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Research Article

Treatment of Experimental Cutaneous Leishmaniasis by the Therapeutic Vaccine SLA-R848-Pam3CSK4

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ABSTRACT

Background: Leishmaniasis is a vector-borne disease affecting 12 million people in the world. Because of the high prevalence of this disease and also problems related to the control and therapy of leishmaniasis, the development of effective and applicable therapeutic approaches in the treatment of leishmaniasis seems to be essential. In recent years, therapeutic vaccines have been considered promising approaches against leishmaniasis. So, we examined the therapeutic efficacy of Soluble *Leishmania* antigen (SLA) in combination with TLR agonists (R848 and Pam3CSK4) as adjuvants using the BALB/c mice model.

Methods: To develop a new therapeutic vaccine for leishmaniasis, SLA and/or Pam3CSK4 and/or R848 were injected one week after infection three times. One week after infection, footpad swelling was monitored weekly. Parasite burden was also assessed by serial dilution 11 weeks post-infection. Before and after vaccination, blood samples were collected, and humoral responses were evaluated using an ELISA assay. Cytokines and NO production were analyzed 11 weeks post-infection in all groups.

Results: Immunological analysis showed that mice treated with SLA-R848-Pam3CSK4, able to control cutaneous leishmaniasis disease and subsequently the smallest lesion size, decreased parasite load, increased IgG2a, IgG2a/IgG1, IFN-γ, and NO production.

Conclusion: The results revealed the effectiveness of SLA-R848-Pam3CSK4 modulation as a therapeutic vaccine in infected-BALB/c mice against *Leishmania major* infection.

Keywords: Soluble *Leishmania* antigen (SLA); R848; Pam3CSK4; leishmaniasis; Therapeutic vaccine

INTRODUCTION

Leishmaniasis is a global vector-borne disease caused by an obligate intracellular protozoan of the genus *Leishmania* [1]. Leishmaniasis has been reported in more than 90 countries. Each year, 1.5 to 2 million new cases are reported around the world [2]. Despite several efforts for providing an effective vaccine against leishmaniasis, there is no approved vaccine for use in humans yet, and a vaccine development program is still underway. Nowadays, immunotherapy is an attractive and successful alternative vaccine for treating cutaneous leishmaniasis [3,4]. The use of TLR agonists as vaccine adjuvants, although previously demonstrated to be efficacious in leishmaniasis therapy [5,6], has limited studies that contributed to the therapeutic vaccines [7–9]. The therapeutic effects of these adjuvants are related to the ligation of TLRs, which activates the nuclear factor-kB (NF-kB) and induces the production of pro-inflammatory cytokines, chemokines, and nitric oxide (NO). The stimulation of these molecules leads to the activation of Th1 cells and control of leishmaniasis [10]. TLR2 (Pam3CSK4), TLR3 (Poly (I:C)), TLRs 4 (MPL), 7/8 (resiquimod and imiquimod), and 9 (CpG) agonists showed protective anti-leishmanial responses in mice models [11–15]. The combinatorial delivery of TLR agonists and *Leishmania* antigen showed strong immune responses against leishmaniasis. Immunization with live *L. major* and Pam3CSK4 showed reduced pathology in BALB/c mice models [14]. Mice treated with L110f plus MPL and CpG agonists showed a TLR synergy in treating cutaneous leishmaniasis [5]. *Leishmania* antigens, such as SLA (soluble *Leishmania* antigen) in combination with an appropriate adjuvant, showed a protective role in treating leishmaniasis [16,17]. We previously treated *L. major*-infected macrophages with a combination of *L. major* antigen (SLA), R848, and Pam3CSK4 [18]. We showed promising results in the induction of immune responses against L. major infection. To the best of our knowledge, immunization containing SLA, R848, and Pam3CSK4 so far have not been used as a therapeutic

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vaccine in leishmaniasis. So, in the current study, we examined the therapeutic effect of this immunization using a *L. major*-infected BALB/c model against cutaneous leishmaniasis. We explored that the combination of SLA, TLR1/2, and TLR7/8 agonists promoted a Th1 cell immunity against cutaneous leishmaniasis compared with *L. major* antigen only or *L. major* antigen and single agonist in *L. major*-infected BALB/c mice.

MATERIALS AND METHODS

Animals

Female BALB/c mice 6-8 weeks were purchased from Pasteur Institute (Tehran, Iran). The mice were maintained under standard conditions. The animals were grouped in 5 groups: PBS (G1); SLA (G2); SLA-R848 (G3); SLA-Pam3CSK4 (G4); SLA-R848-Pam3CSK4 (G5). All experiments were carried out according to the Ethical Committee Acts of Pasteur Institute of IRAN.

Parasites, infection, and SLA

L. major strain (MRHO/IR/75/ER), which was used in this experiment, was described previously [19]. A total of $1\text{-}10^5$ stationary phase metacyclic promastigotes parasites injected s.c. in the right footpad of each BALB/c mice. The progress of the lesion was monitored weekly, one week after infection, by measuring footpad swelling using a dial-gauge caliper (Mitutoyo, Kawasaki, Kanagawa, Japan).

The SLA (Soluble *Leishmania* antigen) of *L. major* was provided by a freeze-thaw and a sonication. In brief, *L. major* promastigotes were washed three times using phosphate-buffered saline (PBS) and then lysed by repeated freeze-thaw method followed by probe sonication (Hielscher®, UP200Ht, Germany). The supernatant of lysate parasites was collected using centrifugation. Next, the SLA was concentrated by a 30 kDa Amicon filter (Amicon®, Ultra 15). The protein concentration of the SLA was determined using the Bradford protein assay [20], and stored at -80 °C until use

Immunization of BALB/c mice

Different groups of mice one week post-infection were immunized subcutaneously in the left hind footpad three times in one-week interval as followed: SLA (25 µg/mouse), SLA-R848 (25 µg/mouse and 10 µg/mouse, respectively), SLA-Pam3CSK4 (25 µg/mouse and 10 µg/mouse, respectively), and SLA-R848-Pam3CSK4 (25 µg/mouse, 10 µg/mouse, and 10 µg/mouse, respectively). R848 and Pam3CSK4 were prepared according to the manufacturer's instructions (InvivoGen, San Diego, CA, USA). The control group received only sterile PBS.

Antibody assessment

Before and after vaccination (4 and 11 weeks post-infection), serum samples were collected from mice and used to assess anti-freeze-thaw (F/T) antigen IgG total, IgG1, and IgG2a antibodies by the ELISA method as described before [21]. Briefly, 96-well plates (Greiner) were coated with 50 µL F/T antigen (10 µg/mL) overnight at 4°C. The plates were washed and blocked with 1% (v/v) of bovine serum albumin (BSA) in PBS-Tween and incubated for 1 h at room temperature. Then, Serum samples were prepared and applied to each well. After washing the plate, levels of IgG total, IgG1, and IgG2a antibodies were evaluated using goat anti-mouse IgG-HRP, goat anti-mouse IgG1, and IgG2a antibodies (Sigma, Germany) were added. After 1 hour, for detection of IgG subclasses, HRP-conjugated rabbit anti-goat antibody (Sigma, Germany) was added, and the plates were incubated for 1 h. Then, the substrate was added (TMB) (Sigma, Germany), and an optical density (OD) was determined at 450 nm using a micro-ELISA reader (Biotek).

Quantitative parasite culture

Parasite load was assessed by limiting dilution assay 11 weeks post-infection. Draining lymph nodes were collected, homogenized, and after cell counting, the triplicate twelve 2-fold serial dilutions were prepared in 96-well plates, which were supplemented with 50 µl of NNN and RPMI 1640 mediums. Then the plates were incubated at 24°C for one week. The number of viable *L. major* parasites was determined by an inverted light microscope, and the parasite load was estimated by SAS software 9.4 M7 (2020, SAS Institute).

Cytokine analysis

Cytokines were assessed in the supernatants of cultured splenocytes with soluble *Leishmania* antigen (37.5 µg/ml) from BALB/c mice infected with *L. major* and treated or not treated with *Leishmania* antigen and TLR agonists. *In vivo* spleen cell responses were evaluated in four mice from each group. At 11 weeks post-infection, mice were sacrificed, and spleens were removed and homogenized. After RBC lysis, splenocytes were resuspended in an appropriate RPMI 1640 medium; 10% FCS (Gibco), 2 mM L-glutamine, and antibiotic (100 IU/ml Penicillin and 100 µg/ml Streptomycin) at a density of 2×10^6 cells/well in triplicate in a 96-well plate. Then plates were incubated at 37°C in 5% CO2 for 96 h. Cytokine measurements were assessed using commercial ELISA kits (Mabtech, Swedish).

Assessment of nitric oxide assay

The supernatant of splenocyte cells from mice was collected after 5 days post-stimulation, and the nitric oxide (NO) production was quantified by the Griess assay. An equal concentration of the Griess reagent (Promega, Madison, WI) was added to the equivalent concentration of samples and standards. The plate was incubated at room temperature for 10 min, and then the absorbance was measured at 540 nm by the microplate reader (BioTek ELx808 Absorbance Microplate Reader). The nitric oxide levels were determined by comparison to a standard curve (different known concentrations of sodium nitrite).

Statistical analysis

The statistical significance of differences between control and treated groups was performed using the two-way ANOVA followed by Dunnett's, or Sidak's multiple comparisons test and one-way analysis of variance (Multiple comparisons Tukey's post hoc test) of the GraphPad Prism software (Prism 8.0.2., 2019, San Diego, CA). Results with a p-value ≤ 0.05 were considered significant, and data expressed as mean+SD.

RESULTS

Footpad swelling measurement

One week after infection with *L. major*, footpad swelling of all mice was measured weekly up to 11 weeks. As shown in Figure 1, Group 5 (SLA-R848-Pam3CSK4) showed the smallest lesion size compared to control and treated groups, although a significant difference with the PBS group was showed after 9 weeks ($p<0.001$). Group 3 (SLA-R848) also showed a significant difference with the PBS control group in 6, 10, and 11 weeks ($p<0.05$). As well as, Group 4 (SLA-Pam3CSK4) at 6 weeks showed a significant difference with the PBS group ($p<0.001$). Group 2 did not show a significant

difference with the PBS control group in any of the weeks.

Reducing parasite load in SLA-R848-Pam3CSK4 treated group

The parasite burden was evaluated in the lymph nodes of all treated and control groups at 11 weeks post-infection. Also, no parasites were observed in the lymph nodes of mice before vaccination (one week post-infection). As shown in Figure 2, all treated groups (G3, G4, and G5 except G2) showed a significant difference in parasite load compared to the control group (G1) ($p<0.01$). Mice treated with SLA only (G2) showed an increase in parasite load compared to dual-(G3 and G4) and triple-(G5) combinations ($p<0.01$). The results indicate that the G5 had the lowest parasite load than the other treated groups ($p<0.0001$), which shows its ability to control parasite propagation.

SLA-R848-Pam3CSK4 immunotherapy induces humoral responses

The production of specific IgG antibodies against F/T antigen was evaluated in the sera of all mice groups by Elisa before and after vaccination (4 and 11 weeks post-infection (Figure 3). IgG titers were detectable after 4 weeks. All treated groups after 4 and 11 weeks post-infection (except G2 at 4 weeks) showed increased IgG levels in comparison to the PBS control group (G1). Significantly higher IgG titers were detected in the sera of mice treated with SLA-R848-Pam3CSK4 (G5) in comparison to G2 (4 and 11 weeks post-infection) and G3 (11 weeks post-infection) ($p<0.05$) (Figure 3A). The SLA-R848-Pam3CSK4 group also induced IgG at 11 weeks after infection compared to the PBS group ($p<0.05$) (Figure 3B).

The mice sera were further examined for F/T *L. major* antigen specific IgG1 and IgG2a antibody isotypes. As shown in Figure 3C, 4 weeks post-infection, the IgG1 levels were significantly higher than IgG2a levels in all groups. The IgG1 titers increased significantly in groups 4 and 5 after 4 weeks compared with a control group (G1) ($p<0.01$). IgG2a titer was almost the same among all groups at week 4. Eight weeks post-infection, IgG1 levels were almost the same in all groups, but IgG2a titer increased significantly in group 5, compared with treated and control groups ($p<0.05$).

The analysis of the IgG2a/IgG1 ratio in Figure 3D showed the lowest ratio in G4 compared to G1 at 4 weeks post-infection ($p<0.05$). Also, there was a significant difference between G3, G4, G5, and PBS group (G1) at 11 weeks post-infection ($p<0.001$). After 11 weeks post-infection, G5 showed significantly the highest level of IgG2a to IgG1 compared with all other groups ($p<0.05$).

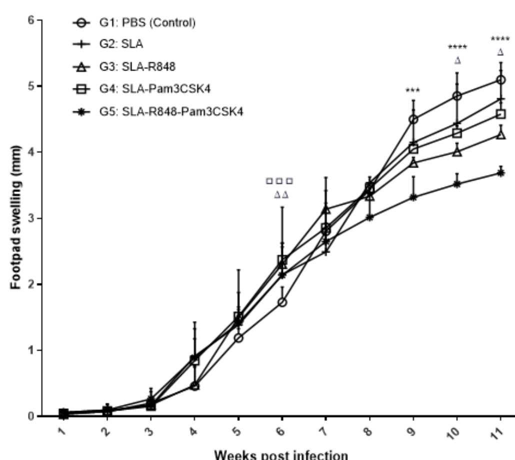

Figure 1: Assessment of footpad swelling in treated and control groups of BALB/c mice. One week after infection with 1×10^5 promastigotes of L. major, mice were vaccinated weekly. The footpad swelling was measured one week after infection for 11 weeks. Results are mean+SD, n=12. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. The following symbols indicate significant differences with PBS control group (G1) and different treated groups: +SLA; Δ SLA-R848; □SLA-Pam3CSK4; *SLA-R848-Pam3CSK4.

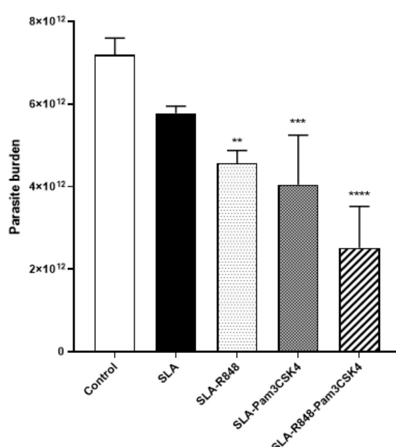

Figure 2: Parasite load in lymph node of treated and control groups. 11 weeks post-infection, the parasite burden was evaluated. Data are mean+SD, n=4 and are representative of three experiments. All groups were compared with control PBS group. **p<0.01; ***p<0.001; ****p<0.0001.

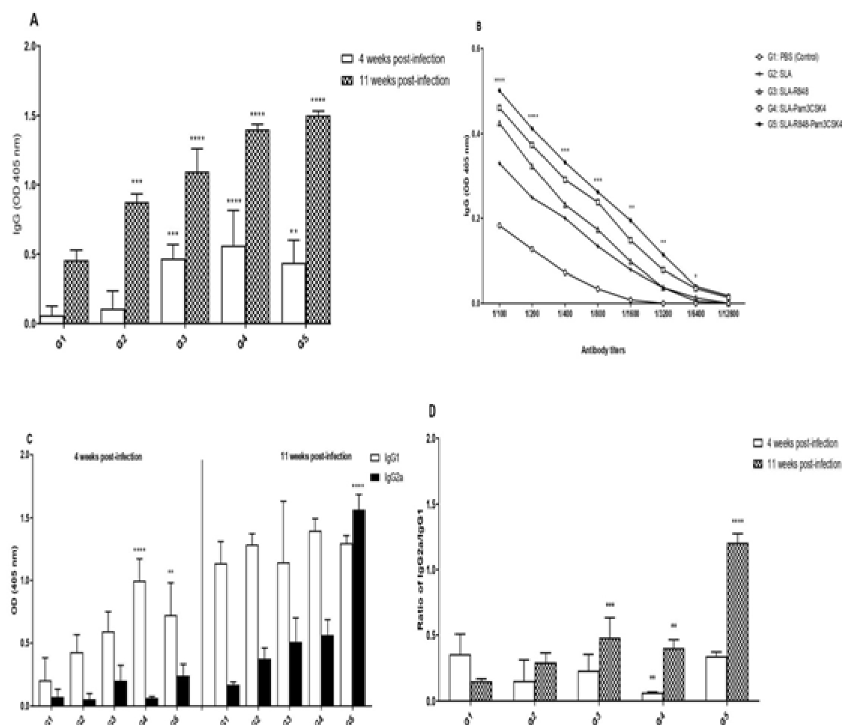

Figure 3: Analysis of humoral response in treated and control groups at different time points. Serum samples were collected at different times (4 and 11 weeks post-infection) and assayed for F/T antigen specific IgG (A), IgG titer in the pooled serum (11 weeks post-infection) (B), IgG1, and IgG2a (C), and the ratio of IgG2a/IgG1 (D) responses by Elisa. Results are mean+SD, n=4. **p<0.01; ***p<0.001; ****p<0.0001 compared with control PBS group.

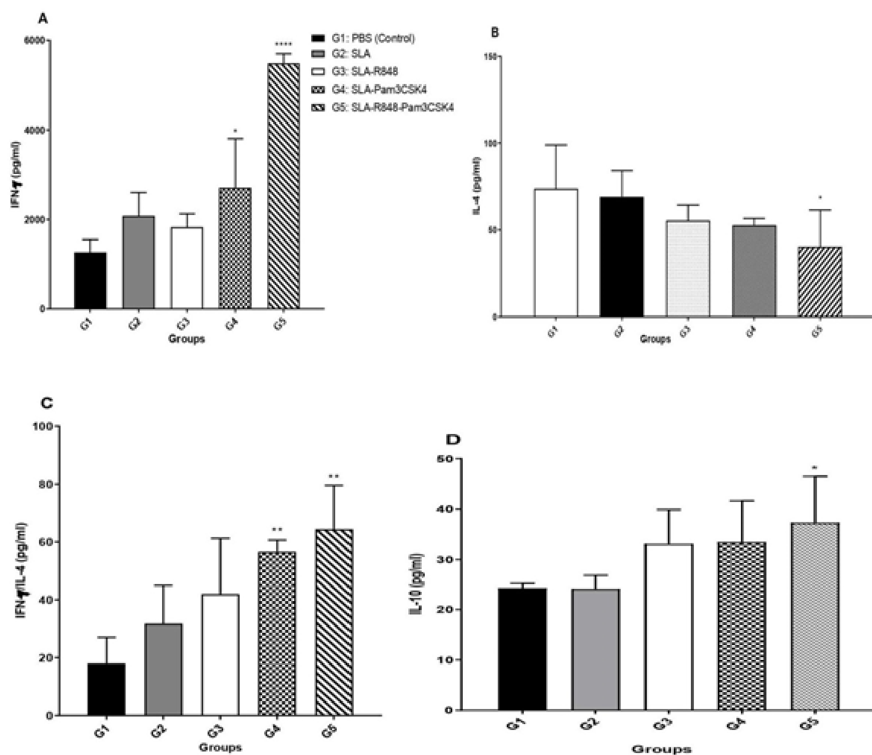

Figure 4: Cellular immune responses in vaccinated and control BALB/c mice in different time points. (A) IFN-γ, (B) IL-4, (C) IFN-γ/IL-4 ratio, and (D) IL-10 production from the splenocytes in different groups (four mice per each group) 11 weeks post-infection. Each sample was examined in triplicate. Data are shown as the mean+SD, which is compared with the PBS control group. Data are indicated by *p<0.05; **p<0.01; ****p<0.0001.

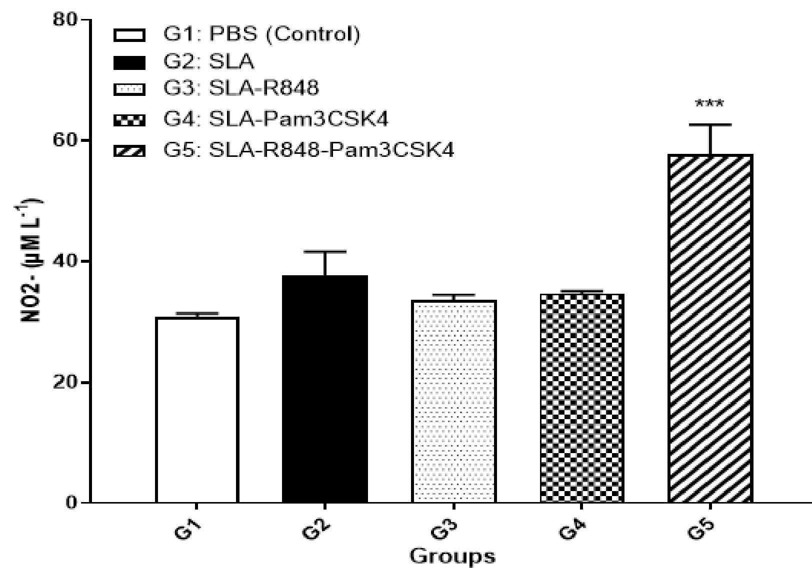

Figure 5: Nitric oxide production in stimulated splenocyte. Nitric oxide production was detected by the Griess reagent at 11 weeks post-infection. Data represented as mean+SD, n=4. ***$p<0.001$ denotes significant changes in NO production compared with the PBS control group.

Immunotherapy with SLA-R848-Pam3CSK4 induces cellular responses

The level of IFN-γ against soluble *Leishmania* antigen (SLA) significantly increased after 11 weeks post-infection compared to before vaccination (Figure 4A). IFN-γ level was significantly higher in the SLA-R848-Pam3CSK4 treated group compared to other treated and control groups 11 weeks post-infection ($p<0.0001$). All treated groups (G2-G5) showed high amounts of IFN-γ in comparison with a control group (G1) after 11 weeks, although there was a significant difference between only G4 and G5 compared to the PBS group.

To investigate the Th2 response, we evaluated the levels of IL-4 in splenocytes from treated and non-treated groups (Figure 4B). At 11 weeks, the highest level of IL-4 production belonged to G1, although there was a significant difference was showed only between G1 and G4 ($p<0.05$). Furthermore, there were no significant differences in IL-4 production between G2–G5 ($p>0.05$).

As shown in Figure 4C, G4 and G5 showed a significant difference in IFN-γ/IL-4 ratio in response to stimulation with SLA compared to the control group (G1) at 11 weeks ($p<0.01$). Meanwhile, G5 treated with SLA-R848-Pam3CSK4 induced significantly higher levels of IFN-γ/IL-4 compared to other treated (G2, G3) and control groups (G1) 11 weeks post-infection ($p<0.05$). Also, there was no significant difference between other treated groups.

IL-10 production was significantly higher in G5 treated with SLA-R848-Pam3CSK4 compared to treated G1 and PBS control group after 11 weeks ($p<0.05$) (Figure 4D).

Induction of NO production in SLA-R848-Pam3CSK4

Since the NO production is crucial for the resolution of the infection [22], we evaluated the NO levels after 11 weeks in all groups. Figure 5 showed the significant difference in NO production between G5 and the control group (G1) ($p<0.001$). Furthermore, the triple combinations of antigen and agonists (G5) had significantly highest level of NO production when compared with the single-(G2) and dual (G3 and G4) combinations ($p<0.01$).

DISCUSSION

Despite the fact that many efforts were made to develop an effective vaccine for the eradication of *Leishmania* infection, there is no effective therapeutic vaccine against leishmaniasis, and studies are limited in the field of prophylactic vaccine [23]. Vaccination with killed parasites, such as soluble *Leishmania* antigens (SLA), which included a mixture of the *Leishmania* antigens, is more potent in stimulating T cells and producing IFN-γ than each of its fractions alone [24]. Administration of *L. major* SLA with suitable adjuvants improves protective immunity against leishmaniasis [25]. The use of TLR agonists as immune adjuvants showed promising results in this disease [26–28]. It was showed that immunotherapy was improved using TLR synergy to expand the immune response against parasites in CL [5]. A protective role of a TLR2 agonist (Pam3CSK4) during infection was approved, and several studies showed the protective and therapeutic effects of this adjuvant against *Leishmania* infection [14,29,30]. In addition, R848 (TLR7/8 agonist) has been assessed in numerous clinical trials to treat various cancers and infectious diseases [31], and the protective role of R848 was approved against *L. major* in mice models too [11].

Effective and sufficient activation of an innate immune response against infection like leishmaniasis is essential for the successful induction of protective T cell responses to better control disease [32]. Our previous study showed the activation of *L. major*-infected macrophages following treatment by SLA, in combination with R848 and Pam3CSK4 [18]. So, in this study, we evaluated the protection and produced immune responses in *L. major*-infected BALB/c mice after immunization with this combination as a therapeutic vaccine.

We showed that SLA-R848-Pam3CSK4 enhanced Th1-associated IgG2a titers more than Th2-associated IgG1 titers (IgG2a/IgG1 ratio), and SLA antigen and both adjuvants were required to stimulate increased humoral immune responses 11 weeks post-infection. The protective immunity against *L. major* depends on the production of IFN-γ derived from CD4+ Th1 cells by IL-12 and

IgG1 antibodies [33].

Based on footpad swelling and parasite burden, vaccination with SLA without adjuvants induced no protection. Scott et al. showed that simultaneous use of antigen and adjuvants effectively stimulates the immune responses [25]. Indeed, the SLA-R848-Pam3CSK4 group showed the lowest tissue damage after 9 weeks, which confirmed the lowest parasite load in this group 11 weeks post-infection. This can result from using R848 and Pam3CSK4 adjuvants in addition to SLA antigen, which protective role was reported in *in vivo* studies [11,34]. Activation of more than one TLR has synergistic effects on innate immune responses and following adaptive immune responses. Studies have shown that the simultaneous use of several TLRs can improve the symptoms of *L. major* infection [34].

Furthermore, Sassi et al. showed that SLA is able to stimulate CD4+ T cells [35]. The therapeutic effects of TLR activation in immunotherapy are associated with the high expression of IL-12 and IFN-γ. In particular, the use of TLR agonists as adjuvants in leishmaniasis has shown promising results. For example, the TLR7 agonist Aldara™ showed anti-Leishmanial activity in mice models and clinical studies [26,36]. The TLR1/2 agonist Pam3CSK4 has also been shown to stimulate a strong Th1 response that protects against leishmaniasis [30]. The activation of the Th1 immune responses was attributed to the simultaneous use of R848 and Pam3CSK4 adjuvants and SLA antigen in G5, contributing to the activation of antigen-presenting cells leading to IL-12 production and IFN-γ secretion that induce Th1-type immune response [37].

The ratio of IFN-γ/IL4 as a marker of Th1 response was lower in the SLA vaccinated group, which is orchestrating with footpad swelling and parasite burden results, although the presence of R848 and Pam3CSK4, in addition to SLA antigen, effectively stimulate the Th1 responses. The Th1 immune response was confirmed by the higher levels of IFN-γ/IL-4 as well as IgG2a/IgG1 titer.

Th1 cells eliminate pathogens but often cause immunopathology responses in hosts. The anti-inflammatory cytokines, such as IL-10, can control these side effects, which have an important role in the immune response against *L. major* [38,39]. Studies showed that SLA and Pam3CSK4 induce mixed production of pro- and anti-inflammatory cytokine responses (IFN-γ, IL-12, and IL-10) [40,41]. It seems that Group 5 immunized with SLA-R848-Pam3CSK4 stimulated the production of IFN-γ, in addition to IL-10, to better control the disease in infected BALB/c mice.

IFN-γ and other inflammatory cytokines are also involved in the upregulation of iNOS expression leading to NO production [42]. Nitric oxide is an important tool for killing intracellular parasites [43]. Effective treatment of leishmaniasis is characterized by increased nitric oxide production [44]. The Pam3CSK4 improved activation of macrophages, which led to increase NO production and parasite killing [14]. A combination of SLA, R848, and Pam3CSK4 can induce NO production, which elicited the induction of Th1 response against *L. major* infection.

CONCLUSION

In the current study, immunization with SLA alone induced no protection in BALB/c mice based on footpad swelling and lymph node parasite burden. In addition, SLA without any adjuvant could not induce Th1 immune response and NO production effectively. The results revealed that activating more than one TLR signaling pathway using TLR agonists synergistically stimulates anti-*Leishmania* responses. It is concluded that when SLA antigen was used in combination with both adjuvants simultaneously, it triggers Th1 cytokine response via TLR1/2 and TLR7/8 signaling to improve immune therapy and enhance protection against leishmaniasis in infected BALB/c mice.

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REFERENCES

1. Torres-Guerrero E, Quintanilla-Cedillo MR, Ruiz-Esmenjaud J, Arenas R. Leishmaniasis: A review. F1000Research. 2017;6:25-36.

2. Reithinger R, Dujardin JC, Louzir H, Pirmez C, Alexander B, Brooker S, et al. Cutaneous leishmaniasis. Lancet Infec Dis. 2007;7(9):581-596.

3. Okwor I, Uzonna JE. Immunotherapy as a strategy for treatment of leishmaniasis: A review of the literature. Immunotherapy. 2000;1:765-776.

4. Osman M, Mistry A, Keding A, Gabe R, Cook E, Forrester S, et al. A third generation vaccine for human visceral leishmaniasis and post kala azar dermal leishmaniasis: First-in-human trial of ChAd63-KH. PLoS neglected tropical diseases. 2017;11(5):e0005527.

5. Raman VS, Bhatia A, Picone A, Whittle J, Bailor HR, O'Donnell J, et al. Applying TLR synergy in immunotherapy: Implications in cutaneous leishmaniasis. J Immunol. 2010;185(3):1701-1710.

6. Coler RN, Goto Y, Bogatzki L, Raman V, Reed SG. Leish-111f, a recombinant polyprotein vaccine that protects against visceral Leishmaniasis by elicitation of CD4+ T cells. Infect Immunity. 2007;75(9):4648-4654.

7. Musa AM, Khalil EA, Mahgoub FA, Elgawi SH, Modabber F, Elkadaru AE, et al. Immunochemotherapy of persistent post-kala-azar dermal leishmaniasis: A novel approach to treatment. Trans R Soc Trop Med Hyg. 2008;102(1):58-63.

8. Gradoni L, Manzillo VF, Pagano A, Piantedosi D, de Luna R, Gramiccia M, et al. Failure of a multi-subunit recombinant leishmanial vaccine (MML) to protect dogs from *Leishmania* infantum infection and to prevent disease progression in infected animals. Vaccine. 2005;23(45):5245-5251.

9. Chakravarty J, Kumar S, Trivedi S, Rai VK, Singh A, Ashman JA, et al. A clinical trial to evaluate the safety and immunogenicity of the LEISH-F1+ MPL-SE vaccine for use in the prevention of visceral leishmaniasis. Vaccine. 2011;29(19):3531-3537.

10. Kawasaki T, Kawai T. Toll-Like Receptor Signaling Pathways. Front Immunol. 2014; 5.

11. Zhang WW, 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 Immunity. 2008;76:3777–3783.

12. Katebi A, Gholami E, Taheri T. *Leishmania* tarentolae secreting the sand fly salivary antigen PpSP15 confers protection against *Leishmania major* infection in a susceptible BALB/c mice model. Molecular Immunol. 2015;67:501–511.

13. Emami T, Rezayat SM, Khamesipour A. 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. Art Cell Nanomed Biotechnol. 2018;46:324–333.

14. Huang L, Hinchman M, Mendez S. Coinjection with TLR2 agonist Pam3CSK4 reduces the pathology of leishmanization in mice. PLoS Negl Trop Dis. 2015;9:e0003546.

15. Sanchez MV, Eliçabe RJ, Genaro MSD. Total *Leishmania* antigens with Poly (I:C) induce Th1 protective response. Parasite Immunol. 2017;39:e12491.

16. Palatnik-de-Sousa CB. Vaccines for leishmaniasis in the fore coming 25 years. Vaccine. 2008;26:1709–1724.

17. Badiee A, Shargh VH, Khamesipour A. Micro/nanoparticle adjuvants for antileishmanial vaccines: Present and future trends. Vaccine. 2013;31:735–749.

18. Katebi A, Varshochian R, Riazi-rad F, Ganjalikhani-Hakemi M, Ajdary S. Combinatorial delivery of antigen and TLR agonists via PLGA nanoparticles modulates *Leishmania major*-infected-macrophages activation. Biomed Pharm. 2021;137:111276.

19. Asadpour A, Riazi-Rad F, Khaze V. Distinct strains of *Leishmania major* induce different cytokine m RNA expression in draining lymph node of BALB/c mice. Parasite Immunol. 2013;35:42–50.

20. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976;72:248–254.

21. Ebrahimpoor S, Pakzad SR, Ajdary S. IgG1 and IgG2a Profile of serum antibodies to *Leishmania major* amastigote in BALB/c and C57BL/6Mice. Ir J Allergy Asthma Immunol. 2013;46:361–367.

22. Blos M, Schleicher U, Rocha FJS. Organ-specific and stage-dependent control of *Leishmania major* infection by inducible nitric oxide synthase and phagocyte NADPH oxidase. Eur J Immunol. 2003;33:1224–1234.

23. Okwor I, Uzonna J. Vaccines and vaccination strategies against human cutaneous leishmaniasis. Human Vac. 2009;5:291–301.

24. Rafati Seyedi Yazdi S, Couty-Jouve S, Alimohamadian MH. Evaluation of cellular immune responses to amastigote soluble *Leishmania major* antigens in recov ered cases of cutaneous Leishmaniasis. Med J Islamic Repub Iran. 1997;11:33–38.

25. Scott P, Pearce E, Natovitz P. 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.

26. Arevalo I, Ward B, Miller R. Successful treatment of drug-resistant cutaneous leishmaniasis in humans by use of imiquimod, an immunomodulator. Clinical Infect Dis. 2001;33:1847–1851.

27. Flynn B, Wang V. Prevention and treatment of cutaneous leishmaniasis in primates by using synthetic type D/A oligodeoxynucleotides expressing CpG motifs. Infect Immunity. 2005;73:4948–4954.

28. Kanzler H, Barrat FJ, Hessel E. Therapeutic targeting of innate immunity with Toll-like receptor agonists and antagonists. Nature Med. 2007;13:552–559.

29. Jayakumar A, Castilho TM, Park E. TLR1/2 activation during heterologous prime-boost vaccination (DNA-MVA) enhances CD8+ T Cell responses providing protection against *Leishmania* (Viannia). PLoS neglected tropical diseases. 2000.

30. Martínez-Orellana P, Quirola-Amores P, Montserrat-Sangrà S. The inflammatory cytokine effect of Pam3CSK4 TLR2 agonist alone or in combination with *Leishmania* infantum antigen on ex-vivo whole blood from sick and resistant dogs. Parasites Vectors. 2017;10:123.

31. Vasilakos JP, Tomai MA. The use of Toll-like receptor 7/8 agonists as vaccine adjuvants. Expert Review Vaccines. 2013;12:809–819.

32. O'neill LA, Golenbock D, Bowie AG. The history of Toll-like receptors—redefining innate immunity. Nature Rev Immunol. 2013;13:453–460.

33. Hölscher C, Arendse B, Schwegmann A. Impairment of alternative macrophage activation delays cutaneous leishmaniasis in nonhealing BALB/c mice. J Immunol. 2006;176:1115–1121.

34. Halliday A, Bates PA, Chance ML. Toll-like receptor 2 (TLR2) plays a role in controlling cutaneous leishmaniasis *in vivo*, but does not require activation by parasite lipophosphoglycan. Parasites Vectors. 2006;9:532.

35. Sassi A, Louzir H, Ben Salah A. Leishmanin skin test lymphoproliferative responses and cytokine production after symptomatic or asymptomatic *Leishmania major* infection in Tunisia. Clin Exp Immunol. 1999;116:127–132.

36. 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.

37. Macatonia SE, Hosken NA, Litton M. Dendritic cells produce IL-12 and direct the development of Th1 cells from naive CD4+ T cells. J Immunol. 1995;154:5071–5079.

38. O'Garra A, Vieira P. TH 1 cells control themselves by producing interleukin-10. Nature Rev Immunol. 2007;7:425–428.

39. Scott P, Novais FO. Cutaneous leishmaniasis: Immune responses in protection and pathogenesis. Nature Rev Immunol. 2016;16:581.

40. Margaroni M, Agallou M, Kontonikola K. PLGA nanoparticles modified with a TNF mimicking peptide, soluble *Leishmania* antigens and MPLA induce T cell priming in vitro via dendritic cell functional differentiation. European J Pharm Biopharm. 2016;105:18–31.

41. Hirata N, Yanagawa Y, Ebihara T. Selective synergy in anti-inflammatory cytokine production upon cooperated signaling via TLR4 and TLR2 in murine conventional dendritic cells. Mol Immunol. 2008;45:2734–2742.

42. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nature Rev Immunol. 2005;5:641–654.

43. Wanasen N, Soong L. L-arginine metabolism and its impact on host immunity against Leishmania infection. Immunol Res. 2001;41:15–25.

44. Kumar R, Pai K, Sundar S. Reactive oxygen intermediates, nitrite and IFN- in Indian visceral leishmaniasis. Clin Exp Immunol. 2001;124:262–265.