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Research Article

Efficient Immunization of BALB/c Mice against Pathogenic *Brucella melitensis* and *B. ovis*: Comparing Cell-Mediated and Protective Immune Responses Elicited by pCDNA3.1 and pVAX1 DNA Vaccines Coding for Omp31 of *Brucella melitensis*

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Background: *Brucella* spp. are intracellular pathogens, therefore cell-mediated immunity is the main response to inhibit survival and growth of the bacteria in vertebrate host.

Objective: Many eukaryotic plasmid vectors are being used in setting up DNA vaccines which may show different efficiencies in same conditions. This is important in designing the vaccines and immunization strategies. We looked into the probable differences of immune responses induced by different eukaryotic DNA plasmid vectors (pcDNA3.1 and pVAX1) harboring the same Omp31 gene of *B. melitensis.*

Materials and Methods: Female BALB/c mice were immunized with pcDNA-*omp31* and pVAX-*omp31* and further boosted with recombinant Omp31. Subclasses of specific serum IgG against the rOmp31 were measured by ELISA. Cytokines responses to rOmp31 in Splenocyte cultures of the immunized mice were evaluated by measuring the production of IL-4, IL-10, IL-12 and IFN-γ. Protective responses of the immunized mice were evaluated by intraperitoneal challenge with pathogenic *Brucella melitensis* 16M and *Brucella ovis* PA76250.

Results: Both DNA vaccine candidates conferred potent Th1-type responses with higher levels of cytokines and immunoglobulins observed in mice immunized with pVAX-*omp31*. Although pcDNA-*omp31* and pVAX-*omp31* both elicited protective immunity, mice immunized with the latter showed a higher protection against both *B. melitensis* and *B. ovis* PA76250.

Conclusion: The results of this study highlight the significant differences between efficiency of diverse plasmid backbones in DNA vaccines which code for an identical antigen. Comparing various plasmid vectors should be considered as an essential part of the studies aiming construction of DNA vaccines for intracellular pathogens.

Keywords: *Brucella*, DNA vaccine, Omp31, pCDNA3.1, pVAX1

1. Background

Brucella spp. are Gram-negative, facultative intracellular pathogens and cause brucellosis in human and animals. This pathogen is mainly localized at the reticuloendothelial system of vertebrate hosts (1-3). *B. melitensis* is the most pathogenic member of the genus and responsible for severe disease in humans, although the preferred hosts are goats, sheep, cows, dogs and camels (4). *Brucella* infections occur mainly after consumption of contaminated dairy or food or by contacts with infected animals (5). There is no licensed vaccines available for prevention of human brucellosis and disease prevention principally relies on control of animal brucellosis by vaccination (6, 7). The most widely used vaccines for control of animal brucellosis are the live attenuated strains, *B. melitensis* Rev1, and *B. abortus* S19 (8). Live attenuated vaccines are pathogenic for humans; they also cause serological interference

with diagnostic methods in vaccinated animals (9, 10). *Brucella* pathogens can escape recognition by the host innate immune responses and further use sophisticated strategies to avoid intracellular destruction after being phagocytosed by host macrophages. This, enables them to survive and establish a persistent infection (2, 11, 12). Since these pathogens survive in macrophages, cell-mediated immunity is necessary for activation of infected macrophages and clearance of the pathogen and formation of active $CD8^+$ cytotoxic T-lymphocytes (2, 13). Many reports are available on using subunit vaccines consisting of *Brucella* outer membrane proteins and their ability to elicit Th1-type responses and partial protection against pathogenic strains (14-16). Many protein antigens including outer membrane proteins and intracellular ones reported to induce potent cytokine and antibody responses especially IFN-γ, IL-12 and IgG2a - immunological mediators which are important for inhibiting *Brucella* infection in hosts (17). The need for induction of more potent cell-mediated immunity has led some researchers to use DNA vaccines as they are believed to elicit Th1-type responses (18-22). A neglected factor remains to be investigated which is the influence of the plasmid vector used for constructing the DNA vaccine. Different plasmid vectors are used to compose DNA vaccines and they may have variable efficiencies in stimulating host immune system. This could be of crucial importance to enlighten whether it is necessary to compare different DNA plasmid backbones for a single antigen or not.

2. Objectives

This study was aimed to present a research model for exploring the efficiency of different eukaryotic DNA plasmids (pVAX1™ and pcDNA™3.1) for eliciting cell-mediated responses against Omp31 (23, 24) – a well-studied antigen of *Brucellae* – as DNA vaccine. In this model, the immunogenic Omp31 is the constant antigen to study the effect of plasmid vector backbone selection on immunological responses elicited in BALB/c mice.

3. Materials and Methods

3.1. Animal Model

Six- to eight-weeks old female BALB/c mice were acquired from Laboratory Animal Production Center (Pasteur Institute, Iran). Mice were maintained under standard laboratory conditions and kept one week for adaptation before experiments (25).

3.2. Bacterial Strains and Culture Conditions

Escherichia coli DH5α was obtained from the culture collection at Pasteur Institute of Iran. E. coli DH5α routinely is cultured using LB medium. *Brucella melitensis* Rev1, *B. melitensis* 16M and *B. ovis* PA76250 were stored in culture collection at Department of Bacteriology (Tarbiat Modares University). *Brucella* strains were cultured routinely on Brucella agar (Merck) and incubated at 37 °C. These pathogenic bacteria were handled according to biosafety level 2 practices and regulations.

3.3. Recombinant Omp31

Recombinant Omp31 (rOmp31) was previously produced in our lab and preserved in -80 °C as a concentrated stock of ~20±1 $mg.mL^{-1}$.

3.4. Construction and Preparation of DNA Vaccines

The complete coding sequence of Omp31 was inserted between *BamH*I and *Xho*I recognition sequences in pVAX1™ (Invitrogen™, V260-20) and pcDNA™3.1 (Invitrogen™, V790-20). Specific primers including restriction sites at the 5' ends were used (bmO31dnF-5'-AAT GGA TCC ACC ACC ATG AAC TCC GTA ATT TTG GCG TCC ATC-5› and bmO31dnR- 5'-ACA CTC GAG TTA GAA CTT GTA GTT CAG ACC GAC GCG-5›) with the Kozak consensus sequence considered before the ATG codon (underlined) (26). Recombinant plasmids named as pVAX-*omp31* and pcDNA-*omp31* were confirmed by sequencing. Recombinant plasmids were amplified in *E. coli* DH5α host and were extracted and purified by EndoFree Plasmid Giga Kit (QIAGEN, Catalog no. 12391) according to the manufacturer instructions. Quality and quantity of the purified plasmids were assessed using NanoDrop spectrophotometer (NanoDrop™ 2000; Thermo Sientific). To confirm the recombinant plasmids can express the inserted *omp*31 gene, they were separately transfected to the COS-7 cell line (ATCC CRL-1651, Pasteur Institute of Iran) by Lipofectamine® 2000 reagent (Life Technologies) and protein expression was traced through western blotting as described previously (24, 27). Rabbit polyclonal antiserum against rOmp31 (Anti-Omp31; produced in our laboratory) (1:5000) was used for tracing Omp31 expression in transfected COS-7 cells. Membranes were then treated with goat anti-rabbit IgG (Sigma) and after being washed thoroughly, developed with enhanced chemiluminescent (ECL) substrate under standard conditions. Appearance of fluorescent bands was recorded on radiographic films (28).

3.5. Immunization of Mice

Six- to eight-week old female BALB/c mice were anesthetized with Ketamine/Xylazine intraperitoneally and immunized intramuscularly with 100μg of

pcDNA-*omp31* (Group I), pVAX-*omp31* (Group II), intact pcDNA3.1 (Group III) or pVAX1 (Group IV) in separate groups of 15 mice. Mice were injected with the same plasmids four times in two-week intervals (24). Thereafter, mice which were immunized with pcDNA-*omp31* (Group I) and pVAX-*omp31* (Group II) were boosted with 20µg of rOmp31, formulated in incomplete Freund's adjuvant (based on our dose determination experiment; data not shown) (Sigma-Aldrich, F5506-10 mL) two weeks and four weeks after the last DNA booster. One group of mice (Group V) were immunized with 20µg of rOmp31 formulated in Freund's adjuvant along with DNA vaccine groups (complete Freund's adjuvant, F5881-10 mL, for priming; incomplete Freund's adjuvant for boosting). A non-immunized group of mice (Group VI) were also considered; they were injected with sterile PBS intramuscularly. For positive control, a group of mice were vaccinated intraperitoneally with 4×10^4 CFU of live *B. melitensis* Rev 1 at day 0 (Group VII).

3.6. Measurement of Specific Serum Immunoglobulins

Serum IgG and IgG subclasses were assessed by indirect ELISA as described before with some minor modifications (27, 29, 30). Mice were bled retroocularly on days 14, 28, 42, 56, 70, 84, and 98 (five mice from group). Nunc MaxiSorp® flat-bottom 96 well plates were coated with 200µL of rOmp31 solution in PBS (5 $\mu g.mL^{-1}$ protein) overnight at 4-6 °C and then blocked with bovine serum albumin 5% in PBS for 2 hrs at 37 °C. Total serum IgG (1:200 dilution) was measured for each mouse in duplicates and for all prepared samples. Omp31-specific IgG1 and IgG2a were also assessed with ISO-2 (Sigma-Aldrich) for samples of day 98 in triplicates for each mouse and OD_{450} value of samples were recorded (27, 30).

3.7. Assessment of Cytokine Responses

The experiment was performed based on a previous report with essential modification (27). Splenocytes from sacrificed mice (5 mice from each group) were extracted and cultured in 24 and 96 well-plates (Jet Biofil®) at 4×10^6 and 2.5×10^5 cells per well, respectively. Cells were cultured in RPMI medium 1640-GlutaMAX™ (Gibco®) supplemented with 10% preheated (56 °C) FBS (Gibco®), 1% Pen-Sterp (Gibco®) and 1X nonessential amino acids (Gibco®). Splenocyte cultures were stimulated with 5 $\mu g.mL^{-1}$ of rOmp31 and were incubated for 72 hrs in humid environment at 37 °C, supplemented with 5% CO_2. Lymphocytic proliferation was assessed in 96-well plates by Cell Proliferation ELISA, BrdU (colorimetric; Roche life sciences). Culture supernatants collected from 24-well plates were examined by specific ELISA for mouse IL-4, IL-10, IL-12 and IFN-γ (R&D) as triplicate for each mouse.

3.8. Protection Assessment

Four weeks after the last booster dose, 8 mice from each group were challenged intraperitoneally with ~10^5 CFU of the pathogenic strain *B. melitensis* 16M, and 8 mice were challenged similarly with the pathogenic strain *B. ovis* PA76250. Mice were maintained under strict biosafety considerations for 4 weeks after which they were sacrificed, their spleens were homogenized and dilutions from spleen suspensions were inoculated and spread on Brucella agar plates (duplicates for each mouse). Plates were incubated at 37 °C for 96 hrs and *Brucella* colonies were counted subsequently.

3.9. Statistical Analysis

Data from all experiments were collected in, and analyzed with the IBM® SPSS® Statistics software (Version 22.0) using the one-way ANOVA analysis, Games-Howell and LSD tests for homogeneity status of variances.

4. Results

4.1. Expression of Omp31 in COS-7 Cells

Two separate COS-7 cell lines which were transfected with pcDNA-*omp31* and pVAX-*omp31* were analyzed by western blotting to trace the recombinant Omp31 expression. Results from this experiment confirmed that both recombinant plasmids (*i.e.* pcDNA-*omp31* and pVAX-*omp31)* can express the inserted coding sequence in eukaryotic host cell (**Fig. 1**).

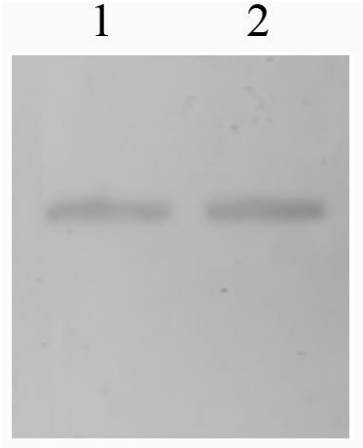

Figure 1. Western blot confirmation of Omp31 expression from DNA plasmid construct in transfected COS-7 cell line. 1: COS-7 transfected with pcDNA-*omp31*, and 2: COS-7 transfected with pVAX-*omp31*.

4.2. Serum Immunoglobulins

Specific serum IgG against rOmp31 and its subclasses were measured by indirect ELISA. Groups of DNA vaccine show high levels of total IgG (**Fig. 2**). Mice immunized with both DNA vaccines did not show significant difference in levels of IgG1 but pVAX-*omp31* elicited significantly higher level of IgG2a (**Fig. 3**).

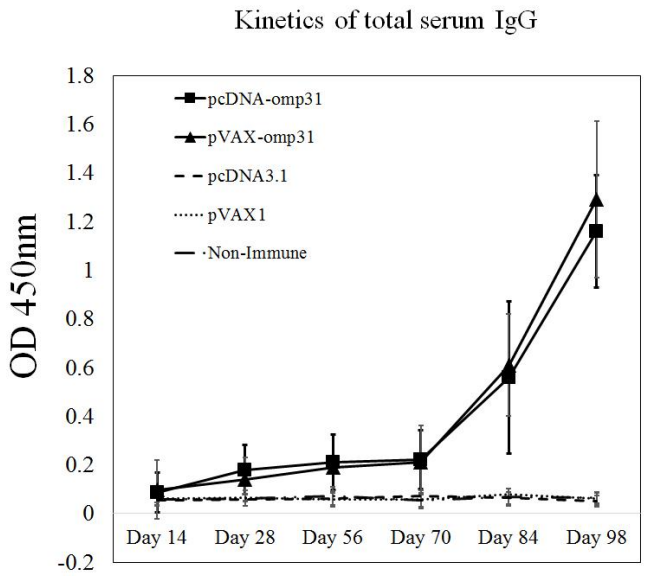

Figure 2. Kinetics of total serum IgG specific to rOmp31. Mice immunized with DNA vaccines show significantly high levels of IgG when boosted with recombinant proteins. There is no significant difference between pcDNA-*omp31*- and pVAX-*omp31*-vaccinated mice in total serum IgG levels.

4.3. Lymphocyte Proliferation and Cytokine Production

Stimulation of splenocyte cultures from mice showed significant levels of lymphocyte proliferation in DNA vaccinated groups (**Fig. 4**). pVAX-*omp31* stimulated a potent proliferation response, significantly higher than pcDNA-*omp31* ($p<0.05$). As depicted in **Figure 5**, cytokine levels also revealed significant production of IL-4, IL-10, IL-12 and IFN-γ as specific responses to stimulation with the rOmp31 in splenocyte cultures from mice which were immunized with both DNA vaccines ($p<0.05$). in the case of IL-10, there was no significant difference between pVAX-*omp31* and pcDNA-*omp31* but pVAX-*omp31* induced higher levels of IL-4, IL-12 and IFN-γ as compared to pcDNA-*omp31* ($p<0.05$).

4.4. Protective Immunity

Mice were challenged intraperitoneally with *B. melitensis* 16M and *B. ovis* PA76250 and results are depicted in **Figure 6**. Mice immunized with live attenuated vaccine strain – *B. melitensis* Rev 1 – showed the best protection as it was expected ($p<0.05$). Protection conferred by either of DNA vaccines which were boosted with rOmp31 were statistically different for each construct against *B. melitensis* 16M and *B. ovis* PA76250. There was significant difference between results from mice immunized with pcDNA-*omp31* and pVAX-*omp31* against both pathogenic strains ($p<0.05$). In general, mice immunized with pVAX-*omp31* showed a better protection than that conferred by pcDNA-*omp31* against *B. melitensis* 16M and *B. ovis* PA76250 ($p<0.05$).

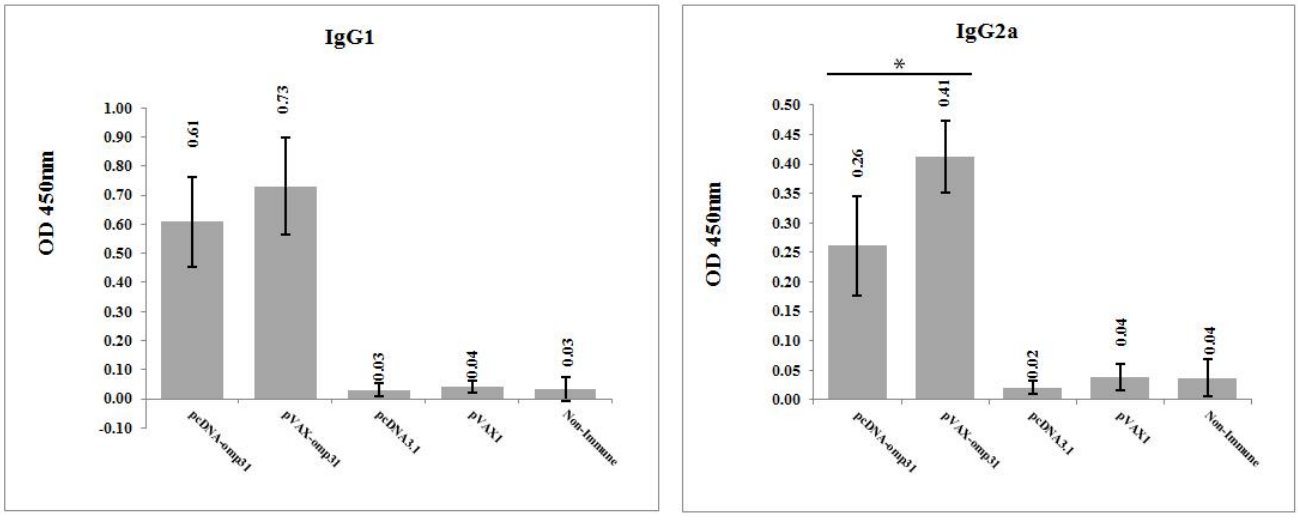

Figure 3. Levels of serum IgG1 and IgG2a specific to rOmp31. DNA vaccine-immunized mice showed significantly high levels of IgG1 and IgG2a as compared to controls of non-immune mice and those injected with intact plasmids. No significant difference was observed in the level of IgG1 elicited by the both DNA vaccines. Mice that were immunized with pVAX-*omp31* elicited significantly higher levels of IgG2a ($p<0.05$).

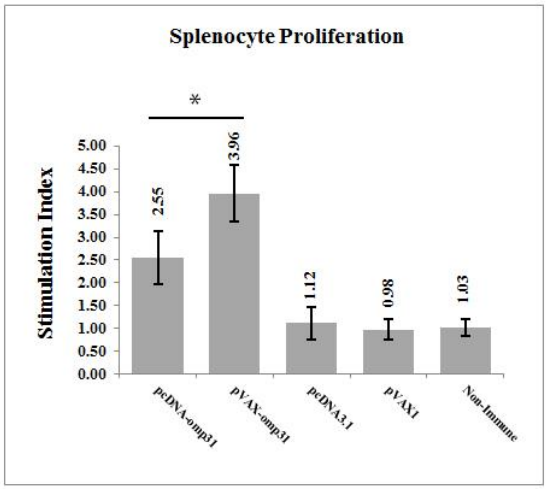

Figure 4. Spleen lymphocyte proliferation response to rOmp31. Both groups immunized with DNA vaccines showed significantly high stimulation indexes (SI) in response to rOmp31. Mice vaccinated with pVAX-*omp31* showed a significantly higher SI than those immunized with pcDNA-*omp31* (p<0.05).

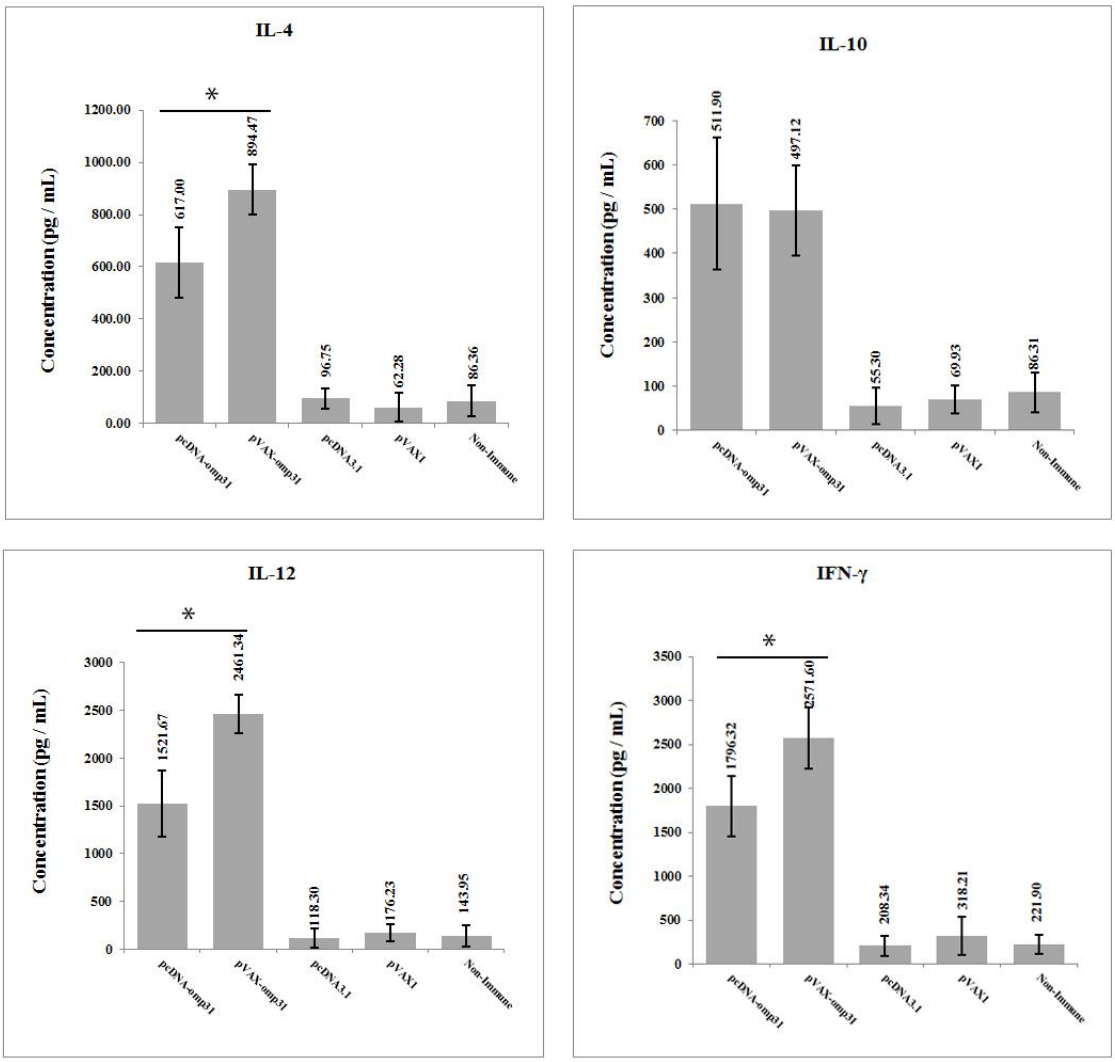

Figure 5. Mice immunized with both DNA vaccines produced significant levels of IL-4, IL-10, IL-12 and IFN-γ in response to stimulation with rOmp31 (p<0.05). Except for IL-10 for which no significant difference was observed between pVAX-*omp31* and pcDNA-*omp31*, other cytokines were produced in significantly higher amounts for mice immunized with pVAX-*omp31* (p<0.05).

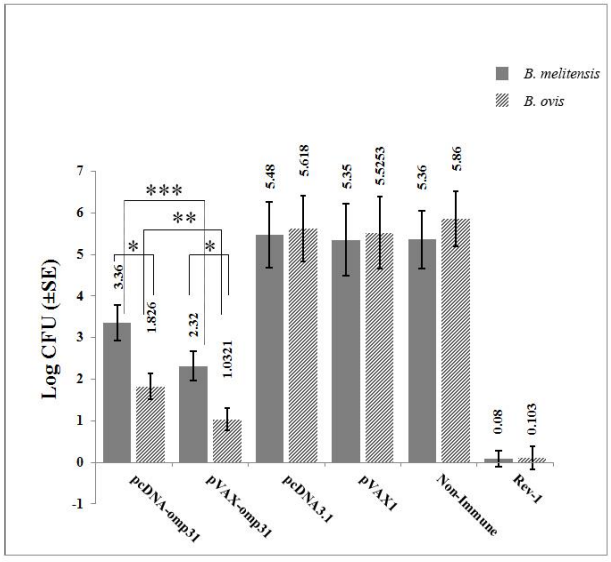

Figure 6. Mice were challenged intraperitoneally with the pathogenic strains *B. melitensis* 16M and *B. ovis* PA76250. *Brucella* cells were quantified in cultures from spleen of mice four weeks post challenge. The best and complete protection was conferred by the live attenuated vaccine strain, *B. melitensis* Rev 1 (p<0.0001). DNA vaccine-immunized groups also showed partial protections against the pathogenic *Brucella* strains which were significantly higher than non-immune controls including those which received intact plasmids (p<0.05). Mice immunized with either of pVAX-*omp31* or pcDNA-*omp31* showed more protection against *B. ovis* PA76250 than *B. melitensis* 16M (*; p<0.05) for each group. Mice vaccinated with pVAX-*omp31* showed more protection than those immunized with pcDNA-*omp31* (**; p<0.05). Protection results for pVAX-*omp31* and pcDNA-*omp31* against *B. melitensis* 16M also showed higher protection elicited by pVAX-*omp31* which is statistically different between two construct (***; p<0.05).

5. Discussion

Brucella pathogens are intracellular in vertebrate hosts and formation of efficient cell-mediated immunity is the principle response to inhibit intracellular survival and growth of the bacteria (1, 14). Many experiments showed that DNA vaccines may stimulate lymphocyte-T mediated immune responses against the encoding gene fragments they are inserted with (18-22, 24). Although remarkable results were reported for various DNA vaccines, but the probable effect of the plasmid backbone on immunoprotective outcome were ignored. Here we presented a comparative research model of different DNA vaccine constructs equally inserted with *omp31* coding sequence (as a famous and fully studied vaccine candidate) from *B. melitensis* (18, 23, 30). We aimed to elucidate whether or not the DNA backbone of plasmids cause any differences in stimulation of cell-mediated immune responses against Omp31 antigen which is present in both *B. melitensis* and *B. ovis*. The coding sequence of this antigen was cloned into pcDNA3.1 and pVAX eukaryotic plasmid vectors, both of which have been reported for their efficiency as DNA vaccine backbones (18, 19, 31-35). Both pcDNA-*omp31* and pVAX-*omp31* induced high levels of cell-mediated responses in BALB/c mice which was consistent with other reports on using DNA vaccines of various protein antigens from the *Brucella*, including its outer membrane proteins (21, 24, 35-42). Many of these antigens are reported to elicit efficient cellular immunity and, in some cases, protective immunity against pathogenic *Brucellae*.

Our constructs, pcDNA-*omp31* and pVAX-*omp31* were used for immunization of mice followed by boosting with rOmp31. This combined immunization was reported to be more effective than using DNA vaccine or protein alone (37). Immunized mice showed high levels of total serum IgG against rOmp31 for both constructs with no significant difference. When IgG subclasses of IgG1 and IgG2a, as common indicators for Th1 and Th2 type responses, were measured, mice immunized with pVAX-*omp31* showed higher levels of IgG2a. Considering higher splenocyte proliferation observed for pVAX-*omp31* (**Fig. 4**) with the corresponding IgG2a production may help us to suggest that there should be more lymphocytic clones elicited against Omp31 vs. mice immunized with pcDNA-*omp31*.

We did not record any significant difference between IL-10 levels as a suppressor of Th-1 responses. This may suggest that both DNA vaccine constructs at least are equal in stimulating Th-1 inhibitory responses. Significantly lower production of IL-4 in mice which were vaccinated with pVAX-*omp31* (**Fig. 5**) implies the lower activation of Th-2 responses in these mice which will be supported by considering the higher levels of

Omp31-specific IgG2a production (**Fig. 3**).

Assessment of cytokine responses from splenocyte cultures revealed that pVAX-*omp31*-vaccinated mice were producing significantly more IL-12 and IFN-γ than those immunized with pcDNA-*omp31* (**Fig. 5**). This finding is also supported with lower IL-4 levels (**Fig. 5**) and higher IgG2a production (**Fig. 3**) in pVAX-*omp31* group. Upon challenge with the virulent strains, *B. melitensis* 16M and *B. ovis* PA76250, pVAX-*omp31* also showed a significantly more potent protection (**Fig. 6**). Th-1 type immune responses are crucial for the host to eliminate the bacterial pathogen in host cells including macrophages as the main infected cells. Higher levels of IFN-γ and specially IL-12 which is important for activation of macrophages (2, 3) is consistent with the higher protection in pVAX-*omp31*-vaccinated mice. Although we observed significant rate of protection, in agreement with previous reports (24, 37) protective responses elicited by both DNA vaccines against pathogenic *B. melitensis* 16M and *B. ovis* PA76250 were partial, probably because of the LPS structure of bacteria that limits the access of host immune system to surface proteins. In this work, similar experimental conditions were used for all mice, leaving the DNA plasmid backbone as the only variable and pVAX-*omp31* induced more effective Th-1 type immune responses specific to the surface protein, Omp31.

6. Conclusion

A comparative approach may elucidate the differences in immunogenicity outcomes related to the selection of various DNA plasmid vectors for construction of DNA vaccines. Outstanding results of this study support us to suggest that in this model of experiment, pVAX-1 acted as a more efficient eukaryotic plasmid vector than pcDNA3.1. Although underlying mechanisms could not be described according to our methods and results, this study model highlights the significant differences between efficiency of diverse constructs which code for an identical antigen in the induction of protective cell-mediated immunity in BALB/c mice. Accordingly, it can be suggested that comparing different eukaryotic plasmids must be considered as an essential part of vaccine investigations.

Disclosure

All authors declare no conflict of interest.

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