Vaccination with Live Attenuated *L. Major* and TLR4 Agonist Promotes aTh1 Immune Response and Induces Protection against *L. Major* Infection in BALB/c Mice

Shamsi Noorpisheh Ghadimi[1], Shirin Farjadian[2], Gholam Reza Hatam[1], Mehdi Kalani[3], Bahador Sarkari[1,4*]

[1]Department of Parasitology and Mycology, [2]Department of Immunology, School of Medicine, [3]Prof. Alborzi Clinical Microbiology Research Center, [4]Basic Sciences in Infectious Diseases Research Center, Shiraz University of Medical Sciences, Shiraz, Iran

ABSTRACT

Background: Toll like receptors play a major role in immune responses against *Leishmania* parasites. **Objective:** To evaluate the efficacy of vaccination with live attenuated *L. major* and TLR4 agonist in protection against *L. major* infection. **Methods:** Attenuated *L. major* was prepared by continuous sub-culturing of the parasite. A total of 90 mice were assigned to 9 groups including 6 groups of BALB/c (G1-6) and 3 groups (G7-9) of C57BL/6 mice. Group 1 was the control groups, group 2 received the wild-type *L. major* promastigotes, group 3 the attenuated line, group 4 the TLR4 agonist, group 5 the wild-type *L. major* and TLR4 agonist, and group 6 the attenuated line along with TLR4 agonist. Group 7 was control, group 8 received wild-type *L. major* and group 9 the wild-type along with TLR4 agonist. Vaccinated mice were then challenged with wild-type of *L. major*. Lesion size, parasite burden, and the expression levels of IL-4, IFN-γ, IL-2, 1L-17A, IL-10, TGF-β and TLR4 were evaluated before the challenge while parasite burden and lesion size were evaluated. **Results:** Vaccinated mice with a TLR4 agonist or attenuated *L. major* plus TLR4 agonist produced the highest levels of IFN-γ, IL-2, and IL-17A. Post-challenge analysis revealed that mice vaccinated with the attenuated line along with TLR4 agonist displayed the lowest lesion size and parasite load. These mice developed a predominant Th1 immune response. **Conclusion:** Vaccination with the attenuated *L. major* along with TLR4 agonist promotes a Th1-mediated immune response which leads to the protection of BALB/c mice against *L. major* infection.

NoorpishehGhadimiS, et al. Iran J Immunol. 2018; 15(2):74-83.

Keywords: Live Attenuated *L. Major,* TLR4 Agonist,Vaccination

---

*Corresponding author: Dr. Bahador Sarkari, Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, e-mail: sarkarib@sums.ac.ir

INTRODUCTION

Leishmaniasis is a protozoan parasitic disease which may present itself in cutaneous, mucocutaneous, or visceral forms. Cutaneous leishmaniasis (CL) is the most common form of the disease, producing lesions on the exposed parts of the body and caused by certain *Leishmania* species (1). Both CL and visceral leishmaniasis (VL) are serious health threats in a few Middle Eastern countries including Iran (2-5). Up to the present, several killed and attenuated vaccines have been designed for CL, yet none of them is capable of effectively precluding the development of leishmaniasis(6,7). Vaccination with live attenuated *Leishmania*, called leishmanization, has been tested on humans with satisfactory results. Leishmanization had been practiced successfully in Middle Eastern countries including Iran up until it was stopped due to safety concerns, as certain individuals developed non-healing lesions (8,9). A key element in the success of vaccine development is selecting the proper adjuvant. Studies over the recent years have demonstrated that co-injection of *Leishmania* parasite with Toll-like receptor (TLR) agonists can reduce pathological lesions when administered to mice (10,11). TLRs are a family of membrane proteins, mainly expressed on macrophage and dendritic cells, regulating the production of inflammatory cytokines (12). TLRs recognize specific pathogen-associated molecular patterns (PAMPs) in different pathogens and signaling through these molecules is one of the primary responses of innate immune systems against *Leishmania* (12,13). Engagement of some TLRs, particularly TLRs 2 and 4, induces T helper (Th)1 differentiation which plays a major part in controlling leishmaniasis (13-15). Srivastava *et al*. showed that the activation of TLR9 in BALB/c mice could reduce both the lesion size and pathogenicity of *Leishmania* in infected mice (16). The use of TLR agonists as adjuvant in immunization against *Leishmania* has proved to entail promising results (10).

TLR4 gets involved in the development of an effective immune response against *Leishmania* by the initial induction of nitric oxide synthesis (17). TLR2 and TLR4 are involved in the formation of effective immune responses in human leishmaniasis (14,15,18,19). Tolouei *et al*. showed that the levels of TLR2 and TLR4 expression in peripheral blood-derived macrophages of patients with healing forms of CL were significantly higher than those with non-healing lesions (19). TLR4 has an essential role as regards controlling *Leishmania* in mice since TLR4-knockout mice cannot effectively prevent wound development caused by *L. major* (15). Recently, it has been determined that leishmanization along with the administration of TLR2 agonist could reduce the parasitic load in the mouse lesions (10). On the other hand, TLR4 agonist injection has been shown to induce dendritic cells to produce high levels of such pro-inflammatory cytokines as IL-12 (20). In view of the protective immune responses induced through inoculating live attenuated *L. major* in humans, it is of utmost importance to study the different ways of optimization along with a particular emphasis on reducing the pathological lesions resulting from the parasites, as well as shortening the duration of the presence of ulcers at the injection site.

Similar to TLR2, the injection of TLR4 agonist with live *L. major* is expected to control parasite growth, reduce the pathological lesions, and create a specific Th1 immune response. Accordingly, the current study was designed to evaluate the efficacy of vaccination with live attenuated *L. major* and TLR4 agonist concerning protection against *L. major* infection in BALB/c mice.

MATERIALS AND METHODS

Preparation of Attenuated *L. major*. Standard *L. major* (MRHO/IR/75/ER strain) was cultivated in RPMI medium supplemented with 10% fetal calf serum, 100 µg/mL streptomycin and 100 U/mL of penicillin. Attenuated *L. major* was prepared by continuous sub-culturing of the parasite (21 times). Wild-type or attenuated promastigotes were adjusted to 10^4/mL in saline for animal inoculation with or without TLR4 agonist synthetic MPLA (Invitrogen, Carlsbad, CA, USA), (20 µg/mL, in saline). Mice were intradermally inoculated with 0.1 mL of promastigotes with/without TLR4 agonist (0.1 mL) at the base of their tails.

Preparation of Soluble *Leishmania* Promastigote Antigens. Soluble *Leishmania* antigen (SLA) was prepared from the late (4-6 days) stationary phase of promastigotes (21). In this regard, 30 mL of parasites was transferred in a 50 mL tube and centrifuged at 2800×g for 15 min at 4°C. The pellets were washed three times in PBS and subjected to three rapid freeze-thaw cycles. Samples were centrifuged at 5000×g for 20 min at 4°C. Supernatants were collected, and stored at -80°C until use.

Mice Immunization and Challenge. In this study, 4 to 6-week-old female BALB/c (n=60) and female C57BL/6 mice (n=30) were kept under controlled conditions at a temperature of 23±1°C, humidity of 50±5%, and 12:12 h light-dark cycles, with regular access to food and water. The study protocol was approved by the Institutional Animal Care and Use Committee of Shiraz University of Medical Sciences.

BALB/c mice were divided into 6 groups (n=10 in each group); G1 mice were the control group with no injection, G2 mice were inoculated with wild-type *L. major* promastigotes, G3 mice were inoculated with the attenuated *L. major*, G4 mice were inoculated with TLR4 agonist, G5 mice were inoculated with wild-type *L. major* promastigotes along with TLR4 agonist, and G6 mice were inoculated with the attenuated *L. major* promastigotes and TLR4 agonist. C57BL/6 mice were further categorized into 3 groups (G7 to G9; 10 mice in each group): G7 was considered as the control group with no injection while G8 mice were inoculated with wild-type *L. major* promastigotes and G9 mice with *L. major* wild-type promastigotes and 20 µg of TLR4 agonist. Within 21 days following the injection of wild-type *L. major*, small and thick nodules appeared at the site of the injection. Direct Giemsa stained smears were taken from the lesions and evaluated microscopically to corroborate the presence of *L. major.* Upon the appearance of the lesions, their sizes were weekly measured using a vernier caliper during the course of infection. Forty-five days post injection, 5 mice were euthanized in each group and the parasite burden was determined in their spleen. Seventy-two hours after stimulation with SLA, the levels of IL-2, IL-4, IL-10, and 1L-17A, IFN-γ, and TGF-β were also evaluated in the supernatant of the mouse splenic mononuclear cells. The production of these cytokines was also examined at mRNA levels of the stimulated cells.

The remaining five mice in each group were intradermally challenged with wild-type *L. major* promastigotes (10^4 cells in 0.1 mL) at the base of their tails. The lesion sizes in the remaining mice were further measured every week. Following 45 days of challenge, the mice were euthanized and their parasite burden was specified.

Evaluation of Cytokine Levels. Mononuclear cells were isolated from mice spleens using Lymphodex (Inno-train, Kronberg, Germany) 45 days post-immunization.

Cells were then stimulated with 3 µg/mL of *L. major* SLA in a 96-well flat bottom cell culture plate (10^6 cells/well) in duplicate. Cells cultured in RPMI 1640 were used as

negative control and those stimulated with 10 μg/mL of concanavalin A (Sigma-Aldrich, Louis, Missouri, USA) were considered as positive control. After 72h, cell culture supernatants were removed and kept at -20°C and cell pellets were stored at -70°C until use. The levels of Th1 (IFN-γ, TNF-α, IL-2), Th2 (IL-4, IL-5, IL-6, IL-10 and IL-13), Th17 (IL-17A, IL-17F, IL-21), Th9 (IL-9) and Th22 (IL-22) cytokines, in the supernatants of spleen cell cultures, were quantified by a multiplex bead-based assay using a commercial kit (BioLegend, San Diego, CA, USA), based on the manufacturer's instructions. Briefly, for each cytokine to be measured, a mixture of antibody-coated beads was incubated with the sample. After covering the cytokine by FITC-labeled beads, which could be differentiated by their sizes and fluorescent intensities, a biotin-conjugated secondary antibody mixture was added followed by incubating with PE-conjugated streptavidin as the detector. The results were visualized with a FACSCalibur flow cytometer (eBioscience, Santa Cruz, CA, USA) and the obtained data were analyzed through the use of FlowCytomix Pro-3.0 software (BioLegend).

Evaluation of TLR4 and Cytokine Gene Expressions. Total RNA was extracted from spleen-derived mononuclear cells using RNeasy Mini Kit (YektaTajhizAzma, Tehran, Iran), according to the manufacturer's instructions. After that, cDNA synthesis was performed using RevertAid Reverse Transcriptase kit (Thermo Scientific, Norcross, GA, USA) and IL-2, IL-4, IL-10, IL-17A, IFN-γ, TGF-β and TLR4 transcript levels were evaluated by real-time PCR using the primers listed in Table 1 (22,23) with β-actin gene employed as calibrator. Real-time PCR was done in a total volume of 20 μL containing 10 μL of Maxima SYBR Green/ROX qPCR Master Mix (2x), 0.5 μL of forward and 0.5 μL of reverse primers, 1.5 μL of cDNA, and 7.5 μL of RNase free water. Real-time PCR conditions for IL-2, IL-17A, IFNγ, TLR4, and β-actin were 10 min at 95°C, 40 cycles of 20 s at 95°C, and 50 s at 60°C. Conditions for the gene amplifications of TGF-β and IL-10 were 10 min at 95□, 40 cycles of 20 s at 95°C, 30 s at 58°C, and 20 s at 60°C. TGF-β gene was amplified under similar conditions except for the annealing temperature which was 59°C. Relative expression of target genes was calculated using $2^{-\Delta\Delta CT}$ method.

Table 1. Primers used for real-time PCR.

Target gene	Accession number	Sequence of primers (5′→3′)	Amplicon size (bp)	Annealing Temp. (°C)	Reference
IFN-γ	NM_008337	**F:** GAGGAACTGGCAAAAGGATGGT **R:** CGCTTATGTTGTTGCTGATGGC	116	60	22
IL-10	NM_010548	**F:** GCAGGACTTTAAGGGTTACTTGGG **R:**GATTTCTGGGCCATGCTTCTC	103	59	22
IL-17A	NM_010552	**F:** AACACTGAGGCCAAGGACTTC **R:** GTCTTCATTGCGGTGGAGAGT	144	60	22
TGF-β	NM_011577	**F:** GCAACAACGCCATCTAT **R:**AAGGTAACGCCAGGAAT	200	59	22
IL-2	NM_008366	**F:** TTCATCAGCAATATCAGAGTAAC **R:** CTCAGAAAGTCCACCACAGT	104	60	Current study
TLR4	NM021297	**F:** GCTTTCACCTCTGCCTTCAC **R:** AGGCGATACAATTCCACCTG	259	58	23
β-actin	NM007393	**F:** GCTACAGCTTCACCACCACAG **R:** GGTCTTTACGGATGTCAACGTC	288	60	23

Evaluation of Parasite Burden. Parasite burden was quantified via amastigote count in Giemsa-stained smears prepared from the lesion (24). Parasite burden was further evaluated in impression smears from the mice spleens and expressed as Leishman–Donovan units (LDU). The LDU index is defined through LDU = amastigote number/ 1000 host cell nuclei × organ weight (g). Also, the ratio of the infected cells (those containing amastigote) to the non-infected cells (those without parasite) and the intensity of infection (mean of parasite number in the infected cells) were determined (25).

Statistical Analysis. Nonparametric statistical tests and one-way analyses of variance (ANOVA) were employed for the statistical analysis of the data. Means of the gene expression, LDU and lesion sizeswere compared among different mouse groups by the Kruskal–Wallis test. Data were analyzed using SPSS 16 and $p<0.05$ was considered as statistically significant. GraphPad Prism version 5 was used for graphical presentations.

RESULTS

Cytokine Production at the mRNA and Protein Level.

As far as the level of IFN-γ and IL-2 is concerned, there existed significant differences between group 1(control group with no injection) and group 2 which received wild-type parasite. The highest IFN-γ level was found in G6 mice which received TLR4 along with attenuated *L. major,* whereas G2 mice receiving wild-type *L. major* had the minimum levels of IFN-γ. Figure.1 shows the RNA transcript level of IFN-γ and IL-2 in different mice groups. G1 and G2 were significantly different in terms of the level of IL-17A. A high level of IL-17A was observed in G6 which received TLR4 and attenuated parasite and the lowest level belonged to G2. Figure.1 shows the level of IL-17A in different mice groups. G1 and G6 had recognizable differences. The IL-10 and TGF-β mRNA expressions were the highest in mice group 2 (BALB/c mice which received wild-type *L. major*), (Figure.1).

It is worth mentioning that concerning the expression level of TGF-β and IL-10, a significant difference was found between groups that received the wild-type of the parasite and those receiving TLR4 or attenuated *L. major*. Th1/Th2 ratio was no different among the mice groups. Nonetheless, mice immunized with attenuated parasite plus TLR4 had the highest Th1 immune response. The cytokine level of IL-4 was specified via Flow cytometry while other cytokines were not detectable. A significant difference was observed between positive controls (treated with Con. A) and sample test (treated with *L. major* antigen). The BALB/c mice group 2 which received the wild type of parasite had the highest level of IL4 production. In G3 and G4, vaccination induced a Th1 response and the level of IL-4 cytokine was very low. Figure.1 shows the differences between different mice groups as regards IL-4 levels.

Expression of TLR4 in mice groups.

Certain changes were observed in TLR4 expression in *L. major* infected splenocytes of susceptible (BALB/c) mice. The highest level of TLR4 expression belonged to G4 of BALB/c mice, followed by G6 which received attenuated line plus TLR4 agonist. Figure 1 shows the TLR4 transcription levels in different mice groups.

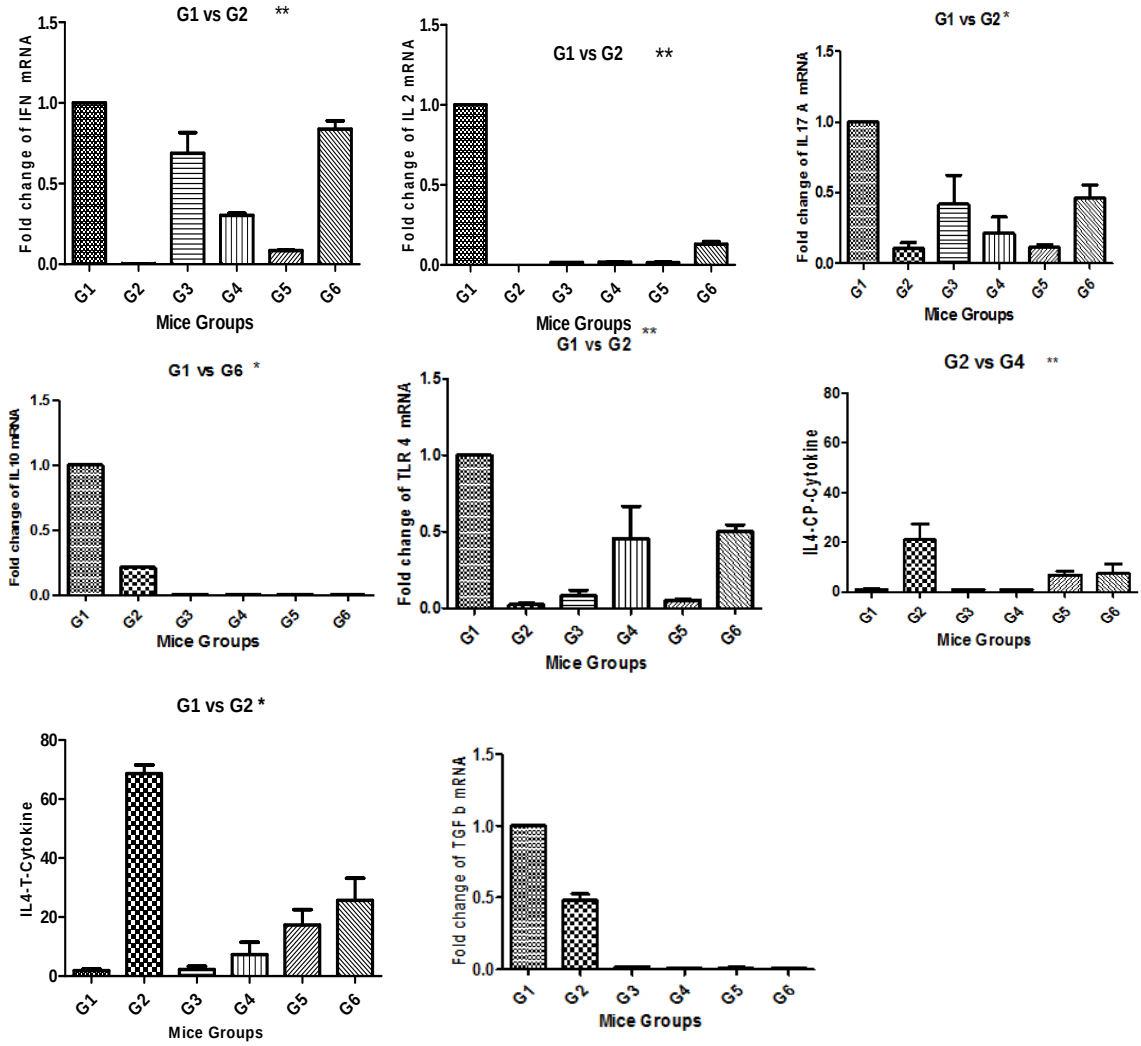

Figure 1. Transcript levels of different cytokines and TLR4 in different BALB/c mice groups. G1: untreated mice; G2: mice inoculated with wild-type *L. major;* G3: mice inoculated with attenuated *L. major*; G4: mice inoculated with TLR4 agonist; G5: mice inoculated with wild-type *L. major* and TLR4 agonist; G6: mice inoculated with attenuated *L. major* and TLR4 agonist (* shows P<0.05, ** shows P<0.01).

In mice injected with different regimens and sacrificed 45 days after, the decrease in parasite burden, in the spleen, was associated with the increase in the levels of IFN- γ, and IL17A and the expression levels of TLR4. In BALB/c mice, the lowest LDUs were respectively observed in G4 and G6, while the maximum LDU was found in G2 which was also host to the highest percentage of infection, with G4 and G6 having the lowest percentage. In C57BL/6 mice, the lowest LDU was seen in group 9 (where no parasites were observed either) which received the wild-type parasite plus TLR4 agonist. Figure 2 illustrates the mean of LDU and the percentage of infection in the spleen of different mice groups.

Lesion Size in different mice groups.

Further evaluated was the protective effect of vaccination with TLR4 adjuvant and

leishmanization against *L. major* infection in BALB/c mice through measuring the lesion size in the basal tail. The lesions were from healing to non-healing and ulcerative forms. Significant differences were observed in the lesion sizes of different mice groups (P<0.005). The highest lesion size belonged to G2 of BALB/c mice which received wild-type parasite, whereas the lowest sizes were observed in both G3 and G4 (Figure. 2).

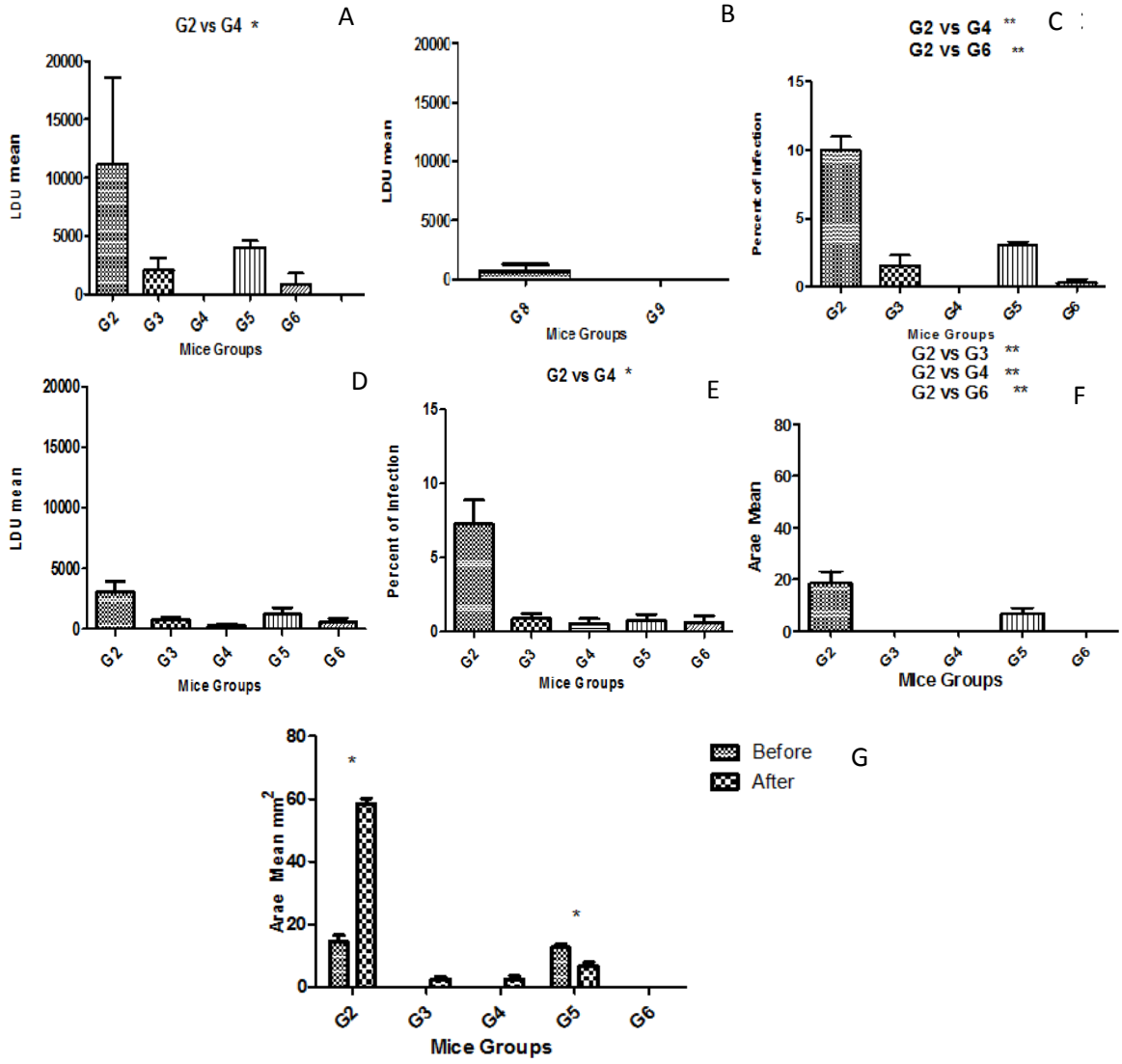

Figure 2. Mean of LDU and percent of infection in the spleen in different group of mice.A and **B**: mean of LDU at 45 days post infection; **C**: percent of infection in the spleen 45 days post infection; **D**: mean of LDU at 90 days post infection; **E**: percent of infection in the spleen at 90 days post infection; **F**: lesion size at 45 days post infection; **G**: lesion size before (at 45 days) and after (at 90 days) challenge. G1: untreated mice; G2: mice inoculated with wild-type *L. major;* G3: mice inoculated with attenuated *L. major*; G4: mice inoculated with TLR4 agonist; G5: mice inoculated with wild-type *L. major* and TLR4 agonist; G6: mice inoculated with attenuated *L. major* and TLR4 agonist.(* shows P<0.05, ** shows P<0.01).

DISCUSSION

To date, no effective vaccine has been developed for cutaneous leishmaniasis. This study demonstrated that leishmanization using attenuated *L. major* parasites in combination with TLR4 agonists prevents the development of lesions and reduces the parasite load in susceptible mice. It is assumed that stimulation with TLR4 agonist increases the proinflammatory cytokines and Th1–mediated immune response which controlled the proliferation of parasite, resulting in smaller lesions and less parasite burden. In this study, the Th2 immune response was observed in both BALB/c and C57BL/6 mice which only received the wild type of the parasite. Of course, in the resistant mice, the immune response was primarily directed toward Th1; however, the production of IL-10 and TGF-β cytokines eventually resulted in an imbalance between Th1 and Th2 which can happen as a result of the increase in Th2 cytokines in mice lacking TLR4 due to the injection of a wild-type strain of the parasite. It is widely accepted that the imbalance between Th1 and Th2 immune responses is a prominent phenomenon which entails CL in mice (26). Vaccination of BALB/c mice with attenuated *L. major* induces a strong Th1 immune response so as to prevent the parasite proliferation. Leishmaniasis is associated with the inactivation of macrophages. LPG is a virulence factor of *Leishmania* and a possible mechanism for the inactivation of macrophages by LPG is the production of IL-10 and TGF-β (27). Both cytokines can deactivate macrophages which results in parasite survival. The co-injection of TLR4 agonist and attenuated line of *L. major* promote a Th1 immune response by the down-regulation of Th2 cytokine expression, mainly TGF-β and IL10. It was found that the expression of TLR4 was higher in vaccinated mice groups, in accordance with previous reports demonstrating that the expression of TLR2, TLR4, and TLR5 in the spleen dendritic cells of BALB/c mice are higher compared with resistant mice (28). Moreover, the findings of our study showed that leishmanization is a factor involved in the increase in the TLR4 levels. Regarding the role of TLRs in cutaneous leishmaniasis, Halliday *et al.* reported larger lesions and higher parasite burdens in both $TLR2^{-/-}$ and $TLR4^{-/-}$ mice compared to the wild-type mice inoculated with *L. major* or *L. Mexicana* promastigotes (14), which is in line with Kropf *et al.* who made use of the same mouse model (11). Murray *et al.* showed that the absence of TLR4 leads to an increase in parasitemia in mice infected with *L. donovan*i and the deviation of the immune response toward Th2 (29). Consistent with the present research, such studies further corroborate the role of TLR4 with regards to cutaneous as well as visceral leishmaniasis. In response to *L. major,* macrophages produce more arginase in mice lacking TLR4 in comparison with TLR4-competent mice. It is assumed that TLR4 plays a role in inhibiting the alternative activation of macrophages which is independent of the acquired immune response (15).

In the study of Komai-Koma, a reduction in the level of IL-4 was observed in the lymph node of mice treated with anti-TLR4 antibodies (18). As expected, the present study also showed a significant difference in IL-4 levels among BALB/c mice inoculated with wild-type of *L. major*, a group where the level of this Th2-associated cytokine was also increased.

It has recently been shown that IL-17 in patients with CL has a pro-inflammatory role (30). On the other hand, IL-17 has been associated with protection against human visceral leishmaniasis (31). In our study, it was found that leishmanization in the presence of TLR4 agonist enhances Th17 immune responses related to the protection in

mice vaccinated with attenuated parasite lines. Earlier studies have suggested that intravenous injection of attenuated parasites (LiΔHSP70-II) to BALB/c mice induce short-term immunity against the subsequent challenge with *L. major*. The provided protection is the result of the attenuated parasites in the liver and the spleen of immunized mice (32).

In conclusion, the present findings indicate that vaccination with live attenuated *L. major* and TLR4 agonist promotes a Th1 immune response in the vaccinated mice via the increase in the levels of IFN-γ, IL-2, and 1L-17A and a decrease in the levels of IL-10 and TGF-β cytokines. We assumed that the absence of IL-10, IL-4 and TGF-β and the induction of strong IFN-γ response led to protection in mice. It can ultimately be concluded that an optimal way for protection against CL would be leishmanization with attenuated *L. major* combined with TLR4 agonists.

ACKNOWLEDGEMENTS

This study was financially supported by the office of vice-chancellor for research of Shiraz University of Medical Sciences (grant No. 94-7638). The results described in this paper were part of Ph.D. student thesis of Shamsi Noorpisheh Ghadimi.

REFERENCES

1. Desjeux P. Leishmaniasis: current situation and new perspectives. Comp Immunol Microbiol Infect Dis. 2004; 27:305-18.
2. Postigo JA. Leishmaniasis in the World Health Organization Eastern Mediterranean Region. Int J Antimicrob Agents. 2010; 36 Suppl 1:S62-5.
3. Sarkari B, Ahmadpour NB, Motazedian MH, et al. Inter- and intraspecific variations of leishmania strains isolated from patients with cutaneous and visceral leishmaniases in Fars Province, South of Iran. Iran J Med Sci. 2016; 41:209-16.
4. Sarkari B, Hatam G, Ghatee M. Epidemiological features of visceral leishmaniasis in Fars province, southern Iran. Iran J Public Health. 2012; 41:94-9.
5. Sarkari B, Naraki T, Ghatee MA, AbdolahiKhabisi S, Davami MH. Visceral leishmaniasis in Southwestern Iran: A retrospective clinico-hematological analysis of 380 consecutive hospitalized cases (1999-2014). PloS One. 2016; 11:e0150406.
6. Gillespie PM, Beaumier CM, Strych U, Hayward T, Hotez PJ, Bottazzi ME. Status of vaccine research and development of vaccines for leishmaniasis. Vaccine. 2016; 34:2992-5.
7. Srivastava S, Shankar P, Mishra J, Singh S. Possibilities and challenges for developing a successful vaccine for leishmaniasis. Parasit Vectors. 2016; 9:277.
8. Hosseini SM, Hatam GR, Ardehali S. Characterization of Leishmania isolated from unhealed lesions caused by leishmanization. East Mediterr Health J. 2005; 11:240-3.
9. Khamesipour A, Dowlati Y, Asilian A, et al. Leishmanization: use of an old method for evaluation of candidate vaccines against leishmaniasis. Vaccine. 2005; 23:3642-8.
10. 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.
11. Kropf P, Freudenberg N, Kalis C, et al. Infection of C57BL/10ScCr and C57BL/10ScNCr mice with Leishmania major reveals a role for Toll-like receptor 4 in the control of parasite replication. J Leukoc Biol. 2004; 76:48-57.
12. Dabbagh K, Lewis DB. Toll-like receptors and T-helper-1/T-helper-2 responses. Curr Opin Infect Dis. 2003; 16:199-204.
13. Dasgupta S, Aghazadeh-Dibavar S, Bandyopadyay M. The role of toll-like receptor agonists in the immunotherapy of leishmaniosis. An update and proposal for a new form of anti-leishmanial therapy. Ann Parasitol. 2014; 60:75-82.

14. Halliday A, Bates PA, Chance ML, Taylor MJ. Toll-like receptor 2 (TLR2) plays a role in controlling cutaneous leishmaniasis in vivo, but does not require activation by parasite lipophosphoglycan. Parasit Vectors. 2016; 9:532.
15. Kropf P, Freudenberg MA, Modolell M, et al. Toll-like receptor 4 contributes to efficient control of infection with the protozoan parasite Leishmania major. Infect Immun. 2004; 72:1920-8.
16. Srivastava S, Pandey SP, Jha MK, Chandel HS, Saha B. Leishmania expressed lipophosphoglycan interacts with Toll-like receptor (TLR)-2 to decrease TLR-9 expression and reduce anti-leishmanial responses. Clin Exp Immunol. 2013; 172:403-9.
17. Chauhan P, Shukla D. Redundant and regulatory roles for Toll-like receptors in Leishmania infection. Clin Exp Immunol. 2017; 190:167-86.
18. Komai-Koma M, Li D, Wang E, Vaughan D, Xu D. Anti-Toll-like receptor 2 and 4 antibodies suppress inflammatory response in mice. Immunology. 2014; 143:354-62.
19. Tolouei S, Hejazi SH, Ghaedi K, Khamesipour A, Hasheminia SJ. TLR2 and TLR4 in cutaneous leishmaniasis caused by Leishmania major. Scand J Immunol. 2013; 78:478-84.
20. Raman VS, Bhatia A, Picone A, et al. Applying TLR synergy in immunotherapy: implications in cutaneous leishmaniasis. J Immunol. 2010; 185:1701-10.
21. Mohammadi-Ghalehbin B, Hatam G, Sarkari B, Mohebali M, Zarei Z, Bohlooli S. Cytokine profile of leishmania infantum Fucose-Mannose ligand in vaccinated dogs in the Northwest of Iran. Iran J Immunol. 2017; 14:293-305.
22. Gholijani N, Amirghofran Z. Effects of thymol and carvacrol on T-helper cell subset cytokines and their main transcription factors in ovalbumin-immunized mice. J Immunotoxicol. 2016; 13:729-37.
23. Yang H, Wei J, Zhang H, et al. Upregulation of Toll-like Receptor (TLR) expression and release of cytokines from mast cells by IL-12. Cell Physiol Biochem. 2010; 26:337-46.
24. Foroughi-Parvar F, Hatam GR, Sarkari B, Kamali-Sarvestani E. Leishmania infantum FML pulsed-dendritic cells induce a protective immune response in murine visceral leishmaniasis. Immunotherapy. 2015; 7:3-12.
25. Hatam G, Rezanezhad H, Motazedian M, Sarkari B. In vitro infectivity of Leishmania major isolated from patients with different clinical forms of cutaneous leishmaniasis and its association with parasite zymodems. Iran J Parasitol. 2009; 4:52-60.
26. Belkaid Y, Hoffmann KF, Mendez S, et al. The role of interleukin (IL)-10 in the persistence of Leishmania major in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. J Exp Med. 2001; 194:1497-506.
27. Spath GF, Epstein L, Leader B, et al. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite Leishmania major. Proc Natl Acad Sci USA. 2000; 97:9258-63.
28. Liu T, Matsuguchi T, Tsuboi N, Yajima T, Yoshikai Y. Differences in expression of Toll-like receptors and their reactivities in dendritic cells in BALB/c and C57BL/6 mice. Infect Immun. 2002; 70:6638-45.
29. Murray HW, Zhang Y, Zhang Y, Raman VS, Reed SG, Ma X. Regulatory actions of Toll-like receptor 2 (TLR2) and TLR4 in Leishmania donovani infection in the liver. Infect Immun. 2013; 81:2318-26.
30. Boaventura VS, Santos CS, Cardoso CR, et al. Human mucosal leishmaniasis: neutrophils infiltrate areas of tissue damage that express high levels of Th17-related cytokines. Eur J Immunol. 2010; 40:2830-6.
31. Wu W, Huang L, Mendez S. A live Leishmania major vaccine containing CpG motifs induces the de novo generation of Th17 cells in C57BL/6 mice. Eur J Immunol. 2010; 40:2517-27.
32. Carrion J, Folgueira C, Soto M, Fresno M, Requena JM. Leishmania infantum HSP70-II null mutant as candidate vaccine against leishmaniasis: a preliminary evaluation. Parasit Vectors. 2011; 4:150.