate at 200, 2000, 20,000, or 200,000-fold dilution for each serum sample. Values are represented as mean ± SD. * indicates significant difference between the groups, ****P < .0001.

2013). Therefore, some efforts are being made to introduce new candidates for the production of the *Leishmania* vaccine. The use of appropriate adjuvants and/or delivery systems in stimulating a proper immune reaction to leishmaniasis is effective in introducing an efficient vaccine against leishmaniasis. Liposomal cargos loaded with protein antigens combined with the described immunostimulatory molecules imitating pathogens in a reductionist mode are interesting formulations to induce protective T cell immunity. The use of TLR agonists and liposomes induces the concurrent antigen presentation and pathways of targeting pattern recognition receptors (PRRs) for the efficient expansion of effector T cells (Bal et al., 2011; Elamanchili et al., 2007). Hence, liposomes containing immunostimulating adjuvants as vaccine delivery systems were provided and applied to deliver soluble *Leishmania* antigens as a first-generation vaccine; the protection and immune reactions were also studied in the mouse model for leishmaniasis.

The innate immune reaction may be stimulated by various pathogen-associated molecular patterns via Toll-like receptors (TLRs). This is a crucial step in inducing the immune response. Using TLR agonists as vaccine adjuvants may be an efficient strategy for the development of vaccines with enhanced protective immunity (Zhang and Matlashewski, 2008). Since imiquimod has been licensed for the topical use in humans and is a TLR agonist, its capacity as a vaccine adjuvant was studied by a well-developed live infection model, i.e. the *Leishmania major* infection in BALB/c mice. Adjuvants could be used to improve and/or prolong immune reactions. Their mechanism should be fully understood, since vaccine adjuvants may act via mechanisms involving the depot formation, the improvement of antigen delivery and uptake, the offering of suitable antigen presenting cells, and the provoking of stimulatory chemokines and cytokines (Perrie et al., 2016).

The elimination of parasitic infections needs various adaptive immune reactions based on the causative pathogen, with intracellular protozoa (such as *Plasmodium* and *Leishmania* spp) requiring a Th1-type immune reaction triggered by IFN-γ production, which activates macrophages and parasite killing (Halliday et al., 2016; Hojatizade et al., 2017). In this study, to evaluate the immune reaction, various parameters, such as the size of footpad swelling, the antibody level in the

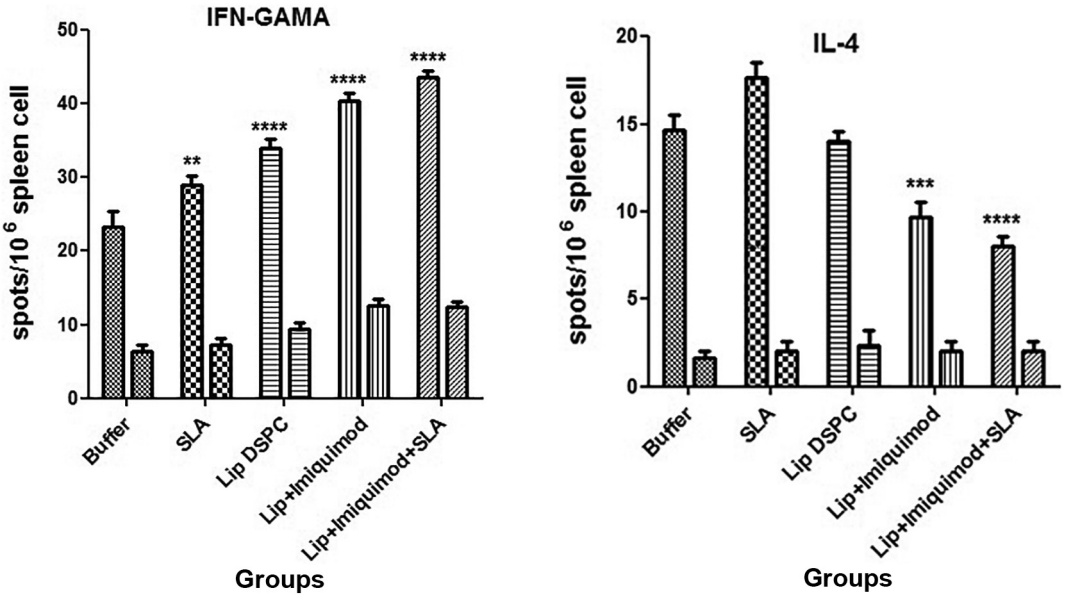


**Fig. 6.** Cytokine level was evaluated through measuring IFN-γ and IL-4 production in immunized mice at week 2 after the last booster injection. Mononuclear splenocytes were cultured in the presence of SLA (10 μg/ml), and the IFN-γ release and IL-4 release from splenocytes induced by different liposomal formulations were determined using ELISpot assay. The data are represented as mean ± SEM (n = 3). * indicates significant difference between the groups, **** (P < .0001), *** (P < .001), ** (P < .01).

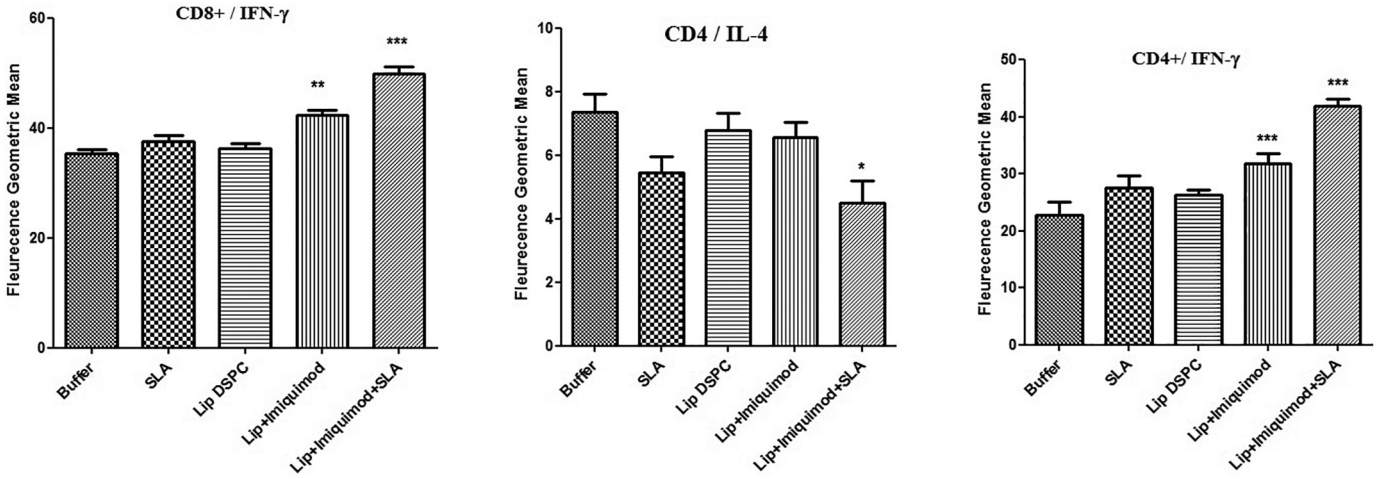


**Fig. 7.** After the last booster, splenocytes were isolated and restimulated, and then stained for surface CD8, CD4, and intracellular IFN-γ and IL-4. Splenocytes were gated by side vs. forward scatter light followed by staining with CD8Pe-cy5 and CD4Pe-cy5. Plots show log fluorescence intensity for IFN-γ- FITC and IL-4-PE. The data are represented as mean ± SEM, (n = 3). *** ($P < .001$), ** ($P < .01$), and * ($P < .05$) denote significant differences from buffer and all other formulations.

sera, and the parasite burden in the footpads or spleens of the infected mice were analyzed and compared among the mouse groups (Jaafari et al., 2007).

In the current study, immunization with SLA did not protect BALB/c mice, taking into account the size of footpad swelling, spleens, the foot parasite burden, and cytokine assays. Exacerbation due to SLA, when there is no Th1-promoting adjuvant, occurs because SLA can develop Th2 reactions by itself (Watanabe et al., 2004). The coexistence of Th1 and Th2 reactions by the immunization of SLA is also in line with the results of other studies (Afonso et al., 1994; Bhowmick et al., 2007). IFN-γ is crucial for the natural protection against and healing of leishmaniasis through the activation of macrophages (Nylen et al., 2003). The level of the cytokine IFN-γ, being indicative of the Th1 reaction in the supernatant of the cultured splenocytes of the mice immunized with Lip + Imiquimod + SLA, stimulates a highly potent Th1 type reaction in comparison with SLA or buffer groups (Nylen et al., 2003).

In this study, the higher IgG2a level was considered as an indicator of resistance to leishmaniasis, indicating that Lip + Imiquimod + SLA stimulated the highest IgG2a level. The least number of live parasites in the footpads and spleens was observed in the Lip + Imiquimod + SLA formulation compared to the other groups. The results showed that the smallest lesion size was observed in the mice immunized with Lip + Imiquimod + SLA with the significant difference of ($P < .05$), 6 weeks after the challenge with other groups. In this respect, the data implied that SLA in association with the liposomes, including DSPC and Imiquimod induced protective reactions to the leishmaniasis in murine model. Numerous adjuvants combined with vaccine candidates to improve the effectiveness of weak immunogenic antigens and induce additive or synergistic T-cell reactions have been shown to be inefficient when there is no adjuvant or subunit vaccine (Badiee et al., 2013). Imiquimod influences are mediated by stimulating natural immunity, especially, the secretion of monocyte/macrophage-derived cytokines, such as IFN- γ and the tumor necrosis factor (TNF) (Kubli-Garfias et al., 2017).

In this study, it was demonstrated that the combination of imiquimod and liposomal proteins triggered the $CD4^+$ T cell reaction, in comparison with liposomal proteins with no imiquimod. Both in vitro and in vivo evaluations showed that the imiquimod adjuvanted liposomal SLA induced more powerful $CD8^+$ T-cell reactions than the reaction induced by the liposomal protein alone. In contrast, poor T-cell reactions were observed when the activation was induced by SLA. These observations showed the specificity of the adjuvanted liposomal formulation to improve the presentation of the entrapped antigen MHC-I. Research shows that a small liposome-encapsulated antigen may activate more T cell reactions than the identical content of the free protein, in vivo and in vitro (Maji et al., 2016). The findings of the current study also indicated that small amounts of antigens were needed for immunization to activate $CD4^+$ and $CD8^+$ T cell reactions efficiently. Developing an effective delivery system is required to target professional APCs for the presentation and processing of encapsulated antigens via MHC complexes to elicit broad spectrum T-cell reactions and resist prevalent infections, such as malaria, tuberculosis, leishmaniasis, viral illnesses, and cancers (Homhuan et al., 2009; Peachman et al., 2005).

Vaccine delivery systems, like liposomes, improve the uptake of the related antigens into APCs. Bilayer composition affects the uptake and interaction of liposomes via APCs and the type of the immune reaction induced (Copland et al., 2005; Nobs et al., 2004). Liposomes provided with DPPC or DSPC having the rgp63 antigen manifested more entrapment effectiveness and triggered the Th1 reaction, unlike liposomes provided with EPC (Badiee et al., 2009; Watson et al., 2012). Distearoyl phosphatidylcholine (DSPC), due to high transition point, along with the helper lipid cholesterol can turn into stable liposomes to inhibit their simple removal from the blood. The formulation of the DSPC/Chol liposome was selected, since liposome rigidity increased through substituting the underivatized phosphatidyl choline with phospholipids at an elevated transition temperature (Tc), like DSPC (Tc = 54 °C) (Frezard, 1999). Antigens entrapped in DSPC liposomes showed a unique approach to protecting and delivering encapsulated antigens to trigger antigen-specific cellular immune reactions. Antigens encapsulated in liposomes might trigger protective immune reactions against experimental visceral leishmaniasis; however, the immunostimulation pathways are not identified (Maji et al., 2016). Liposomes with a high Tc improved the adjuvant activity, since they had higher membrane rigidity in vivo, so the entrapped solutes (e.g. antigens) remained with the carrier for a long time; hence, they triggered a more powerful immune reaction than the ones entrapped in phospholipids with a low Tc (Watson et al., 2012). Moreover, the cholesterol in the vesicles simplified the cytoplasmic secretion of the antigens and inhibited lysosomal degradation. The in vivo-elevated stability of the vesicles stimulate the CD8 + T-cell reaction better (Ignatius et al., 2000). DSPC liposomes were effectively taken up by APCs, being crucial for the induction of the protective Th1 type of immunological reactions and required for protection against leishmaniasis.

In general, liposomes containing the imiquimod adjuvant trigger protection against the challenge of L. *major* in the BALB/c mice. The complicated effects of lipid compositions and immunostimulatory adjuvants on the triggered immune reactions should be considered.

## Competing interests

The authors declare that they have no competing interests.

## Acknowledgments

This study was part of MSc dissertation of Maryam Rezaei Nasab with grant NO. K/142 and code of ethics IR.Zaums.IREC.1392.1818. The financial support of the Infectious Diseases and Tropical Medicine Research Center, Resistant Tuberculosis institute, Zahedan University of Medical Sciences, Zahedan, Iran, are gratefully acknowledged.

## References

Afonso, L.C., Scharton, T.M., Vieira, L.Q., Wysocka, M., Trinchieri, G., Scott, P., 1994. The adjuvant effect of interleukin-12 in a vaccine against Leishmania major. In: Science-AAAS-Weekly Paper Edition-including Guide to Scientific Information. vol. 263. pp. 235–236.

Alimohammadian, M.H., Hakimi, H., Nikseresht, M., 1993. The p reparation and evaluation of reference leishmanin from leishmania major for use in man for diagnostic and experimental purposes. Med. J. Islam. Repub. Iran 7, 23–28.

Alving, C.R., 1995. Liposomal vaccines: clinical status and immunological presentation for humoral and cellular immunity. Ann. N. Y. Acad. Sci. 754, 143–152.

Badiee, A., Jaafari, M.R., Khamesipour, A., 2007. Leishmania major: immune response in BALB/c mice immunized with stress-inducible protein 1 encapsulated in liposomes. Exp. Parasitol. 115, 127–134.

Badiee, A., Jaafari, M.R., Samiei, A., Soroush, D., Khamesipour, A., 2008. Coencapsulation of CpG oligodeoxynucleotides with recombinant Leishmania major stress-inducible protein 1 in liposome enhances immune response and protection against leishmaniasis in immunized BALB/c mice. Clin. Vaccine Immunol. 15, 668–674.

Badiee, A., Jaafari, M.R., Khamesipour, A., Samiei, A., Soroush, D., Kheiri, M.T., Barkhordari, F., McMaster, W.R., Mahboudi, F., 2009. Enhancement of immune response and protection in BALB/c mice immunized with liposomal recombinant major surface glycoprotein of Leishmania (rgp63): the role of bilayer composition. Colloids Surf. B: Biointerfaces 74, 37–44.

Badiee, A., Shargh, V.H., Khamesipour, A., Jaafari, M.R., 2013. Micro/nanoparticle adjuvants for antileishmanial vaccines: present and future trends. Vaccine 31, 735–749.

Bainor, A., Chang, L., McQuade, T.J., Webb, B., Gestwicki, J.E., 2011. Bicinchoninic acid (BCA) assay in low volume. Anal. Biochem. 410, 310–312.

Bal, S.M., Hortensius, S., Ding, Z., Jiskoot, W., Bouwstra, J.A., 2011. Co-encapsulation of antigen and Toll-like receptor ligand in cationic liposomes affects the quality of the immune response in mice after intradermal vaccination. Vaccine 29, 1045–1052.

Bhowmick, S., Ravindran, R., Ali, N., 2007. Leishmanial antigens in liposomes promote protective immunity and provide immunotherapy against visceral leishmaniasis via polarized Th1 response. Vaccine 25, 6544–6556.

Buates, S., Matlashewski, G., 2001. General suppression of macrophage gene expression during Leishmania donovani infection. J. Immunol. 1, 3416–3422.

Coler, R.N., Skeiky, Y.A., Bernards, K., Greeson, K., Carter, D., Cornellison, C.D., Modabber, F., Campos-Neto, A., Reed, S.G., 2002. 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. 70, 4215–4225.

Copland, M.J., Rades, T., Davies, N.M., Baird, M.A., 2005. Lipid based particulate formulations for the delivery of antigen. Immunol. Cell Biol. 83, 97.

Darrah, P.A., Patel, D.T., De Luca, P.M., Lindsay, R.W., Davey, D.F., Flynn, B.J., Hoff, S.T., Andersen, P., Reed, S.G., Morris, S.L., 2007. Multifunctional T H 1 cells define a correlate of vaccine-mediated protection against Leishmania major. Nat. Med. 13, 843.

Davis, D., Gregoriadis, G., 1987. Liposomes as adjuvants with immunopurified tetanus toxoid: influence of liposomal characteristics. Immunology 61, 229.

Elamanchili, P., Lutsiak, C.M., Hamdy, S., Diwan, M., Samuel, J., 2007. "Pathogen-mimicking" nanoparticles for vaccine delivery to dendritic cells. J. Immunother. 30, 378–395.

Evans, K.J., Kedzierski, L., 2011. Development of vaccines against visceral leishmaniasis. J. Trop. Med. 2012.

Frezard, F., 1999. Liposomes: from biophysics to the design of peptide vaccines. Braz. J. Med. Biol. Res. 32.

Gradoni, L., 2015. Canine Leishmania vaccines: still a long way to go. Vet. Parasitol. 208, 94–100.

Greenblatt, C.L., 1980. The present and future of vaccination for cutaneous leishmaniasis. In: The Present and Future of Vaccination for Cutaneous Leishmaniasis, pp. 259–285.

Halliday, A., Turner, J.D., Guimarães, A., Bates, P.A., Taylor, M.J., 2016. The TLR2/6 ligand PAM2CSK4 is a Th2 polarizing adjuvant in Leishmania major and Brugia malayi murine vaccine models. Parasit. Vectors 9, 96.

Hartley, M.-A., Drexler, S., Ronet, C., Beverley, S.M., Fasel, N., 2014. The immunological, environmental, and phylogenetic perpetrators of metastatic leishmaniasis. Trends Parasitol. 30, 412–422.

Heidari-Kharaji, M., Taheri, T., Doroud, D., Habibzadeh, S., Rafati, S., 2016. Solid lipid nanoparticle loaded with paromomycin: in vivo efficacy against Leishmania tropica. Appl. Microbiol. Biotechnol. 100, 7051–7060.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., Horiuchi, T., Tomizawa, H., Takeda, K., Akira, S., 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. Nat. Immunol. 22, 196–200.

Hojatizade, M., Badiee, A., Khamesipour, A., Mirshafiey, A., Akhtari, J., Mehravaran, A., Alavizadeh, S.H., Abbasi, A., Saberi, Z., Nikpoor, A.R., 2017. DDA/TDB liposomes containing soluble Leishmania major antigens induced a mixed Th1/Th2 immune response in BALB/c mice. Nanomed. J. 4, 71–82.

Homhuan, A., Kogure, K., Nakamura, T., Shastri, N., Harashima, H., 2009. Enhanced antigen presentation and CTL activity by transduction of mature rather than immature dendritic cells with octaarginine-modified liposomes. J. Control. Release 136, 79–85.

Ignatius, R., Mahnke, K., Rivera, M., Hong, K., Isdell, F., Steinman, R.M., Pope, M., Stamatatos, L., 2000. Presentation of proteins encapsulated in sterically stabilized liposomes by dendritic cells initiates CD8+ T-cell responses in vivo. Blood 96, 3505–3513.

Iwasake, A., Medzhitov, R., 2004. Toll-like receptor control of the adaptive immune response. Nat. Immunol. 5, 987–995.

Iwasaki, A., Medzhitov, R., 2004. Toll-like receptor control of the adaptive immune responses. Nat. Immunol. 5, 987–995.

Jaafari, M.R., Badiee, A., Khamesipour, A., Samiei, A., Soroush, D., Kheiri, M.T., Barkhordari, F., McMaster, W.R., Mahboudi, F., 2007. The role of CpG ODN in enhancement of immune response and protection in BALB/c mice immunized with recombinant major surface glycoprotein of Leishmania (rgp63) encapsulated in cationic liposome. Vaccine 25, 6107–6117.

Joshi, M.D., Unger, W.J., Storm, G., van Kooyk, Y., Mastrobattista, E., 2012. Targeting tumor antigens to dendritic cells using particulate carriers. J. Control. Release 161, 25–37.

Jurk, M., Heil, F., Vollmer, J., Schetter, C., Krieg, A.M., Wagner, H., Lipford, G., Bauer, S., 2002. Human TLR7 or TLR8 independently confer responsiveness to the antiviral compound R-848. Nat. Immunol. 3, 499.

Kataoka, K., Harada, A., Nagasaki, Y., 2012. Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv. Drug Deliv. Rev. 64, 37–48.

Khamesipour, A., Rafati, S., Davoudi, N., Maboudi, F., Modabber, F., 2006. Leishmaniasis vaccine candidates for development: a global overview. Indian J. Med. Res. 123, 423.

Krishnan, L., Sad, S., Patel, G.B., Sprott, G.D., 2003. Archaeosomes induce enhanced cytotoxic T lymphocyte responses to entrapped soluble protein in the absence of interleukin 12 and protect against tumor challenge. Cancer Res. 63, 2526–2534.

Kubli-Garfias, C., Vázquez-Ramírez, R., Trejo-Muñoz, C., Berber, A., 2017. Insights on the mechanism of action of immunostimulants in relation to their pharmacological potency. The effects of imidazoquinolines on TLR8. PLoS One 12, e0178846.

Maji, M., Mazumder, S., Bhattacharya, S., Choudhury, S.T., Sabur, A., Shadab, M., Bhattacharya, P., Ali, N., 2016. A lipid based antigen delivery system efficiently facilitates MHC Class-I antigen presentation in dendritic cells to stimulate CD8+ T cells. Sci. Rep. 6.

Mazumder, S., Maji, M., Ali, N., 2011. Potentiating effects of MPL on DSPC bearing cationic liposomes promote recombinant GP63 vaccine efficacy: high immunogenicity and protection. PLoS Negl. Trop. Dis. 5, e1429.

Mehravaran, A., Jaafari, M.R., Jalali, S.A., Khamesipour, A., Tafaghodi, M., Hojatizade, M., Abbasi, A., Badiee, A., 2015a. Cationic immune stimulating complexes containing soluble Leishmania antigens: preparation, characterization and in vivo immune response evaluation. Iran. J. Immunol. 12, 274.

Mehravaran, A., Jaafari, M.R., Jalali, S.A., Khamesipour, A., Tafaghodi, M., Hojatizade, M., Badiee, A., 2015b. The role of surface charge of ISCOMATRIX nanoparticles on the type of immune response generated against Leishmaniasis in BALB/c mice. Nanomedicine. Journal 2, 249–260.

Mehravaran, A., Jaafari, M.R., Jalali, S.A., Khamesipour, A., Ranjbar, R., Hojatizade, M., Badiee, A., 2016. The role of ISCOMATRIX bilayer composition to induce a cell mediated immunity and protection against leishmaniasis in BALB/c mice. Iran. J. Basic Med. Sci. 19, 178.

Moon, J.J., Suh, H., Bershteyn, A., Stephan, M.T., Liu, H., Huang, B., Sohail, M., Luo, S., Um, S.H., Khant, H., 2011. Interbilayer-crosslinked multilamellar vesicles as synthetic vaccines for potent humoral and cellular immune responses. Nat. Mater. 10, 243.

Mutiso, J.M., Macharia, J.C., Kiio, M.N., Ichagichu, J.M., Rikoi, H., Gicheru, M.M., 2013. Development of Leishmania vaccines: predicting the future from past and present experience. J. Biomed. Res. 27, 85.

Myler, P.J., Fasel, N., 2008. Leishmania: After the Genome. Horizon Scientific Press.

Nadim, A., Javadian, E., Tahvildar-Bidruni, G., Ghorbani, M., 1983. Effectiveness of leishmanization in the control of cutaneous leishmaniasis. In: Bulletin de la Société de Pathologie Exotique et de ses Filiales. vol. 76. pp. 377–383.

Noazin, S., Modabber, F., Khamesipour, A., Smith, P.G., Moulton, L.H., Nasseri, K., Sharifi, I., Khalil, E.A., Bernal, I.D.V., Antunes, C.M., 2008. First generation leishmaniasis vaccines: a review of field efficacy trials. Vaccine 26, 6759–6767.

Nobs, L., Buchegger, F., Gurny, R., Allémann, E., 2004. Current methods for attaching targeting ligands to liposomes and nanoparticles. J. Pharm. Sci. 93, 1980–1992.

Nordly, P., Agger, E.M., Andersen, P., Nielsen, H.M., Foged, C., 2011. Incorporation of the TLR4 agonist monophosphoryl lipid A into the bilayer of DDA/TDB liposomes: physico-chemical characterization and induction of CD8+ T-cell responses in vivo. Pharm. Res. 28, 553–562.

Nylen, S., Maasho, K., Söderström, K., Ilg, T., Akuffo, H., 2003. Live Leishmania promastigotes can directly activate primary human natural killer cells to produce interferon-gamma. Clin. Exp. Immunol. 131, 457–467.

O'Hagan, D.T., Singh, M., 2003. Microparticles as vaccine adjuvants and delivery systems. Exp. Rev. Vaccines 2, 269–283.

Patere, S.N., Pathak, P.O., Shukla, A.K., Singh, R.K., Dubey, V.K., Mehta, M.J., Patil, A.G.,

Gota, V., Nagarsenker, M.S., 2017. Surface-modified liposomal formulation of amphotericin B: in vitro evaluation of potential against visceral leishmaniasis. AAPS PharmSciTech 18, 710–720.
Peachman, K.K., Rao, M., Alving, C.R., Palmer, D.R., Sun, W., Rothwell, S.W., 2005. Human dendritic cells and macrophages exhibit different intracellular processing pathways for soluble and liposome-encapsulated antigens. Immunobiology 210, 321–333.
Perrie, Y., Crofts, F., Devitt, A., Griffiths, H.R., Kastner, E., Nadella, V., 2016. Designing liposomal adjuvants for the next generation of vaccines. Adv. Drug Deliv. Rev. 99, 85–96.
Raman, V.S., Duthie, M.S., Fox, C.B., Matlashewski, G., Reed, S.G., 2012. Adjuvants for Leishmania vaccines: from models to clinical application. Front. Immunol. 3, 144.
Rhee, E.G., Mendez, S., Shah, J.A., Wu, C.-y., Kirman, J.R., Turon, T.N., Davey, D.F., Davis, H., Klinman, D.M., Coler, R.N., 2002. Vaccination with heat-killed Leishmania antigen or recombinant leishmanial protein and CpG oligodeoxynucleotides induces long-term memory CD4+ and CD8+ T cell responses and protection against Leishmania major infection. J. Exp. Med. 195, 1565–1573.
Scott, P., Pearce, E., Natovitz, P., Sher, A., 1987. Vaccination against cutaneous leishmaniasis in a murine model. I. Induction of protective immunity with a soluble extract of promastigotes. J. Immunol. 139, 221–227.
Smith, D.M., Simon, J.K., Baker Jr., J.R., 2013. Applications of nanotechnology for immunology. Nat. Rev. Immunol. 13, 592–605.
Soto, M., Ramírez, L., Pineda, M.A., González, V.M., Entringer, P.F., de Oliveira, C.I., Nascimento, I.P., Souza, A.P., Corvo, L., Alonso, C., 2009. Searching genes encoding Leishmania antigens for diagnosis and protection. Sch. Res. Exch. 2009.
Tafaghodi, M., Eskandari, M., Khamesipour, A., Jaafari, M.R., 2016. Immunization against cutaneous leishmaniasis by alginate microspheres loaded with Autoclaved Leishmania Major (ALM) and Quillaja Saponins. Iran. J. Pharm. Res. 15, 573.
Takeda, K., Kaisho, T., Akira, S., 2003. Toll-like receptors. Annu. Rev. Immunol. 21, 335–376.
Taswell, C., 1981. Limiting dilution assays for the determination of immunocompetent cell frequencies. I. Data analysis. J. Immunol. 126, 1614–1619.
Titus, R.G., Marchand, M., Boon, T., Louis, J., 1985. A limiting dilution assay for quantifying Leishmania major in tissues of infected mice. Parasite Immunol. 7, 545–555.
Torchilin, V.P., 2005. Recent advances with liposomes as pharmaceutical carriers. Nat. Rev. Drug Discov. 4, 145–160.
Uzonna, J.E., Wei, G., Yurkowski, D., Bretscher, P., 2001. Immune elimination of Leishmania major in mice: implications for immune memory, vaccination, and reactivation disease. J. Immunol. 167, 6967–6974.
Watanabe, T., Kitani, A., Murray, P.J., Strober, W., 2004. NOD2 is a negative regulator of Toll-like receptor 2–mediated T helper type 1 responses. Nat. Immunol. 5, 800–808.
Watson, D.S., Endsley, A.N., Huang, L., 2012. Design considerations for liposomal vaccines: influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens. Vaccine 30, 2256–2272.
WHO, 2016. World Health Statistics 2016: Monitoring Health for the SDGs Sustainable Development Goals. World Health Organization.
Wickelgren, I., 2006. Targeting the Tolls. American Association for the Advancement of Science.
Zhang, W.-W., Matlashewski, G., 2008. Immunization with a Toll-like receptor 7 and/or 8 agonist vaccine adjuvant increases protective immunity against Leishmania major in BALB/c mice. Infect. Immun. 76, 3777–3783.