Brucella melitensis 16MΔTcfSR as a potential live vaccine allows for the differentiation between natural and vaccinated infection

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Abstract. Brucellosis is a zoonotic disease that poses a serious threat to public health and safety. Although the live attenuated vaccines targeting brucellosis, such as M5-90, are effective, there are a number of drawbacks to their use. For example, the vaccines are unable to differentiate between the natural and vaccinated forms of the infection, and these vaccines have also been shown to cause abortion in pregnant animals. Therefore, a safer and more potent vaccine is required. In the present study, a *B. melitensis* 16M TcfSR promoter mutant (16MΔTcfSR) was constructed in an attempt to overcome these drawbacks. A TcfSR mutant was derived from *B. melitensis* 16M and tested for virulence and protection efficiency. Levels of immuoglobulin G (IgG), and cytokine production were determined. In addition, TcfS was assessed as a diagnostic marker for brucellosis. The survival capacity of the 16MΔTcfSR mutant was shown to be attenuated in the RAW 264.7 murine macrophage cell line and BALB/c mice, and the vaccination was shown to induce a high level of protective immunity in BALB/c mice. In addition, the 16MΔTcfSR vaccination elicited an anti-*Brucella*-specific IgG response and induced the secretion of interferon-γ. Thus, the TcfS antigen allowed for the serological differentiation between the natural and vaccinated infection in animals. In conclusion, the results demonstrated that the 16MΔTcfSR mutant was attenuated in murine macrophage cells and BALB/c mice; therefore, 16MΔTcfSR is a potential candidate for a live attenuated vaccine against *B. melitensis* infection.

Introduction

Brucella organisms are facultative, intracellular bacteria of animals and humans that can cause diseases of worldwide significance (1,2). *Brucella* infections can result in a variety of acute diseases, such as epididymitis or abortion in animals, and fever, arthritis, dementia and meningitis in humans (3-5). Currently, an effective and safe vaccine targeting *Brucella* for animals and humans does not exist. Therefore, low virulence and high protective vaccines are important to prevent the spread of disease.

Brucella melitensis M5-90 is the only approved vaccine currently available for protection against *B. melitensis* infection in China (6). Vaccination with M5-90 induces significant protection in sheep and goats. In addition, M5-90 administration has decreased the incidence of brucellosis in animals and humans, and is routinely administered to sheep and goats to prevent brucellosis. However, the M5-90 vaccine has a number of disadvantages. For example, the vaccination has been found to cause abortions if administered to pregnant animals. Furthermore, M5-90 can cause local hypersensitivity reactions in cases of accidental inoculation. Therefore, the development of a less virulent and more efficient vaccine to prevent and control brucellosis is crucial. The deletion of virulence genes is required for the development of live vaccines against *B. melitensis* infection that are superior to M5-90 (7).

The two-component regulatory system (TCS) is one of the most important virulence regulatory systems in *Brucella*, and genome sequencing has revealed 21 putative TCSs in the *Brucella* genus (8). TcfSR is one of TCSs, and is located in chromosome II (9). TCSs can coordinate an intricate network of virulence genes to allow the host cells to sense environmental varieties and to subsequently exert an appropriate response in *Brucella*.

In the present study, the effect of the *B. melitensis* 16M TcfSR promoter mutant (16MΔTcfSR) on virulence was investigated. The aim of the current study was to determine whether 16MΔTcfSR may be useful as an attenuated live *B. melitensis* vaccine.

Materials and methods

Bacterial strains, plasmids, cells and mice. B. melitensis strain 16M and the M5-90 vaccine strain were obtained from the Center of Chinese Disease Prevention and Control (Beijing, China). *Brucella* was cultured in tryptone soya agar (TSA) or tryptone soya broth (Sigma-Aldrich, St. Louis,

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MO, USA), while *Escherichia coli* strain DH5α cells were grown on Luria-Bertani medium. The pGEM-7Zf$^+$ plasmid was purchased from Promega Corporation (Madison, WI, USA) and a RAW 264.7 murine macrophage cell line was purchased from the Cell Resource Center at the Institute of Basic Medical Sciences of the Chinese Academy of Medical Sciences/Peking Union Medical College (Beijing, China). A total of 290 BALB/c female mice (age, 6 weeks) were obtained from the Experimental Animal Center of the Academy of Military Medical Science (Beijing, China). All experimental procedures and animal care protocols were performed in compliance with institutional animal care regulations. The present study was approved by the ethics committee of Shihezi University (Shihezi, China).

Construction of the 16MΔTcfSR mutant. The sequence of the TcfSR promoter region was predicted using Neural Network Promoter Prediction software (http://www.fruitfly.org/seq_tools/promoter.html). The specific DNA sequences for TcfSR and homologous arms were screened from GenBank (http://www.ncbi.nlm.nih.gov/nuccore/17986243?from=1053312&to=1054655&sat=4&sat_key=105779979), and Primer 5.0 software (Premier Biosoft, Palo Alto, CA, USA) was used to design all polymerase chain reaction (PCR) primers. Two pairs of primers with restriction sites at the 5' ends were designed for amplification of the upstream (1,026 bp) and downstream (1,024 bp) arms of the *B. melitensis* 16M TcfSR promoter, in which the *Xho*I, *Kpn*I and *Sac*I (underlined) sites were integrated into the two PCR fragment ends. The primer sequences were as follows: TcfSR-N-terminal forward, CTC GAG AGC CGC TAT TAT ACC GGA, and reverse, GGT ACC TTG GCC GAT AAT GAT TGC; TcfSR-C-terminal forward, GGT ACC ATG AGA ATT ATC CTC ATC GAA G, and reverse, GAG CTC GTC TGG AAA CCC ATG GTG. The two arms of the 16M TcfSR promoter were cloned into a T-Vector pMD19 simple vector (Takara Bio, Inc., Tokyo, Japan) for sequencing, and subsequently subcloned into the pGEM-7Zf$^+$ plasmid to generate the suicide plasmid, pGEM-7Zf$^+$-TcfSR. In addition, one pair of primers with restriction sites at the 5' end were designed for *SacB* DNA fragment amplification. The primer sequences were as follows: *SacB* forward, GAG CTC GGG CTG GAA GAA GCA GAC CGC TA (*Xho*I site), and reverse, GAG CTC GCT TAT TTG TTA ACT GTT AAT TGT CC (*Xho*I site). The 1,475-bp fragment was amplified using a PCR method from *Bacillus subtilis*. Briefly, genomic DNA was isolated from *B subtilis* using a commercial kit (Omega Bio-Tek, Norcross, GA, USA), according to the manufacturer's instructions. The PCR reaction system contained the following: 1.5 μl 10X buffer, 0.2 μl dNTP (10 mmol/l), 1 μl DNA (20 ng/μl), 0.2 μl Taq enzyme, 0.2 μl primers x2 (20 μmol/l) 0.6 μl $MgCl_2$ (25 mmol/l) (TIANGEN Biotech Co., Ltd., Beijing, China) and 11.1 μl H_2O. The total volume was 15 μl (60˚C; 30 cycles). The PCR reaction conditions were as follows: 5 min at 95˚C, followed by 30 cycles at 65˚C for 40 sec and 72˚C for 1 min, and 10 min at 72˚C. The PCR products were analyzed using 2% agarose gel electrophoresis (voltage, 150 V; 15 min). Next, the *SacB* fragment was subcloned into the pGEM-7Zf$^+$-TcfSR plasmid to generate the pGEM-7Zf$^+$-TcfSR-SacB plasmid. The competent *B. melitensis* 16M strain was subjected to electroporation with pGEM-7Zf$^+$-TcfSR-SacB, and the potential TcfSR deletion mutant, 16MΔTcfSR, was isolated using its ampicillin resistance and sucrose phenotypes. The mutant was further confirmed by PCR amplification using the following primers: TcfSR-I forward, GCT CTG CGG GTT GAT CTT GG, and reverse, TGA CAG GCG TGG AAC AGC, which were located on the upstream and downstream homologous arm of the TcfSR promoter, respectively. The PCR products were sequenced by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China), to confirm the sequence. In addition, the deletion mutant was further confirmed by PCR amplification and reverse transcription PCR sequencing, as described previously (10). The RNA of parental 16M and mutant 16MΔTcfSR was extracted using RNAprotect Bacteria Reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. RNA was reverse transcribed into cDNA using an Omniscript RT kit (Invitrogen Life Technologies), according to the manufacturer's instructions. The mutant was detected and confirmed as correct using PCR. The primer sequences were as follows: TcfSR forwad, GGCGGCTTGTGGCGCAG, and reverse, GCCTTGGTCGTTCCTGCTTG. Briefly, total RNA was isolated from *Brucella* parental strain and mutant strain using a commercial kit (Omega Bio-Tek), according to the manufacturer's instructions. RNA concentration and purity were determined using 2% agarose gel electrophoresis, and the RNA was measured at an optical density of 260/280, with an absorption ratio of >1.8 (ELx808; Bio-Tek Instruments, Inc., Winooski, VT, USA). cDNA was synthesized in a 20 μl reaction mixture, containing 2 μg total RNA, using the Omniscript Reverse Transcription kit (Takara Cio, Inc.) and oligo(dT) primers (forward, ATGATGCGCCCGCGCAG and reverse, CTAATGCAGCACGCGCCC), according to the manufacturer's instructions. The total PCR reaction volume was 15 μl. The PCR reaction conditions were as follows: 5 min at 95˚C, followed by 30 cycles at 65˚C for 40 sec and 72˚C for 1 min, and 10 min at 72˚C.

Evaluation of the 16MΔTcfSR mutant survival capacity in RAW 264.7 macrophages. A RAW 264.7 murine macrophage cell line was used to assess the survival capability of 16MΔTcfSR, M5-90 or the *B. melitensis* 16M parental strain. RAW 264.7 murine macrophages at a density of $2x10^6$ cells/well were cultured in a six-well plate for 24 h at 37˚C and 5% CO_2. The cells were infected with *Brucella* at a multiplicity of infection (MOI) of 100. At 45 min post-infection, the cells were washed three times with phosphate-buffered saline (PBS) and incubated with 50 μg/ml gentamicin (Invitrogen Life Technologies) for 1 h to eliminate any extracellular bacteria. Subsequently, the culture was replaced with Dulbecco's modified Eagle's medium (Gibco Life Technologies, Carlsbad, CA, USA) containing 25 μg/ml gentamicin. At 0, 4, 8, 12 and 24 h post-infection, the supernatant was discarded and the cells were lysed by incubation in PBS containing 0.1% (v/v) Triton X-100. The number of colony forming units (CFU) was determined by plating serial dilutions of the lysates on TSA plates. All assays were performed in triplicate and repeated at least three times (11).

Evaluation of the 16MΔTcfSR mutant survival capability in mice. BALB/c mice (age, 6 weeks; n=50 per group) were inocu-

lated intraperitoneally (i.p.) with a 200-μl sample of 1x10^6 CFU 16MΔTcfSR, M5-90 or 16M, or 200 μl PBS for the control mice. The virulence of the bacteria in the mice was evaluated by enumeration of the bacteria in the spleens at different time points post-inoculation. At weeks 2, 4, 6, 8 and 10 post-inoculation, the mice (n=10/time point per group) were euthanized by CO_2 asphyxiation and the spleens were removed aseptically. The splenocytes were homogenized in 1 ml PBS containing 0.1% (v/v) Triton X-100, serially diluted and plated on TSA plates. All the assays were repeated twice with similar results.

Evaluation of the protection efficiency induced by 16MΔTcfSR in mice. Groups of female BALB/c mice (age, 6 weeks; n=20 per group) were injected i.p. with 1x10^6 CFU (200 μl) 16MΔTcfSR (experimental vaccine group) or M5-90 (reference vaccine control group), or with 200 μl PBS (unvaccinated control group). At week 11 post-vaccination, the mice were challenged i.p. with 1x10^6 CFU per mouse (200 μl) of the 16M virulent strain. The mice (n=10/time point per group) were euthanized at weeks 2 and 4 after the challenge, and bacterial CFU in the spleens were determined, as aforementioned. A mean value for each spleen count was obtained following logarithmic conversion. Log units of protection were obtained by subtracting the mean log CFU for the experimental group from the mean log CFU for the control group, as previously described (12). The experiments were repeated twice.

Evaluation of antibody production. To determine the antibody production of sera from the inoculated mice, serum samples were obtained from the mice at 2, 4, 6, 8, and 10 weeks post-immunization. Immunoglobulin G (IgG) levels were determined using the ELISA Quantikine Mouse kit (R&D Systems, Inc., Minneapolis, MN, USA) (13). Briefly, heat-killed and sonicated *B. melitensis* 16M whole-cell antigen was used to coat 96-well plates at a concentration of 25 μg protein/well. Following overnight incubation at 4˚C, the plates were washed once with 100 μl PBS containing 0.05% Tween-20, and blocked with 200 μl blocking buffer [10% heat-inactivated fetal bovine serum (Gibco Life Technologies) in PBS, pH 7.4] for 2 h at 37˚C. Mice serum samples in dilution buffer (1:300) were added to the wells in triplicate and incubated for 2 h at 37˚C. Following 2 h incubation, 100 μl rabbit anti-mouse IgG-horseradish peroxidase (1:3,000) was added, and the plates were incubated at 37˚C for 30 min. After two washes with wash solution, 100 μl TMB substrate solution was added to each well and incubated at 37˚C in the dark for 15 min. The reaction was terminated following the addition of 50 μl H_2SO_4 and the absorbance was measured at 450 nm (Scan 500; Interscience, Saint-Nom-la-Bretèche, France). All assays were performed in triplicate and repeated at least three times.

Cytokine production assay. Briefly, 10 weeks post-vaccination, the BALB/c mice were sacrificed and their spleens were aseptically removed. Single cell suspensions were obtained from the spleens by homogenization, as described previously. The cells were suspended in complete RPMI 1640 medium (Gibco Life Technologies) supplemented with 2 mM L-glutamine (Solarbio Science & Technology, Co., Ltd., Beijing, China) and 10% (v/v) heat-inactivated fetal bovine serum. Splenocytes were cultured in 96-well plates (4x10^5 cells/well); the cultures were

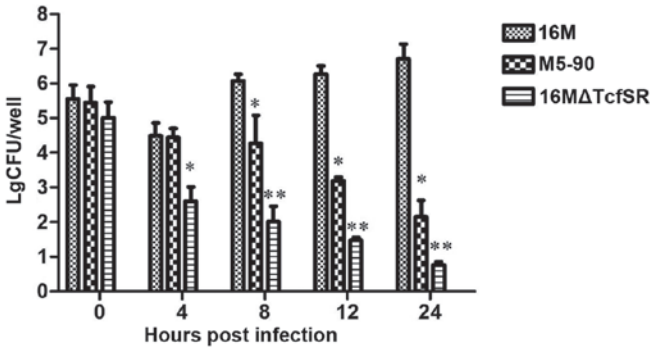

Figure 1. Survival capability of different *Brucella* strains in RAW 264.7 murine macrophages. Macrophages were infected with 16MΔTcfSR, M5-90 and 16M at a multiplicity of infection of 100. At the indicated time points, macrophages were lysed and the amount of bacteria was quantified by plating serial dilutions on tryptone soy agar plates. *P<0.05 and **P<0.01, vs. 16M-injected mice. CFU, colony forming units.

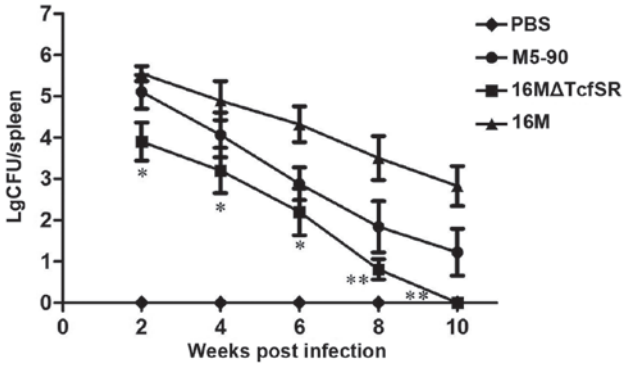

Figure 2. Clearance of different *Brucella* strains following infection. Mice were intraperitoneally inoculated with 1x10^6 CFU 16M, 16MΔTcfSR or M5-90, or PBS in the control group. At the indicated time points, the mice (n=10/time point) were euthanized and the spleens were removed aseptically. The bacteria were calculated by plating serial dilutions on tryptone soy agar plates, from which the bacteria numbers in the spleens were determined. *P<0.05 and **P<0.01, vs. all other groups. PBS, phosphate-buffered saline; CFU, colony forming units.

stimulated by adding 25 μg heat-killed *B. melitensis* 16M lysate/well, 0.5 μg ConA (positive control), or medium alone (negative control). The cells were then incubated at 37˚C with 5% CO_2 for 72 h. The plates were centrifuged at 350 x g for 10 min, and the clear culture supernatants were collected and stored at -20˚C. Interferon (IFN)-γ levels were estimated using an iELISA. The detection of IFN-γ was conducted as previously described (14). IFN-γ levels were determined using an ELISA Quantikine Mouse kit (R&D Systems, Inc.), according to the manufacturer's instructions.

Cloning, expression and purification of the recombinant protein. The open reading frames of TCFS and L7/L12 were amplified by PCR using the DNA from the *B. melitensis* 16M strain (14). Subsequently, the amplified DNA fragments were cloned into the pET-32a vector (Novagen®; EMD Biosciences, Inc., Madison, WI, USA) and expressed in *E. coli* BL21 (DE3) cells (Novagen®; EMD Biosciences, Inc.,) as an N-terminal His-tagged recombinant protein. The recombinant proteins were separated and analyzed with SDS-PAGE (12%). The

Table I. Evaluation of the protective efficacy of 16MΔTcfSR and M5-90 vaccinations against *Brucella melitensis* 16M infection in BALB/c mice.

	Log CFU spleen[a]		Units of protection[b]		Uninfected/total mice[c]	
Vaccination	Week 2	Week 4	Week 2	Week 4	Week 2	Week 4
16MΔTcfSR	5.10±0.13[d]	4.83±0.11[d]	2.02	1.76	2/10	2/10
M5-90	5.48±0.16[d]	4.98±0.11[d]	1.44	1.63	1/10	1/10
PBS	7.12±0.19	6.61±0.15	-	-	0/10	0/10

[a]Results are expressed as the mean ± standard deviation. [b]Log units of protection = average log CFU in spleens of control unvaccinated mice - average log CFU in spleens of vaccinated mice. [c]Number of mice found free from the *B. melitensis* 16M challenging strain at necropsy with respect to the total number of mice challenged. [d]P<0.01, vs. PBS unvaccinated controls. PBS, phosphate-buffered saline; CFU, colony forming units.

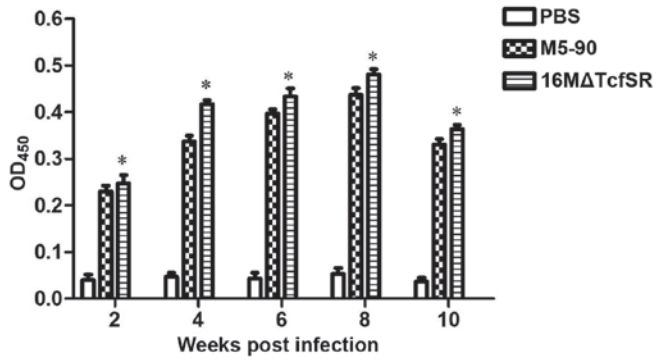

Figure 3. Humoral immune response in the serum of mice immunized with different *Brucella* strains. Mice were intraperitoneally inoculated with 1x10^6 CFU 16MΔTcfSR or M5-90, while the control group received PBS. At weeks 2, 4, 6, 8 and 10 post-vaccination, serum samples were collected and the levels of immunoglobulin G antibodies were determined using an enzyme-linked immunosorbent assay. Results are expressed as the mean ± standard deviation (n=10/time point) of the absorbance values at 450 nm (OD_{450}). *P<0.05, vs. PBS unvaccinated controls. PBS, phosphate-buffered saline; CFU, colony forming units; OD, optical density.

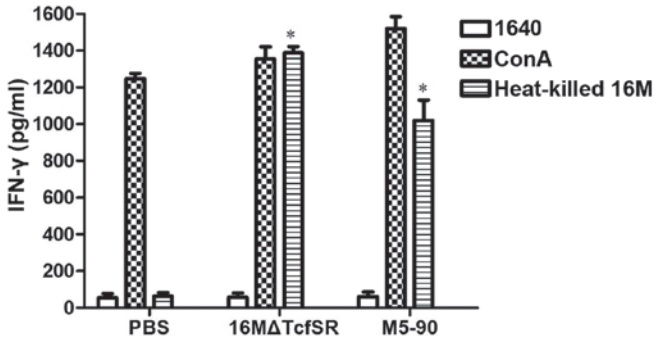

Figure 4. Production of cytokines in the stimulated spleen cells from different *Brucella* strains. Spleens were collected from mice that had been intraperitoneally inoculated with 1x10^6 CFU 16MΔTcfSR or M5-90, while the control group received PBS. At week 10 post-vaccination, splenocytes were recovered and stimulated with heat-killed *B. melitensis* 16M, ConA or RPMI 1640. The splenocyte culture supernatants were harvested and IFN-γ production (pg/ml) was assessed using an enzyme-linked immunosorbent assay. *P<0.05, vs. PBS unvaccinated controls subjected to the same stimulus. PBS, phosphate-buffered saline; CFU, colony forming units; IFN, interferon; ConA, concanavalin A.

recombinant proteins, TCFS and L7/L12, were purified using affinity chromatography with Ni^{2+}-conjugated Sepharose (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

Western blot analysis. Cell lysates of the recombinant proteins, TCFS and L7/L12, were analyzed by western blot analysis, as previously described (15). The purified recombinant TCFS and L7/L12 proteins were separated by 12% SDS-PAGE and electrotransferred to nitrocellulose membranes (Solarbio Science & Technology, Co., Ltd.) using a Mini Trans-Blot Cell (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 200 mA for 1 h. The membranes were blocked for 2 h with 5% nonfat milk in TBST (100 mM trus-HCl; 150 mM NaCl; 0.05% Tween 20, pH 7.2) at 37˚C. The membranes were then washed three times with TBST and incubated with a primary *Brucella*-vaccinated sera (1:300) for 1 h at 37˚C, and a sheep anti-mouse IgG horseradish peroxidase (HRP)-conjugated secondary antibody (1:5,000; cat. no. ab6808; Abcam, Cambridge, UK) for 1 h at 37˚C. The membrane was developed using an enhanced HRP-3,3'-diaminobenzidine substrate color kit (Beyotime Institute of Biotechnology, Haimen, China).

TCFS iELISA. Serum samples were obtained from the mice infected with the various *Brucella* strains. Antibody responses to the purified recombinant TCFS protein were estimated using a TCFS-based indirect ELISA (R&D Systems, Inc.), as previously described (16).

Statistical analysis. Bacterial survival in the macrophage cell line and in the mice was expressed as the mean ± standard deviation (SD) of the log CFU. Furthermore, the antibody response and cytokine production results are expressed as the mean ± SD of the optical density value at 450 nm. The differences between groups were analyzed by analysis of variance using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

16MΔTcfSR is attenuated compared with B. melitensis 16M for survival in RAW 264.7 murine macrophages. RAW 264.7 murine macrophages were infected with 16MΔTcfSR, M5-90 and *B. melitensis* 16M, and the survival capacity and repli-

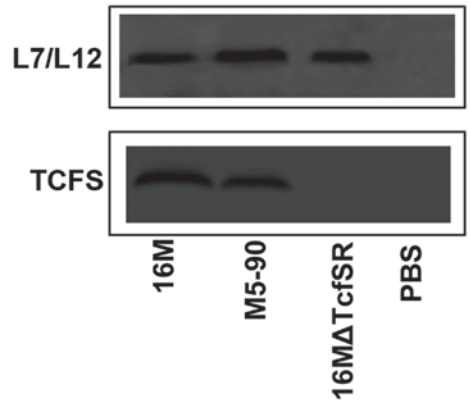

Figure 5. Immune response of TCFS and L7/L12 to 16MΔTcfSR immunization sera. Sera from mice immunized with 16MΔTcfSR, 16M, M5-90 and PBS were collected, and western blot analysis was used to detect the specific expression of antibodies against TCFS and L7/L12. TCFS protein was shown to react with the 16M- and M5-90-inoculated mice serum; however, no reaction was observed with the 16MΔTcfSR-inoculated mice serum. PBS, phosphate-buffered saline.

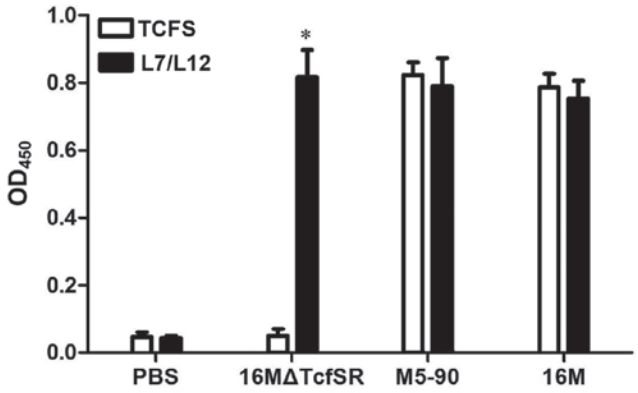

Figure 6. Humoral immune responses to TCFS and L7/L12 were assessed in the serum using an indirect enzyme-linked immunosorbent assay. Humoral immune responses against the TCFS protein were not detected in the serum collected from 16MΔTcfSR-inoculated mice. PBS, phosphate-buffered saline; OD, optical density.

cation capability of the *Brucella strains* in the macrophage cell line were determined. The macrophages were infected with the three strains at a MOI of 100, and the surviving bacteria were calculated. At 0 h post-infection, no statistically significant difference in the amount of bacteria was observed among the three strains. However, at 4 h post-infection, there was a 1.89-log and 1.84-log decrease ($P<0.01$) in the bacteria number of 16MΔTcfSR when compared with that of 16M and M5-90, respectively. By 8 h post-infection, a 4.08-log and 2.27-log decrease ($P<0.01$) was observed in the bacteria number of 16MΔTcfSR when compared with that of 16M and M5-90, respectively. Furthermore, at 12 h post-infection, there was a 4.79-log and 1.71-log decrease ($P<0.01$) in the bacteria number of 16MΔTcfSR compared with that of 16M and M5-90, respectively. Finally, at 24 h post-infection, a 5.94-log and 1.38-log decrease ($P<0.01$) was observed in the bacteria number of 16MΔTcfSR when compared with that of 16M and M5-90, respectively (Fig. 1). These results indicated that the 16MΔTcfSR mutant had a decreased survival capability in RAW 264.7 murine macrophages compared with the 16M and M5-90 strains, indicating that 16MΔTcfSR was attenuated compared with *B. melitensis* 16M for survival in RAW 264.7 murine macrophages.

16MΔTcfSR is attenuated in BALB/c mice. To determine the survival capability of the various *Brucella* strains in the BALB/c mice, the mice were inoculated i.p. with $1x10^6$ CFU 16MΔTcfSR or M5-90. When compared with M5-90 and 16M, the number of splenic CFU in the 16MΔTcfSR-infected mice was significantly reduced ($P<0.01$) at weeks 2, 4, 6, 8 and 10. In addition, at week 10 post-inoculation, 16MΔTcfSR was shown to be completely cleared in the spleens of the mice (Fig. 2). Thus, the results demonstrated that the 16MΔTcfSR mutant was attenuated in the BALB/c mice.

16MΔTcfSR induces immune protection against a challenge with B. melitensis 16M. In order to determine the protection efficiency of 16MΔTcfSR, the mice were vaccinated i.p. with $1x10^6$ CFU 16MΔTcfSR or M5-90, or PBS as the control. At week 11 post-vaccination, the mice were challenged i.p. with $1x10^6$ CFU (200 μl) of the 16M virulent strain. The mice immunized with 16MΔTcfSR exhibited significantly fewer splenic *Brucella* colonies when compared with the non-immunized mice at weeks 2 (2.02-log) and 4 (1.76-log) following the challenge ($P<0.05$; Table I). In addition, a similar CFU of protection was observed in the mice immunized with 16MΔTcfSR compared with those immunized with M5-90 ($P<0.05$). The 16MΔTcfSR vaccination exhibited a similar protective efficacy compared with that of the M5-90 vaccination (Table I). Thus, the results indicated that 16MΔTcfSR was able to provide a similar protection efficacy against the challenge with 16M to that of the M5-90 vaccine strain.

16MΔTcfSR induces humoral and cytokine responses. Serum samples from the mice inoculated with 16MΔTcfSR, M5-90 or PBS were obtained from the immunized mice at selected intervals following immunization to monitor the total IgG levels using an ELISA. For the mice inoculated with 16MΔTcfSR and M5-90, the total IgG levels peaked at week 8 post-inoculation, and there was no statistically significant difference between these two groups ($P>0.05$). However, these two groups demonstrated significantly higher IgG levels when compared with the control group ($P<0.01$; Fig. 3).

To characterize the cellular immune response, the IFN-γ levels in the splenocytes of the 16MΔTcfSR- and M5-90-vaccinated mice were evaluated at week 10 following the vaccination. Eight weeks after immunization, splenocytes were obtained from the mice and the levels of IFN-γ in the culture supernatants were determined in triplicate. As a positive control, the nonspecific mitogen ConA was used. Spleen cells from 16MΔTcfSR or M5-90 vaccinated animals were induced to secrete high levels of IFN-γ after stimulation. As expected, ConA stimulation induced the production of IFN-γ in spleen cells from all three groups, and no cytokine production was induced by PBS stimulation in any of the groups. The IFN-γ levels in the splenocytes of the 16MΔTcfSR-vaccinated mice were shown to be significantly higher, as compared with those in the PBS-injected mice, and slightly higher as compared with those in the M5-90-vaccinated mice (Fig. 4).

Differentiation of 16MΔTcfSR immunization from infection using the protein TCFS as a test antigen. To consider whether the TCFS protein may be used as a diagnostic marker antigen for the differentiation between vaccinated and infected mice, the recombinant purified protein, TCFS, was interacted with 16MΔTcfSR-, 16M- and M5-90-inoculated sera. Western blot analysis was performed using immunogenic L7/L12 protein as positive control to determine whether antibodies against TCFS and L7/L12 were induced in these sera. For the positive control, an L7/L12 reaction band was observed in the serum of the 16MΔTcfSR-, 16M- and M5-90-infected mice. In addition, the TCFS protein was shown to react with the 16M and M5-90-inoculated mice serum to produce specific bands. However, the TCFS protein was not shown to react with the 16MΔTcfSR-inoculated mice serum (Fig. 5). Antibodies against the two proteins were also detected using an iELISA, and the results from the iELISA were similar to that from the western blot analysis (Fig. 6). Furthermore, antibodies against L7/L12 were detected in the sera of the 16MΔTcfSR-, M5-90- and 16M-vaccinated mice, whereas antibodies against TCFS were only detected in the sera of the M5-90- and 16M-vaccinated mice. These results indicated that the TCFS protein had good reactogenicity; thus, TCFS may be used to differentiate the vaccination from a natural infection.

Discussion

The majority of the currently licensed vaccines have numerous drawbacks, including residual virulence, induction of splenomegaly, and interference with serodiagnosis (17-20). With regard to these limitations, significant effort has been made to develop novel vaccines. The TCS, TcfSR, is a regulatory system that controls gene expression and is involved in the virulence for *Brucella*. In the present study, the 16MΔTcfSR mutant was constructed and the virulence and protection efficacies were evaluated in a macrophage cell line and mice to assess the ability of 16MΔTcfSR in maintaining protective efficacy.

Thus, a deletion mutant of TcfSR was constructed with the aim to confirm that the reduced survival capability of the mutant was directly associated with the deletion of the promoter for TcfSR. The 16MΔTcfSR was evaluated for survival and attenuation in a RAW 264.7 murine macrophage cell line and BALB/c mice. As demonstrated by the present study, the 16MΔTcfSR mutant was much more susceptible to eradication in the macrophage cell line compared with the wide-type 16M strain. Moreover, clearance of 16MΔTcfSR was observed within 10 weeks in the BALB/c mice, which was faster compared with M5-90. These results are consistent with hypothesis that TcfSR is involved in the virulence of *Brucella*.

An ideal *Brucella* live attenuated vaccine combines survival capability with persistence in the host (21). Therefore, in the present study, the protective efficacy of the 16MΔTcfSR mutant was investigated. The results demonstrated that vaccination with 16MΔTcfSR was able to provide good protective efficacy against a challenge with the wild-type 16M strain. In addition, the 16MΔTcfSR vaccination conferred a level of protection that was equivalent to that conferred by the M5-90 vaccination.

The cytokine profiles and antibody responses were also investigated to evaluate the protection conferred by the 16MΔTcfSR vaccination. *Brucella* is a facultative, intracellular parasitic pathogen. The organism infects the host cells and primarily provokes cell-mediated immunity. IFN-γ is produced by T lymphocytes and is a potent macrophage-activating factor. The T helper 1 immune responses characterized by IFN-γ production are known to be associated with the protective immunity against *Brucella*, and these responses are stimulated most effectively by live vaccines (22). IFN-γ plays an important role in eradicating intracellular *Brucella* (23). IFN-γ exerts antibacterial effects; thus, the current study detected the host secretion levels of IFN-γ in order to evaluate the antimicrobial capacity and cellular immunity of the host. A previous study demonstrated that IFN-γ is a critical cytokine required for macrophage bactericidal activity (24). The results of the present study demonstrated that treatment with 16MΔTcfSR induced a higher secretion of IFN-γ compared with that observed following treatment with M5-90. In addition, high levels of IgG in the host humoral response can prevent *Brucella* from entering the cells, thereby reducing the injury on the body. Levels of specific IgG antibodies in the serum are important for evaluating the immunogenicity of brucellosis. In the present study, the results with regard to the humoral immune response revealed that mice infected with 16MΔTcfSR produced anti-*Brucella* IgG. In addition, vaccination with 16MΔTcfSR conferred levels of IgG that were at least similar to that conferred by the M5-90 vaccination.

Serological diagnosis using a variety of techniques, such as the Rose Bengal plate test, serum agglutination test and iELISA, is the most convenient method for brucellosis diagnosis. These methods use hot saline extract and lipopolysaccharide (LPS) as antigens of smooth *Brucella*. *Brucella* LPS is the most important antigen during the immune response in brucellosis (25). However, differentiating between the serum of vaccinated animals and the serum of infected animals using LPS-based serological tests is difficult. Thus, the present study evaluated the possibility of using TCFS protein as a diagnostic antigen marker. Recombinant protein expression of TCFS was conducted, and the protein was used to detect the antibody profiles in the different serum samples. The results revealed that a humoral immune response to TCFS was detected in the serum of mice infected with 16M and M5-90; however, a reaction was not observed in the 16MΔTcfSR-vaccinated serum or in the PBS-treated controls. These results indicated that TCFS may be used as a diagnostic marker antigen for the serological diagnosis of brucellosis. Furthermore, the presence of antibodies against TCFS following 16MΔTcfSR vaccination was investigated using an iELISA. The results indicated that the mice infected with the 16M and M5-90 strains tested positive for the presence of TCFS antibodies, whereas the mice infected with 16MΔTcfSR exhibited negative expression. Therefore, vaccination with 16MΔTcfSR enables the differentiation between vaccination and infection. The TCFS protein may allow for the distinction and differentiation of the vaccination from infection; however, confirmation is required in further studies.

In the present study, the 16MΔTcfSR mutant of the TcfSR TCS in *Brucella* was successfully constructed. The 16MΔTcfSR mutant exhibited a reduced survival capacity in the macrophage

RAW 264.7 cell line and BALB/c mice, while providing a level of protection similar to that provided by the M5-90 vaccine strain against a *B. melitensis* virulence 16M challenge. In addition, immunization with the 16MΔTcfSR vaccination induced humoral and cytokine responses. Furthermore, the present study demonstrated that TCFS protein is an ideal diagnostic antigen for the differentiation of immunization from infection using an iELISA. Therefore, 16MΔTcfSR enables the differentiation between the vaccination and infection. In conclusion, 16MΔTcfSR is a potential vaccine candidate with reduced virulence that provides high protection efficiency. In addition, TCFS protein may be used to differentiate between infected and vaccinated animals by serological diagnosis.

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