



A Laboratory Strain of *Leishmania major*: Protective Effects on Experimental Leishmaniasis

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Received: 10 July 2018 / Accepted: 6 May 2019 / Published online: 20 May 2019
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Abstract

Purpose Leishmaniasis, as one of the most important vector-borne and zoonotic diseases, can be seen in different forms and is more prevalent in developing countries worldwide. Due to the absence of effective strategies in its prevention, treatment, and control, investigation of effective control strategies against the disease is necessary. In this research, we evaluated the immunogenicity of a cold-adapted laboratory strain of *Leishmania major* (LMC) in the mouse model.

Methods Twenty BALB/c mice were divided into two groups. LMC group received 4×10^6 of LMC strain in 0.5 ml DMEM, and VLM group, as the control group, received 0.5 ml Dulbecco's modified Eagle's medium. Both groups were challenged with virulent *L. major* 3 weeks after inoculation.

Results The data obtained from the analysis of immune responses and histopathological changes interestingly revealed protection against *L. major* in immunized mice. Compared with the VLM group, the mice immunized with LMC strain of *L. major* in the LMC group showed a significant increase in IFN- γ and IgG2a levels ($P < 0.05$) which are important indexes for Th1-related immune responses. Additionally, significant differences in concentration of IgG1 and IgG total before and after the challenge was observed in LMC group ($P < 0.05$). Furthermore, the immunized mice showed a significant reduction in mean sizes of skin lesion and liver damage compared to the VLM group.

Conclusion Based on the present findings on immunogenicity of LMC strain, it seems this strain is able to induce both humoral and cellular immunity and a significant protection against *L. major* in the mouse model.

Keywords *Leishmania major* · Cold-adapted laboratory strain · Immunogenicity · Histopathology · BALB/c mice

Introduction

Leishmaniasis is considered endemic in 98 countries, including some of the poorest population in the world. Based on the World Health Organization (WHO) reports, approximately more than 12 million people are currently infected

with leishmaniasis and over 350 million people are at the risk of catching the disease around the world. The disease is transmitted by the bite of phlebotomine sand flies. Cutaneous leishmaniasis (CL), caused by *Leishmania major*, is the most common form of the disease as it is the case in Iran, Afghanistan, Syria, Saudi Arabia, and Algeria [1, 41, 43]. The skin sores caused by CL appear as papules or nodules on the sand fly bite and may end up as skin ulcers and scab formation. Various forms of drugs are available for the treatment of human leishmaniasis; however, treatments such as pentavalent antimonials compounds are toxic and very expensive for those infected. Moreover, treatment failures and incidence of drug resistance are common. The aforementioned side effects and problems in chemical treatment strategies highlight the need for a valuable and safe vaccine, which produces long life immunity [22, 30, 43]. Therefore, many studies have focused on the development of an effective vaccine as a feasible solution. Over the past

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two decades, different vaccine forms and molecule candidates such as the entrapped-in-liposomes recombinant gp63 [18], killed whole-parasites as first generation vaccines [17, 19, 20], and *L. major* secreted–excreted exogenous antigens have shown signs of protecting animals against experimental leishmaniasis [41]. Nevertheless, to date, there is no licensed vaccine available against any form of human leishmaniasis [10, 18, 24]. More recent investigations in *L. major*-resistant mouse have indicated complexities in the responsible mechanisms for acquired immunity, which highlights the need to redesigning of vaccines against *Leishmania* that require sustained cell-mediated immune responses [33, 34]. Live-non-attenuated vaccines (an old strategy) in the hyper-endemic Asian countries are known as an effective way to protect people against further lesions. In these endemic areas, leishmanization (LZ) was used to develop a self-healing lesion and consequently to protect people against lesions on the face and other exposed parts of the body, especially in children [9, 20, 26, 28]. Adverse side effects of LZ including the development of large, persistent lesions led to the discontinuation of LZ in many countries and the focus of vaccine development projects, consequently, changed towards killed or live-attenuated vaccines [7, 12, 16]. Nowadays, genetic manipulation of *Leishmania* parasites to reduce virulence, together with maintaining immunogenicity, is an appealing prospect in *Leishmania* vaccine research. Live-attenuated vaccination is closely similar to a natural infection that may lead to the same immune responses, and there is minimal risk associated with infection by live virulent parasites [12, 16].

Recently, an interesting strain of *L. major* was prepared by a long-term serial passage in low temperature condition in Razi Vaccine and Serum Research Institute, Shiraz branch, Iran. This cold-adapted laboratory strain of *Leishmania major* was named *L. major* cold strain (LMC). Previous pilot studies have indicated LMC was immunogenic and no pathogenic effect was observed [2]. In the present study, the ability of LMC strain to induce an immune response and as a protection against *L. major* infection in susceptible BALB/c mice was investigated.

Materials and Methods

Preparation of the Parasites

The LMC strain was maintained in the Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich) with 2% fetal bovine serum (FBS) (Sigma-Aldrich), supplemented with 50 µg/ml gentamicin (Sigma-Aldrich), 100 U/ml penicillin G potassium salt (Sigma-Aldrich), and 100 µg/ml streptomycin sulfate salt (Sigma-Aldrich). In addition, Live *L. major* parasite (for a challenge infection) was taken from the Razi

vaccine and Serum Institute (Karaj—Iran), in RPMI 1640 medium (Sigma-Aldrich) with 10% FBS (Sigma-Aldrich) supplemented with 100 U/ml penicillin G potassium salt (Sigma-Aldrich), 50 µg/ml gentamicin (Sigma-Aldrich), and 100 µg/ml streptomycin sulfate salt (Sigma-Aldrich) [3, 42, 44].

Study Design

Twenty male inbred BALB/c mice at 4 weeks of age, provided by the Pasteur Institute (Tehran, Iran), were housed in separate laboratory cages with optimal conditions (25–27 °C, light/dark cycles in 12-h interval). Commercial laboratory rodent pellets and water were also available freely. The mice were divided into two groups. LMC group received 4×10^6 of LMC strain in 0.5 ml DMEM and VLM group, as the control group, received 0.5 ml DMEM. All doses were injected subcutaneously into the groin. Three weeks later, both groups were challenged subcutaneously at the base of the tail with 2×10^6 of virulent amastigotes of *L. major* isolated from fresh skin lesion.

Antigen Preparation

Leishmania major antigens were prepared as described previously [35, 41] with some modifications as follows: promastigotes were gained from culture and then washed two times with cold phosphate-buffered saline (PBS). Thereafter, centrifugation was performed twice at 9000g for 30 min. The parasites were then sonicated at 1 °C (3 × 60 s at 65 W). The concentration of proteins in the supernatant was determined by Lowry's method [23] and were stored at –80 °C until used.

Immune Response Assay

ELISA assay was performed to measure anti-*Leishmania* antibodies (total IgG, IgG1 and IgG2a), before and 14 weeks after the challenge. Briefly, ELISA plate wells were coated with 50 µl of 0.5 µg/ml of antigen suspension at 4 °C overnight. Plates were washed and blocked with 100 µl of 1% BSA in PBS–Tween/well for 1 h at 37 °C. Serum samples were diluted 1:100 with PBS–Tween and each well was filled with 100 µl of diluted serum. Then, anti-mouse IgG isotype was added to the plates according to the manufacturer's instructions (Sigma, USA). Then plates were incubated at 37 °C for 1 h. Thereafter, plates were washed and charged with 100 µl of substrate and chromogen mixture and incubated at room temperature in dark for 15 min. The reaction was stopped by adding 100 µl of stop solution, that is, H₂SO₄ 12.5%, and the ODs of wells were read at 450 nm by ELISA reader. The cut-off value was defined as the mean value of the control group plus two standard deviations.

All mice from each group were killed humanely 14 weeks after the challenge. The spleens of sacrificed mice were aseptically removed and lymphocytes were extracted via high-pressure pumping. The lymphocytes were cultured using 1×10^6 cells per well supplemented with 0.5% mouse normal sera and DMEM and incubated at 37 °C with 5% CO₂ [21, 31, 41]. Afterward, cells were stimulated with a 50- μ g antigen of parasites separately. Secreted IFN- γ and IL-4 in supernatants were measured 72 h after stimulation using a commercial kit based on enzyme-linked immunosorbent assay (ELISA) (Shanghai Crystal Day Biotech Co., LTD, Shanghai, China).

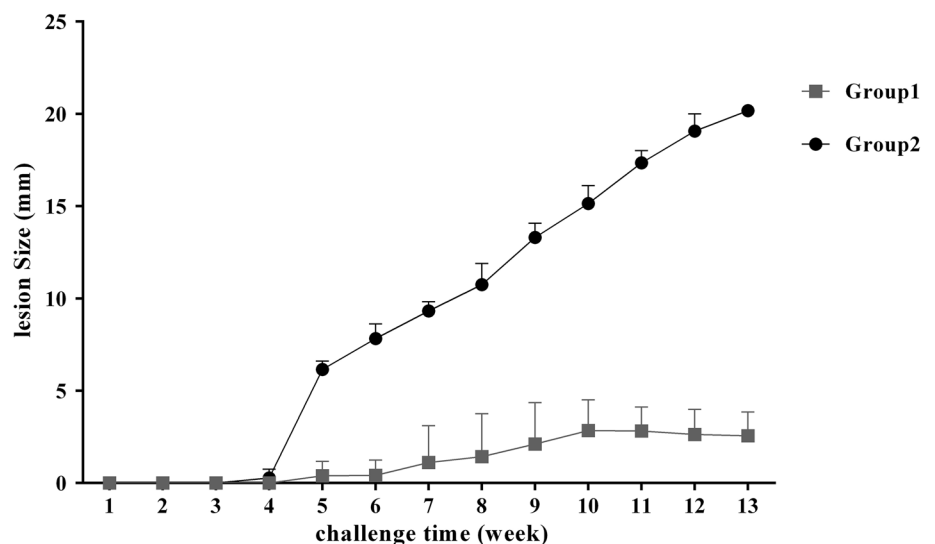
Histopathological Analysis

Sampling was performed from the injection site, lesions, and liver in all groups. Next, small samples were fixed in 10% formalin-buffer, embedded in paraffin, sectioned and stained with hematoxylin and eosin (H&E), and Giemsa stain. A semi-quantitative scoring method was used to evaluate the tissue lesions and following grades were considered: none (0, normal), mild (1, 0–33% of tissue affected), moderate (2, 34–66% of tissue affected), and severe (3, 67–100% of tissue affected) [14].

Statistical Analysis

Statistical analysis was performed using SPSS v.16 and Graph Pad Prism 6.0. One-way ANOVA, followed by Tukey test, and two-tailed Student's *t* test were used. Results with $P < 0.05$ were considered to be statistically significant. Values were expressed as mean \pm SD.

Fig. 1 Lesion development was recorded from 3 weeks after challenge until week 14 with regular intervals. Lesion size rapidly developed in the VLM group, whereas lesion size showed a decreasing trend in LMC group. VLM virulent *L. major*, LMC *L. major* cold strain



Animal Ethics

This experiment was accomplished under the approval of the state committee on animal ethics, Shiraz University, Shiraz, Iran. Also, the recommendations of European Council Directive (86/609/EC) of November 24, 1986 were used regarding the standards in the protection of animals used for experimental purposes.

Results

Clinical Findings

Lesion development was recorded from 3 weeks after the challenge started, with 1-week interval until week 14 post challenge. Lesion size development in VLM group was at a more rapid rate than LMC group. The ulcer area indicated a decreasing trend in LMC group in comparison with VLM group. Moreover, significant differences were seen between LMC and VLM groups from 7 weeks post challenge ($P < 0.05$) (Fig. 1).

Histopathological Findings

The tissue sections of the VLM group showed a moderate to severe diffuse granulomatous dermatitis and mild to severe diffuse hepatitis with the presence of numerous parasites within the lymphocytes, macrophages, and outside the cells (Fig. 2a, b). The skin sections of the LMC group revealed a mild diffuse granulomatous dermatitis and hepatitis. Parasites were rarely observed in cutaneous sections (Fig. 2c, d). The mean \pm SD of histopathological scores was showed in Table 1.

Fig. 2 Skin sections of different groups. **a** Tissue section of a mouse receiving *L. major* shows severe diffuse granulomatous dermatitis with many macrophages and lymphocytes infiltration (asterisks), H&E, $\times 180$. **b** There are a large number of Leishman bodies within the macrophages and extracellular (arrows) in a mouse receiving *L. major*, Giemsa, $\times 720$. **c** Tissue section of a mouse received 4×10^6 LMC strain and challenged by *L. major* (LMC group) shows mild diffuse granulomatous dermatitis with few inflammatory cells (asterisks), H&E, $\times 180$. **d** Parasites (arrow) are rarely observed in the cutaneous section of the mouse of LMC group, H&E, $\times 720$

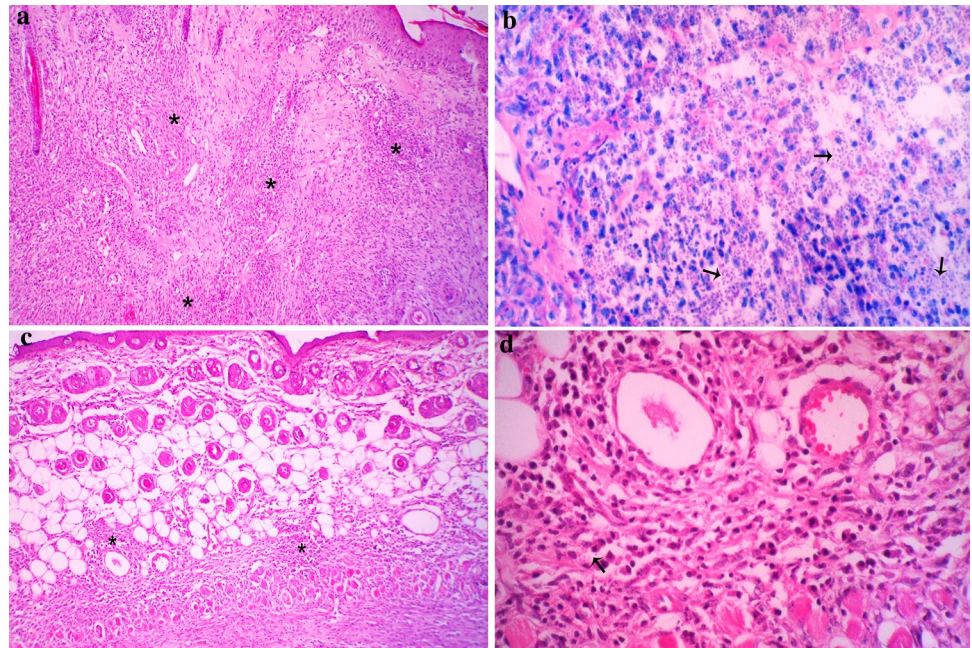


Table 1 Comparison of histopathological scores in experimental groups (mean \pm SD)

Groups	Skin lesions	Liver lesions
VLM group	2.80 \pm 0.42 ^a	2.40 \pm 0.51 ^a
LMC group	0.90 \pm 0.31 ^b	0.50 \pm 0.52 ^b

Different letters indicate statistically significant differences ($P < 0.05$)
VLM virulent *L. major*, LMC *L. major* cold strain

Immune Response Assay

In the VLM group, the IgG1 and IgG total levels increased and the IgG2a decreased. Also, IgG1 titer was found to be statistically significant ($P < 0.05$) in this group. The concentration of IgG1, IgG total, and especially IgG2a, increased in LMC group, which showed a significant difference before and after the challenge ($P < 0.05$) (Figs. 3, 4 and 5).

The increasing amount of IFN- γ was observed in the mice of LMC group which showed a significant difference from the VLM group ($P < 0.05$). Although the IL-4 level increased after the challenge with *L. major*, no significant difference was found between the groups ($P > 0.05$) (Table 2) (Fig. 6).

Discussion

Given that leishmaniasis is one of the major public health concerns, nowadays prevention, control, and treatment aspects of this disease are the common topics in the recently published research around the world. Due to the various limitations in control strategies and the low efficacy of common

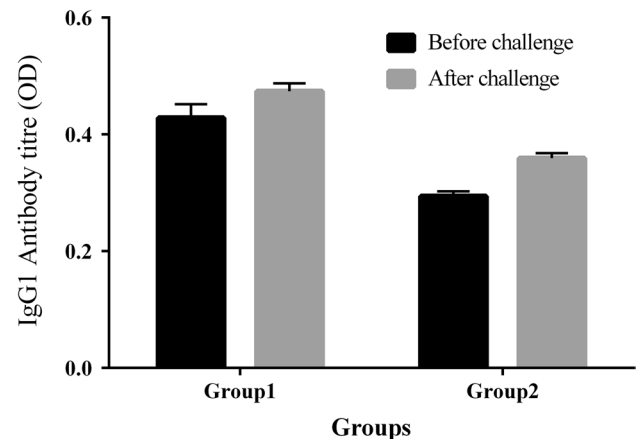


Fig. 3 The IgG1 level in BALB/c mice, before and 14 weeks after the challenge. VLM virulent *L. major*, LMC *L. major* cold strain

chemotherapy, developing an effective vaccine against three different forms of leishmaniasis seems to be the most logical and effective policy in control strategy [13, 18, 27]. However, despite recent substantial efforts by many researchers on different types of *Leishmania* vaccines, including killed parasites, mosquito salivary gland antigens, recombinant *Leishmania* antigens such as *L. major* stress-inducible protein 1 (LmSTI1) [3, 6], glycoprotein 63 (gp63), and membrane glycoprotein 46 (gp46) [8, 25] in experimental models, there is no effective commercial vaccine available [15, 39]. Based on recent investigations, a high degree of protection can be gained by immunization with live-attenuated or first-generation vaccine and/or drug-sensitive strains of *Leishmania* [13, 29, 37, 38]. Based on the previous studies

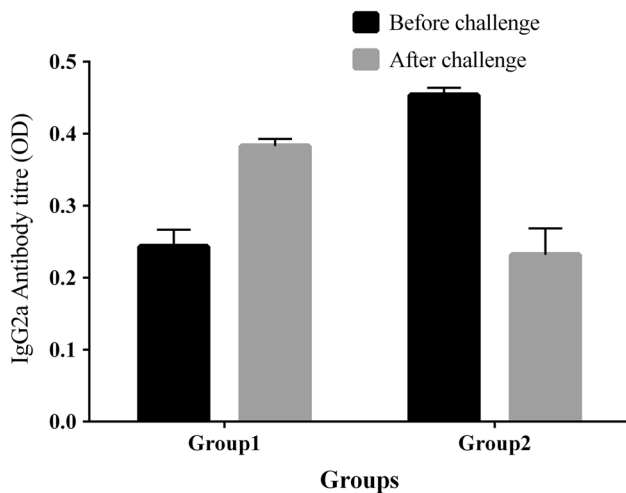


Fig. 4 The IgG2a level in BALB/c mice, before and 14 weeks after the challenge. VLM virulent *L. major*, LMC *L. major* cold strain

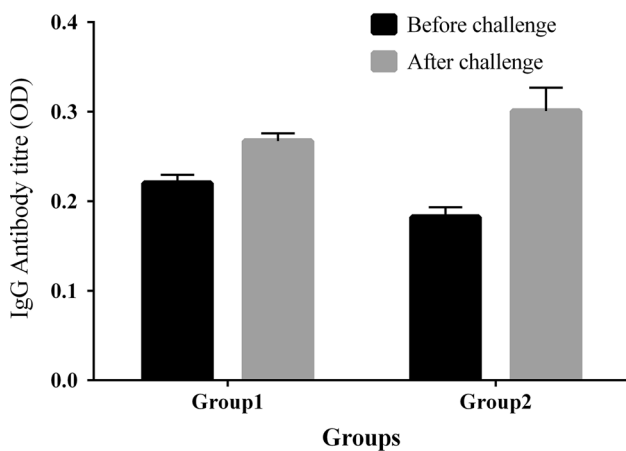


Fig. 5 The IgG total level in BALB/c mice, before and 14 weeks after the challenge. VLM virulent *L. major*, LMC *L. major* cold strain

Table 2 The mean \pm SD of parameter values in experimental groups

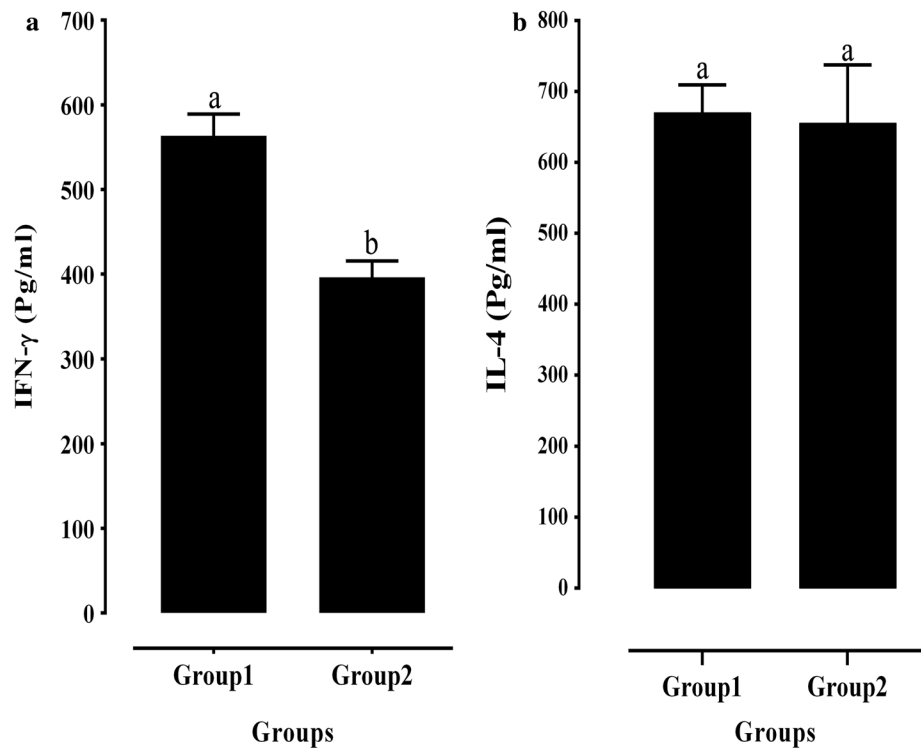
Groups	IFN- γ (Pg/ml)	IL-4 (Pg/ml)
VLM group	396.35 \pm 42.535 ^b	655.67 \pm 183.49 ^a
LMC group	563.56 \pm 57.35 ^a	670.41 \pm 86.91 ^a

Different letters indicate statistically significant differences

VLM virulent *L. major*, LMC *L. major* cold strain

on host immune response against cutaneous leishmaniasis (caused by *L. major*), protection and healing of the lesions occurred when a high level of IFN- γ , IL-12, IL-2 and low levels of IL-4 and IL-5 were produced as a result of Th1 immune response activation [5, 18, 33]. The challenge of susceptible BALB/c mice with virulent amastigotes of *L. major* followed by 4×10^6 LMC strain as a live-attenuated variety of *L. major* in the present study revealed a significant increase in IFN- γ concentration. The findings of the present study demonstrated that induction of Th1 response by live-attenuated variety of *L. major* leads to milder histopathological alterations with a small number of Leishman bodies in skin lesions and liver sections in LMC group versus VLM group. Previous studies have presented a correlation between high levels of IgG2a with Th1 response and disease control [4, 40]. High concentration of IgG2a in LMC group showed that immunization of mice with 4×10^6 LMC strain induced high levels of immunogenicity and protection; therefore, protection was achieved in the experimental challenge group. Additionally, significant progress in wound healing was recorded in LMC group, whose lesion size at the injection site decreased when compared to lesions of VLM group. A similar study proved that dogs immunized with live-attenuated of *L. donovani* recovered from *Leishmania* infection in a very short period of time duration [13]. Data obtained from the present investigation are also in agreement with the previous studies [32, 36, 38]. Also, our results are in consistency with the data obtained from immunization of BALB/c mice with live attenuated *L. donovani* [11]. In accordance with the results obtained from this study and the aforementioned studies, live-attenuated vaccines can be regarded as a potential strategic alternative against leishmaniasis. Live-attenuated vaccines with majority antigens and epitopes similar to a natural infection are capable of stimulating the host immune responses and induce the innate immune response of antigen-presenting cells [38]. In this study, LMC strain practically showed this leishmaniasis novel control approach could delete parasites and simultaneously enhance protective immunity against leishmaniasis. Consequently, we suggest that, in the future studies, more attention be directed at the attenuated vaccines for immunization against *Leishmania* parasites so that they be regarded as a potentially suitable vaccine candidate for the protection of the host against this disease.

Fig. 6 **a** The IFN- γ concentration produced by spleen cells in BALB/c mice. **b** The IL-4 concentration produced by spleen cells in BALB/c mice. VLM virulent *L. major*, LMC *L. major* cold strain



Acknowledgements This work was supported by the Research Council of Shiraz University and School of Veterinary Medicine, Shiraz University (94GCU5M83832) and the Razi Vaccine and Serum Research Institute, Shiraz Branch, Agricultural Research, Education and Extension Organization (AREEO).

Compliance with Ethical Standards

Conflict of Interest The authors have no competing interests.

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