RESEARCH PAPER

Liposomes containing the imiquimod adjuvant as a vaccine in the cutaneous leishmaniasis model

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ABSTRACT

Objective(s): Attempts to produce vaccines for leishmaniasis need adjuvants to trigger the kind of immune reaction required for protection. In this study, we examined the properties of the TLR7 agonist imiquimod, a vaccine adjuvant, making use of a live model of infection where the immune reactions could be identified prior to and following the challenge of infection.

Materials and Methods: The liposomes of EPC containing the imiquimod adjuvant were prepared and characterized for protein concentration, surface charge, and particle size. Vaccination was done using the soluble Leishmania antigen (SLA) as a first-generation vaccine model in the liposomal state to vaccinate BALB/c mice against the challenge of leishmania major. BALB/c mice were vaccinated subcutaneously, three times at a two-week interval. Parasite burden, footpad swelling, IgG isotype, as well as the level of IL-4 and IFN-γ were assessed as the protection criteria.

Results: The group of mice vaccinated by Lip+Imiquimod+SLA demonstrated a lower amount of footpad swelling and parasite burden than the buffer group. In addition, the highest level of IFN-γ and the lowest level of IL-4 production was noticed in the splenocytes of the mice vaccinated with the formulation of Lip+Imiquimod+SLA.

Conclusion: These results imply that imiquimod added to the formulation of liposomes is able to modulate the immune reaction of the BALB/c mice vaccinated preferably to a Th1 reaction rather than a Th2 reaction which can also lead to partial protection against the challenge of Leishmania.

Keywords: *Adjuant, Leishmaniasis, Imiquimod, Liposome, Vaccine*

INTRODUCTION

Leishmaniasis, is a parasitic vector-borne disease, which poses a significant public health threat globally. Based on a report by the WHO, this disease affects approximately 12 million individuals in 88 world countries, with about 350 million other individuals being in danger [1].

Leishmaniasis is an ignored tropic disease, which affects the poverty-stricken people, for whom getting access to effective treatment and diagnosis is hard. Individuals inflicted with the *Leishmania* show many symptoms, including the self-healing dermal lesion (CL) as well as the possibly deadly visceral type of the disease, titled 'visceral leishmaniasis[2] .Many efforts have been made to discover new medicines for treating this disease, yet pentavalent antimonials are the most prevalent compounds to treat the disease, which were introduced more than 50 years ago. Medicines utilized for the treatment of leishmaniasis have some constraints, including resistance development, long treatment time, strong side effects and high toxicity. In spite of the latest advancements in molecular immunology and pharmaceutics, no authorized vaccine is present against leishmaniasis up to now [3].

In all leishmaniasis types, T lymphocytes

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chiefly mediate pathology and immunity. In the traditional infection pattern of mice with *L. major*, healing and controlling the infection are dependent in general on developing the Th1 (T-helper 1) induced immune reaction featured by the high generation of interferon-gamma (IFN-γ) and interleukin-12 (IL-12). In the group of mice, the cells of CD4+ Th1 induce resistance in the mice infected with *Leishmania major*, while the cells of CD4 Th2 induce susceptibility [4] . Some other research imply that resistant mice getting infected with *L. major* promote the *in vivo* generation of IL-12, and that IL-12 is required for the inducing of defensive Th1 reaction. In addition, the absorption of *L. major* by means of DCs (dendritic cells) results in the generation of IL-12 and the following Th1 cells priming. It has been suggested that the elective failure of the signaling of IL-12 by antigen specific CD4+ T cells leads to the vulnerability of the BALB/c mice to infection with *L. major* [5]. Most recent studies also confirm that a more complicated CMI (cell-mediated immune response) affects the consequences of leishmaniasis, especially CL [6].

As against the curative phenotype, the immune response of the Th2 type is in charge of the persistence and susceptibility of the disease. This non-healing phenotype is in charge of the preferred development of Th2 cells, featured by the generation of IL-13, IL-5, and IL-4[7].

Immunity response generated by vaccines depends on the potentials of the vaccines to induce the suitable immune reaction capable of eliminating or controlling the pathogen. It appears that the low effectiveness of test vaccines is because of the lack of a delivery system or the appropriate adjuvant. Delivery systems based on particles, including liposomes, have attracted a lot of attention as efficient adjuvants and stable carriers in delivering vaccines. Liposomes are closed synthesized vesicles consisted of lipid concentric split up by aqueous media, which are used as systems for the delivery of peptides, DNA, drugs and proteins, which could also be utilized as immunoadjuvants to generate immune reactions to different antigens. All kinds of antigens, such as proteins, peptides, nucleic acids, carbohydrates, as well as small hapten molecules can be involved in the formulations of liposomes with appropriate modifications to vesicle features to accommodate the antigen's charge and size [8]. The benefits of utilizing liposomes depend on an improvement in

the adding of target principles, antigen's stability, as well as the controlled release of the antigen [9].

The co-administration of efficient and safe adjuvants is required for increasing the uptake, persistence and presentation of the antigen, as well as providing immunity stimulation. Therefore, the combining of immunopotentiating adjuvants and delivery systems has turned into an effective strategy for the rational design of vaccines [10, 11].

Some techniques, including liposomes, archaeosomes, micelles, polymersomes, and ISCOMs are utilized to transfer the antigens of proteins to professional APCs [12-19].

The utilization of a delivery system and an adjuvant is required for any advanced vaccines, especially the ones used against leishmaniasis. A lot of adjuvants of different features and types of effects have been utilized in producing vaccines for leishmaniasis. Some of these adjuvants are classified as immunostimulatory adjuvants, including imiquimod, MPL (Monophosphoryl lipid A), cytokines (IL-2, IL-12, GM-CSF), MDP/MTP-PE (muramyl di- or tripeptides and derivatives), CpG oligonucleotides, and saponins (QuilA, QS-21) [20]. Imiquimod with its respective compound S-28463, being the members of the imidazoquinolines, have been successful on FDA-approved Toll-like receptor (TLR) 7/8 agonist in clinical studies on cutaneous leishmaniasis [21-23].

Imiquimod acts by its immunomodulatory effects on different cells engaged in the immune system, where it is demonstrated to prompt the discharge of some cytokines, including tumor necrosis factor (TNF)-α, interferon (IFN)-α, IL-6, interleukin (IL)-1β, and IL-8. Macrophages and monocytes are the major target cells of imiquimod. Since imiquimod has been proven to modulate the activity of macrophages and monocytes, leading to antiviral effects, it is verified as a safe and efficient factor against dermal lesions generated by viral infections [24].

In this study, we examined the adjuvant features of the vaccine of the TLR7 agonist imiquimod, making use of a live model of infection, in which immune reactions could be identified prior to and following the challenge of infection. In order to vaccinate BALB/c mice against the challenge *of L. major*, vaccination was done using SLA (soluble *Leishmania* antigen) as a model for the first-generation vaccine in the form of liposomes.

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MATERIALS AND METHODS
Animals, ethics statement

Female 6–8 week old BALB/c mice were performed from Laboratory of Animal Research Center of Zahedan University of Medical Sciences. The mice were kept in the animal care equipment in pathogen-free condition. The protocol of experimental design 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.

Imiquimod, parasites and soluble leishmania antigen (SLA)

Imiquimod (R837) was provided from Invivogen Company *L. major* strain (MRHO/IR/75/ER) used in this experiment was previously used for the preparation of experimental *Leishmania* vaccine, leishmanin test in Iran [25, 26]. The SLA preparation was done by the protocol established with minor modification. In brief, the parasites were harvested at stationary phase and rinsed three times using HEPES buffer (10 mM + sucrose 10%, pH 7.4) [27]. Afterwards, the number of promastigotes was set to 1.2×10^9 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 protein concentration of the preparation was indicated by BCA (bicinchoninic acid) protein assay kit (Thermo Scientific, Waltham, USA) [28].

Liposome preparation and characterization

Liposomes encapsulating SLA were prepared by lipid film procedure. The lipid phase consisting of EPC (1, 2-dimyristoyl-sn-glycero-3-ethylphosphocholine (chloride salt) (20 mM; Avanti polar lipids, USA) and cholesterol (10 mM; Avanti polar lipids, USA) (2:1 molar ratio) was dissolved in chloroform in a sterile tube. The solvent was removed using rotary evaporation (Hettich, Germany), causing deposition of a thin lipid film over the tube's wall. The lipid film was then freeze–dried (TAITEC, Japan) overnight to remove the solvent. The lipid film was hydrated and dispersed in sterile buffer (HEPES buffer 10 mM pH 7.4) having SLA (2 mg/mL). The multilamellar vesicles (MLVs) were converted to unilamellar vesicles under argon employing bath sonicator (Bandelin, Germany) at 20 ºC for 15 min. The dispersion of liposome was extruded 13 times via 400 and 200 nm polycarbonate membranes, respectively (Avestin, Canada). Dynamic Light Scattering Instrument (Nano-ZS, Malvern, UK) was used to determine the zeta potential and particle size of liposome. Formulations were measured by Particle sizes were indicated as the mean±standard deviation and poly dispersity index (PDI) (n=3). Zeta potentials were reported as the means±zeta deviation (n=3) [29].

Characterization of the prepared formulations

The SLA concentration encapsulated in liposomes and characterize the antigen were indicated by BCA protein assay kit (Thermo Scientific, Waltham, USA). Analytical SDS-PAGE was done to qualitatively calculate the SLA encapsulated in the liposomal SLA (Lip-SLA). The discontinuous system included 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. The gels were stained with silver to detect protein after electrophoresis [30].

Immunization of BALB/c mice

Different groups of mice, 10 mice in each group, were immunized subcutaneously (SC) three times at a 3-week interval in the footpad (RF) intervals with one these formulations: HEPES buffer, SLA, Lip EPC, Lip+ imiquimod, Lip+ Imiquimod + SLA, in final volume of 50µl [31].

Challenge with L. major promastigotes

The immunized mice and control groups were challenged with 1×10^6 late stationary phase *L. major* promastigotes two weeks after the last booster injection. Parasites were injected subcutaneously into the right footpad in a volume of 50 µl. Lesion progression was weekly accompanied by measurement of the thickness of

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the infected footpad in comparison to the same footpad thickness before infection employing a digital caliper (Mitutoyo Measuring Instruments, Japan) [32].

Quantitative parasite burden after challenge

The number of viable *L. major* parasites in the spleens/ footpad of mice was obtained by restricting the dilution assay procedure [32]. The mice were killed at week 9 after challenge. The feet were removed aseptically and homogenized in RPMI 1640 supplemented with 2 mM glutamine, 10% v/v heat inactivated FCS (Eurobio, Scandinavie), 100 units of penicillin per ml, and 100 µg/ml of streptomycin sulfate (RPMI-FCS). The homogenate was diluted with the media in eight serial 10-fold dilutions and put in every well of flat-bottom 96-well microtiter plates (Nunc, Denmark), having solid layer of rabbit blood agar in tetraplicate and was 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 viable parasites in every spleen and infected footpad were indicated using GraphPad Prism software, a statistical method for limiting dilution assay.

Antibody isotype assay

The levels of antigen-specific serum IgG subclasses were indicated through a standard enzyme linked immunosorbent assay (ELISA) technique. Samples of blood were obtained from mice before and 8 weeks post challenge, and the sera were separated and stored at -20 °C. The evaluation of IgG1, anti-SLA IgG total, and IgG2a was carried out to identify bound antibodies [33]. Microtiter plates (Nunc, Denmark) were covered with 50 µl of SLA (10 µg/ml) in PBS buffer (0.01 M, pH 7.3) and serum serial dilutions overnight at 4°C. HRP-rabbit anti-mouse IgG isotype was administered to the plates based on the manufacturer's protocol (Invitrogen Inc., USA). Optical density (OD) was indicated at 450 nm by 630 nm as the criterion wavelength.

ELISpot assay

ELISpot assessment was done by mouse ELISpot kits from U-cytech (Utrecht, the Netherlands). At week 2, three mice from every group after the last booster injection (before challenge) were killed. Their splenocytes were separated and restimulated in vitro via mitogen Concanavalin A (Con A) as a positive control or SLA as a recalled antigen. ELISpot plates were covered with antibodies of anti-IL-4 or anti-IFN-γ and incubated overnight at 4 °C. The splenocytes (5 × 105 cells/well) were cultured in triplicate in 200 µl volume with DMEM (as background responses), medium having Con A (as positive controls), or medium having 10 µg/ml of SLA in the pre-coated plates. Spot counting was conducted by Kodak 1D software (Version 3.5, Eastman Kodak, and Rochester, New York) after incubation (37°C, 5% CO_2) for 24 hr (for IFN-γ assay) or 48 h (for IL-4 assay). The average number of spots ± SD in triplicate wells was estimated and demonstrated as spot-forming units (SFU) per 105 splenocytes.

Flow cytometry

The mechanism of cellular uptake of liposomes was quantified by fluorescence activated cell sorting (FACS) using various biochemical inhibitors. For identification of cellular uptake of formulations, splenocytes were seperated 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™ and Fixation/Permeabilization Kit. Splenocytes (10^6 cells/ml) in medium having Golgi Plug™ (1 µl/ml) were triggered with PMA/ionomycin cocktail (2 µl/ml) at 37 °C for 4 h. 10^5 splenocytes were added to flowcytometry tubes after stimulation and rinsed twice with stain buffer (2% FCS in PBS). 1 µl anti-CD8a-PE-cy5 antibody and 1 µl anti CD4-PE-cy5 antibody in isolated tubes were used to stain splenocytes at 4 °C for 30 min. The cells were rinsed with stain buffer and fixed by Cytofix/Cytoperm™ solution. The fixed cells were rinsed twice using Perm/Wash™ buffer and stained with 1 µl anti-IFN-γ- FITC antibody at 4 °C for 30 min. CD4 cells were stained with 1 µl anti-IL-4-PE antibody. The cells were rinsed with Perm/Wash™ buffer and suspended in 300 µl stain buffer for flow cytometric analysis Calibur (BD Biosciences, USA).

Statistical analysis

The statistical analysis was conducted through the statistical program GraphPad Prism. One-way ANOVA assessed the variations among different groups. In the case of significant *P*-value, Tukey–Kramer multiple comparisons were done as a post-test to evaluate the average values in various mice groups. *P*<0.05 was assumed as statistically meaningful.

RESULTS

Liposome characterization

Liposomes used in this study were homogenous vesicles in average diameter with the size ranging from 108 to 146 nm and polydispersity index (PDI) from 0.15 to 0.22. The zeta potentials calculated by particle size analyzer were -15.3 ± 8.2 mV for Lip EPC+ Imiquimod+SLA, -10.5 ± 15.7 mV for Lip EPC+ Imiquimod and -9 ± 11.4 mV for Empty Lip EPC formulations, respectively (n=3). The entrapment of SLA in Liposome was estimated % 29 ± 5.6 (n = 3) (Table 1).

The SLA concentration in the formulations was set to 50 µg per 50 µL prior to injection. SLA and liposomal SLA characterization was done using SDS-PAGE electrophoresis (Fig 1).

SLA SDS-PAGE analysis showed different protein bands with various ranges10 to 80kDa. The analysis of liposomal SLA showed nearly each band similar to free SLA, revealing that proteins with SLA get entrapped into the formulation after the preparation of liposomes.

Table 1. Particle size, polydispersity index (PDI), zeta potential and antigen entrapment of various liposomal formulations (mean±SD, n= 3)

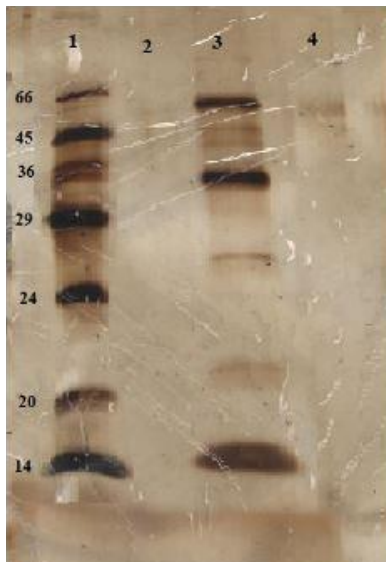| Formulation	Particle size(nm)	PDI	Zeta potential(Mv)	Antigen entrapment (%)
Empty Lip EPC	108.8 ± 8.2	0.15 ± 0.09	-9 ± 11.4	-
Lip EPC+ Imiquimod	139 ± 11.4	0.16 ± 0.08	-10.5 ± 15.7	-
Lip EPC+ Imiquimod+SLA	146 ± 9.8	0.22 ± 0.03	-15.3 ± 8.2	% 29 ± 5.6

Fig 1. SDS-PAGE analysis of Soluble Leishmania antigens (SLA) alone and liposomal SLA. Lane 1: low-range protein standard (Sigma, USA), Lane 2: Empty liposome, Lane 3: liposome containing SLA, Lane 4: liposome containing imiquimod

Challenge results

To investigate the extent of protection, the immunized mice were challenged with *L. major* promastigotes and the size of lesion developed in footpad was recorded weekly (Fig 2). The lesion size developed at an abrupt rate in mice which were immunized buffer or SLA in comparison with the mice group immunized with Lip EPC, Lip EPC + Imiquimod, and Lip EPC + Imiquimod +SLA after challenge. Totally, the groups of mice immunized with Lip EPC + Imiquimod +SLA showed the smallest footpad swelling compared with other mice, but there was no significant difference in footpad swelling between the groups of mice immunized with different formulations.
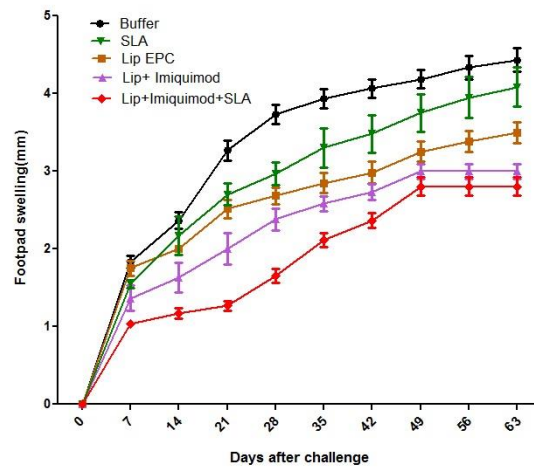

Fig 2. Footpad swelling in BALB/c mice immunized SC, three times in 3-week intervals, with SLA, Lip EPC, Lip+ Imiquimod, Lip+Imiquimod+SLA or buffer alone. The footpad thickness of each mouse was measured on both footpads for 63 days. Each point represents the average increase in footpad thickness ± SEM (n=7)

Parasite burden in footpad after challenge

The number of viable *L. major* was estimated in the infected footpad of various mice groups 63 days after challenge (Fig 3A). Results of parasite burden showed that the group of mice immunized with Lip+Imiquimod+SLA had the least parasite, which was significantly ($P<0.05$) lower than the control group, but no meaningful variation was observed in parasites' number in all vaccinated groups in comparison to the control.

Parasite burden in spleen

The number of viable *L. major* parasites was estimated in the spleen of various groups of mice at 9 weeks post challenge (Fig 3B).
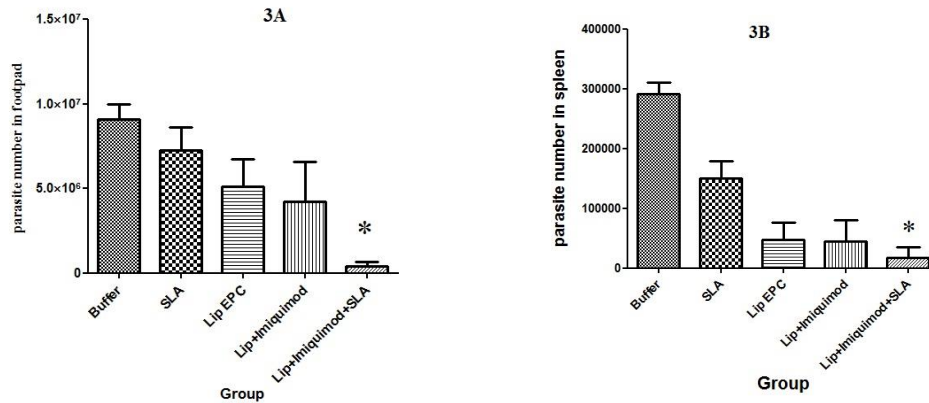
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Fig 3. Footpad parasite burden (3A) and spleen parasite burden (3B) in BALB/c mice. Mice immunized SC, three times in 3-week intervals with SLA, Lip EPC, Lip+Imiquimod, Lip+ Imiquimod+SLA or buffer alone after challenge with L. major promastigotes. 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)

Mice which were immunized with Lip+Imiquimod+SLA demonstrated the least live parasites compared with control group (*P*<0.05). However, the difference in number of spleen parasites among the groups vaccinated with Liposome EPC or Lip+Imiquimod compared with control group were not significant.

Antibody response

The type of immune response generated in immunized mice was determined by titration of anti-SLA-specific IgG, IgG1 and IgG2a antibodies before (Fig 4A-C) and after (Fig 5A-C) challenge with *L. major* promastigotes. Before challenge as shown in Fig 4A–C, the highest IgG2a level was observed in the mice sera immunized with Lip EPC, Lip EPC+Imiquimod and Lip EPC+Imiquimod+SLA; it was notably (*P*<0.0001) more than the control receiving HEPES buffer(1/200,1/2000 or 1/20000 serum dilutions).About IgG1 and IgG Abs levels, there was a meaningful difference (*P*<0.0001) among the mice group immunized with Lip EPC, Lip EPC+Imiquimod and Lip EPC+Imiquimod+SLA and the group receiving HEPES buffer (1/200 serum dilutions).After challenge, there was a meaningful difference in the IgG1, IgG2a, and IgG Abs levels in the mice sera immunized with different formulations in comparison to the control receiving HEPES buffer after challenge with *L. major* promastigotes (Fig 5A-C).Result show that, the mice sera immunized with Lip EPC, Lip EPC+Imiquimod and Lip EPC+Imiquimod+SLA generated considerably (*P*<0.0001) the greatest IgG1, IgG2a, and IgG Abs titers in comparison to the groups receiving HEPES buffer (notably 1/200 or 1/2000 serum dilutions).
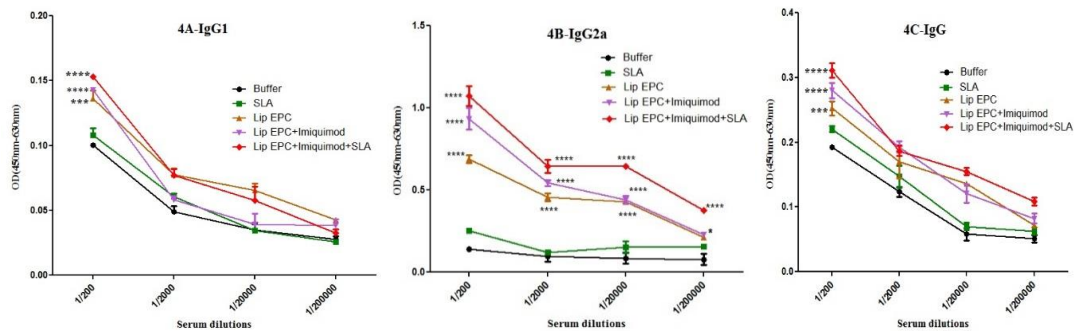

Fig 4. The levels of anti-SLA specific IgG1 (A), IgG2a (B), and IgG (C) antibodies based on mean absorbance in sera of BALB/c mice before challenge. Mice immunized SC, three times in 3-week intervals, with SLA, Lip EPC, Lip EPC+ Imiquimod, Lip EPC+Imiquimod+SLA or buffer alone. Blood samples were collected 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<0.05), *** (P<0.001), **** (P<0.0001)
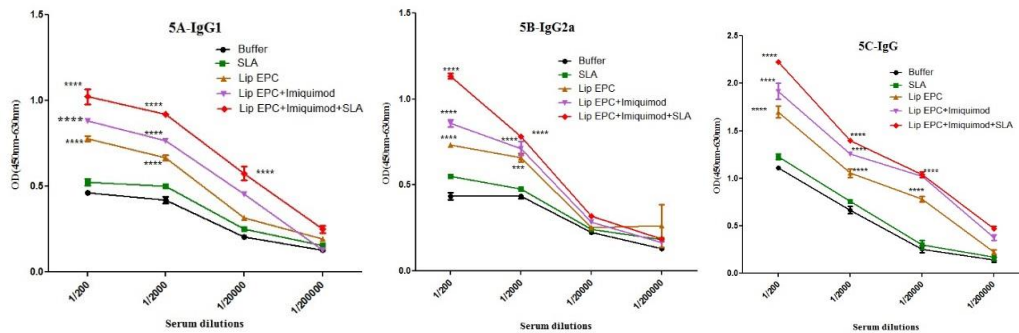
Fig 5. Levels of anti-SLA specific IgG1 (A), IgG2a (B), and IgG (C) in sera of BALB/c mice; Mice immunized SC, three times in 3-week intervals, with, SLA, Lip EPC, Lip EPC+ Imiquimod, Lip EPC+Imiquimod+SLA or buffer alone; Blood samples were collected from the mice 2 weeks after the last booster and 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<0.001), **** (P<0.0001)

ELISpot results

Splenocytes of immunized mice were isolated in the day before challenge, and cultured in vitro in medium alone (as a negative control), or with Con A (as a positive control), or restimulated with either SLA as recalled antigens (Fig 6). The findings of ELISpot assays revealed that splenocytes isolated from the mice immunized with Lip+Imiquimod and Lip+Imiquimod+SLA secreted meaningfully greater IFN-γ amounts (*P*<0.05) (*P*<0.001) respectively, compared to the mice immunized with HEPES buffer. In terms of IL-4 secretion, the significantly (*P*<0.05) lowest level of IL-4 was seen in the cell supernatant of mice immunized with Lip+Imiquimod+SLA when compared with the mice immunized with other formulations.

Flow cytometry results

Splenocytes were isolated at 2 weeks after the last booster injection to determine the antigen-specific T cell responses in different groups of mice. Extracellular staining was employed for surface markers of CD4 and CD8. Intracellular cytokine staining was employed for IFN-γ and IL-4 cytokines accompanied by flow cytometry analyses.

Fig 7 shows that, the CD8+/IFN-γ cells frequency in the mice immunized with Lip+Imiquimod+SLA was significantly (*P*<0.05) higher than that of others formulations.

The frequency of CD4+/IFN-γ cells in the group of mice immunized with Lip+Imiquimod and Lip+Imiquimod+SLA were significantly (*P*<0.001) greater than the other groups, while flow
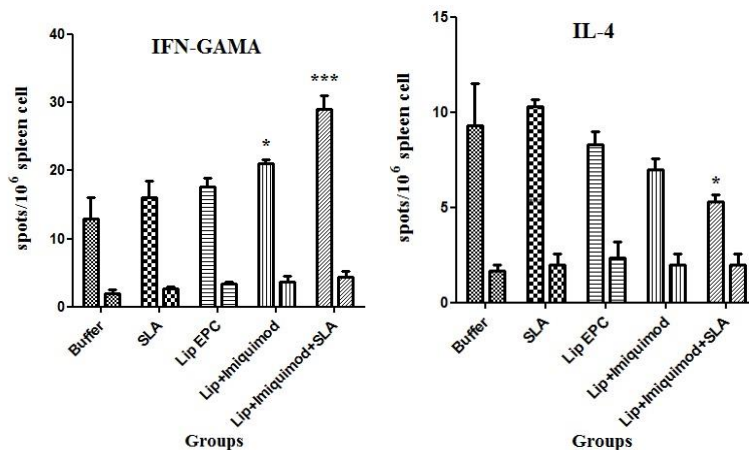

Fig 6. Cytokine level was evaluated through measuring IFN-γ and IL-4 production in immunized mice at week 2 after the last booster injection. Mononuclear splenocytes were cultured in the presence of SLA (10 µg/ml), and the IFN-γ release and IL-4 release from splenocytes induced by different liposomal formulations were determined using ELISpot assay. The data are represented as mean ± SEM (n=3)
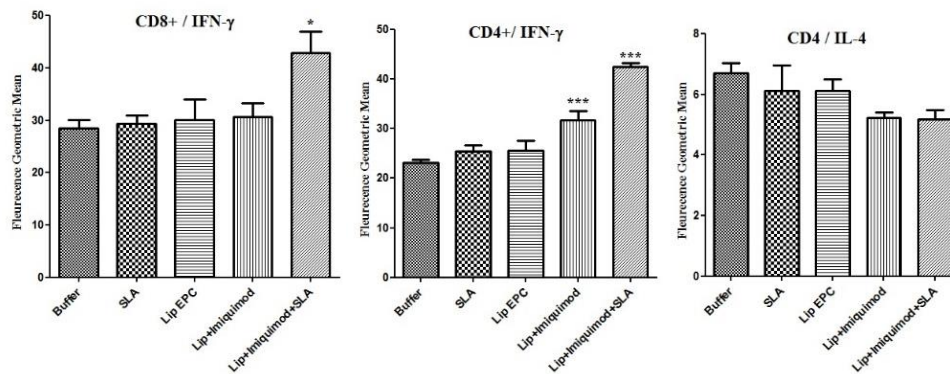
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Fig 7. After the last booster, splenocytes were isolated and restimulated, and then stained for surface CD8, CD4, and intracellular IFN-γ and IL-4. Splenocytes were gated by side vs. forward scatter light followed by staining with CD8Pe-cy5 and CD4Pe-cy5. Plots show log fluorescence intensity for IFN-γ- FITC and IL-4-PE. The data are represented as mean ± SEM, (n= 3). *** (P<0.001) and * (P<0.05) denote significant differences from buffer and all other formulations

cytometric results also showed IL-4 production in CD4 cells, that implies T cell-dependent humoral immunity was not induced significantly in any groups when compared with control group.

DISCUSSION

Major methods for controlling leishmaniasis depend principally on the prompt detection of cases as well as chemotherapy, which are impeded by an increase in the resistance to first-line medicines (pentavalent antimonials) or side-effects and toxicity connected with second-line medicines (amphotericin B and pentamidine). The control of the vector and the reservoir host is hard because of the presence of operational problems and recurrent relapses in the host [34].

Hence, it seems that developing an efficient vaccine against leishmaniasis is the most efficient tool for controlling leishmaniasis. Some experimental vaccines have been tested for Leishmania in clinical trials for humans. Nevertheless, the findings were not consistent in some studies, and in general, they presented low prophylactic efficiency, having been as such because of some reasons, such as the lack of a delivery system and a suitable adjuvant [35-37].

In developing efficient vaccines against leishmaniasis, a delivery system or a suitable adjuvant is required to prompt an appropriate immune reaction against leishmaniasis. This is a major concern in developing secure vaccine adjuvants. Imiquimod (R837) is principally a TLR7 agonist used in human beings [38]. On the basis of these test findings and since treatment with

imiquimod received FDA's confirmation in 1997 (to treat cervical dermal warts created by the infection with HPA), a small-scale human study was done so as to treat CL patients [38]. The main goal of this study was to find out if TLR7-activating molecules would be as efficient as vaccine adjuvants, making use of a verified live model of infection, i.e. infection with *Leishmania major*, in BALB/c mice.

Imiquimod would prompt the generation of anti-virus cytokines, such as IL-1b (interleukin-1b), IFN-α (interferon-α), TNF-a (tumour necrosis factor-a), and IL-6 in monocytes and macrophages [24]. In addition, Imiquimod could increase the immune response of Th1 by prompting the discharge of IFN-γ and IL-12 from macrophages [39]. It has been demonstrated that imiquimod reduces the level of intracellular Leishmania by prompting the generation of nitric oxide, yet it exerts no direct impact on the parasite [40]. Using a gene array method, it has also been shown that, in line with the capability of imiquimod for eradicating intracellular *Leishmania* amastigotes, its relevant compound, i.e. S-28463, prompts the expression of genes connected with the activation of macrophages as well as an inflammatory reaction, including IL-1, NF-κB, MIP-1, and iNOS [41].

The results showed that the amount of the swelling of the footpad in Lip+Imiquimod+SLA - immunized mice on the 63rd day from the challenge was lower than that of other mice, yet no significant difference existed in the swelling of the footpad between the two classes of mice

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vaccinated with various formulations (Fig 2). To design a vaccine for leishmaniasis, the measuring of the parasite burden of the spleen and feet is crucial in specifying the effectiveness of the vaccine. The findings of the parasite burden of the spleen and feet verified that the mice vaccinated with Lip+Imiquimod+ SLA demonstrated a lower parasite burden than the buffer group, having been significantly (P <0.05) less than that of the control group (Fig 3A and 3B).

The assessment of the antibody isotypes of IgG2a and IgG1 is utilized as an indicator of the immune responses of Th1 and Th2, respectively. In the present study, prior to the challenge, as Figure 4A–C demonstrates, the highest amount of IgG2a was seen in the sera of the mice immunized with Lip+Imiquimod+SLA, Lip EPC+Imiquimod, and Lip EPC, having been significantly higher (P<0.0001) than that of the control group which received the buffer of HEPES. In addition, concerning the level of IgG1, a significant difference (P<0.0001) was observed in the group of the mice immunized with Lip EPC+Imiquimod+SLA, Lip EPC+Imiquimod, and Lip EPC, and the mice which received the buffer of HEPES. After the challenge, the results demonstrated that the sera of the mice immunized with Lip EPC+Imiquimod+SLA, Lip EPC+Imiquimod, and Lip EPC produced (P<0.0001) a significantly higher amount of IgG Abs, IgG2a, and IgG1titers than the group which received the buffer of HEPES (Fig 5A- C).

The IFN-a's level, which is a cytokine indicating the Th1 reaction, in evaluation of the cytokine showed that Lip+Imiquimod+SLA and Lip+Imiquimod formulations discharged a higher amount of IFN-γ (P<0.05), (P<0.0001) than the mice group which was immunized with the buffer (Fig 6). Besides, the lowest IL-4 level was identified in the splenocytes of the mice group which was immunized with Lip+Imiquimod+SLA; in addition, a significant difference (P<0.05) was observed in the IL-4 level in the control group and different vaccinated groups (Fig 6). CD4 and CD8 indices indicate the prevalence of the IL-4 and IFN-γ generating cells in Th2 and Th1 populations, respectively. The findings demonstrated that the Lip+Imiquimod+SLA formulations prompted a considerably (P<0.05) greater amount of IFN-γ in the lymphocytes of CD8$^+$, which indicated a greater number of cells which produced IFN-γ in the population of CD8$^+$ than the other ones (Fig 7). The prevalence rates of the cells of CD4$^+$/

IFN-γ in the mice group which was immunized with Lip+Imiquimod and Lip+Imiquimod+SLA were considerably (P<0.001) higher than that of other groups (Fig 7); however, the results of flowcytometry demonstrated that the lowest production of IL-4 in the cells of CD4 was prompted considerably in no group than the control one (Fig 7).

Leishmaniasis resistance is coupled with a dominant Th1 reaction with the production of IFN by the CD4$^+$T antigen-specific lymphocyte population. Besides, the activity of the population of CD8$^+$ T cells has demonstrated to have a significant role in post-recovery protection against infection with *L. major* as well as in efficient vaccinations against murine experimental leishmaniasis [42]. In contrast, the immune responses of Th2, being featured by the production of IL-4 is accompanied by the worsening of the disease [42].

In past research, liposomes provided using the DPPC or DSPC-containing antigen of rgp63 demonstrated more entrapment efficacy and were capable of stimulating a better Th1 response than liposomes provided using EPC-containing rgp63, as a recombined antigen that prompts a Th2 kind of immune reaction [43].

It is worth noting that an immune reaction is a complicated response to infection, with both Th2 and Th1 phenotypic cells being most often identified in the course of the immune reaction. Therefore, the biological composition of the immune reaction is identified by the dominance of one type of cell over another, not easily by the absence or presence of Th2- or Th1-type immune cells [42]. Primary characterization research shows that imiquimod is able to prompt the generation of antiviral cytokines, including IL-1β, TNF and IFN-α from monocytes. In addition, it could increase the Th1's immune reaction by prompting the discharge of IFN-γ and IL-12 from macrophages [21]. It has also been shown that the treatment of macrophages infected with *L. donovani* by imiquimod leads to the eradication of intracellular amastigotes, with this depending on NO generation by the macrophages treated [40]. Just later, it was found out that imiquimod was similar to the single-stranded RNA in structure, being able to activate macrophages by triggering the pathway of TLR7 [44]. Our past research showed that imiquimod could prompt the Th1 immune reaction by inducing the discharge of IFN-γ from macrophages, having been defending

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against the challenge posed by *Leishmania major* [45, 46]. Emami et al. had been used soluble *Leishmania* antigens (SLA), monophosphoryl lipid A (MPL) and imiquimod (IMQ) for vaccination with liposomal carrier DSPC and DSPG, their reports indicated that this composition could be an appropriate delivery system to induce cellular immunity pathway against leishmaniasis. In the Emami study, DSPC and DSPG nanoparticles were used that have Tm = 55 °C, while our research uses the nanoparticles consisted of EPC that have Tm 0°C. The phase transition temperature of lipids has a significant effect on the performance of nanoparticles, following the subcutaneous administration. In general, it can be said that the kinetics of these nanoparticles are different, and the reason for their selection is based on the type of function and the permanence in the circulatory system, how it is opsonized by macrophages, and so on. Nonetheless our findings are consistent with the report by Emami et al [47].

CONCLUSION

Based on the parasite burden, challenge, as well as cytokine assessment results, one can suggest that imiquimod added to the formulation of liposome can lead to the modulation of the immune reaction by the BALB/c mice vaccinated, preferably to a Th1-type reaction rather than a Th2-type one, being able to prompt limited protection against the challenge of Leishmania. The activation of Th2-cell as shown by the production of IL-4 might describe the imperfect protection seen in the present experimental system. The present study implies that more emphasis should be placed on the use of imiquimod as an adjuvant in human vaccines against intracellular pathogens, especially in cases where a Th1 reaction is necessary for protective immunity.

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REFERENCES

1. WHO/Leishmaniasis,WHO,http://www.who.int/ leishmaniasis/en/. 2017; Accessed date: 8 November 2017.
2. Modabber F. Leishmaniasis vaccines: past, present and future. Int J Antimicrob Agents. 2010; 36: S58-S61.
3. Evans KJ, Kedzierski L. Development of vaccines against visceral leishmaniasis. J Trop Med. 2012; 2012: 892817
4. Askarizadeh A, Jaafari MR, Khamesipour A, Badiee A. Liposomal adjuvant development for leishmaniasis vaccines. Ther Adv Vaccines. 2017; 5: 85-101.
5. Sacks D, Noben-Trauth N. The immunology of susceptibility and resistance to Leishmania major in mice. Nat Rev Immunol. 2002; 2: 845-58.
6. Scott P, Novais FO. Cutaneous leishmaniasis: immune responses in protection and pathogenesis. Nat Rev Immunol. 2016; 16: 581-592.
7. Martins VT, Chávez-Fumagalli MA, Costa LE, Martins AM, Lage PS, Lage DP, Duarte MC, Valadares DG, Magalhães RD, Ribeiro TG. Antigenicity and protective efficacy of a Leishmania amastigote-specific protein, member of the super-oxygenase family, against visceral leishmaniasis. PLoS Negl Trop Dis. 2013; 7: e2148.
8. Watson DS, Endsley AN, Huang L. Design considerations for liposomal vaccines: influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens. Vaccine. 2012; 30: 2256-2272.
9. Apostolopoulos V. Vaccine delivery methods into the future. Multidisciplinary Digital Publishing Institute; 2016.
10. Nordly P, Agger EM, Andersen P, Nielsen HM, Foged C. 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. 2011; 28: 553-562.
11. Moon JJ, Suh H, Bershteyn A, Stephan MT, Liu H, Huang B, Sohail M, Luo S, Um SH, Khant H. Interbilayer-crosslinked multilamellar vesicles as synthetic vaccines for potent humoral and cellular immune responses. Nat Mater. 2011; 10: 243-251.
12. Kataoka K, Harada A, Nagasaki Y. Block copolymer micelles for drug delivery: design, characterization and biological significance. Adv Drug Deliv Rev. 2012; 64: 37-48.
13. Mazumder S, Maji M, Ali N. Potentiating effects of MPL on DSPC bearing cationic liposomes promote recombinant GP63 vaccine efficacy: high immunogenicity and protection. PLoS Negl Trop Dis. 2011; 5: e1429.
14. Joshi MD, Unger WJ, Storm G, van Kooyk Y, Mastrobattista E. Targeting tumor antigens to dendritic cells using particulate carriers. J Control Release. 2012; 161: 25-37.
15. Smith DM, Simon JK, Baker Jr JR. Applications of nanotechnology for immunology. Nat Rev Immunol. 2013; 13: 592-605.
16. Krishnan L, Sad S, Patel GB, Sprott GD. Archaeosomes induce enhanced cytotoxic T lymphocyte responses to entrapped soluble protein in the absence of interleukin 12 and protect against tumor challenge. Cancer Res. 2003; 63: 2526-2534.
17. Mehravaran A, Jaafari MR, Jalali SA, Khamesipour A, Tafaghodi M, Hojatizade M, Badiee A. The role of surface charge of ISCOMATRIX nanoparticles on the type of immune response generated against Leishmaniasis in BALB/c mice. Nanomed J. 2015; 2: 249-260.
18. Mehravaran A, Jaafari MR, Jalali SA, Khamesipour A, Tafaghodi M, Hojatizade M, Abbasi A, Badiee A. Cationic immune stimulating complexes containing soluble Leishmania antigens: preparation, characterization and in vivo immune response evaluation. IJI. 2015; 12: 274-287.
19. Mehravaran A, Jaafari MR, Jalali SA, Khamesipour A, Ranjbar R, Hojatizade M, Badiee A. The role of ISCOMATRIX

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bilayer composition to induce a cell mediated immunity and protection against leishmaniasis in BALB/c mice. IJBMS. 2016; 19: 178-86.

20. Bhowmick S, Ali N. Recent developments in leishmaniasis vaccine delivery systems. Expert Opin Drug Deliv. 2008; 5: 789-803.

21. Raman VS, Reed SG, Duthie MS, Fox CB, Matlashewski G. Adjuvants for Leishmania vaccines: from models to clinical application. Front Immunol. 2012; 3: 144.

22. Miranda-Verástegui C, Llanos-Cuentas A, Arevalo I, Ward B, Matlashewski G. Randomized, double-blind clinical trial of topical imiquimod 5% with parenteral meglumine antimoniate in the treatment of cutaneous leishmaniasis in Peru. Clin Infect Dis. 2005; 40: 1395-1403.

23. Arevalo I, Ward B, Miller R, Meng T-C, Najar E, Alvarez E, Matlashewski G, Alejandro L-C. Successful treatment of drug-resistant cutaneous leishmaniasis in humans by use of imiquimod, an immunomodulator. Clin Infect Dis. 2001; 33: 1847-1851.

24. Dockrell D, Kinghorn G. Imiquimod and resiquimod as novel immunomodulators. J Antimicrob Chemother. 2001; 48: 751-755.

25. Khamesipour A, Rafati S, Davoudi N, Maboudi F, Modabber F. Leishmaniasis vaccine candidates for development: a global overview. Indian J Med Res. 2006; 123: 423-38.

26. ALIMOHAMMADIAN MH, HAKIMI H, NIKSERESHT M. The P Reparation And Evaluation Of Reference Leishmanin From Leishmania Major For Use In Man For Diagnos Tic And Experimental Purposes. MJIRI. 1993; 7: 23-28.

27. Scott P, Pearce E, Natovitz P, Sher A. Vaccination against cutaneous leishmaniasis in a murine model. I. Induction of protective immunity with a soluble extract of promastigotes. J Immunol. 1987; 139: 221-227.

28. Bainor A, Chang L, McQuade TJ, Webb B, Gestwicki JE. Bicinchoninic acid (BCA) assay in low volume. Anal Biochem. 2011; 410: 310-312.

29. Torchilin PV, Torchilin V, Torchilin V, Weissig V. Liposomes: a practical approach: Oxford University Press; 2003.

30. Firouzmand H, Badiee A, Khamesipour A, Heravi Shargh V, Alavizadeh SH, Abbasi A, Jaafari MR. Induction of protection against leishmaniasis in susceptible BALB/c mice using simple DOTAP cationic nanoliposomes containing soluble Leishmania antigen (SLA). Acta Trop. 2013; 128: 528-535.

31. de Barros NB, Aragao Macedo SR, Ferreira AS, Tagliari MP, Kayano AM, Nicolete LDF, Soares AM, Nicolete R. ASP49-phospholipase A2-loaded liposomes as experimental therapy in cutaneous leishmaniasis model. Int Immunopharmacol. 2018; 55: 128-132.

32. Shargh VH, Jaafari MR, Khamesipour A, Jaafari I, Jalali SA, Abbasi A, Badiee A. Liposomal SLA co-incorporated with PO CpG ODNs or PS CpG ODNs induce the same protection against the murine model of leishmaniasis. Vaccine. 2012; 30: 3957-3964.

33. Hojatizade M, Soleymani M, Tafaghodi M, Badiee A, Chavoshian O, Jaafari MR. Chitosan Nanoparticles Loaded with Whole and Soluble Leishmania Antigens, and Evaluation of Their Immunogenecity in a Mouse Model of Leishmaniasis. IJI. 2018; 15: 281-293.

34. Jafari I, Heravi Shargh V, Shahryari M, Abbasi A, Jaafari MR, Khamesipour A, Badiee A. Cationic liposomes formulated

with a novel whole Leishmania lysate (WLL) as a vaccine for leishmaniasis in murine model. Immunobiology. 2018; 223: 493-500.

35. Das A, Ali N. Correction: Combining Cationic Liposomal Delivery with MPL-TDM for Cysteine Protease Cocktail Vaccination against Leishmania donovani: Evidence for Antigen Synergy and Protection. PLoS Negl Trop Dis. 2015; 9: e0004185.

36. Badiee A, Heravi Shargh V, Khamesipour A, Jaafari MR. Micro/nanoparticle adjuvants for antileishmanial vaccines: present and future trends. Vaccine. 2013; 31: 735-749.

37. Iborra S, Solana JC, Requena JM, Soto M. Vaccine candidates against leishmania under current research. Expert Rev Vaccines. 2018; 17:323-334.

38. Zhang W-W, Matlashewski G. 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. 2008; 76: 3777-3783.

39. Khamesipour A. Therapeutic vaccines for leishmaniasis. Expert Opin Biol Ther. 2014; 14:1641-9.

40. Buates S, Matlashewski G. Treatment of experimental leishmaniasis with the immunomodulators imiquimod and S-28463: efficacy and mode of action. J Infect Dis. 1999; 179: 1485-1494.

41. Buates S, Matlashewski G. Identification of genes induced by a macrophage activator, S-28463, using gene expression array analysis. Antimicrob Agents Chemother. 2001; 45:1137-1142.

42. Naseri H, Eskandari F, Jaafari M, Khamesipour A, Abbasi A, Badiee A. PEG ylation of cationic liposomes encapsulating soluble Leishmania antigens reduces the adjuvant efficacy of liposomes in murine model. Parasite Immunol. 2017; 39: e12492.

43. Badiee A, Jaafari MR, Khamesipour A, Samiei A, Soroush D, Kheiri MT, Barkhordari F, McMaster WR, Mahboudi F. 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. 2009; 74: 37-44.

44. Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K, Akira S. Small anti-viral compounds activate immune cells via the TLR7 MyD88–dependent signaling pathway. Nat Immunol. 2002; 3: 196-200.

45. Mehravaran A, Nasab MR, Mirahmadi H, Sharifi I, Alijani E, Nikpoor AR, Akhtari J. Protection induced by Leishmania Major antigens and the imiquimod adjuvant encapsulated on liposomes in experimental cutaneous leishmaniasis. Infect Genet Evol. 2019; 70: 27-35.

46. Mehravaran A, Rezaei Nasab M, Mirahmadi H, Sharifi I, Alijani E, Nikpoor AR, Akhtari J, Hojatizade M. Immunogenicity and protection effects of cationic liposome containing imiquimod adjuvant on leishmaniasis in BALB/c mice. IJBMS. 2019; 22: 1-10.

47. Emami T, Rezayat SM, Khamesipour A, Madani R, Habibi G, Hojatizade M, Jaafari MR. The role of MPL and imiquimod adjuvants in enhancement of immune response and protection in BALB/c mice immunized with soluble Leishmania antigen (SLA) encapsulated in nanoliposome. Artif Cells Nanomed Biotechnol. 2018; 46: 324-333.

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