



# A lentiviral vaccine expressing KMP11-HASPB fusion protein increases immune response to *Leishmania major* in BALB/C

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## Abstract

Hydrophilic acylated surface protein B (HASPB) is an immunogenic *Leishmania*-specific protein that antibodies are produced against it in the sera of *Leishmania*-infected individuals. Kinetoplastid membrane protein 11 (KMP11) is another *Leishmania* antigen and considered as the suitable candidate for vaccine development Leishmaniasis. It is a highly conserved surface protein expressed in both promastigotes and amastigotes. In this study, KMP11 and HASPB coding sequences were cloned into a pCDH-cGFP lentiviral vector as a fusion protein to be used as a DNA vaccine against *L. major*. The KMP11-HASPB fusion protein was successfully expressed as evidenced by RT-PCR and Western blot assays. The effect of the vaccine was determined by evaluating the level of IFN- $\gamma$ , IL-10, IgG1, and IgG2a performed using ELISA as well as determining the parasite load after challenge with *L. major* in vaccinated mice. The results revealed that IFN- $\gamma$ , IL-10, IgG1, and IgG2a significantly increased after vaccination using KMP11-HASPB-expressing lentiviruses in BALB/c mice. It is noteworthy that the level of IFN- $\gamma$  and IgG2a was higher than that of IL-10 and IgG1, respectively, which indicates the activation Th1 cells, macrophages, and cellular immunity. Moreover, the parasite load in the spleen and lymph node of vaccinated mice after challenge was significantly lower than that of controls.

**Keywords** KMP11 · HASPB · Recombinant lentiviral · Leishmaniasis vaccines

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## Introduction

Leishmaniasis is an important zoonotic disease caused by facultative intracellular protozoa belonging to *Leishmania* genus, a Kinetoplastida that infects the macrophages of vertebrates and has more than 30 species, more than 20 of which are pathogenic for human (Alvar et al. 2012). According to the World Health Organization (WHO), nearly 350 million individuals are susceptible to the infection, and nearly 12 million are infected annually, among which 1.5–2 million and 500,000 suffer from cutaneous and visceral Leishmaniasis (Vlach et al.), respectively (Conter et al. 2017).

The disease has been observed sporadically in rural parts of Palestine, Jordan, Senegal, Mali, Sudan, northern Nigeria, and Angola (Akilov et al. 2007). In Iran, the disease is endemic in north and northeast of Isfahan, Torkaman Sahra, Gonbad, Sarakhs, and Esferaien. The most important endemic region, however, is northern Isfahan (Talari et al. 2006; Hashemi et al. 2016).

The drugs of choice in Leishmaniasis treatment include antimony sodium gluconate, pentostam, antimony-containing liposomes, and liposomal amphotericin B (Khamesipour 2014). Nonetheless, treatment with some of these drugs has been limited because of toxicity, not to mention they are expensive (Sadeghian and Nilforoushzadeh 2006).

More than 30 different protein antigens have been considered for vaccine development, such as Lipophosphoglycan (LPG), Thiol-specific antioxidant antigen (Memariani et al.), Glycoprotein 63 (gp63), *Leishmania* homolog of receptors for activated C-kinase (LACK), *Leishmania major* stress-inducible protein 1 (LmST1), and combinations of them. These antigens have been used as either second-generation vaccines or third-generation vaccines, i.e., DNA vaccines. DNA vaccines have several advantages over the first- and second-generation vaccines. For example, they can induce both CD4<sup>+</sup> and CD8<sup>+</sup> T cells, several DNA sequences of antigenic proteins can be incorporated into a single DNA vaccine, and their production is more convenient and cheaper than other types of vaccines (Rivas-Santiago and Cervantes-Villagrana 2014; Engwerda and Matlashewski 2015).

Hydrophilic acylated surface protein B (HASP B) is a membrane protein of *Leishmania*, which is a proper candidate for vaccine development. It is highly immunogenic, and antibodies against it rise rapidly in the serum of CL and VL patients (Mohapatra et al. 2010). In canine models, HASP B DNA vaccine induced long-lasting immunity against *L. donovani* (Zackay et al. 2013).

Kinetoplastida membrane protein-11 (KMP11) is another immunogenic protein of *Leishmania*, which was introduced by Jardim and colleagues, for the first time, as a T cell-interacting protein (Jardim et al. 1991). KMP11 is a Kinetoplastida-specific protein, which is

capable of inducing both innate and acquired immunity against *Leishmania*. In addition, it has a low homology with human proteins, which makes it a suitable candidate for vaccine development (Jardim et al. 1995). Therefore, it has been evaluated extensively in immune protection studies against *Leishmania*, and it was indicated that KMP11 has a strong potential to induce immune protection in mouse (Stebeck et al. 1995; Guha et al. 2013). Moreover, in 2012, Maroof et al. found out that the adenoviral DNA vaccine harboring KMP11 and HASP B resulted in a protective immune response against *L. donovani* in mice (Maroof et al. 2012). The latest study, first in the human trial, showed that ChAd63-KH, an adenovirus expressing a novel synthetic gene (KH) encoding two *Leishmania* proteins KMP-11 and HASP B, is safe and induces cytokine-producing CD8<sup>+</sup> T cells.

Our results in the past study indicated that lentiviral vaccine expressing the KMP11-HASP B fusion protein of *L. infantum* is capable of inducing the immune system to produce cytokines and specific antibodies against *L. infantum* and resulted in decreased parasite burden (Mortazavidehkordi et al. 2016). Since these two proteins have been detected in different species of *Leishmania* and have a high degree of similarity among species, therefore, for the first time, we developed a lentiviral DNA vaccine containing KMP11-HASP B coding sequence of *L. major* and evaluated its potentials in a mouse model of *L. major*.

## Materials and methods

### Cell cultures, parasite culture, and animals

HEK293T cells (human embryonic kidney, ATCC CRL-3216), purchased from Mede Bioeconomy Company, Iran, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA). The medium was supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and penicillin (100 units/ml)/streptomycin (100 mg/ml) (Invitrogen, Carlsbad, CA, USA). The cells were incubated at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

*L. major* (MHRO/IR/75/ER) was obtained from Pasteur Institute of Iran, and the promastigote form of the parasite was cultured in RPMI 1640 (Gibco, UK), supplemented with 20% FBS and penicillin (100 units/ml)/streptomycin (100 µg/ml) (Invitrogen, Carlsbad, CA, USA) to obtain sufficient number of promastigotes, as described elsewhere (Hashemi et al. 2018).

To evaluate the recombinant vaccine, 30 inbred, 8–10-week-old BALB/c mice, which were purchased from Pasteur Institute of Iran, were maintained in the laboratory. Animal maintenance and sample collections were performed

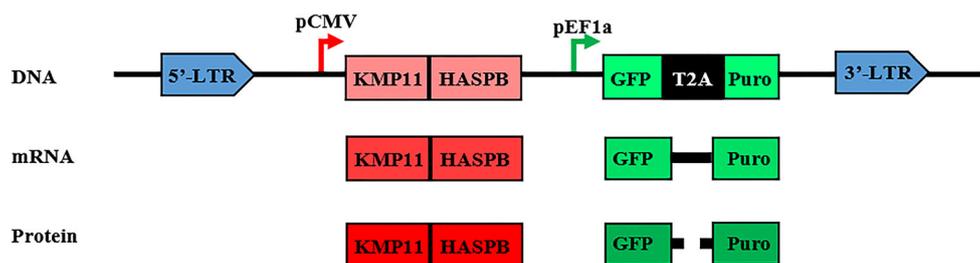
according to the National Animal Care approved by the Institutional animal care of Isfahan University of medical sciences based on the National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education (MOHME) of Iran (2005).

### Design, synthesis, and confirmation of the construct

The coding sequences (CDS) of HASPB and KMP11 (Accession numbers AJ237587.1 and M\_003722567, respectively) of *L. major* (Friedlin) were retrieved from GeneBank, NCBI. After codon optimization, the sequences were synthesized by Genscript, USA, and incorporated into pUC57 plasmid. As shown in Fig. 1, the KMP11-HASPB fusion protein and GFP are expressed under pCMV and eEF promoters, respectively. The transfer vector expresses three proteins, KMP11-HASPB fusion protein from one transcript, and GFP and puromycin, separated from another one by T2A (Fig. 1). The plasmid was used to transform competent *E. coli* DH5 $\alpha$  cells. The transformed bacteria were then cultured on ampicillin-containing LB agar at 37 °C for 24 h. Colonies appeared on the plates were used for plasmid extraction. Plasmid was extracted using Miniprep plasmid extraction kit (Qiagen, Germany) according to the manufacturer's instructions. The plasmid was then digested with *HindIII* and *EcoRI*, electrophoresed on 1% agarose gel. Finally, it was sequenced to further confirm the cloning procedure.

### Construction of KMP11-HASPB-containing recombinant lentiviral vector

Previously cloned in pUC57, KMP11-HASPB fragment was sub-cloned in pCDH513B (pCDH-KH hereafter) using *XbaI* and *BamHI*. The recombinant pCDH-KH was digested using *XbaI* and *BamHI* restriction enzymes and then sequenced in order to confirm the cloning procedure.



**Fig. 1** Schematic presentation of the KMP11-HASPB construct. The sequences of KMP11 (Accession#: XM\_003722566) and HASPB (Accession#: AJ237587.1) were retrieved from GeneBank, NCBI. The sequences were synthesized by Genscript, USA, and were incorporated into pUC57 plasmid. In this construct, the KMP11-HASPB is transcribed

### Virus production (rLentivirus-KH and lentivirus-empty)

According to Trono protocol, HEK293T cells were transfected using transfer lentiviral vector (pCDH-KH or pCDH-empty) in conjunction with three plasmids (pLP1, pLP2, and VSV-G) using calcium phosphate transfection method (Barde et al. 2010). On the first day,  $5 \times 10^6$  HEK293T cells were cultured in a 10-cm plate containing DMEM supplemented with 10% FBS. On the second day, 21  $\mu$ g of the transfer vector (pCDH-KH or pCDH-empty), 15  $\mu$ g the pLP1 vector, 6  $\mu$ g pLP2, and 5  $\mu$ g of VSV-G vector were mixed with transfection buffer and added to the cells drop-wise. The medium was replaced with fresh medium within 14 h after transfection. GFP expression was assessed after 24 h using a fluorescent microscope. Subsequently, the packaged recombinant lentiviruses (named rLentivirus-KH hereafter) were harvested from the supernatant 24, 48, and 72 h post-transfection by centrifugation at 350g at 4 °C for 5 min. The supernatant was then filtered through a 0.22- $\mu$ m filter. The recombinant viruses were stored at  $-70$  °C for subsequent experiments. To determine the titer of packaged viruses, the number of GFP positive cells were counted using flow cytometry based on the equation “TU (Transduction Unite/ml) =  $[F \times C/V] \times D$ ” in which F is the frequency of GFP-positive cells, C is the total number of cells in the well at the time of transduction, V is the volume of inoculum in mL, and D is the lentivirus dilution factor. The control vector, not containing the synthetic gene, was named Lentivirus-empty. All steps have been previously described in detail (Kutner et al. 2009).

### Western blot

Expression of KMP11-HASPB fusion protein was confirmed by performing Western blot analysis on transduced HEK293T cells as described elsewhere (Khorashadizadeh et al. 2015; Hosseini et al. 2017). Briefly, rLentivirus-KH and Lentivirus-empty were used to transduce HEK293T

under CMV (Cytomegalovirus) promoter and GFP is expressed under eEF (eukaryotic elongation factor) promoter. Transfer vector expresses three proteins (KMP11-HASPB, GFP, and puromycin resistance factor) from two mRNAs

cells. The cells were then treated with 2 µg/mL puromycin (Sigma-Aldrich, USA). Subsequently, the transduced HEK293T cells (r293T-KH) were cultured in 10-cm plates. Non-transduced HEK293T cells (293T-N) were used as the control group. r293T-KH and 293T-N cells were trypsinized after 72 h and centrifuged at 190g for 5 min at 4 °C. The cells were lysed in 200 µL of protease-inhibitor-containing RIPA buffer (Thermo Fisher Scientific, IL). The lysates were centrifuged at 8800g for 12 min at 4 °C. Then, the protein concentration of the supernatants was determined using a BCA Protein Assay Kit (Thermo Fisher Scientific, IL). Lysates/lane of 30 µg were loaded onto a 12% SDS-PAGE gel and then transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, CA). Anti-KMP11 mouse monoclonal antibody (Abcam, USA) and mouse anti β-actin antibody (Abcam, USA) at 1:1000 dilution were used for immunoblotting. Five percent non-fat milk was used to block the membranes. The second antibody was Anti-mouse IgG monoclonal rabbit antibody (Abcam, USA) at the dilution of 1:2000. Finally, chemiluminescence (ECL) method was used to visualize the protein band, and the obtained integrated optical density (IOD) of each band was measured and normalized using β-actin internal standard.

### Reverse transcription PCR

HEK293T cells pretreated with rLentivirus-KH and Lentivirus-empty for 72 h were subjected to total RNA extraction using RNeasy® Plus Mini Kit (Alameda, CA, United States). cDNA synthesis was performed using QuantiTect Rev. Transcription Kit (Qiagen, Germany). Specific primers for the gene construct were Forward: 5'ATGGCCACCACGTACGA3' and Reverse: 5'CACGCTGCCGCAACTAG3'. PCR thermal cycling was 1 cycle of 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, and a final extension at 72 °C for 7 min. After electrophoresis on 1% agarose gel, the PCR products, stained with DNA safe stain, were visualized with a UV trans-illuminator (SABZ biomedical, Iran) and digitally photographed.

### Immunization of mice

Thirty inbred, 8-week-old BALB/c mice were divided randomly into three groups ( $n = 10$ )—group 1 (rLentivirus-KH), group 2 (lentivirus-empty), and group 3 (PBS). The mice in group 1 were subcutaneously injected twice during 1 week (with a 3-day interval) with  $1 \times 10^6$  TU of recombinant rLentivirus-KH ( $2 \times 10^6$  TU/ml). The control mice in groups 2 and 3 were also injected subcutaneously with  $1 \times 10^6$  TU of recombinant lentivirus-empty ( $1 \times 10^7$  TU/ml) or 0.1 ml PBS,

respectively. Three weeks after the immunization, immunized mice of each group were used for cytokine assay. Then, immunized mice from each group were challenged subcutaneously with  $1 \times 10^7$  stationary-phase *L. major* (MHRO/IR/75/ER) promastigotes, in the right hind footpad. The development of infection was followed by measuring footpad swelling using a metric caliper. The lesion size was defined as the mean of thickness of footpad.

### Cytokine analysis

Splenocytes of five mice were isolated and analyzed 3 weeks after challenge for the expression of cytokines as described elsewhere (Pirdel et al. 2014). Briefly,  $2 \times 10^6$  splenocytes/well of mice from all three groups were recovered after homogenization of the spleen. The cells were plated in a 96-well plate in duplicate after three washing steps and stimulated with *L. major* lysate, SLA (soluble *Leishmania* antigen), or medium alone. After 72 h, supernatants were collected, and IFN-γ as well as IL-10 were evaluated in triplicate by sandwich ELISA kits (Abcam, USA) according to the manufacturer's instructions.

### Antibody ELISA

Three weeks after challenge with *L. major*, the humoral immune response was assessed as described elsewhere (Kaur et al. 2015; Seyedeh et al. 2015). The presence of antibodies in the sera of five mice was analyzed in triplicate. 96-well flat-bottom plates were coated with promastigotes of *L. major* obtained from the stationary phase of the culture. The plates were blocked using 1% ( $w/v$ ) BSA. The sera were then diluted 1:100 and applied to the plates. Afterwards, the plate was incubated with peroxidase-conjugated goat anti-mouse IgG1 (Abcam, USA) and peroxidase-conjugated goat anti-mouse IgG2a (Abcam, USA) and developed with peroxidase substrate, tetramethylbenzidine (TMB), to detect the attached antibodies. The plate was read at 450 nm using an ELISA microplate reader (Bio-Rad, USA).

### Parasite burden

The five mice were subcutaneously injected 3 weeks after the last vaccination with  $1 \times 10^7$  stationary-phase *L. major* promastigotes. The parasite burden of the lymph node and spleen of the vaccinated mice were quantified 8 weeks after infection by limiting dilution, as described previously (Soudi et al. 2011). Using a glass grinder, the tissues were homogenized, and the suspensions were serially diluted (two-fold) with RPMI culture medium in 96-well microplates. Ten days after plating, each well was evaluated for the presence of parasites. Calculation of the

number of parasites in the original tissues was performed based on the dilution factor of the last positive well using this formula:

$$\text{Parasite burden} = -\log(\text{parasite number}/\text{tissue weight})$$

### Statistical analysis

Mann-Whitney statistical test was used to analyze the data using SPSS (V.18). A  $p$  value  $< 0.05$  was considered as statistically significant.

## Results

### Construction of the KMP11-HASPB recombinant lentiviral vector

The construct used in this study has been shown in Fig. 1. KMP11-HASPB sequence was sub-cloned in pCDH-cGFP successfully. *XbaI* and *BamHI* restriction enzymes were used to digest the construct. Two fragments of 8200 bp length (pCDH) and nearly 800 bp length (KMP11-HASPB) were obtained as shown in Fig. 2. Finally, the cloning of the construct was further confirmed by sequencing.

### Packaging and titration of the recombinant lentiviral particles

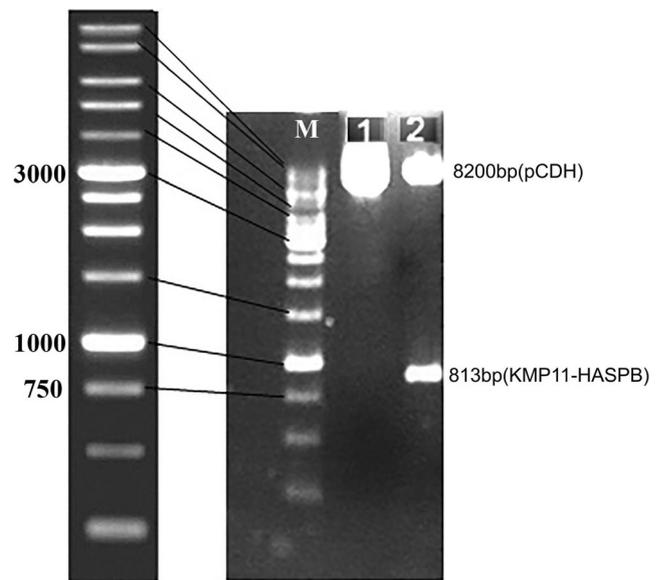
cGFP expression using fluorescent microscopy was assessed to verify the packaging of the recombinant lentivirus. After 72 h, more than 90% of HEK 293T cells was shown to be successfully transfected using lentivirus containing pCDH-KMP11-HSABP (Fig. 3a). The titer of the recombinant virus (rLentivirus-KH) was  $2 \times 10^6$  TU/mL based on the cGFP expression. Lentivirus-empty had a titer over  $1 \times 10^7$  TU/mL.

### Transduction and expression of KMP11-HASPB

RT-PCR and Western blot methods were used to evaluate the expression of KMP11-HASPB mRNA and the fusion protein, respectively, in transduced HEK293T cells (Fig. 3). The presence of nearly 800 bp (Fig. 3b) and of KMP11-HASPB fusion protein bands confirmed the expression of the fusion protein at mRNA and protein levels, respectively (Fig. 3c). In controls, transduced with the lentivirus-empty and non-transduced, bands were observed in neither RT-PCR nor Western blot assays.

### IgG2a and IgG1 assay

Three weeks post-injection, the titer of IgG1 and IgG2a antibodies against *L. major* in the sera of the rLentivirus-



**Fig. 2** Construction of recombinant lentiviral vector. Analysis of recombinant pCDH-KH by restriction enzyme digestion using *XbaI* and *BamHI* showed two fragments with 8200 bp (pCDH) and nearly 800 bp length (KMP11-HASPB). Lane 1: undigested plasmid, Lane 2: digested plasmid, M: 1 kb DNA marker

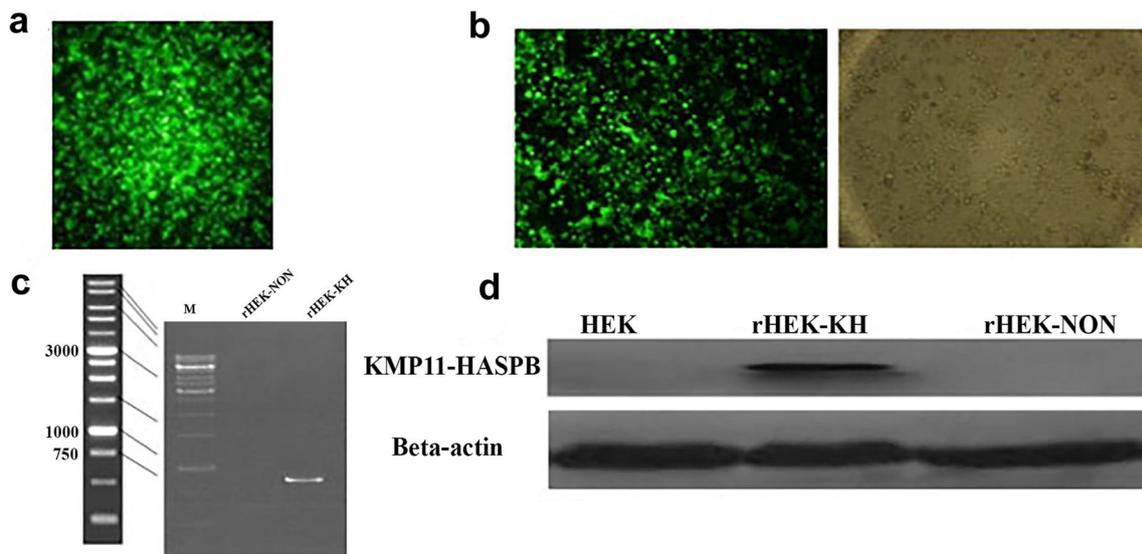
KH-immunized mice were detected by ELISA method to determine the immune-response-induction ability of the fusion protein. Mice that received PBS or lentivirus-empty were used as controls. According to the results shown in Fig. 4a, mice vaccinated with rLentivirus-KH showed a significant increase ( $p < 0.05$ ) of IgG1 and IgG2a levels in comparison with control groups. Furthermore, as shown in Fig. 4, IgG2a level was significantly higher than IgG1 level ( $p < 0.05$ ).

### IL-10 and IFN- $\gamma$ concentration

The levels of IFN- $\gamma$  (Th1) and IL-10 (Th2) were measured in the supernatant of splenocytes of mice vaccinated with rLentivirus-KH in presence of SLA to determine whether the rLentivirus-KH vaccine was able to induce Th1/Th2 immune response (Fig. 5). The results indicated that the level of IFN- $\gamma$  and IL-10 produced by splenocytes of rLentivirus-KH-immunized mice were higher than that of controls significantly ( $p < 0.05$ ). It should be mentioned that the level of IFN- $\gamma$  was significantly higher than that of IL-10 ( $p < 0.05$ ).

### Protection against *L. major*

We evaluated the efficacy of the rLentivirus-KH vaccine against experimental challenge with *L. major*. To do so, the immunized mice were challenged by subcutaneous injection of  $1 \times 10^7$  *L. major* promastigotes. Compared with those receiving PBS or lentivirus-empty, a significant decrease of parasites was observed in rLentivirus-KH-



**Fig. 3** Transfection of HEK293T by three plasmids (pCDH, psPAX, and pMD2), transduction of HEK293T by lentiviral particles, RT-PCR, and Western blot. **a** HEK293T 72 h after transfection. **b** HEK293T cells prior to transduction (left) and transduced HEK293T cells by rLentivirus-KH. Comparison of both pictures showed the high transduction efficiency higher than 90%. **c** RT-PCR detected the expression of KMP11-HASPB mRNA in HEK293T cells after transfection. Nearly 800 bp band indicates the presence of the mRNA of the fusion protein KMP11-HASPB in transduced cells with recombinant virus (rLentivirus-KH). **d** Equal

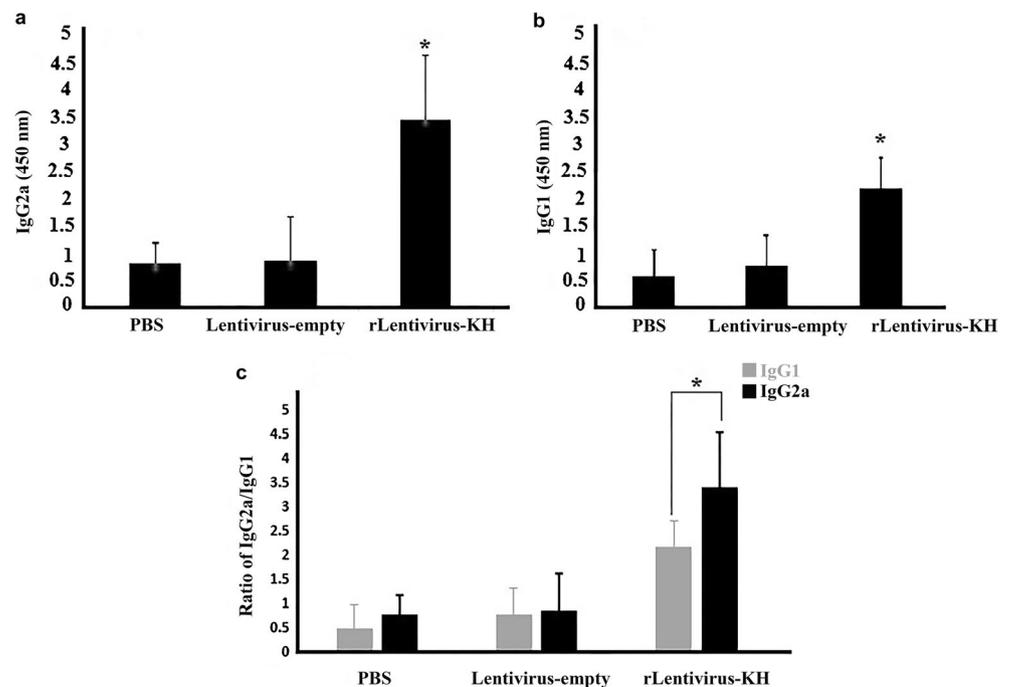
amount of the cell extracts (rLentivirus-KH, lentivirus-empty, and mock) were loaded on all lanes. Immunoblotting was performed using mouse monoclonal antibody against KMP11 (Abcam, USA) and mouse anti  $\beta$ -actin antibody (Abcam, USA). A band of KMP11-HASPB confirmed the presence of the fusion protein (KMP11-HASPB) in the HEK293T cell lysate transduced with rLentivirus-KH. However, there was no detectable band in the control cells (transduced with the lentivirus-empty and non-transduced)

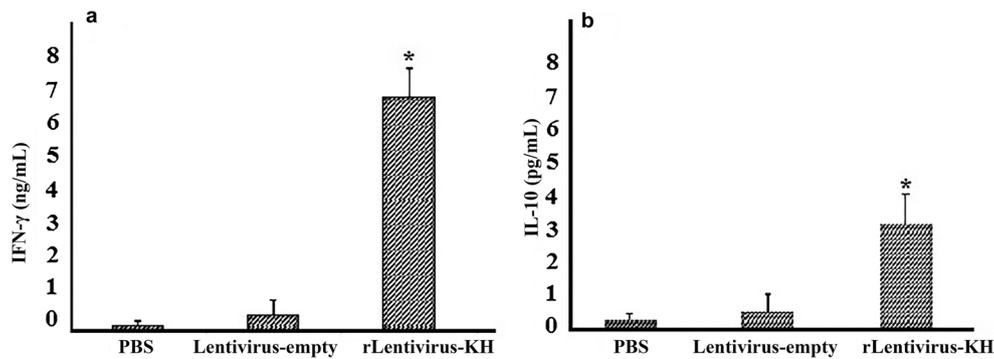
immunized mice (Fig. 6). The number of parasites in spleen and lymph node of the rLentivirus-KH-immunized mice was significantly lower than that of PBS and lentivirus-empty groups. At 8 weeks post-infection, there are significant differences in footpad swelling between mice immunized in comparison with control groups.

## Discussion

In the present study, for the first time, we evaluated the effects of a lentiviral vaccine, expressing KMP11-HASPB fusion protein, on the induction of the immune system in mice against *L. major*.

**Fig. 4** The level of KMP11-HASP IgG antibody in the sera of immunized mice. Three weeks post-injection, the titer of IgG2a (a) and IgG1 (b) antibodies against *L. major* in the sera of the mice immunized with rLentivirus-KH were detected by ELISA method. IgG1 and IgG2a concentration produced by rLentivirus-KH-immunized mice were significantly higher than that of controls (rLentivirus-empty and PBS). The results showed that the vaccinated mice had a significant increase in IgG2a production in comparison with IgG1. Data are presented as means  $\pm$  SD. All tests were performed in triplicate. \* $P < 0.05$





**Fig. 5** Analysis of IFN- $\gamma$  and IL-10 levels in the supernatants of splenocytes stimulated with lysate of *L. major*. **a** IFN- $\gamma$  produced by splenocytes of rLentivirus-KH-immunized mice was significantly higher than that of controls (lentivirus-empty and PBS). **b** IL-10 produced by

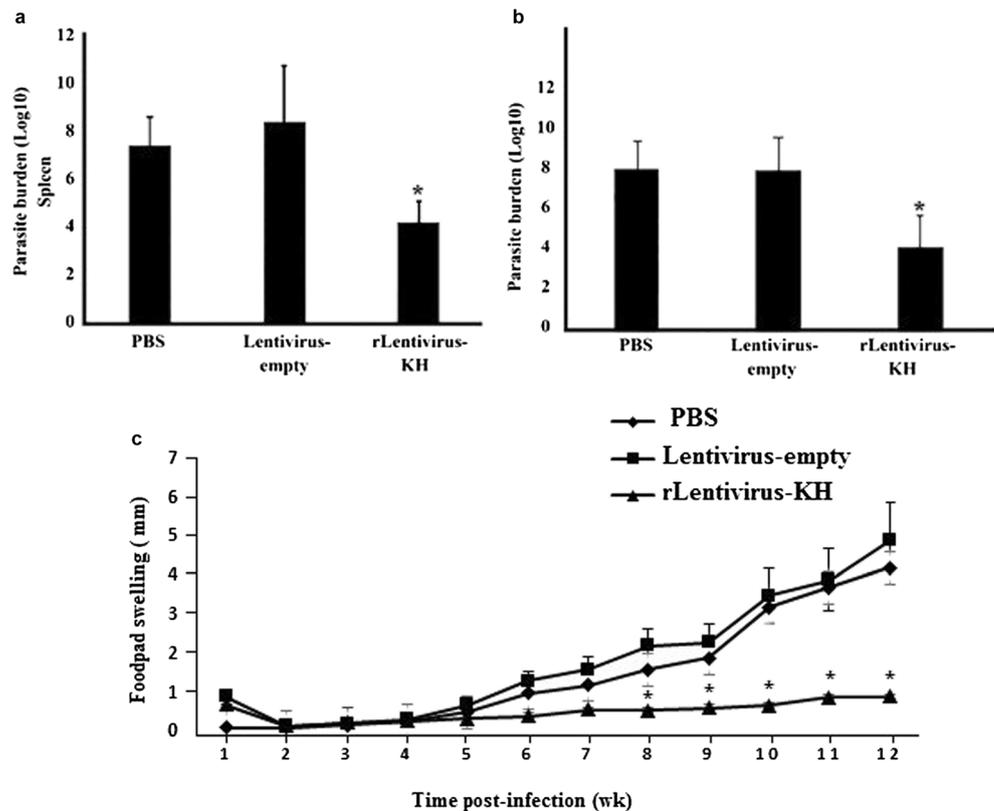
splenocytes of rLentivirus-KH-immunized mice was significantly higher than that of controls (lentivirus-empty and PBS). It is noteworthy that the level of IFN- $\gamma$  was significantly higher than that of IL-10. Data are presented as means  $\pm$  SD. All tests were performed in triplicate, \* $P < 0.05$

HASPB is an immunogenic protein found in all kinetoplastids. Sera from patients suffering from either VL or CL react with recombinant HASPB with high specificity and sensitivity (Jensen et al. 1996; Jensen et al. 1999). A hydrophilic acylated protein recently found in *L. braziliensis*, the ortholog of HASPB of other *Leishmania* species, is also detected by sera taken from *L. braziliensis*-infected patients (Depledge et al. 2010). This indicates that HASPB family of proteins is ubiquitously present in *Leishmania* species and is a major immunogenic protein of *Leishmania*. Vaccination with recombinant *L. donovani* HASPB at a low dose can induce long-term protection when administered in the absence of

exogenous antigen in mice (Stager et al. 2000; Stager et al. 2003). It has also been observed that recombinant HASPB is immunogenic in dogs and is capable of inducing a considerable protection against canine Leishmaniasis in canine animal models (Moreno et al. 2007).

KMP11, as mentioned earlier, is a kinetoplastida-specific protein (Jardim et al. 1991). Since it is not similar to human antigens, it is capable of stimulating both innate and acquired immunity. All vaccinations using KMP11, either as a recombinant protein or DNA vaccine, confirmed the potential of this antigen in the induction of immune system. For example, Basu and colleagues showed that KMP11 can protect mice

**Fig. 6** Protection against *L. major* infection by rLentivirus-KH vaccination. Mice inoculated with PBS, lentivirus-empty, or rLentivirus-KH were challenged with *L. major*. The number of parasites in the spleen (a) and lymph node (b) was measured 8 weeks after the infection. The immunized mice showed a reduction in the number of parasites in spleen and lymph node compared with PBS and lentivirus-empty groups. **c** Footpad swelling was measured weekly. According to the results shown in Fig. 6c, mice vaccinated with rLentivirus-KH showed a significant increase of footpad swelling in comparison with control groups. Data are presented as means  $\pm$  SD. All tests were performed in triplicate, \* $P < 0.05$



against the parasite by increasing the level of IL-12, IFN- $\gamma$ , and TNF and decreasing IL-10 level (Basu et al. 2005). In addition, KMP11-expressing DNA vaccine used in hamsters after challenge with *L. chagasi* resulted in the lowered parasite burden in the lymph node and spleen and increased level of IFN- $\gamma$ , compared to IL-10 (da Silva et al. 2011). In the majority of the studies, the immunogenicity of this antigen was evaluated in VL including *L. infantum* and *L. donovani*. In the present study, we have assessed the protection efficacy of KMP11-HASPB against *L. major* in a mouse model.

In previous studies, it was demonstrated that suitable protein antigens used for *Leishmania* vaccination should be capable of inducing the immune system to produce IgG2a. To assess the efficacy of the fusion protein, the level of specific antibodies (IgG1 and IgG2a) was measured in immunized and control mice. The results demonstrated that the level of specific IgG1 and IgG2a significantly increased in vaccinated mice compared with the control group ( $p < 0.05$ ). In addition, the serum level of IgG2a was more than that of IgG1 ( $p < 0.05$ ). These findings show the proper stimulation of immune system against *Leishmania* by rLentivirus-KH vaccine.

Since cytokines play an important role in the type of immunity developed against *L. major*, we examined the level of IFN- $\gamma$  and IL-10 produced by mice splenocytes. As expected, splenocytes of vaccinated mice produced a significant level of IFN- $\gamma$  compared to the control group and that of IL-10 ( $p < 0.05$ ). Based on previous research, effective vaccination against *Leishmania* leads to the production of Th1-related cytokines such as IFN- $\gamma$  which in turn stimulates the differentiation of Th1 cells and activation of TC, natural killer cells (NK), and M1 macrophages. Subsequently, activation of M1 macrophages results in the production of TNF and NO, which play a key role in the activation of macrophages and their abilities to eradicate pathogens (Podinovskaia and Descoteaux 2015). In fact, one of the main reasons why immune system cannot successfully eliminate *Leishmania* is its ineffective activation which results from the insufficient production of cytokines that activate them. Therefore, an important parameter in evaluating the efficacy of a vaccine is the level of pro-inflammatory cytokines such as IFN- $\gamma$ , TNF, and IL-12 as well as TGF- $\beta$  and IL-10 (Latifynia et al. 2013). In a desirable vaccination, the first three cytokines should be produced at high levels, and the last one should be produced at low levels. Nonetheless, the cytokine pattern of the immune system against *Leishmania* is not perfectly clear, and both inflammatory and anti-inflammatory cytokines have been detected in the sera of Leishmaniasis patients (Kautz-Neu et al. 2012). Maroof et al. and Mortazavidehkordi et al. who evaluated DNA vaccines against *L. donovani* and *L. infantum*, respectively, showed that KMP11 and HASPB are highly capable of stimulating mice immune system and can protect mice against the parasite (Maroof et al. 2012; Mortazavidehkordi et al. 2016). In the present study, we showed that KMP11-HASPB fusion protein induced the immune system of mice against *L. major* in that

the parasite load in the lymph node and spleen of the vaccinated mice was significantly lower than that of controls ( $p < 0.05$ ). Our results are in line with other researchers', and this indicates that a promising fusion protein has been probably discovered for vaccination against different species of *Leishmania*. Since these two proteins have been detected in different species of *Leishmania* and have a high degree of similarity among species, it seems that, by bioinformatics means, we can find common cross-species epitopes that can be used as a single potent vaccine against *Leishmania*. However, since the course of infection and the immune system of human and mouse are completely different, which prevents researchers to properly evaluate vaccines, more suitable animal models are needed. Moreover, to stimulate the immune system more effectively, adjuvants should also be considered in vaccination and evaluation of vaccines.

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### Compliance with ethical standards

Animal maintenance and sample collections were performed according to the National Animal Care approved by the Institutional animal care of Isfahan University of medical sciences based on the National Ethical Guidelines for Biomedical Research issued by the Research and Technology Deputy of Ministry of Health and Medicinal Education (MOHME) of Iran (2005).

**Conflict of interest** The authors declare that they have no conflicts of interest.

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