Vaccine 37 (2019) 3221–3228

Contents lists available at ScienceDirect

Vaccine

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

Leishmania major p27 gene knockout as a novel live attenuated vaccine candidate: Protective immunity and efficacy evaluation against cutaneous and visceral leishmaniasis in BALB/c mice

Samira Elikaee [a], Mehdi Mohebali [a,b,*], Sassan Rezaei [a], Hamid Eslami [c], Ali Khamesipour [d], Hossein Keshavarz [a,b], Mohammad Reza Eshraghian [e]

[a] Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[b] Research Center for Endemic Parasites of Iran, Tehran University of Medical Sciences, Tehran, Iran
[c] Department of Medical Biotechnology, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran
[d] Center for Research and Training in Skin Diseases and Leprosy, Tehran University of Medical Sciences, Tehran, Iran
[e] Department of Epidemiology and Biostatistics, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article history:
Received 2 December 2018
Received in revised form 1 April 2019
Accepted 24 April 2019
Available online 29 April 2019

Keywords:
Leishmania
Gene knockout
Vaccine
Immunity
Cross protection
BALB/c mice

ABSTRACT

Leishmaniasis is a growing health problem in many parts of the world and efforts to find vaccine against the disease are a public health priority. Live attenuated vaccines are the gold standard for protection against intracellular pathogens such as *Leishmania* spp. Defined genetic alteration of the *Leishmania* genome can be achieved using a gene-targeted disruption strategy that allows for the selection of parasites lacking genes essential for long-term survival and virulence. Previously, we demonstrated that genetically modified live attenuated *Leishmania major*, lacking the p27gene (*Lmp27*$^{-/-}$) is safe and induces cellular immunity in BALB/c mice. p27 is a component of the COX complex that is responsible for ATP synthesis. In the current study, the *Lmp27*$^{-/-}$ strain was assessed as a live attenuated vaccine. Overall protective immunity and efficacy were evaluated at various time periods following *Leishmania major* (*L. major*) and *Leishmania infantum* (*L. infantum*) challenges separately in BALB/c mice. Cytokine and anti-*Leishmania* antibody levels, splenocyte proliferation, delayed type hypersensitivity (DTH), skin lesion development, and parasite burden in the liver and spleen were the measured variables. The results demonstrated that immunized mice had a significant T-helper type 1 (Th1) response, smaller skin lesions and lower parasite burdens in their liver and spleens following a *L. major* challenge. Furthermore, the *Lmp27*$^{-/-}$ mutant also granted cross-protection against *L. infantum* infection.

These results suggest that immunization with *Lmp27*$^{-/-}$ parasites provide significant protective immunity and efficacy against infection with homologous as well as heterologous species of *Leishmania* parasites.

1. Introduction

Protozoan parasites of the genus *Leishmania* cause a wide spectrum of clinical manifestations known as leishmaniases, which affect millions of people worldwide. Cutaneous leishmaniasis (CL) is the most common forms of this disease in the Old World and New World [1]. In Iran, two forms of CL have been reported: zoonotic (ZCL) and anthroponotic (ACL). ZCL caused by *Leishmania major* (*L. major*) is endemic in most parts of Iran [2]. Viceral leishmaniasis (VL) has been reported sporadically in Iran, but the disease is endemic in northwestern and southern areas[3].

There is no effective vaccine available for Leishmaniasis, and existing drug treatments are expensive and have toxic side effects [4–6]. Leishmaniasis is a disease that is most likely to be controlled by a successful vaccination program [5]. The fact that recovery from a primary infection renders the host resistant to subsequent infections indicates that a vaccine is feasible [7]. A common consent about *Leishmania* vaccine suggests that for effective protective response against *leishmania* infections, parasite persistence may be significant and immunization with live attenuated parasites could be effective in this issue [8,9].

Different methods have been used in the preparation of live attenuated *Leishmania* parasites as a vaccine, including chemical

* Corresponding author at: Department of Medical Parasitology and Mycology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran.
E-mail address: mehdimohebali.tums@gmail.com (M. Mohebali).

https://doi.org/10.1016/j.vaccine.2019.04.068
0264-410X/

mutagenesis, irradiation, long-term *in vitro* culture and selection for temperature sensitivity [9,10]. Although these attenuated vaccines have shown appropriate levels of protection in animal models, due to possible reversion to the virulent form and/or long term persistence, they have not been used in clinical trials yet. Because of these issues live attenuated vaccines with targeted gene deletion were considered for the development of vaccines [11]. Several genetically modified *Leishmania* parasites lacking essential genes were tested and shown to induce different protections against virulent challenge [12–16]. Since the *Leishmania donovani* p27 gene knockout (*Ldp27*$^{-/-}$) strain showed desirable protective results against *Leishmania* infections [17], we developed a *Leishmania major* p27 gene knockout (*Lmp27*$^{-/-}$) strain that was safe and immunogenic in BALB/c mice [18]. In this study, we assessed *Lmp27*$^{-/-}$ protective immunity and efficacy against homologous (*L. major*) and heterologous (*L. infantum*) *Leishmania* species.

2. Materials & methods

2.1. Ethical consideration

The experiments using animals were performed in strict accordance with the protocols reviewed and approved by the Institutional Animal Care and Research Advisory Committee of Tehran University of Medical Sciences under Grant ID 92-01-160-21598.

2.2. Mice and parasites

Female BALB/c mice were obtained from Razi Vaccine and Serum Research Institute (Alborz, Iran) and used at 6–8 weeks of age. All mice (4–5 mice/subgroup) were maintained at Tehran University of Medical Sciences. Wild type (Wt) *L. major* and Wt *L. infantum* and *Lmp27*$^{-/-}$ mutant parasites (MRHO/IR/75/ER) were grown in RPMI1640, supplemented with 100 U/ml Pen/Strep and 10% (v/v) heat-inactivated FCS (all from Gibco).

2.3. Immunization and challenge

Mice (n = 90) were divided in two groups of *Lmp27*$^{-/-}$ immunized and PBS injected. The *Lmp27*$^{-/-}$ group were immunized subcutaneously (s.c.) with 3×10^6 stationary phase *Lmp27*$^{-/-}$ mutant promastigotes [18] and 0.1 ml PBS was used in PBS group. Four weeks after immunization half of mice (n = 20) in each group were s.c. challenged with 10^5 stationary-phase Wt *L. major* parasites and the others intrapritonealy (IP) challenged with 10^5 stationary phase Wt *L. infantum* [17]. Ten mice were considered for DTH evaluation post challenge with *L. major*. All evaluations were assessed at 4 and 12 weeks post challenged and at least 4–5 mice were used for each sub group. Division of mice was shown in Fig. 1.

2.4. Protective immunity evaluation

2.4.1. Lymphocyte proliferation

At weeks four and twelve after inoculation, splenocytes were seeded in 96-well plates (NUNC) at a density of 10^5 cells/ well and stimulated with 50 μg/ml soluble *Leishmania* antigen (SLA) for 24 h at 37 °C. The positive control group was stimulated with ConA (5 μg/ml). Then, 20 μl of MTT (Sigma) solution (5 mg/ml) was added to each well at indicated times, and incubation was continued for another 4 h. After discarding the supernatant, intracellular formazan crystals were dissolved by addition of 200 μl of dimethyl sulfoxide (DMSO; Sigma) in each well. Cell proliferation was determined by measuring the absorbance at 490 nm on an ELISA reader (Thermo Labsystems, Waltham, MA, USA).

2.4.2. Cytokine production

At weeks four and twelve after Challenge, splenocytes were separated into single cell suspensions and red blood cells were lysed with 0.17 M NH4Cl pH 7.2. Splenocytes were then resuspended at 2×10^6 cells/mL in RPMI 1640 supplemented with 10% FCS with 1:100 Pen/Strep (Gibco). Cells were either stimulated at 37°C in 5% CO2 with 50 μg/ml of SLA, or they were left unstimulated (null) for 72 h. The concentrations of IFN-γ and IL-4 cytokines in the culture supernatant were determined by sandwich ELISA according to the manufacturer's instructions (eBioscience). The SLA was prepared from stationary phase *L. major* and *L. infantum* promastigotes. Briefly, 2×10^8 promastigotes/ml were washed three times in 5 ml of cold sterile phosphate-buffered saline (PBS). After five cycles of freezing and thawing, the suspension was centrifuged at 8000*g* for 20 min at 4 °C and supernatant containing SLA was collected and stored at −70 °C. The protein concentration was determined via the Bradford reaction (BioRad).

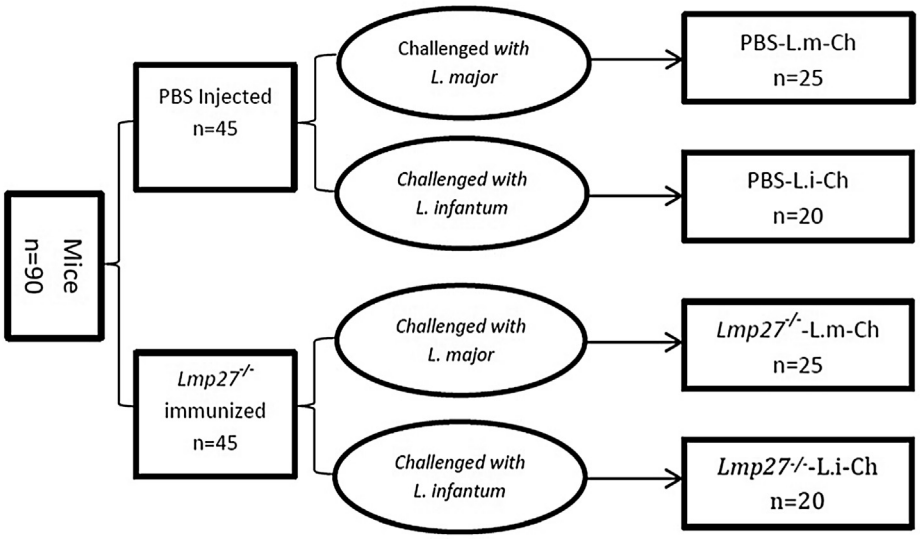

Fig. 1. Groups and sub-groups of mice. PBS-L.m-Ch, PBS-*L. major* challenged; *Lmp27*$^{-/-}$-L.m-Ch, *Lmp27*$^{-/-}$-immunized *L. major* challenged; PBS-L.i-Ch, PBS-*L. infantum* challenged; *Lmp27*$^{-/-}$-L.i-Ch, *Lmp27*$^{-/-}$-immunized *L. infantum* challenged; n, Number of mice.

2.4.3. Antibody (Ab) responses

At weeks 4 and 12 after inoculation, different groups of mice were tail-bled and the levels of anti-Leishmania IgG1 and IgG2a Abs were determined using ELISA. Briefly, 96-well microtiter plates (Nunc, Denmark) were coated with 50 μl of 10 μg/ml of SLA overnight at 4 °C. Plates were washed and blocked with 1% bovine serum albumin in Tween 20 (PBS–Tween). Serum samples were diluted to 1:200 with PBS–Tween 20 and applied to the plates. The plates were then treated with anti-mouse IgG isotype (Zymed Laboratories Inc., San Francisco, CAS, USA) according to the manufacturer's instructions. Optical density was determined at 450 nm, using 630 nm as the reference wavelength [19].

2.4.4. Delayed type hypersensitivity (DTH)

The DTH reaction was measured at 12 weeks post challenge by intradermal injection of freeze-thawed (FT) *L. major* (2×10^6 promastigotes in 50 μl per injection) into the right hind footpad, whereas left hind footpad served as control. FT *L. major* promastigotes were prepared by repeating a freeze (−196 °C)/thaw (37 °C) cycle ten times. The induration was measured in two perpendicular directions 72 h after injection.

2.5. Efficacy evaluation

Lesion development was measured using dial calipers at 1, 4, 8 and 12 weeks after *L. major* inoculation. Parasite burden in the liver and spleen was carried out at 4 and 12 weeks after *L. major* and *L. infantum* challenges. This was done using the limiting dilutions method [20].

2.6. Statistical analyses

Statistical analysis was performed by Compared Means, Independent Samples T-Test using SPSS Statistics 21.0 software. A p value ≤ 0.05 was considered the cutoff value for statistical significance.

3. Results

3.1. Proliferation assay

The results of the spleen cell proliferative response as measured by MTT test against SLA antigens at a 50-μg/well concentration 4 and 12 weeks post-challenge showed significantly higher Stimulation Index (SI) in *Lmp27*$^{-/-}$-L.m-Ch group, the difference was statistically significant ($p < 0.001$) (Fig. 2A).

3.2. Cytokine assay in challenge with L. Major

To evaluate immune responses in BALB/c mice after an inoculation challenge, the production of *L. major* Ag–specific cytokines was analyzed in splenocyte culture supernatant.

Results showed that four weeks after the challenge with Wt *L. major*, Th1-associated IFN-γ was significantly enhanced in *Lmp27*$^{-/-}$-L.m-Ch group as well as the ratio of IFN-γ/IL-4. Twelve weeks after their initial exposure to Wt *L. major* parasites, IFN-γ and IL-4 production decreased in immunized mice (Fig. 3A, B). These results suggest that *Lmp27*$^{-/-}$ mutant induces strong Th1 immune response in immunized mice (Fig. 3E).

3.3. Humoral responses in challenge with L. Major

The IgG1 and IgG2a humoral responses in the immune and inoculated BALB/c mice were evaluated by ELISA to determine Th1/Th2 immune responses.

Sera from BALB/c mice were collected at 4 and 12 weeks post-challenge. Results demonstrated that after 4 weeks IgG2a level increased significantly in *Lmp27*$^{-/-}$-L.m-Ch mice and after 12 weeks, although serum levels of IgG2a and IgG1 were lower but the IgG2a/IgG1 ratio was significantly higher in *Lmp27*$^{-/-}$immunized mice (Fig. 3C, D). In the group that was *Lmp27*$^{-/-}$-immunized and inoculated with *L. major*, there was a strong increase in IgG2a level compared with the PBS-*L. major* challenged groups after 4 weeks; and after twelve weeks the IgG2a/IgG1 ratio was significantly higher than in the PBS challenged mice. Increased IgG2a and decreased IgG1 levels are considered typical for Th1 and Th2 cell responses respectively (Fig. 3F). The data suggest that *Lmp27*$^{-/-}$ immunization induced a Th1 response.

3.4. DTH response

Twelve weeks post-challenge the *Lmp27*$^{-/-}$-L.m-Ch group exhibited significantly higher DTH responses compared with PBS-L.m-Ch mice. DTH was measured after 72 h of *L. major* Ag stimulation by injection of freeze-thawed *L. major* (2×10^6 promastigotes in 50 μl per injection) into the footpad (Fig. 4).

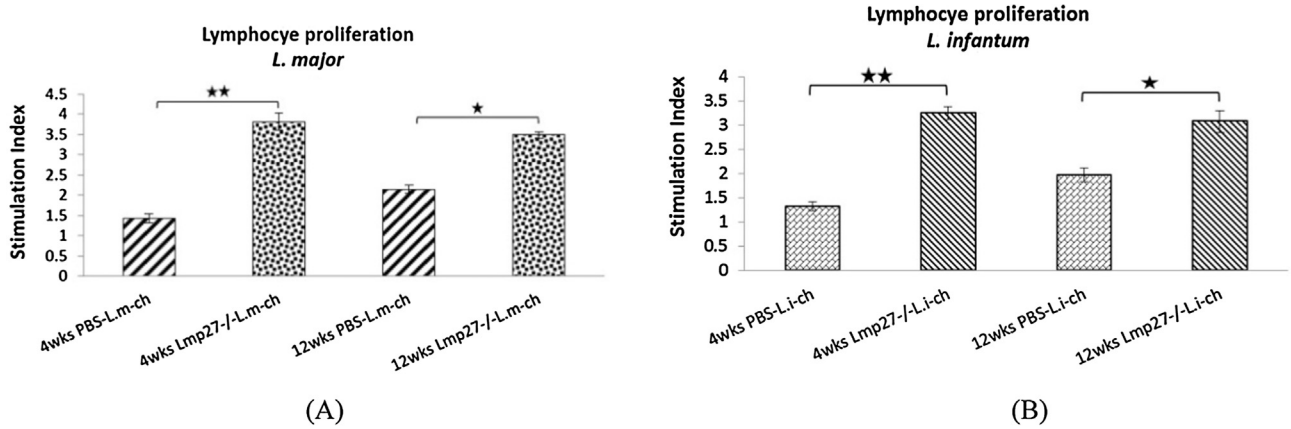

Fig. 2. *L. major* Ag–stimulated lymphocyte proliferation from PBS and immunized challenged mice with (A) Wt *L. major* and (B) Wt *L. infantum*. Four weeks post immunization, *Lmp27*$^{-/-}$-immunized and PBS mice were challenged with Wt parasites and 4 and 12 weeks post challenge mice were euthanized, and spleens were collected and lymphocyte proliferation evaluated using MTT assay, * $p < 0.01$, ** $p < 0.001$. PBS-L.m-Ch, PBS-*L. major* challenged; *Lmp27*$^{-/-}$-L.m-Ch, *Lmp27*$^{-/-}$-immunized *L. major* challenged; PBS-L.i-Ch, PBS-*L. infantum* challenged; *Lmp27*$^{-/-}$-L.i-Ch, *Lmp27*$^{-/-}$-immunized *L. infantum* challenged; 4wks, 4 weeks; 12wks, 12 weeks.

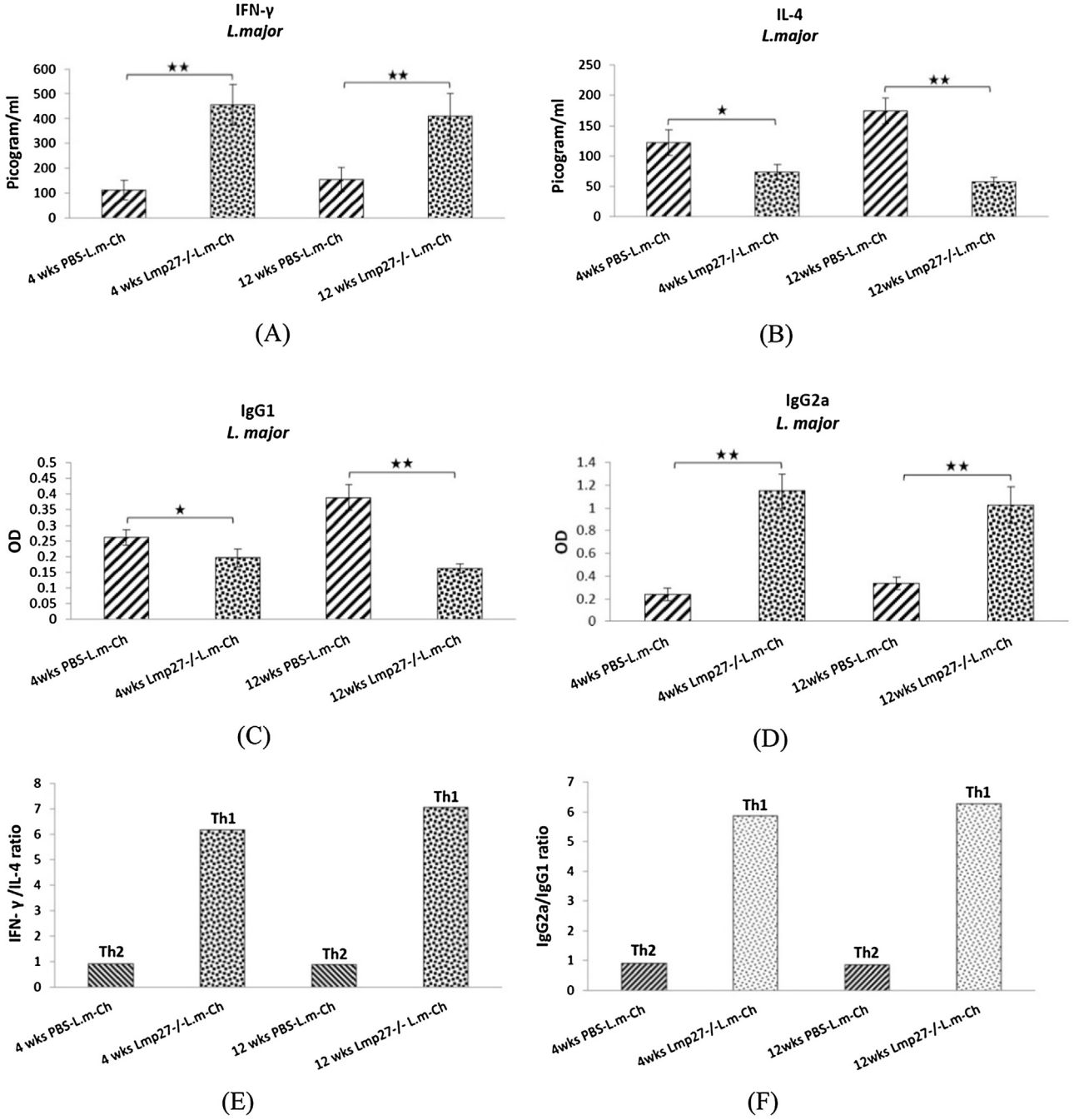

Fig. 3. Cytokine profiles in splenocyte culture supernatants and humoral responses of BALB/c mice 4 and 12 weeks post challenge with Wt *L. major*. ELISA measurement of the concentrations of (A) IFN- γ, (B) IL-4 cytokines in culture supernatants and (C) IgG1, (B) IgG2a Abs in sera of the mice and Th1/Th2 skewing, (E) IFN-γ/IL-4 and (F) IgG2a/IgG1 ratios were shown, * $p < 0.01$, ** $p < 0.001$. PBS-L.m-Ch, PBS-*L. major* challenged; *Lmp27*$^{-/-}$-L.m-Ch, *Lmp27*$^{-/-}$-immunized *L. major* challenged; 4wks, 4 weeks; 12wks, 12 weeks.

3.5. Efficacy of Lmp27$^{-/-}$ *mutant against challenge with Wt L. Major*

BALB/c mice were immunized with *Lmp27*$^{-/-}$ and 4 weeks later, together with the PBS group, were challenged with Wt. *L. major*. Lesion development was monitored for 1, 4, 8 and 12 weeks post-challenge. Results demonstrated that PBS mice developed lesions upon Wt. *L. major* challenge, whereas *Lmp27*$^{-/-}$-immunized mice either developed no lesions at all, or alternatively developed smaller nodules than their PBS counterparts (Fig. 5A). Parasite burdens in the liver and spleen that were measured 4 and 12 weeks post-challenge were also significantly decreased in *Lmp27*$^{-/-}$-immunized group (Fig. 5B, C).

3.6. Protective immunity of Lmp27$^{-/-}$ *in challenge with Wt L. Infantum*

For determination of whether immunization with the *Lmp27*$^{-/-}$ mutant strain could provide cross protective immunity against infection involving heterologous species of *Leishmania*, 4 weeks post immunization with *Lmp27*$^{-/-}$, immunized and PBS group BALB/c mice were challenged with Wt *L. infantum* via intraperitoneal inoculation. Immunological evaluation revealed that at 4 and 12 weeks post-challenge, the immunized group's IFN- γ/IL-4 (Fig. 6A, B) ratio, the ratio of IgG2a/IgG1 (Fig. 6C, D), and the degree of lymphocyte proliferation were significantly higher when

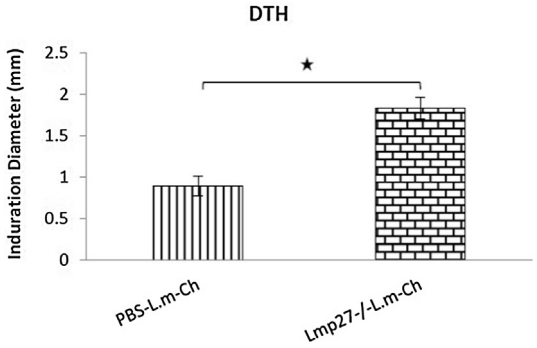

Fig. 4. At 12 weeks post immunization and post challenged DTH reaction was checked by measurement of footpad induration 72 h after injection of freeze-thawed *L. major* into the footpad of all Wt, immunized, PBS and *Lmp27*$^{-/-}$ immunized challenged mice, * $p < 0.01$, ** $p < 0.001$. *Lmp27*$^{-/-}$-imm, *Lmp27*$^{-/-}$-immunized; PBS-L.m-Ch, PBS-*L. major* challenged; *Lmp27*$^{-/-}$-L.m-Ch, *Lmp27*$^{-/-}$-immunized *L. major* challenged.

compared to the PBS *L. infantum*-challenged group (Fig. 2B). This indicates that *Lmp27*$^{-/-}$ mutant could induce a Th1 response to common antigens which lead to cross protection against *L. infantum* challenge (Fig. 6E, F).

3.7. Cross protection of *Lmp27*$^{-/-}$ against Wt L. Infantum

At 4 and 12 weeks after being challenged with *L. infantum*, the immunized mice showed significantly lower parasite burdens both in the liver and spleen when compared with similarly challenged PBS mice. Parasite burden was lower at 12 weeks compared to 4 weeks in the *Lmp27*$^{-/-}$-L.i-Ch group (Fig. 7A, B).

4. Discussion

Historically the most successful vaccines against intracellular pathogens have been based on live attenuated organisms. Studies using attenuated strains of *Leishmania* have shown that this is a good strategy for producing a long-lasting protective immune response [21,22] as they closely mimic the natural course of infection. A live attenuated vaccine strain would present a full complement of *Leishmania* antigens to the host immune system along with appropriate pattern-recognition molecules for the parasite [23]. Previous work from our laboratories has shown that the *Lmp27*$^{-/-}$ live attenuated mutant is safe and immunogenic in BALB/c mice [18]. Due to differences in virulence factors between *Leishmania* species [24], the mechanisms of protective immunity and efficacy against such homologous and heterologous *Leishmania* parasites remained unexplored. Hence, in this study, the *Lmp27*$^{-/-}$ strain was used to determine its protective immune responses and efficacy against Wt *L. major* as well as its cross protection against Wt *L. infantum* in BALB/c mice.

Four weeks after exposure in the *Lmp27*$^{-/-}$ immunized groups, both the pro-inflammatory cytokine (IFN-γ) and anti-inflammatory cytokine (IL-4) increased. However, in order to prevent tissue damage, achieving a balance between the primary inflammatory and anti-inflammatory cytokines is essential [25,26]. With regard to *L. donovani* infection, it has been reported

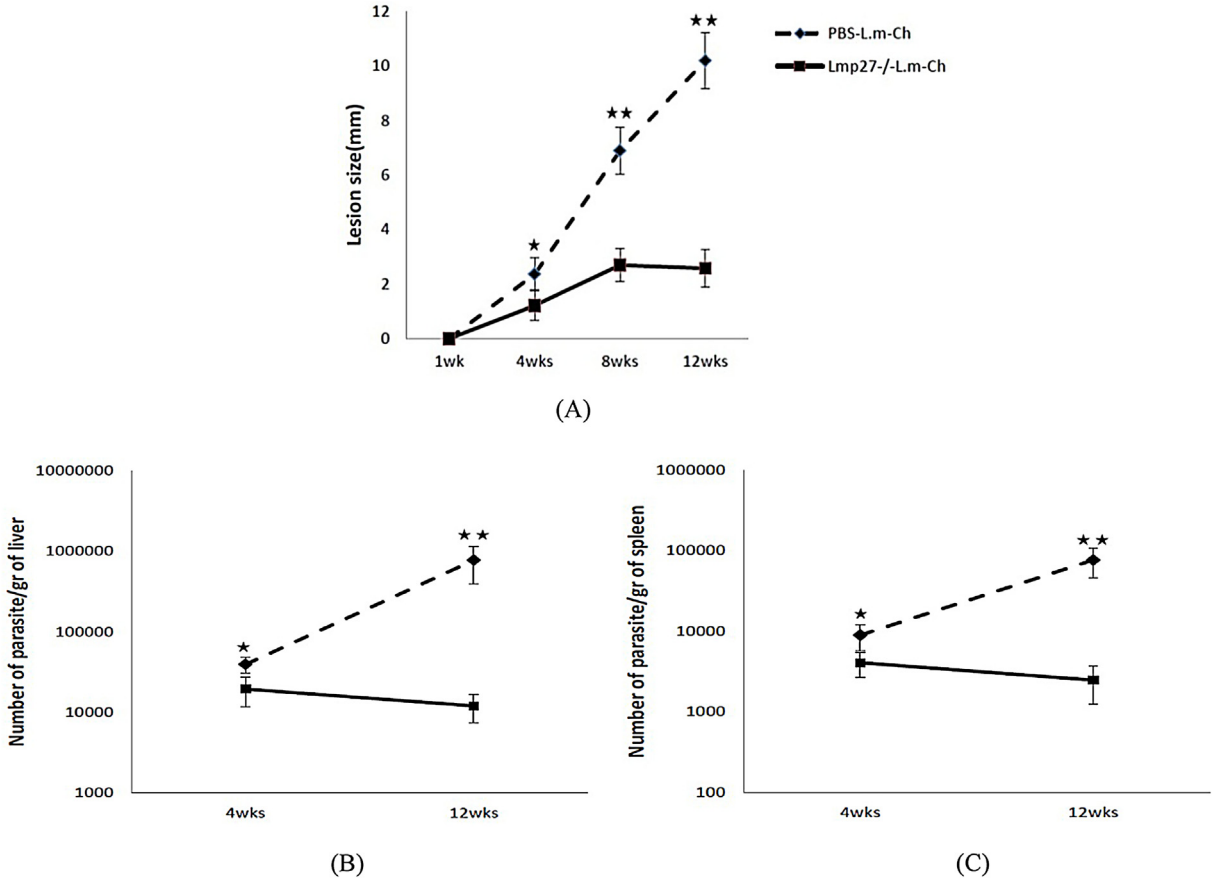

Fig. 5. Lesion size and parasite burden in PBS and *Lmp27*$^{-/-}$ immunized mice were assessed post challenge with Wt *L. major* parasites. (A) Lesion size diameters were measured 1, 4, 8 and 12 weeks after inoculation, Parasite burdens in the (B) liver and (C) spleen 4 and 12 weeks post challenge were shown. Each group contained at least 4 mice. * $p < 0.01$, ** $p < 0.001$. PBS-L.m-Ch, PBS-*L. major* challenged; *Lmp27*$^{-/-}$-L.m-Ch, *Lmp27*$^{-/-}$-immunized *L. major* challenged; 4wks, 4 weeks; 12wks, 12 weeks.

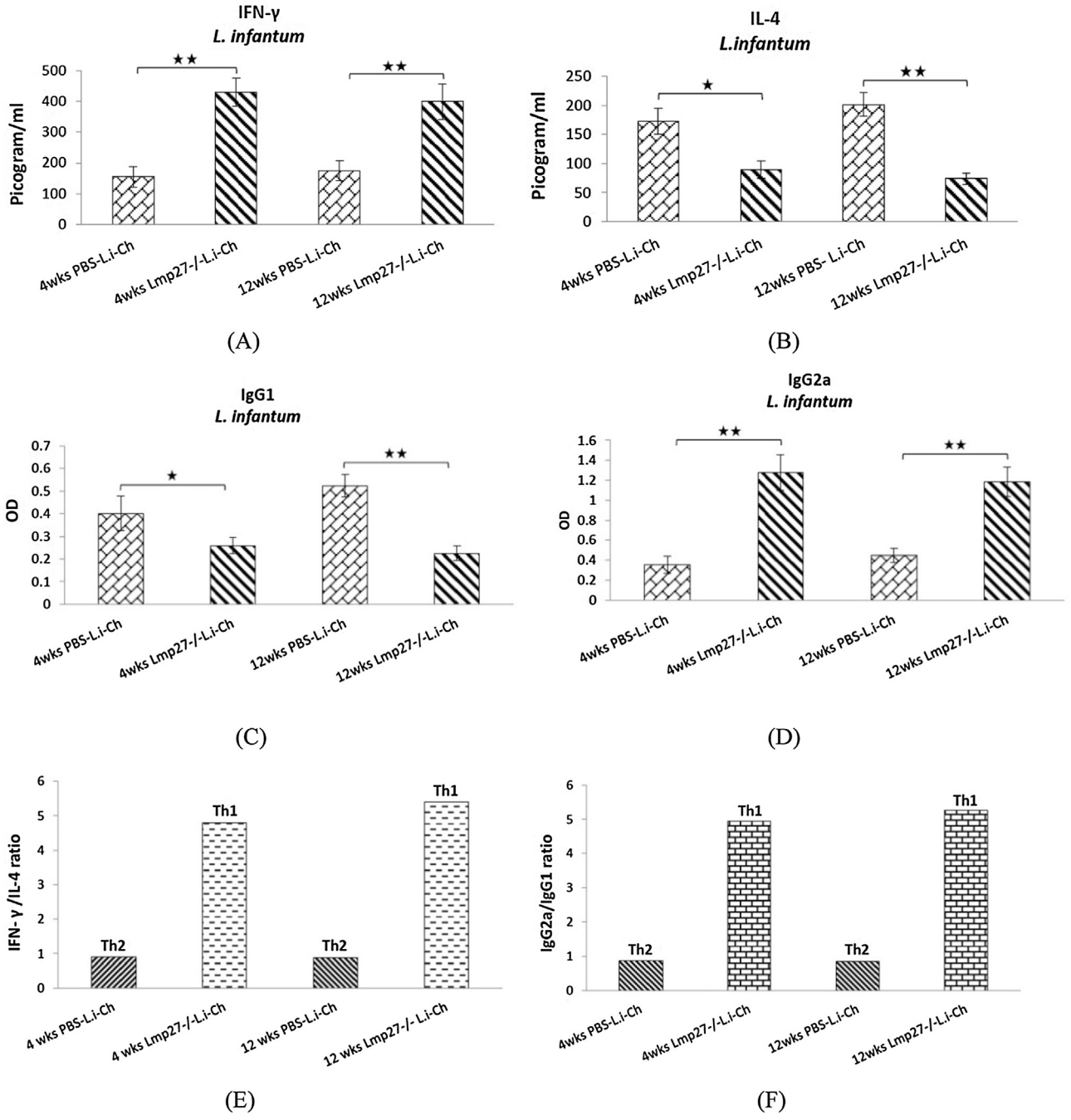

Fig. 6. Cytokines and antibodies levels in PBS and *Lmp27*$^{-/-}$ immunized mice were measured using ELISA 4 and 12 weeks post challenge with Wt *L. infantum* parasites. Concentrations of (A) IFN-γ and (B) IL-4 cytokines, (C) IgG1 and (D) IgG2a Abs in sera of the mice and Th1/Th2 skewing, (E) IFN- γ /IL-4 and (F) IgG2a/IgG1 ratios were shown, * $p < 0.01$, ** $p < 0.001$. PBS-L.i-Ch, PBS-*L. infantum* challenged; *Lmp27*$^{-/-}$-L.i-Ch, *Lmp27*$^{-/-}$-immunized *L. infantum* challenged; 4wks, 4 weeks; 12wks, 12 weeks.

that the balance between the pro-inflammatory and anti-inflammatory cytokines determines parasite clearance from the liver and spleen [27] and may be important in the progression of liver granuloma maturation [17].

This study showed that *Lmp27*$^{-/-}$ was able to induce significant levels of IFN-γ, when challenged with *L. major* and *L. infantum* infections. It is well established that IFN-γ is a key effector cytokine which is crucial in the elimination of *Leishmania* parasites [28]. Four weeks post challenge; IFN-γ levels were significantly ($p = 0.033$) higher in challenge with *L. major* compared to *L. infantum*, suggesting more antigenic similarity with *L. major*.

12 weeks after both challenges, IL-4, due to the immune system's control of the infection, declined significantly, whereas it increased in the PBS groups.

The IgG2a (associated with Th1) response increased significantly following *L. major* and *L. infantum* challenges, confirming the development of a protective immune response resulting from immunization as was observed in *L. infantum*–infected dogs and hamsters after immunization with *LdCen*$^{-/-}$ [13,29] and other studies [17,30].

The protective immunity in leishmaniasis was steered by Th1 polarization [31]. *Ldp27*$^{-/-}$, *LdCen*$^{-/-}$ and *Leishmania infantum*

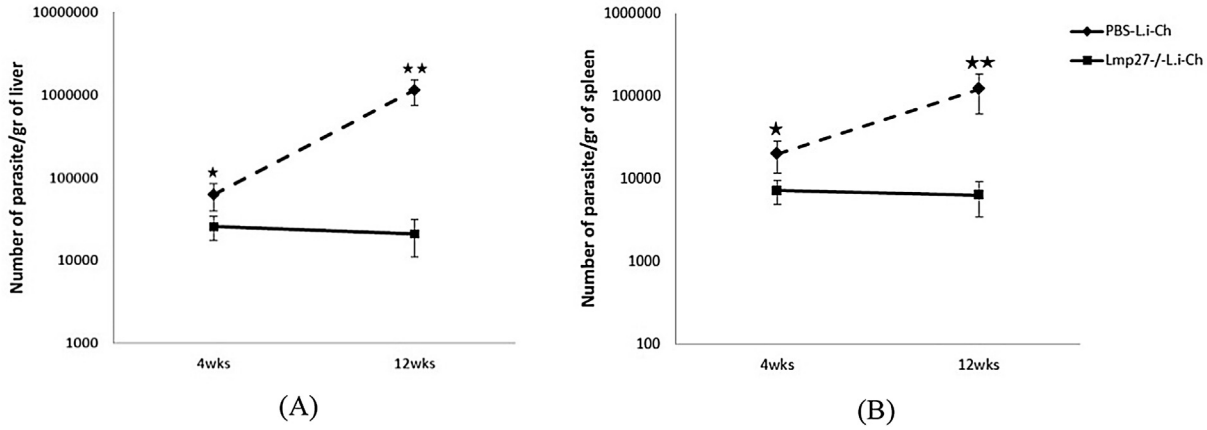

Fig. 7. Parasite burden in Liver and Spleen in PBS and *Lmp27*$^{-/-}$ immunized mice were measured 4 and 12 weeks post challenge with Wt *L. infantum* parasites. Parasite burdens in the (A) liver and (B) spleen were shown. Each group contained at least 4 mice; * $p < 0.01$, ** $p < 0.001$. PBS-L.i-Ch, PBS-*L. infantum* challenged; *Lmp27*$^{-/-}$-L.i-Ch, *Lmp27*$^{-/-}$-immunized *L. infantum* challenged; 4wks, 4 weeks; 12wks, 12 weeks.

KHARON1 null mutants have been shown to exhibit typical upregulation of Th1 cytokines and antibodies [17,32,33]. In accordance with these earlier findings, our data also demonstrated a significant increase in the INF-γ/ IL-4 and IgG2a/IgG1 ratios found in *Lmp27*$^{-/-}$ immunized groups after an *L. major* challenge when compared to their respective PBS groups. This data suggests that the mutant parasite was able to orient the immune system toward Th1 cellular immunity. Similar results have been observed in challenges with *L. infantum* [17,30,34].

Following inoculation, the high lymphocyte proliferation seen in *Lmp27*$^{-/-}$ vaccinated groups indicates that the *Lmp27*$^{-/-}$ mutant parasite induces a strong immune response in spite of being cleared after 12 weeks compared to Wt parasite [18], suggesting the strong induction of memory responses by *Lmp27*$^{-/-}$ parasites. Similar results were obtained using mutant strains of *L. donovani* without the ascorbic acid gene [35] or centrin gene [32] when challenged with wild type *L. donovani* , *Ldp27*$^{-/-}$, and dhfr-ts^{-} *L. major* after heterologous challenges [16,17]. Moreover, *Lmp27*$^{-/-}$-immunized mice also showed significantly high DTH responses against *L. major* Ag as well as what was observed in *Ldp27*$^{-/-}$ immunized mice [17].

The smaller lesion sizes and the reduction in parasitic load in the *Lmp27*$^{-/-}$ immunized groups as compared to the PBS groups demonstrate that the mutant parasite is effective in inducing both protection and cross-protection. Similar findings were obtained in the study of *L. major*-infected mice after immunization with lpg2 mutants [15]. Cross protections have also been observed in some studies [30,36–39], while in others it has not been reported [37,40]. This matter may be related to an inherent difference between different species and their interactions with the immune system. Genome sequencing, however, has revealed that 90% of the genome is conserved between *L. major* and *L. infantum*, and 99% of the genes are syntonic [41,42], suggesting a strong likelihood of shared antigens between the two.

In conclusion, this study reveals that in BALB/c mice, *Lmp27*$^{-/-}$ is able to stimulate the cellular immune system, and induces protection against Wt *L. major* and cross-protection against Wt *L. infantum* challenges. A study is underway to evaluate its protective immunity and efficacy in dogs against challenges by virulent *Leishmania* parasites. These results are suggestive that *Lmp27*$^{-/-}$ parasites can serve as *Leishmania* vaccine candidate.

Acknowledgements

The authors are appreciative to Dr. Behnaz Akhoundi, Mrs. Sorour Charehdar, Mr. Quinn Kern Allely, Dr. Vahid Nasiri and Mr. Mohammad Taghi Satvat for their enormous contributions. We would like to thank Dr. Hira Nakhasi for his helpful suggestions during the course of the study and also thanks to Prof. Farrokh Modabber for careful consideration of the manuscript and provided critical suggestions and revision.

Source of funding

This study was financially supported by Tehran University of Medical Sciences (Projects No 92-01-160-21598 and 97-03-160-39686).

Conflicts of interest

The authors declare no conflict of interest.

References

[1] World Health Organization. Manual for case management of cutaneous leishmaniasis in the WHO Eastern Mediterranean Region; 2014.
[2] Holakouie-Naieni K, Mostafavi E, Boloorani AD, Mohebali M, Pakzad R. Spatial modeling of cutaneous leishmaniasis in Iran from 1983 to 2013. Acta Trop 2017;166:67–73.
[3] Mohebali M. Visceral leishmaniasis in Iran: review of the epidemiological and clinical features. Iran J Parasitol 2013;8:348–58.
[4] Alvar J, Croft S, Olliaro P. Chemotherapy in the treatment and control of leishmaniasis. Adv Parasitol 2006;61:223–74.
[5] De Luca PM, Macedo ABB. Cutaneous leishmaniasis vaccination: a matter of quality. Front Immunol 2016;7:151.
[6] Selvapandiyan A, Dey R, Gannavaram S, Lakhal-Naouar I, Duncan R, Salotra P, et al. Immunity to visceral leishmaniasis using genetically defined live-attenuated parasites. J Trop Med 2012;2012:631460.
[7] Okwor I, Liu D, Beverley SM, Uzonna JE. Inoculation of killed *Leishmania major* into immune mice rapidly disrupts immunity to a secondary challenge via IL-10-mediated process. Proc Natl Acad Sci USA 2009;106:13951–6.
[8] Birnbaum R, Craft N. Innate immunity and *Leishmania* vaccination strategies. Dermatol Clin 2011;29:89–102.
[9] Selvapandiyan A, Duncan R, Debrabant A, Lee N. Genetically modified live attenuated parasites as vaccines for leishmaniasis. Indian J Med Res 2006;123:455.
[10] Silvestre R, Cordeiro-da-Silva A, Ouaissi A. Live attenuated *Leishmania* vaccines: a potential strategic alternative. Arch Immunol Ther Exp (Warsz) 2008;56:123–6.

[11] Titus RG, Gueiros-Filho FJ, De Freitas L, Beverley SM. Development of a safe live *Leishmania* vaccine line by gene replacement. Proc Natl Acad Sci USA 1995;92:10267–71.
[12] Bhattacharya P, Dey R, Dagur PK, Joshi AB, Ismail N, Gannavaram S, et al. Live attenuated *Leishmania donovani* Centrin knock out parasites generate non-inferior protective immune response in aged mice against Visceral Leishmaniasis. PLoS Negl Trop Dis 2016;10:e0004963.
[13] Fiuza JA, da Costa Santiago H, Selvapandiyan A, Gannavaram S, Ricci ND, Bueno LL, et al. Induction of immunogenicity by live attenuated *Leishmania donovani* centrin deleted parasites in dogs. Vaccine 2013;31:1785–92.
[14] Papadopoulou B, Roy G, Breton M, Kündig C, Dumas C, Fillion I, et al. Reduced infectivity of a *Leishmania donovani* biopterin transporter genetic mutant and its use as an attenuated strain for vaccination. Infect Immun 2002;70:62–8.
[15] Uzonna JE, Späth GF, Beverley SM, Scott P. Vaccination with phosphoglycan-deficient *Leishmania major* protects highly susceptible mice from virulent challenge without inducing a strong Th1 response. J Immunol 2004;172:3793–7.
[16] Veras PST, Brodskyn CI, Balestieri FMP, De Freitas L, Ramos A, Queiroz A, et al. A dhfr-ts-*Leishmania major* knockout mutant cross-protects against *Leishmania amazonensis*. Mem Inst Oswaldo Cruz 1999;94:491–6.
[17] Dey R, Dagur PK, Selvapandiyan A, McCoy JP, Salotra P, Duncan R, et al. Live attenuated *Leishmania donovani* p27 gene knockout parasites are nonpathogenic and elicit long-term protective immunity in BALB/c mice. J Immunol 2013;190:2138–49.
[18] Elikaee S, Mohebali M, Rezaei S, Eslami H, Khamesipour A, Keshavarz H, et al. Development of a new live attenuated *Leishmania major* p27 gene knockout: Safety and immunogenicity evaluation in BALB/c mice. CellImmunol 2018;332:24–31.
[19] Badiee A, Jaafari MR, Khamesipour A. *Leishmania major:* immune response in BALB/c mice immunized with stress-inducible protein 1 encapsulated in liposomes. Exp Parasitol 2007;115:127–34.
[20] Titus R, Marchand M, Boon T, Louis J. A limiting dilution assay for quantifying *Leishmania major* in tissues of infected mice. Parasite Immunol 1985;7:545–55.
[21] Selvapandiyan A, Dey R, Gannavaram S, Solanki S, Salotra P, Nakhasi HL. Generation of growth arrested *Leishmania* amastigotes: a tool to develop live attenuated vaccine candidates against visceral leishmaniasis. Vaccine 2014;32:3895–901.
[22] Fiuza JA, Gannavaram S, da Costa Santiago H, Selvapandiyan A, Souza DM, Passos LSA, et al. Vaccination using live attenuated *Leishmania donovani* centrin deleted parasites induces protection in dogs against *Leishmania infantum*. Vaccine 2015;33:280–8.
[23] Evans KJ, Kedzierski L. Development of vaccines against visceral leishmaniasis. J Trop Med 2011;2012:892817.
[24] McMahon-Pratt D, Alexander J. Does the *Leishmania major* paradigm of pathogenesis and protection hold for New World cutaneous leishmaniases or the visceral disease? Immunol Rev 2004;201:206–24.
[25] Launois P, Maillard I, Pingel S, Swihart KG, Xénarios I, Acha-Orbea H, et al. IL-4 rapidly produced by Vβ4 Vα8 CD4+ T cells instructs Th2 development and susceptibility to *Leishmania major* in BALB/c mice. Immunity 1997;6:541–9.
[26] Noben-Trauth N, Paul WE, Sacks DL. IL-4-and IL-4 receptor-deficient BALB/c mice reveal differences in susceptibility to *Leishmania major* parasite substrains. J Immunol 1999;162:6132–40.
[27] Stäger S, Alexander J, Carter KC, Brombacher F, Kaye PM. Both interleukin-4 (IL-4) and IL-4 receptor α signaling contribute to the development of hepatic granulomas with optimal antileishmanial activity. Infect Immun 2003;71:4804–7.
[28] Kima PE, Soong L. Interferon gamma in leishmaniasis. Front Immunol 2013;4:156.
[29] Fiuza JA, Dey R, Davenport D, Abdeladhim M, Meneses C, Oliveira F, et al. Intradermal immunization of *Leishmania donovani* centrin knock-out parasites in combination with salivary protein LJM19 from sand fly vector induces a durable protective immune response in hamsters. PLoS Negl Trop Dis 2016;10:e0004322.
[30] Dey R, Natarajan G, Bhattacharya P, Cummings H, Dagur PK, Terrazas C, et al. Characterization of cross-protection by genetically modified live-attenuated *Leishmania donovani* parasites against *Leishmania mexicana*. J Immunol 2014;193:3513–27.
[31] Singh RK, Gannavaram S, Ismail N, Kaul A, Gedda MR, Nakhasi HL. Centrin-Deleted *Leishmania donovani* Parasites Help CD4+ T Cells to Acquire Th1 Phenotype and Multi-Functionality Through Downregulation of CD200–CD200R Immune Inhibitory Axis. Front Immunol 2018;9:1176.
[32] Selvapandiyan A, Dey R, Nylen S, Duncan R, Sacks D, Nakhasi HL. Intracellular replication-deficient *Leishmania donovani* induces long lasting protective immunity against visceral leishmaniasis. J Immunol 2009;183:1813–20.
[33] Santi AMM, Lanza JS, Tunes LG, Fiuza JA, Roy G, da Silva Orfanó A, et al. Growth arrested live-attenuated *Leishmania infantum* KHARON1 null mutants display cytokinesis defect and protective immunity in mice. Sci Rep 2018;8:11627.
[34] Alves-Silva MV, Nico D, Morrot A, Palatnik M, Palatnik-de-Sousa CB. A chimera containing CD4+ and CD8+ T-cell epitopes of the *Leishmania donovani* nucleoside hydrolase (NH36) optimizes cross-protection against *Leishmania amazonesis* infection. Front Immunol 2017;8:100.
[35] Anand S, Madhubala R. Genetically engineered ascorbic acid-deficient live mutants of *Leishmania donovani* induce long lasting protective immunity against visceral leishmaniasis. Sci Rep 2015;5:10706.
[36] Carrión 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.
[37] Tonui WK, Titus RG. Cross-protection against *Leishmania donovani* but not *L. braziliensis* caused by vaccination with *L. major* soluble promastigote exogenous antigens in BALB/c mice. Am J Trop Med Hyg 2007;76:579–84.
[38] Gicheru M, Olobo J, Anjili C. Heterologous Protection by *Leishmania donovani* for *Leishmania major* Infections in the Vervet Monkey Model of the Disease. Exp Parasitol 1997;85:109–16.
[39] McCall LI, Zhang WW, Ranasinghe S, Matlashewski G. Leishmanization revisited: immunization with a naturally attenuated cutaneous *Leishmania donovani* isolate from Sri Lanka protects against visceral leishmaniasis. Vaccine 2013;31:1420–5.
[40] Nation CS, Dondji B, Stryker GA. Previous exposure to a low infectious dose of *Leishmania major* exacerbates infection with *Leishmania infantum* in the susceptible BALB/c mouse. Parasitol Res 2012;111:1407–15.
[41] Peacock CS, Seeger K, Harris D, Murphy L, Ruiz JC, Quail MA, et al. Comparative genomic analysis of three *Leishmania* species that cause diverse human disease. Nat Genet 2007;39:839–47.
[42] Rochette A, Raymond F, Ubeda JM, Smith M, Messier N, Boisvert S, et al. Genome-wide gene expression profiling analysis of *Leishmania major* and *Leishmania infantum* developmental stages reveals substantial differences between the two species. BMC Genomics 2008;9:255.