Vaccine 32 (2014) 6659–6666

Contents lists available at ScienceDirect

Vaccine

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

Immunization of mice with a novel recombinant molecular chaperon confers protection against *Brucella melitensis* infection

Amir Ghasemi[a], Mahmood Jeddi-Tehrani[b,**], Josef Mautner[c], Mohammad Hossein Salari[d], Amir-Hassan Zarnani[e,f,*]

[a] Department of Microbiology and Immunology, Faculty of Medicine, Kashan University of Medical Sciences, Kashan, Iran
[b] Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
[c] Technische Universität München & Helmholtz Zentrum München, Munich, Germany
[d] Department of Pathobiology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran
[e] Nanobiotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
[f] Immunology Research Center, Iran University of Medical Sciences, Tehran, Iran

ARTICLE INFO

Article history:
Received 24 June 2014
Received in revised form 4 September 2014
Accepted 5 September 2014
Available online 19 September 2014

Keywords:
Brucella
Recombinant protein
Vaccine
Molecular chaperon

ABSTRACT

Brucella spp. are zoonotic Gram-negative intracellular pathogens with the ability to survive and replicate in phagocytes. It has been shown that bacterial proteins expressed abundantly in this niche are stress-related proteins capable of triggering effective immune responses. BMEI1549 is a molecular chaperone designated DnaK that is expressed under stress conditions and helps to prevent formation of protein aggregates. In order to study the potential of DnaK as a prospective *Brucella* subunit vaccine, immunogenicity and protective efficacy of recombinant DnaK from *Brucella melitensis* was evaluated in BALB/c mice. The *dnak* gene was cloned, expressed in *Escherichia coli*, and the resulting recombinant protein used as subunit vaccine. DnaK-immunized mice showed a strong lymphocyte proliferative response to *in vitro* antigen stimulation. Although comparable levels of antigen-specific IgG2a and IgG1 were observed in immunized mice, high amounts of IFN-γ, IL-12 and IL-6, no detectable level of IL-4 and very low levels of IL-10 and IL-5 were produced by splenocytes of vaccinated mice suggesting induction of a Th1 dominant immune response by DnaK. Compared to control animals, mice vaccinated with DnaK exhibited a significant degree of protection against subsequent *Brucella* infection ($p < 0.001$), albeit this protection was less than the protection conferred by Rev.1 ($p < 0.05$). A further increase in protection was observed, when DnaK was combined with recombinant Omp31. Notably, this combination, as opposed to each component alone, induced statistically similar level of protection as induced by Rev.1 suggesting that DnaK could be viewed as a promising candidate for the development of a subunit vaccine against brucellosis.

1. Introduction

Brucella melitensis is a zoonotic Gram-negative pathogen that is an important etiological agent causing abortion and infertility in domestic animals, and undulant fever, migratory arthralgia, myalgia and osteomyelitis in humans [1,2]. Because of the severe economic and medical burden of brucellosis, vaccination of all vulnerable hosts and culling of infected animals is the only way of controlling the disease [3]. The live attenuated *B. melitensis* Rev.1 strain is the most broadly used vaccine in control programs against brucellosis in the livestock [4]. It has been shown that Rev.1 can be useful for eradicating this disease [5]. Thus, it is considered in widespread vaccination programs in many countries [6]. Nevertheless, availability of such vaccines as Rev.1 does not obviate the need for development of new vaccines due to some problems associated with application of this vaccine, included among them are eliciting long lasting immune responses against the O polysaccharide making it difficult to differentiate vaccinated animals from those naturally infected, induction of abortion when administered during pregnancy, pathogenicity for humans and resistance to streptomycin [7]. These problems have stimulated scientists to find alternative ways to protect the livestock from *Brucella* infection.

In order to increase safety, subunit vaccines have been developed but these depend on the identification of antigens able to confer protection against brucellosis. Numerous protein antigens

* Corresponding author at: Nanobiotechnology Research Center, AvicennaResearch Institute, ACECR, Tehran, Iran PO. Box: 19615-1177.
** Corresponding author at: Monoclonal Antibody Research Center, AvicennaResearch Institute, ACECR, Tehran, Iran PO. Box: 19615-1177.
E-mail addresses: Mahjed@avicenna.ac.ir (M. Jeddi-Tehrani), zarnani@avicenna.ac.ir, zarnania@gmail.com (A.-H. Zarnani).

http://dx.doi.org/10.1016/j.vaccine.2014.09.013
0264-410X/

are shown to stimulate protective immune response in mice model. The recent examples comprise HspA [8], ribosomal protein L9 [9], BLSOmp31 [10], rF278a [11], FlgJ and FliN [12], Omp31, Omp16 and BP26 expressed by invasive *Escherichia coli* vaccines [7], CobB and AsnC [13], Omp28 formulated with CpG oligonucleotides [14], P39 protein formulated with CpG oligodeoxynucleotides [15], Ado-Hcyase [16] and Rs-α [17], combination of Omp16 and Omp19 [18]. Although some of these antigens have been recently identified, the protection conferred is low in most settings. Thus, to develop efficient subunit vaccines, screening and assessment of new protective antigens is essential.

Brucella is able to infect macrophages and to persist and replicate in the intracellular environment [19]. Identifying those bacterial proteins that are necessary for intracellular survival of *Brucella* may provide new insights into mechanisms of pathogenesis and immune protection, and candidate antigens for vaccine design. We previously described that sera from Rev.1-immunized rabbits strongly reacted with the molecular chaperone DnaK of *B. melitensis*, which is also expressed in other strains [20]. The molecular chaperone DnaK (BMEI1549) is a member of the highly conserved 70-kilodalton heat-shock protein (hsp70) family [21]. Under stress conditions, DnaK assists in protein folding, translocation and interaction by binding to unfolded polypeptide domains [22]. However, No data about the immunological properties of BMEI1549 product has been reported yet. Importantly, the gene coding for this molecular chaperone is different from the previously described *Brucella* gene BMEI2002 that encodes a protein also designated DnaK. It was shown that the latter confers a partial protection against *Brucella abortus* infection in mice [23] and is necessary to resistant of *Brucella suis* to bacterial killing of macrophages [24]. In the present study, we evaluated for the first time the immunogenicity and protective efficacy of the purified recombinant DnaK (BMEI1549) in mice. Protection against subsequent infection was evaluated after vaccination with DnaK alone, or in combination two well-known protective antigens of *B. melitensis* recombinant outer membrane protein, Omp31 [20,25,26] and cytoplasmic protein, Trigger Factor (TF) [27–30]. We hypothesized that inclusion of such antigens in vaccine formulation could potentially augment the protective efficacy of each antigen alone. With this in mind, the combination of different panel of antigens was tested in our experiments.

2. Materials and methods

2.1. Bacterial strains

E. coli TOP10 and BL21 (DE3) (a gift from Dr. Pourmand, Tehran University of Medical Sciences) were used for expression of DnaK. Bacterial strains were routinely grown at 37 °C in LB broth or agar. *B. melitensis* 16 M (virulent strain) or *B. melitensis* Rev.1 (vaccine strain) were cultured in *Brucella* agar (HiMedia, Delhi, India) as described elsewhere [31].

2.2. Production and purification of DnaK

Cloning, expression, and purification of DnaK from *B. melitensis* in *E. coli* BL21 and its purification have been described previously [20]. Briefly, the *dnak* gene was amplified by PCR from genomic DNA of *B. melitensis* 16 M (Forward: 5′ CATATGACACCTT CTG 3′, Reverse: 5′ GGATCCTACCGACCAGCG 3′). The amplified DNA fragment was directly inserted into pTZ57R (InsTAclone™ PCR Cloning Kit) (Fermentas, Vilnius, Lithuania) and then subcloned into the pET28a+ vector (Novagen, Madison, WI, USA). 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was used to induce expression of DnaK. Purification of DnaK was performed under denaturing condition as previously described [32]. Contaminating endotoxins were eliminated during the purification step by 0.1% Triton X-114 in washing buffers [33–35]. Vaccine protein should be in its native form as far as effective blocking immune responses are concerned. In our experiment we first solubilized the protein in 8 M urea for the purpose of purification and at the next step we re-folded the protein in a stepwise dialyzing process with decreasing gradient of urea. Finally purified protein was dialyzed against PBS.

2.3. The SDS-PAGE and Western blotting

The purity of the recombinant protein and its identity was assessed by SDS-PAGE, Coomassie blue staining and Western blotting [20]. Briefly, purified recombinant protein was size-separated by SDS-PAGE and the proteins transferred to a nitrocellulose membrane (BioRad, USA). Next, the membrane was incubated with anti-6-His peroxidase (Roche, Mannheim, Germany) (1/40,000) for 1 h. Finally, the bound conjugates were detected using diaminobenzidine (DAB) (Sigma, NY, USA). Only purified recombinant protein with an endotoxin content of less than 0.05 endotoxin units per mg of protein (evaluated by Limulus amebocyte lysate analysis kit, Lonza, Basel, Switzerland) was used. The concentration of recombinant protein was determined by the Bradford method [36].

2.4. Mice

Six-to-eight weeks-old female BALB/c mice were purchased from Pasteur Institute of Iran. Mice were handled under best possible conditions of temperature, hygiene, humidity and light (cycles of 12 h dark/light). All experimental procedures on animals were accepted by the ethical committee of Avicenna Research Institute. After Rev.1 inoculation, mice were kept in biosafety level 3 animal facilities.

2.5. Immunization

Mice were randomly divided into seven groups. Three groups with 15 mice each received DnaK, PBS or Rev.1 vaccine only to study immunogenicity and protective efficacy. Two groups including 10 mice each received Omp31 and TF to assess lymphocyte proliferation and conferred protection. The other groups consisting of five mice each were used to evaluate and protection induced by antigen cocktails. Mice were anaesthetized with methoxyfuor ane (Mallinckrodt, Phillipsburg, NJ, USA) and immunized intraperitoneally (i.p.) either with 30 μg of DnaK, TF or Omp31, 30 μg DnaK and 30 μg TF [28], or 30 μg DnaK and 30 μg Omp31 [20], or PBS (negative control) on day 0 and 15 as described previously [23]. Briefly, mice were injected with proteins or PBS in Complete Freund's Adjuvant (CFA) (Sigma) on day 0 and with incomplete Freund's adjuvant (IFA) (Sigma) on day 15. For comparison, a control group was immunized by the subcutaneous route (s.c.) at day 0 with 8×10^8 formalin-killed Rev.1 in IFA. Sera were obtained 0, 15, 30, and 45 days after the first immunization. On day 45 after the first immunization, five mice from each group were challenged intraperitoneally *B. melitensis* 16 M, five mice were sacrificed to assess immune responses including cytokine production and proliferation assay, and the remaining five mice were bled on day 75 to monitor memory responses.

2.6. Humoral immune responses

The titers of DnaK-specific IgG1 and IgG2a antibodies in mouse sera were investigated by ELISA as previously reported [20]. In order to find a cut-off value for this test, the mean specific OD plus 3 S.D. from 20 sera from PBS-immunized mice at 1:100 dilutions was determined. Serum titers are denoted as the reciprocal of the last serum dilution giving an OD higher than the cut-off [23].

2.7. Preparation and culture of splenocytes

Thirty days after the last immunization, spleens were removed from the mice immunized with DnaK, Omp31, TF, PBS or Rev.1 and homogenized with a syringe in 10 ml PBS containing 5 mM ethylene diamine tetraacetic acid (PBS-EDTA) on ice. The cells were washed twice with PBS-EDTA and mononuclear cells (MNCs) were isolated by Ficoll–Paque (GE Healthcare, Uppsala, Sweden) discontinuous gradient centrifugation. The cells were cultured in RPMI 1640 based media (RPMI 1640 supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated FBS) at 37 °C in 5% CO_2.

2.8. Lymphocyte proliferation and cytokine assay

Mouse splenocytes were adjusted to 2×10^6 cells/ml and 100 μl of this suspension were added per well of 96-well culture plates either alone (negative control), or together with 0.25–1 μg/ml of purified DnaK, Omp31, TF, or 3 μg/ml of concanavalin A (Con A). The cells were cultured for 2 days and then incubated for 4 h with 100 μl of 1 mg/ml 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-((phenylamino) carbonyl)-2*H*-tetrazolium hydroxide (XTT) (Sigma) containing 25 μl of 5 mM phenazine methosulfate (PMS) (Sigma) per well as previously reported [8]. The optical density was read at 492 nm (Bio-Tek Instruments). The stimulation index (SI) was calculated as the ratio between the optical density values of stimulated to unstimulated cells using the following formula:

$$SI = \frac{\text{mean OD of stimulated culture} - \text{mean OD of blank control}}{\text{mean OD of unstimulated culture} - \text{mean OD of blank control}}$$

To assess cytokine production, 2×10^6 splenocytes in 2 ml of complete RPMI 1640 medium were brought out per well of a 24-well flat-bottom plate. Cells were then incubated with 1 μg/ml DnaK or 3 μg/ml Con A at 37 °C in 5% CO_2 for 48 h [10]. Control wells received PBS instead of antigen. Supernatants were collected after 48 h and stored at −70 °C for cytokine assay. Levels of interferon-gamma (IFN-γ), interleukins-(IL) 12 (p70), 10, 6, 5 and 4 were measured according to the manufacturer's instructions (BD Pharmingen). Minimal detection levels of the aforesaid cytokine sets were 31.3 pg/ml, 62.5 pg/ml, 31.3 pg/ml, 15.6 pg/ml, 15.6 pg/ml, 7.8 pg/ml, respectively.

2.9. Analysis of lymphocyte subtypes by flow cytometry

Splenocytes (1×10^6 cells/ml) were stained with fluorescein isothiocyanate (FITC) labeled anti-mouse CD3, FITC labeled anti-mouse CD4 and Phycoerythrin (PE) labeled anti-mouse CD8. FITC and PE-conjugated mouse IgG1 were used as isotype controls. Using a Partec flow cytometer (Partec PAS, Germany), lymphocytes were first gated based on their forward and sideward scatters. Data analysis was performed with FlowMax software (Partec PAS, Germany).

2.10. Protection experiments

Four weeks after the last immunization, five immunized mice per group were challenged by i.p. injection of 0.2 ml *B. melitensis* 16 M suspension containing 1×10^4 bacteria. Thirty days after bacterial challenge, mice were sacrificed and their spleens removed. Each spleen was homogenized in 1 ml 0.9% NaCl containing 0.1% Triton X-100, serially diluted and plated on Brucella agar in triplicates and incubated at 37 °C for 3–4 days [17,37]. The results were expressed as the mean log CFU ± S.D. per group.

2.11. Statistical analysis

Data among several groups was analyzed and compared by one way one factor analysis of variance (ANOVA) and Turkey's post hoc test in SPSS. p values <0.05 were considered as statistically significant.

3. Results

3.1. Production of recombinant DnaK

In order to obtain large amounts of recombinant DnaK, *E. coli* was transformed with the pET28-*dnak* plasmid and expression of the 6xhistidine-tagged protein induced with IPTG. Recombinant DnaK was purified from bacterial lysates using Ni-NTA agarose. Identity of the purified protein of 48 kDa as DnaK was verified by SDS-PAGE and Western blotting (Fig. 1). Twenty mg of the recombinant protein was obtained from 1 l of liquid culture.

3.2. Humoral responses induced by DnaK

Specific antibody titers against DnaK were measured by ELISA in sera obtained at different days after the first immunization of mice with DnaK. DnaK-specific humoral responses became detectable during the second week, peaked after six weeks, and maintained at high levels until the eleventh week after the first immunization (Fig. 2). Throughout the entire observation period, although not statistically significant, IgG2a titers were consistently higher than IgG1 titers (IgG1mean titer: 18,666; IgG2a mean titer: 20, 750). The mice vaccinated with Rev.1 also produced considerable amounts of DnaK-specific IgG1 and IgG2a responses. However, there was a significant difference between DnaK-specific IgG1 and IgG2a titers elicited in the mice immunized with DnaK compared to the amount of specific IgG1 and IgG2a against DnaK produced in Rev.1-vaccinated mice, respectively ($p < 0.001$) (Fig. 2).

3.3. Cellular immune response

To further characterize the immune responses and, splenocytes from mice immunized with DnaK, PBS, Omp31,TF or Rev.1 were isolated 45 days after the first immunization, and incubated with different concentrations of DnaK followed by analysis of lymphocyte proliferation and cytokine production. In comparison to PBS, specific proliferative responses were observed with cells from DnaK- or Rev.1-vaccinated animals (Fig. 3). We found that splenocytes of DnaK-immunized mice were more responsive to recall cognate antigen stimulation (0.25 μg/ml) compared to the TF-immunized mice. More specifically, *in vitro* proliferation assay clearly showed that as low as 0.25 μg/ml of DnaK induced the same levels of antigen-specific proliferation compared to when splenocytes from the TF-immunized mice were re-stimulated with 1 μg/ml of TF antigens. Splenocytes from TF-immunized mice did not show significant proliferative response when stimulated with 0.25 μg/ml cognate antigen (Fig. 3). Although splenocytes of Rev. 1-immunized mice did not proliferate in response to stimulation with up to 2.5 μg/ml TF, they responded to stimulation with TF in concentrations more than 2.5 μg/ml (data not shown). As positive control, splenocytes of all groups proliferated in response to ConA stimulation (data not shown).

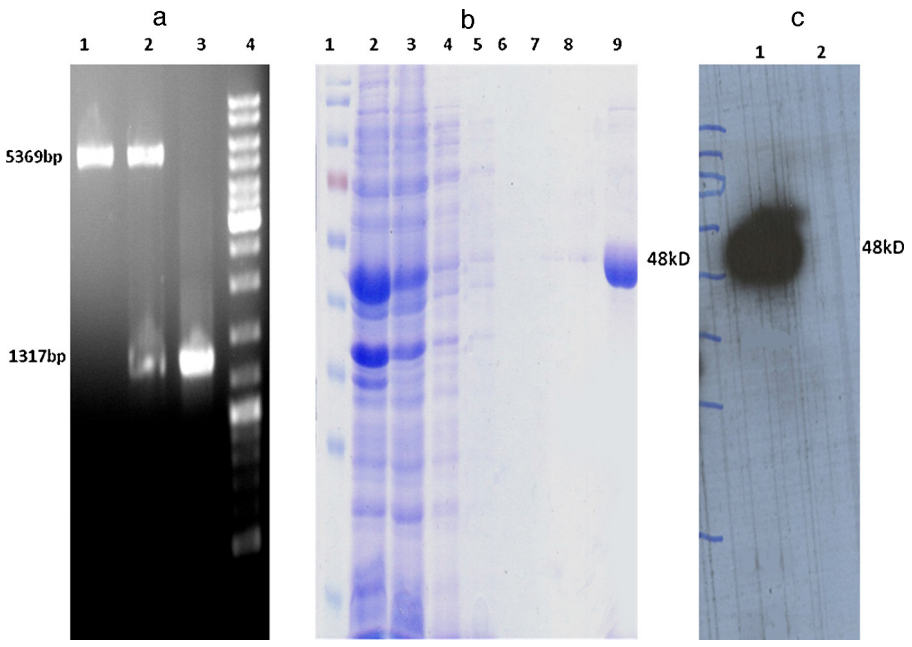

Fig. 1. Confirmation of cloning, expression and purification of DnaK. (a) Digestion of pET-*dnak* with *Nde*I and *BamH*I Lane 1: pET-28a+ digested with *Nde*I and *BamH*I Lane 2: pET-*dnak* digested with *Nde*I and *BamH*I, Lane 3: PCR product of *dnak* gene, Lan 4: molecular marker. (b) SDS-PAGE analysis of DnaK purification. Lane 1: molecular weight marker, Lane 2: after IPTG induction, Lane 3: flow through, Lanes 4 and 5: the wash with buffer containing 20 mM Imidazole, Lanes 6–8: the wash with buffer containing 40 mM Imidazole, Lane 9: eluted recombinant protein with elution buffer containing 1 M Imidazole. (c) Western blot analysis of DnaK with anti-His tag monoclonal antibody. Lane1: purified DnaK, Lane 2: lysate of untransfected bacteria.

Lymphocytes from DnaK and Rev.1-vaccinated mice secreted significantly higher amounts of IFN-γ, IL-12 and IL-6 than PBS-immunized group ($p < 0.001$), which only produced background levels of these cytokines. Such immunizations did not trigger detectable levels of IL-4 production. DnaK induced very low levels of IL-10 and IL-5 production (Fig. 4). In response to ConA, splenocytes from all groups produced IFN-γ, IL-12, IL-10, IL-5, and IL-4, with no significant differences among the groups (data not shown). In order to investigate the involvement of T cells in the DnaK-specific immune responses, CD3+, CD4+ and CD8+ T lymphocyte in mouse spleens were analyzed by flow cytometry. As shown in Fig. 5, the percentage of CD3+, CD4+ and CD8+ cells in the DnaK-vaccinated group was significantly increased in comparison to PBS-immunized mice ($p < 0.05$).

3.4. Protection assay

To analyze the level of protection induced by DnaK in mice, three groups of mice were immunized with DnaK, Rev.1, or PBS. In addition, four other groups of mice received Omp31 or TF alone, or in combination with DnaK. On day 45 after the first immunization, mice were challenged with 1×10^4 *B. melitensis* 16 M

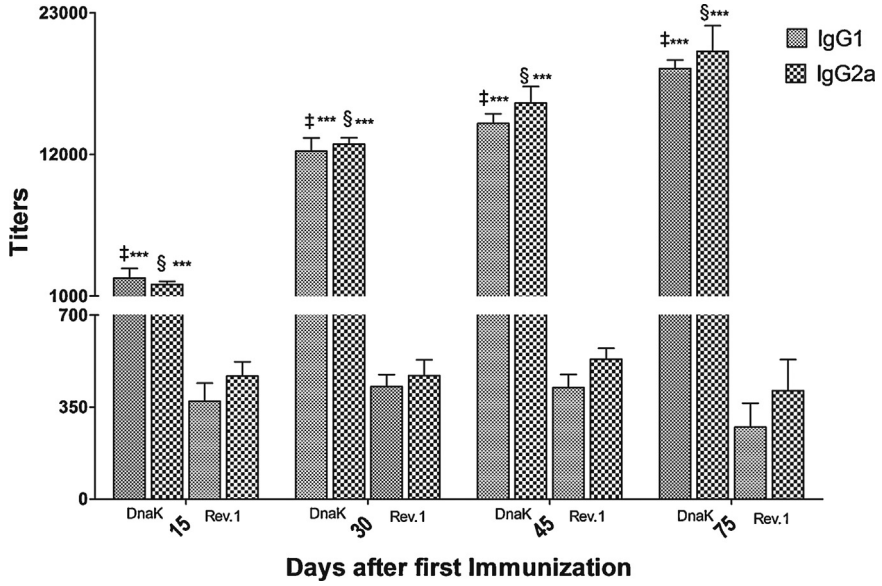

Fig. 2. Kinetics of the IgG1 and IgG2a responses elicited after immunization with DnaK. Mice were immunized with DnaK or Rev.1 and bled retroorbitally on the indicated days. Specific IgG1 and IgG2a antibody titers against DnaK were evaluated by ELISA. Titer values represent the mean ± SD of sera from five animals with three repeats. ‡ Comparison of antigen-specific anti-DnaK IgG1 in DnaK-and Rev.1-immunized mice, comparison of antigen-specific anti-DnaK IgG2a in DnaK and Rev.1-immunized mice, *** $p < 0.001$.

Table 1
Protection against *B. melitensis* infection conferred by DnaK immunization.

Immunized group ($n=5$)	Adjuvant	Log_{10}[a] CFU of *Brucella melitensis*	Units of protection[b]
PBS	CFA/IFA	4.96 ± 0.42	0
DnaK	CFA/IFA	3.327 ± 0.38[c]	1.633
TF	CFA/IFA	2.75 ± 0.16[c,d,e]	2.2
Omp31	CFA/IFA	3.3 ± 0.29[c]	1.66
DnaK + TF	CFA/IFA	3.12 ± 0.46[c]	1.84
DnaK + Omp31	CFA/IFA	3.08 ± 0.28[c]	1.88
Rev.1	IFA	2.78 ± 0.22[c,d,f]	2.18

[a] The bacteria content in spleens is represented as the mean log CFU ± S.D. per group.
[b] Units of protection were determined by deducting the mean log CFU of the immunized groups from the mean log CFU of the control (PBS-immunized) group.
[c] Significantly different from PBS-immunized mice, $p < 0.001$.
[d] Significantly different from Omp31, $p < 0.05$.
[e] Significantly different from DnaK, $p < 0.01$.
[f] Significantly different from DnaK, $p < 0.05$.

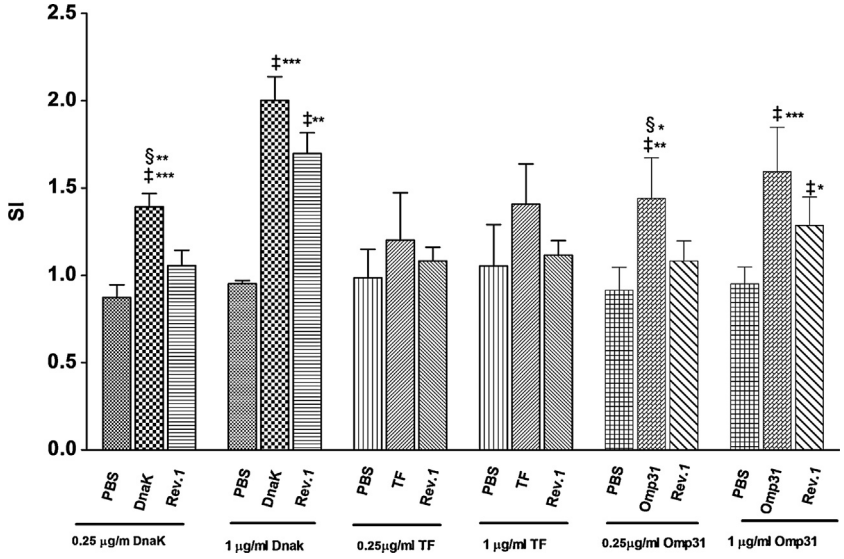

Fig. 3. Proliferative responses of spleen cells from mice immunized with DnaK. BALB/c mice were immunized with DnaK, Omp31, TF or Rev.1. Mice immunized with PBS were used as controls. Spleen cells from immunized mice were stimulated *in vitro* with 0.25–1 μg/ml purified DnaK and 3 μg/ml ConA for 48 h and the extent of proliferation was assayed by XTT. Each bar symbolizes the stimulation index (SI) computed as the ratio between the obtained mean absorbance values of stimulated cells to the unstimulated cells. The data are the mean SI ± SD of five individual mice from each group with three repeats. ‡ Comparison to PBS group and, § comparison to Rev.1 group. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

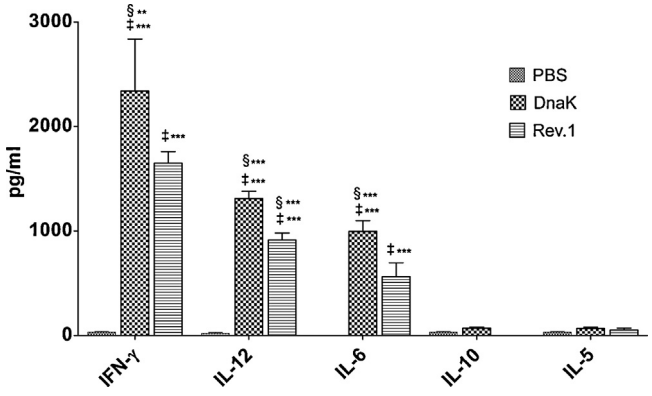

Fig. 4. Cytokine production in spleen cells from mice immunized with PBS, DnaK and Rev.1 vaccine. Spleen cells of PBS-, DnaK- or Rev.1-immunized mice were stimulated *in vitro* with 1 μg/ml DnaK for 48 h. Cytokine concentrations in culture supernatants were measured by sandwich ELISA. The data are the mean ± SD of five individual mice from each group with two repeats. ‡ Comparison of DnaK to PBS and, § comparison of Rev.1 to PBS group. ** $p < 0.01$; *** $p < 0.001$.

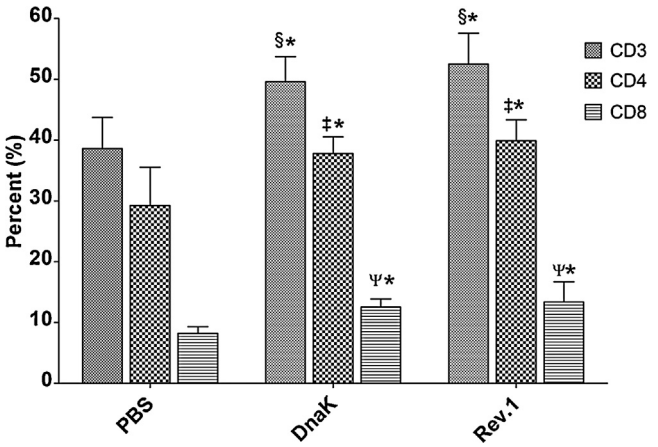

Fig. 5. Analysis of T lymphocyte subsets in DnaK-vaccinated mice. Thirty days after last immunization, splenocytes from BALB/c mice immunized with DnaK, Rev.1 or PBS were collected and labeled with FITC-anti-mouse CD3, CD4 and PE-anti-mouse CD8. Lymphocytes were gated based on forward and side scatter pattern and the percentage of CD4 and CD8 cells were quantified using FlowMax software. Each bar symbolizes the mean ± SD of the percentage of CD4+ or CD8+ T cells from five individual mice with two repeats. Comparison of CD3 percent with PBS group, ‡ comparison of CD4 percent with PBS group, Ψ comparison of CD8 percent with PBS group * $p < 0.05$.

live bacteria and protection was evaluated by measuring bacterial colony forming units in the spleen of mice one month later. In these experiments, protection was defined as a significant reduction of splenic bacterial load as compared to mice injected with PBS alone. The vaccine efficacy was calculated as the $\log_{10}$ of protection. DnaK-immunized mice exhibited a significant degree of protection compared to control mice that had received PBS ($p < 0.001$). However, this protection was lower than that induced by the Rev.1 vaccine ($p < 0.05$) (Table 1). Of note, the combination of Dnak with Omp31 enhanced protection against infection in mice as compared to DnaK or Omp31 alone, but this increase did not yet reach statistical significance. Although the level of protection conferred by DnaK plus TF was higher than protection conferred in mice immunized by DnaK alone, the immunization with DnaK plus TF did not exceed protection compared to TF alone (Table 1).

4. Discussion

The identification of immunodominant antigens is an important step toward the development of safe and effective subunit vaccines. In this regard, many studies on numerous cell surface and intracellular components of *Brucella* have been performed, but only few antigens have shown significant protective potential including: L7/L12, Omp16, TF, Omp31, P39 and BLSOmp31 [10,18,27,38–41].

Immunity against *Brucella* is mainly mediated by mucosal immunity (MI) and acquired T cell-mediated immunity (CMI); including IFN-γ producing CD4+ T lymphocytes [42–44] and CD8+ T lymphocytes killing infected macrophages [45–47]. IFN-γ has been shown to play a key role in the control of brucellosis by activating macrophages and skewing antibody responses toward protective IgG2a [44]. We hypothesized that abundantly expressed proteins that are essential for infection might be good candidates for subunit vaccines. Molecular chaperones such as DnaK are proteins that assist in protein folding and prevent protein aggregation and thereby protect bacteria against conditions of cellular stress [48–50]. Consistent with this, immunization of mice with a DnaK protein from *B. abortus* has been shown previously to provide partial protection against subsequent *B. abortus* infection [23]. The molecular chaperon DnaK evaluated in this study, however, only shares 12.5 percent identity with the DnaK protein from *B. abortus*.

Cell-mediated immune responses were assessed in mice vaccinated with DnaK by measuring antigen-specific proliferation and cytokine production of splenocytes *in vitro*. Splenocytes from DnaK-immunized mice showed significant levels of proliferation *in vitro* in response to stimulation with DnaK ($p < 0.001$), compared to PBS-stimulated splenocytes. Of note, the proliferative response of Rev.1-vaccinated mice to1 μg/ml DnaK was significant higher compared to control mice ($p < 0.01$), indicating that the attenuated live-vaccine elicited immune responses against DnaK.

The cytokines produced by splenocytes from vaccinated animals were predominantly of Th1 type (IL-12 and IFN-γ). In addition, high amounts of IL-6 were produced by splenocytes of DnaK-immunized mice. IL-6 involvement in innate and subsequently adaptive immune responses is that this cytokine is a key signal in the transition from the initial innate immune response to infection to a more sustained adaptive immune response. Regarding the fact the production of this cytokine is mainly triggered by the PAMP-mediated TLR signaling cascade and induces many favorable immune functions leading to pathogen elimination; it is conceivable that this cytokine serve a role in immunity against brucellosis. Moreover, IL-6 is one of the key determinants in causing naive T cells to differentiate into Th17 cells, together with transforming growth factor (TGF)-β. Beside the role that IL-17 plays in controlling infection, this cytokine suppresses development of regulatory T cells thereby induces favorable anti-infection immunity [51]. Interestingly, it has been reported that Interleukin-6-deficient mice are highly susceptible to intracellular pathogen, *Listeria monocytogenes* infection [52]. In this context, immnunogenic brucella antigens, like DnaK, capable of inducing IL-6 production are desired.

To further investigate the Th1/Th2 profile of the elicited immune responses, production of antigen-specific IgG1 and IgG2a antibodies was examined. Although recombinant DnaK induced humoral immune responses predominantly of IgG2a isotype, comparable IgG1 titers were also produced. There are other reports showing that protective immune responses against *Brucella* vaccines are associated with high levels of Th1 cytokines and high IgG2and IgG1 levels [26,27,53–55]. Further recent examples include a well-designed study conducted by Jain et al. [9] showing that splenocytes from mice immunized with L9 based DNA vaccine (pVaxL9) secreted Th1 type cytokines including IFN-γ, IL-2, TNF-α but not IL-4 after re-stimulating with recombinant L9 *in vitro* while immunization of mice with pVaxL9 elicited specific antibody response of both IgG1 and IgG2a isotypes against L9. Another study performed by Al-Maririet et al. [15] showed that immunization of mice with *E. coli* BL21 (DE3) pEt15b-p39 with or without CpG ODN induced IFN-γ but not IL-5 secretion in response to P39 antigen *in vitro* while both IgG1 and IgG2a were elicited against P39. Why do some antigens induce production of IgG1 in spite of no IL-4 or IL-5 production? The reason for this phenomenon is not clear at the moment but different kinetics of cytokine and antigen-specific IgG1/2a production may be one explanation.

In the spleen of DnaK-immunized mice, we also observed a significant increase in the frequency of CD3+, CD4+ cells and a higher CD4+/CD8+ cell ratio, implying an antigen-specific T cell activation *in vivo*.

We next examined the protective capacity of DnaK vaccination in a challenge assay of mouse model of brucellosis. Our results showed that DnaK-immunized mice, although inferior to Rev.1 or TF vaccination, conferred protective immunity to mice. In our point of view the level of protection is not the only determining factor in terms of superiority of one type of vaccine over the other one. Interestingly, *in vitro* proliferation assay clearly showed that as low as 0.25 μg/ml of DnaK induced the same levels of antigen-specific proliferation compared to when splenocytes from TF-immunized mice were stimulated with 1 μg/ml of TF antigens. This implies that threshold level of bacterial load for infection establishment is higher in DnaK-immunized mice in comparison to the TF-immunized mice. In other words, DnaK-immunized mice compared to TF-immunized mice are more likely to be resistant to infection establishment when challenged with a low bacterial load.

Despite lower levels of cytokines and splenocyte proliferation obtained in Rev.1 vaccine group compared with DnaK group, the conferred protection was much higher with Rev.1 vaccine. The rational behind this finding is that splenocytes of all groups were re-stimulated only with DnaK *in vitro*. In this regard it is imaginable that DnaK-immunized mice produced higher amounts of cytokines and exhibited higher proliferation in response to immunizing antigen than those that had been immunized with whole bacteria containing multiple immunodominant antigens.

To investigate whether the protective capacity of DnaK is further enhanced when combined with other antigens expressed at different phases of the pathogen's life cycle, mice were vaccinated with DnaK in combination with two well-known antigens, Omp31 [20] or TF [28].

Combination of DnaK and Omp31 induced a greater, but not statistically significant, protection than the levels conferred by each component alone. Notably, this combination, as opposed to each component alone, induced statistically similar level of protection as induced by Rev.1. Formulation of *Brucella* vaccines containing two different components have been already tested with different set of Brucella antigens. It has been reported that immunization of mice with unlipidated Omp16 plus 19 formulated with IFA could

enhance the protection against *B. abortus* infection compared to single components although difference was not statistically significant [18]. In another work conducted by Cassataro et al. [56], it has been demonstrated that immunized mice with rBLS plus $Omp31_{48-74}$ show an elevated protection against *B. melitensis* infection which was at the same level as induced by Rev.1.

Although administration of DnaK plus TF caused an increase in protection as compared to DnaK alone, this combination unexpectedly protected less efficiently than TF alone (Table 1). In a similar observation, it has been reported that mice immunized simultaneously with DnaK and SurA didn't show any synergic consequence on protection compared to each component alone [23]. Why the combination of different antigens may have beneficial or detrimental effects on vaccine efficacy is not well understood. Interestingly, cocktail vaccines containing antigens that are expressed at different phases of the pathogen's life cycle may show increased efficacy [57] that is attributable to combination of DnaK with Omp31. In addition, interference of epitopes may cause the lack of synergy *in vivo* [18] which likely happened in combination of DnaK with TF. In course of immune responses against a pathogenic microorganisms containing multiple immunogenic determinants, most of the immune responses are usually focused against immunodominat one resulting to lower immune responses against subdominants ones. Logistically, this can be viewed as an approach through which host concentrate its energy for an effective response. We found that splenocytes of DnaK-immunized mice were more responsive to recall cognate antigen stimulation compared to those of TF-immunized mice. This implies higher immunogenicity of DnaK compared to TF (although the level of protection was higher in TF-immunized mice). In this context it is conceivable that DnaK can diminish or even mask the immune responses against TF which is less immunogenic.

In summary, our results show that DnaK is able to elicit cellular responses of Th1-dominant type and to confer significant protection against subsequent *Brucella* infection. Moreover, the combination of DnaK with Omp31 provided an increased protection compared to the administration of the single components. Future studies shall address, which combinations of antigens are most effective. Ultimately, these studies may lead to the development of multivalent subunit vaccines that provide high level of protection against *B. melitensis* infection.

Acknowledgements

We would like to thank Farhad Hosseini and Farhad Khani (Animal Lab, Avicenna Research Institute, ACECR, Tehran, Iran) for technical assistances. This work was supported by grants from Avicenna Research Institute (Grant no. 88-49) and Tehran University of Medical Sciences (Grant no. 8723). This work was a part of the Ph.D. thesis of Dr. Amir Ghasemi.

References

[1] Van der Henst C, de Barsy M, Zorreguieta A, Letesson JJ, De Bolle X. The *Brucella pathogens* are polarized bacteria. Microbes Infect 2013;15:998–1004.
[2] Pappas G, Akritidis N, Bosilkovski M, Tsianos E, Brucellosis. N Engl J Med 2005;352:2325–36.
[3] Olsen SC. Recent developments in livestock and wildlife brucellosis vaccination. Rev Sci Tech 2013;32:207–17.
[4] Avila-Calderon ED, Lopez-Merino A, Sriranganathan N, Boyle SM, Contreras-Rodriguez A. A history of the development of *Brucella* vaccines. Bio-Med Res Int 2013;2013:743509.
[5] Garin-Bastuji B, Blasco JM, Grayon M, Verger JM. *Brucella melitensis* infection in sheep: present and future. Vet Res 1998;29:255–74.
[6] Refai M. Incidence and control of brucellosis in the near east region. Vet Microbiol 2002;90:81–110.
[7] Gupta VK, Radhakrishnan G, Harms J, Splitter G. Invasive *Escherichia coli* vaccines expressing *Brucella melitensis* outer membrane proteins 31 or 16 or periplasmic protein BP26 confer protection in mice challenged with *B. melitensis*. Vaccine 2012;30:4017–22.
[8] Ghasemi A, Zarnani AH, Ghoodjani A, Rezania S, Salari MH, Jeddi-Tehrani M. Identification of a new immunogenic candidate conferring protection against *Brucella melitensis* infection in mice. Mol Immunol 2014;62:142–9.
[9] Jain S, Afley P, Dohre SK, Saxena N, Kumar S. Evaluation of immunogenicity and protective efficacy of a plasmid DNA vaccine encoding ribosomal protein L9 of *Brucella abortus* in BALB/c mice. Vaccine 2014;32:4537–42.
[10] Clausse M, Diaz AG, Ghersi G, Zylberman V, Cassataro J, Giambartolomei GH, et al. The vaccine candidate BLSOmp31 protects mice against *Brucella canis* infection. Vaccine 2013;31:6129–35.
[11] Riquelme-Neira R, Retamal-Diaz A, Acuna F, Riquelme P, Rivera A, Saez D, et al. Protective effect of a DNA vaccine containing an open reading frame with homology to an ABC-type transporter present in the genomic island 3 of *Brucella abortus* in BALB/c mice. Vaccine 2013;31:3663–7.
[12] Li X, Xu J, Xie Y, Qiu Y, Fu S, Yuan X, et al. Vaccination with recombinant flagellar proteins FlgJ and FliN induce protection against *Brucella abortus* 544 infection in BALB/c mice. Vet Microbiol 2012;161:137–44.
[13] Fu S, Xu J, Li X, Xie Y, Qiu Y, Du X, et al. Immunization of mice with recombinant protein CobB or AsnC confers protection against *Brucella abortus* infection. PLoS One 2012;7:e29552.
[14] Kaushik P, Singh DK, Kumar SV, Tiwari AK, Shukla G, Dayal S, et al. Protection of mice against *Brucella abortus* 544 challenge by vaccination with recombinant OMP28 adjuvanted with CpG oligonucleotides. Vet Res Commun 2010;34:119–32.
[15] Al-Mariri A, Mahmoud NH, Hammoud R. Efficacy evaluation of live *Escherichia coli* expression *Brucella* P39 protein combined with CpG oligodeoxynucleotides vaccine against *Brucella melitensis* 16M, in BALB/c mice. Biologicals 2012;40:140–5.
[16] Yang Y, Yin J, Guo D, Lang X, Wang X. Immunization of mice with recombinant *S*-adenosyl-L-homocysteine hydrolase protein confers protection against *Brucella melitensis* infection. FEMS Immunol Med Microbiol 2011;61:159–67.
[17] Yang Y, Wang L, Yin J, Wang X, Cheng S, Lang X, et al. Immunoproteomic analysis of *Brucella melitensis* and identification of a new immunogenic candidate protein for the development of brucellosis subunit vaccine. Mol Immunol 2011;49:175–84.
[18] Pasquevich KA, Estein SM, Garcia Samartino C, Zwerdling A, Coria LM, Barrionuevo P, et al. Immunization with recombinant *Brucella* species outer membrane protein Omp16 or Omp19 in adjuvant induces specific CD4+ and CD8+ T cells as well as systemic and oral protection against *Brucella abortus* infection. Infect Immun 2009;77:436–45.
[19] Hamer I, Goffin E, De Bolle X, Letesson JJ, Jadot M. Replication of Brucella abortus and *Brucella melitensis* in fibroblasts does not require Atg5-dependent macroautophagy. BMC Microbiol 2014;14:223.
[20] Ghasemi A, Salari MH, Zarnani AH, Pourmand MR, Ahmadi H, Mirshafiey A, et al. Immune reactivity of *Brucella melitensis*-vaccinated rabbit serum with recombinant Omp31 and DnaK proteins. Iran J Microbiol 2013;5:19–23.
[21] Bardwell JC, Craig EA. Major heat shock gene of Drosophila and the *Escherichia coli* heat-inducible dnaK gene are homologous. Proc Natl Acad Sci USA 1984;81:848–52.
[22] Zhu X, Zhao X, Burkholder WF, Gragerov A, Ogata CM, Gottesman ME, et al. Structural analysis of substrate binding by the molecular chaperone DnaK. Science 1996;272:1606–14.
[23] Delpino MV, Estein SM, Fossati CA, Baldi PC, Cassataro J. Vaccination with Brucella recombinant DnaK and SurA proteins induces protection against *Brucella abortus* infection in BALB/c mice. Vaccine 2007;25:6721–9.
[24] Kohler S, Teyssier J, Cloeckaert A, Rouot B, Liautard JP. Participation of the molecular chaperone DnaK in intracellular growth of *Brucella suis* within U937-derived phagocytes. Mol Microbiol 1996;20:701–12.
[25] Estein SM, Cassataro J, Vizcaino N, Zygmunt MS, Cloeckaert A, Bowden RA. The recombinant Omp31 from *Brucella melitensis* alone or associated with rough lipopolysaccharide induces protection against *Brucella* ovis infection in BALB/c mice. Microbes Infect 2003;5:85–93.
[26] Cassataro J, Estein SM, Pasquevich KA, Velikovsky CA, de la Barrera S, Bowden R, et al. Vaccination with the recombinant Brucella outer membrane protein 31 or a derived 27-amino-acid synthetic peptide elicits a CD4+ T helper 1 response that protects against *Brucella melitensis* infection. Infect Immun 2005;73:8079–88.
[27] Yang X, Hudson M, Walters N, Bargatze RF, Pascual DW. Selection of protective epitopes for *Brucella melitensis* by DNA vaccination. Infect Immun 2005;73:7297–303.
[28] Ghasemi A, Salari MH, Zarnani AH, Pourmand MR, Ahmadi H, Shirazi MH, et al. Immunogenicity assessment of *Brucella mellitensis* HSP and TF proteins by immunized rabbit serum. Iran J Allergy Asthma Immunol 2013;12:192–4.
[29] Ghasemi A, Ranjbar R, Amani J. In silico analysis of chimeric TF, Omp31 and BP26 fragments of *Brucella melitensis* for development of a multi subunit vaccine candidate. Iran J Basic Med Sci 2014;17:172–80.
[30] Yang X, Walters N, Robison A, Trunkle T, Pascual DW. Nasal immunization with recombinant *Brucella melitensis* bp26 and trigger factor with cholera toxin reduces *B. melitensis* colonization. Vaccine 2007;25:2261–8.
[31] Contreras-Rodriguez A, Ramirez-Zavala B, Contreras A, Schurig GG, Sriranganathan N, Lopez-Merino A. Purification and characterization of an immunogenic aminopeptidase of *Brucella melitensis*. Infect Immun 2003;71:5238–44.
[32] Ghasemi A, Salari MH, Pourmand MR, Zarnani AH, Ahmadi H, Shirazi MH, et al. Optimization and efficient purification in production of *Brucella melitensis* recombinant HSP A and TF proteins with low endotoxin contents. Jundishapur J Microbiol 2013;6:e6875.

[33] Aida Y, Pabst MJ. Removal of endotoxin from protein solutions by phase separation using Triton X-114. J Immunol Methods 1990;132:191–5.
[34] Liu CL, Nikas YJ, Blankschtein D. Novel bioseparations using two-phase aqueous micellar systems. Biotechnol Bioeng 1996;52:185–92.
[35] Reichelt P, Schwarz C, Donzeau M. Single step protocol to purify recombinant proteins with low endotoxin contents. Protein Expr Purif 2006;46:483–8.
[36] Stoscheck CM. Quantitation of protein. Methods Enzymol 1990;182:50–68.
[37] Gonzalez D, Grillo MJ, De Miguel MJ, Ali T, Arce-Gorvel V, Delrue RM, et al. Brucellosis vaccines: assessment of *Brucella melitensis* lipopolysaccharide rough mutants defective in core and *O*-polysaccharide synthesis and export. PLoS One 2008;3:e2760.
[38] Al-Mariri A, Tibor A, Mertens P, De Bolle X, Michel P, Godefroid J, et al. Protection of BALB/c mice against *Brucella abortus* 544 challenge by vaccination with bacterioferritin or P39 recombinant proteins with CpG oligodeoxynucleotides as adjuvant. Infect Immun 2001;69:4816–22.
[39] Cassataro J, Velikovsky CA, de la Barrera S, Estein SM, Bruno L, Bowden R, et al. A DNA vaccine coding for the *Brucella* outer membrane protein 31 confers protection against *B. melitensis* and *B. ovis* infection by eliciting a specific cytotoxic response. Infect Immun 2005;73:6537–46.
[40] Luo D, Ni B, Li P, Shi W, Zhang S, Han Y, et al. Protective immunity elicited by a divalent DNA vaccine encoding both the L7/L12 and Omp16 genes of *Brucella abortus* in BALB/c mice. Infect Immun 2006;74:2734–41.
[41] Tabynov K, Kydyrbayev Z, Ryskeldinova S, Yespembetov B, Zinina N, Assanzhanova N, et al. Novel influenza virus vectors expressing *Brucella* L7/L12 or Omp16 proteins in cattle induced a strong T-cell immune response, as well as high protectiveness against *B. abortus* infection. Vaccine 2014;32:2034–41.
[42] Cheers C. Pathogenesis and cellular immunity in experimental murine brucellosis. Dev Biol Stand 1984;56:237–46.
[43] Murphy EA, Sathiyaseelan J, Parent MA, Zou B, Baldwin CL. Interferon-gamma is crucial for surviving a *Brucella abortus* infection in both resistant C57BL/6 and susceptible BALB/c mice. Immunology 2001;103:511–8.
[44] Zhan Y, Cheers C. Endogenous gamma interferon mediates resistance to *Brucella abortus* infection. Infect Immun 1993;61:4899–901.
[45] Baldwin CL, Parent M. Fundamentals of host immune response against *Brucella abortus*: what the mouse model has revealed about control of infection. Vet Microbiol 2002;90:367–82.
[46] Paranavitana C, Zelazowska E, Izadjoo M, Hoover D. Interferon-gamma associated cytokines and chemokines produced by spleen cells from *Brucella*-immune mice. Cytokine 2005;30:86–92.
[47] Ko J, Splitter GA. Molecular host-pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans. Clin Microbiol Rev 2003;16:65–78.
[48] Gusev NB, Bogatcheva NV, Marston SB. Structure and properties of small heat shock proteins (sHsp) and their interaction with cytoskeleton proteins. Biochemistry (Moscow) 2002;67:511–9.
[49] Munchbach M, Nocker A, Narberhaus F. Multiple small heat shock proteins in rhizobia. J Bacteriol 1999;181:83–90.
[50] Haslbeck M, Buchner J. Chaperone function of sHsps. Prog Mol Subcell Biol 2002;28:37–59.
[51] Naugler WE, Karin M. The wolf in sheep's clothing: the role of interleukin-6 in immunity, inflammation and cancer. Trends Mol Med 2008;14:109–19.
[52] Dalrymple SA, Lucian LA, Slattery R, McNeil T, Aud DM, Fuchino S, et al. Interleukin-6-deficient mice are highly susceptible to *Listeria monocytogenes* infection: correlation with inefficient neutrophilia. Infect Immun 1995;63:2262–8.
[53] Clapp B, Skyberg JA, Yang X, Thornburg T, Walters N, Pascual DW. Protective live oral brucellosis vaccines stimulate Th1 and th17 cell responses. Infect Immun 2011;79:4165–74.
[54] Al-Mariri A. Protection of BALB/c mice against *Brucella melitensis* 16 M infection induced by vaccination with live *Escherchia coli* expression Brucella P39 protein. Vaccine 2010;28:1766–70.
[55] Commander NJ, Spencer SA, Wren BW, MacMillan AP. The identification of two protective DNA vaccines from a panel of five plasmid constructs encoding *Brucella melitensis* 16M genes. Vaccine 2007;25:43–54.
[56] Cassataro J, Pasquevich KA, Estein SM, Laplagne DA, Velikovsky CA, de la Barrera S, et al. A recombinant subunit vaccine based on the insertion of 27 amino acids from Omp31 to the N-terminus of BLS induced a similar degree of protection against *B. ovis* than Rev.1 vaccination. Vaccine 2007;25:4437–46.
[57] Igietseme JU, Eko FO, Black CM. Contemporary approaches to designing and evaluating vaccines against chlamydia. Expert Rev Vaccines 2003;2:129–46.