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Protective immunity against *Rickettsia heilongjiangensis* in a C3H/HeN mouse model mediated by outer membrane protein B-pulsed dendritic cells

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Rickettsia heilongjiangensis is an obligate intracellular bacterium that causes Far-Eastern tick-borne spotted fever. Outer membrane protein B (OmpB) is an important surface protein antigen of rickettsiae. In the present study, the *ompB* gene of *R. heilongjiangensis* was divided into four fragments, resulting in four recombinant proteins (OmpB-p1, OmpB-p2, OmpB-p3, and OmpB-p4). Each OmpB was used *in vitro* to stimulate murine bone marrow-derived dendritic cells (BMDCs) of C3H/HeN mice, and the OmpB-pulsed BMDCs were transferred to naïve C3H/HeN mice. On day 14 post-transfer of BMDCs, the mice were challenged with *R. heilongjiangensis* and the rickettsial loads in the mice were quantitatively determined on day 7 post-challenge. Mice receiving BMDCs pulsed with OmpB-p2, OmpB-p3, or OmpB-p4 exhibited significantly lower bacterial load compared with mice receiving OmpB-p1-pulsed BMDCs. $CD4^+$ and $CD8^+$ T cells isolated from the spleen of C3H/HeN mice receiving BMDCs pulsed with each OmpB were co-cultured with BMDCs pulsed with the respective cognate protein. In flow cytometric analysis, the expression level of CD69 on $CD4^+$ or $CD8^+$ T cells from mice receiving BMDCs pulsed with OmpB-p2, OmpB-p3, or OmpB-p4 was higher than that on cells from mice receiving OmpB-p1-pulsed BMDCs, while the expression level of tumor necrosis factor (TNF)-α on $CD8^+$ T cells and interferon (IFN)-γ on the $CD4^+$ and $CD8^+$ T cells from mice receiving OmpB-p2, -p3, or -p4 was significantly higher than on cells from mice receiving OmpB-p1-pulsed BMDCs. Our results suggest that the protective OmpBs could activate $CD4^+$ and $CD8^+$ T cells and drive their differentiation toward $CD4^+$ Th1 and $CD8^+$ Tcl cells, respectively, which produce greater amounts of TNF-α and, in particular, IFN-γ, to enhance rickettsicidal activity of host cells.

***Rickettsia heilongjiangensis*, dendritic cells, outer membrane protein, protective immunity**

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Rickettsiae are obligate intracellular gram-negative bacteria that require various host cells for growth and replication. The genus *Rickettsia* has been divided into three groups: the *R. bellii* group, the typhus group, and the spotted fever group (SFG). The *R. bellii* group contains *R. bellii* and *R. canadensis* and is not known to be pathogenic; the typhus group includes *R. prowazekii* and *R. typhi* that cause louse- and flea-borne typhus, respectively; the SFG is made up of more than 20 recognized species that are transmitted mainly by ticks, but also by fleas and mites [1]. *Rickettsia heilongjiangensis*, the causative agent of Far-Eastern spotted fever (FESF), was first isolated from *Dermacentor silvarum* ticks in the Northeastern area of China in 1983 and classified within the *R. japonica* subgroup of SFG [2]. FESF, an emerging tick-borne disease, has been diagnosed in patients

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in northeastern China [3], Siberia, and the Far East of Russia [4–6], and Honshu island of Japan [7]. Most of the patients naturally infected by *R. heilongjiangensis* had fever, chills, headache, dizziness, myalgias, arthralgias, and anorexia, after which most also presented with a macular or maculopapular rash, while certain patients had a primary lesion (eschar) at the site of tick attachment and lymphadenopathy regional to the eschar [6]. In our recent study, *R. heilongjiangensis* was used to infect BALB/c mice by inoculation of the retro-orbital venous plexus to imitate a blood infection caused by tick biting. In a murine model, after inoculation of the retro-orbital venous plexus, *R. heilongjiangensis* rapidly entered the circulation for systemic dissemination and made its way into the liver, spleen, lungs, and brain; severe pathological lesions were observed in these organs [8].

Dendritic cells (DCs) are antigen-presenting cells, which take up and process exogenous antigens for presentation to the host immune system. Immature DCs are particularly potent in antigen uptake and efficiently capture invading pathogens and then migrate to draining secondary lymphoid tissues, where they present antigens to naïve T cells and NK cells [9]. Following antigen stimulation, immature DCs undergo maturation or an activation process with high expression of major histocompatibility complex (MHC) and costimulatory molecules, and the mature (activated) DCs present antigens to and activate naïve T cells, eliciting adaptive immune responses against invading pathogens through the direction of T-helper (Th) cell differentiation and processing of pathogen molecules [10].

OmpA and OmpB have been demonstrated to be immunodominant surface proteins of SFG rickettsiae, and both proteins, particularly OmpB, have been shown to be important protective antigens in immune responses against spotted fever caused by *R. rickettsii* and/or *R. conorii* [11–13]. In the present study, the *ompB* gene (4875 bp) of *R. heilongjiangensis* was divided into four fragments, resulting in four recombinant OmpB proteins (OmpB-p1, OmpB-p2, OmpB-p3, and OmpB-p4). In our previous studies, murine bone marrow derived DCs (BMDCs) pulsed by protective antigen proteins of *Coxiella burnetii* could mediate specific immune responses against Q fever [14,15]. Therefore, in the current study, the four OmpB proteins were used to pulse BMDCs from C3H/HeN mice *in vitro* and the pulsed BMDCs were transferred to naïve C3H/HeN mice. After challenge with viable *R. heilongjiangensis*, the rickettsial loads in the recipient mice were quantitatively determined to evaluate the efficacy of passive protection mediated by BMDCs pulsed with various OmpB proteins. The mechanism of protection against *R. heilongjiangensis* infection conferred by the OmpB-activated BMDCs was investigated by analysing the levels of activation and related cytokine expression in $CD4^+$ and $CD8^+$ T cells from mice receiving BMDCs pulsed with various OmpB proteins.

1 Materials and methods

1.1 Mice

Specific pathogen-free male C3H/HeN mice at 6–7 weeks of age were purchased from the Vital River Laboratories (Beijing, China). The animal usage was approved by the Beijing Administrative Committee for Laboratory Animals and the animal care met the standards of this committee.

1.2 Culture and purification of *R. heilongjiangensis*

Rickettsia heilongjiangensis (054 strain) was grown in Vero cells in Dulbecco's minimal essential medium supplemented with 5% heat-inactivated fetal bovine serum and 2 mmol L^{-1} L-glutamine at 33°C in 5% CO_2. The infected cells were harvested and rickettsiae were purified from the host cells by centrifugation and Renografin gradient centrifugation according to the process described previously [8]. The purified rickettsiae were collected and suspended in culture media. The amount of rickettsiae was determined by quantitative polymerase chain reaction (qPCR) [8], and the viable organisms were ~95% of the collected organisms that were measured using a bacterial viability kit (LIVE/DEAD BacLightBacterial Viability Kits, Invitrogen, Carlsbad, CA). One part of the purified organisms was treated at 60°C for 30 min and the heat-inactivated organisms were used as whole cell antigen (WCA) of *R. heilongjiangensis*.

1.3 Recombinant protein antigens

Four fragments of the *ompB* gene were amplified from the genomic DNA of *R. heilongjiangensis* using four pairs of primers, *ompB*-1 (5′-GAGGATCCCACATAGTTGACGTTGGT-3′ and 5′-GAGCTCGAGAATTTCTGCAAGTGGAT-3′), *ompB*-2 (5′-GCGGGATCCGCCGGTACAAATTTAGG-3′ and 5′-GCGTCGACA AGAGTAATTTTACCGTCA-3′), *ompB*-3 (5′-CGCGGATCCCAAGTAACGTTTACTACAG-3′ and 5′-GCGTCGACATCAACAGCCTCGTCACCT-3′), and *ompB*-4 (5′-CGCGGATCCTCTGAAGCGGGAGCAAT-3′ and 5′-GCGTCGACAGAGTACCTTGATGTG-3′). The sequences of GGATCC, CTCGAG, and GTCGAC represent the restriction enzyme sites of *Bam*H I, *Xho* I, and *Sal* I, respectively. The four amplified gene fragments (*ompB-1*, *ompB-2*, *ompB-3*, and *ompB-4*) were cloned into plasmid pET-32a(+) and expressed in *Escherichia coli* BL21 (DE3) (Novagen, Madison, WI) as previously described [16]. The resulting recombinant OmpB proteins (OmpB-P1, OmpB-P2, OmpB-P3, and OmpB-P4) and TrxA (*E. coli* thioredoxin), a fusion tag expressed by the pET-32a(+) vector, were purified by affinity chromatography with Ni-NTA resin (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. The purified recombinant OmpB proteins were analyzed by immunoblot-

ting with sera obtained from C3H/HeN mice experimentally infected with *R. heilongjiangensis* on day 21 post-infection as described previously [15]. Then the purified recombinant proteins were treated with Triton X-114 (Amresco, Solon, OH) to remove lipopolysaccharide (LPS) as described previously [17]. The recombinant proteins were used to pulse BMDCs only when the LPS level was below 0.005 endotoxin units/mL determined using the *Limulus* amoebocyte assay (Sigma-Aldrich, St. Louis, MO, USA).

1.4 Preparation of BMDCs

BMDCs were isolated from bone marrow of C3H/HeN mice as described previously [14,18]. Briefly, a single-cell suspension from bone marrow was prepared from the mouse femurs and the bone marrow cells thus obtained were suspended in red blood cell lysis buffer (139.6 mmol L^{-1} NH_4Cl, 16.96 mmol L^{-1} Tris, pH 7.2) and incubated at 37°C for 5 min. After washing with PBS, the cell suspension was incubated with mouse monoclonal antibodies (anti-CD4 (L3T4), anti-CD8a, anti-CD45R, anti-MHC-II (eBioscience, CA, USA), and rabbit serum complements (Merck, Germany) at 37°C for 1 h to remove lymphocytes and MHC-II^+ cells. After a PBS wash, the remaining cells were suspended in complete RPMI-1640 medium containing 10% fetal bovine serum, 2 mmol L^{-1} glutamine, 50 μmol L^{-1} 2-mercaptoethanol, 100 μg mL^{-1} streptomycin sulfate, and 100 U mL^{-1} penicillin. Approximately 1×10^6 cells were added to each well of a six-well plate and granulocyte-macrophage colony-timu- ting factor (GM-CSF, 20 ng mL^{-1}; Peprotech, NJ, USA) and interleukin (IL)-4 (10 ng mL^{-1}, Peprotech) were added to the culture medium. After 6 days' culture, nonadherent cells were collected and suspended in complete 1640 medium.

1.5 BMDC activation assay

Suspensions of BMDCs were dispensed into wells of the plate (1×10^6 cells/well) and stimulated with 10 μg mL^{-1} of rickettsial WCA or protein antigen (OmpB-p1, OmpB-p2, OmpB-p3, or OmpB-p4) at 37°C and 5% CO_2 for 24 h [14,15]. In parallel, the BMDCs were stimulated with 10 μg mL^{-1} of TrxA or antigen dilution buffer alone (mock stimulation). After stimulation, the BMDCs were harvested from wells using cell scraper and stained with a combination of anti-mouse monoclonal antibodies (BD Pharmingen, San Jose, CA, USA), including anti-CD11b-FITC, anti-CD11c-PE, anti-CD40-APC, anti-CD80-APC, anti-CD86-APC, and anti-MHC-II-APC according to the manufacturer's protocol. Stained cells were assessed using a FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and data were analyzed with CellQuest software (version 5.2, BD Biosciences).

1.6 BMDC adaptive transfer and mouse challenge

After 24 h of antigen stimulation, BMDCs were harvested and suspended in RPMI-1640 medium at a concentration of 1×10^6 cells mL^{-1}. Five C3H/HeN mice were injected intraperitoneally with BMDCs pulsed with each antigen (5×10^5 cells per mouse). On day 14 post-transfer (pt) of BMDCs, mice were challenged intraperitoneally with *R. heilongjiangensis* (1×10^6 organisms per mouse). On day seven post-challenge, the mice were sacrificed and organs (lung, spleen, liver, and brain) were collected. DNA was extracted from each organ using a DNeasy blood and tissue kit (Qiagen GmbH). The DNA samples were determined by qPCR specific for *R. heilongjiangensis* to measure the rickettsial loads in mice as per the method described previously [8].

1.7 Antigen-specific T cells induced by antigen-pulsed BMDCs

On day 14 pt, three C3H/HeN mice receiving BMDCs pulsed with each antigen (WCA, OmpB-P1, OmpB-P2, OmpB-P3, OmpB-P4, TrxA, or dilution buffer alone) were sacrificed and their spleens were collected. The spleen tissue was ground and disintegrated into single cells on a 200-mesh sterile copper grid and the lymphocytes was separated from the cell suspension using lymphocyte separation medium (TBD, Tianjin, China) based on a previously described method [14]. The $CD4^+$ or $CD8^+$ T cells were isolated from the lymphocytes using a mouse $CD4^+$ or $CD8^+$ T cells Isolation Kit II and MACS (MiltenyiBiotec GmbH, BergischGladbach, Germany) according to the manufacturer's protocols. The purity of the isolated T cells was greater than 97%, as assessed by a flow cytometric measurement with anti-CD3e-PerCP and anti-CD4-APC/anti-CD8a-APC. The $CD4^+$ or $CD8^+$ T cells were cultured with incomplete RPMI-1640 medium containing 10% fetal bovine serum. The cognate-antigen-pulsed BMDCs were added to co-culture with $CD4^+$ or $CD8^+$ T cells at 1:5 BMDC/T-cell ratio. After 18 h of co-culture, cells were collected for the flow cytometry assay.

For the flow cytometry assay of CD69, the $CD4^+$ or $CD8^+$ T cells were stained with anti-CD3e-PerCP, anti-CD4-APC/anti-CD8a-APC, and anti-CD69-PE at room temperature for 15 min. For detection of intracellular cytokines, brefeldin A (Golgi Stop; BD Biosciences) was added to the cell cultures for 6 h prior to cell harvest. T cells were then harvested and stained with anti-CD3e-PerCP, anti-D4-APC/anti-CD8a-APC, and anti-IL-4-PE/anti-interferon (INF)-γ-PE/anti-tumor necrosis factor (TNF)-α-PE at room temperature for 15 min after washing in permeabilization buffer (0.5 mL/10^6cells). The reagents used for flow cytometry were all produced by BD Pharmingen. After antibody staining, the expression of IFN-γ, TNF-α and IL-4 on $CD4^+$ or $CD8^+$ T cells was determined by flow cytometry.

1.8 Statistical analysis

Data from the flow cytometer were analyzed using CellQuest software (BD Biosciences). The between-group differences in expression level of co-stimulatory molecules and intracellular cytokines were assessed using a one-way analysis of variance, followed by the Student-Newman-Keuls (SNK) test. The results of qPCR were analyzed by the Kruskal-Wallistest, followed by the SNK test using SAS 9.1 software (SAS Institute Inc., Cary, NC, USA). *P*-values of <0.05 were considered significant.

2 Results

2.1 Activation of BMDCs induced by various antigens

The *ompB* gene (GenBank: CP002912) of *R. heilongjiangensis* was divided into four fragments based on immunogenic epitope prediction of OmpB using Lasergene 8.1.3 software (DNASTAR, Madison, WI, USA; Figure S1 in Supporting Information). Four fragments of OmpB were expressed in *Escherichia coli* cells and the resulting recombinant OmpBs reacted with sera obtained from C3H/HeN mice infected with *R. heilongjiangensis* on day 21 post-infection [8] (Figure 1). The recombinant OmpBs and WCA of *R. heilongjiangensis* were used to *in vitro* pulse BMDCs, after which the MHC class II molecules (MHC II) and costimulatory molecules (CD40, CD80, and CD86) in BMDCs were analyzed by flow cytometry. As a result, the expression levels of the costimulatory molecules (except CD80) in BMDCs treated with WCA, OmpB-p1, OmpB-p2, OmpB-p3, OmpB-p4, or TrxA were 100% higher than those in mock-pulsed BMDCs (P<0.05) (Figure 2), while the expression level of MHC II in BMDCs treated with WCA, OmpB-p1, OmpB-p2, OmpB-p3, or OmpB-p4 was significantly greater than that in TrxA- or mock-pulsed BMDCs. However, the expression levels of the costimulatory molecules and MHC-II were not significantly different between BMDCs pulsed with different OmpBs (Figure 2).

2.2 Protection against *R. heilongjiangensis* mediated by antigen-pulsed BMDCs

The antigen-pulsed BMDCs were transferred into naïve C3H/HeN mice and the recipient mice were subsequently challenged with viable *R. heilongjiangensis* on day 14 pt. Seven days after challenge with *R. heilongjiangensis*, the rickettsial load in organs (lung, liver, spleen, or brain) of mice receiving BMDCs pulsed with WCA, OmpB-p2, OmpB-p3, or OmpB-p4 were at least 70% lower than mice receiving OmpB-p1-, TrxA-, or mock-pulsed BMDCs (Figure 3, P<0.05). For each group, the rickettsial load in the lung was significantly higher (P<0.05) than that in the spleen, while the rickettsial load in the liver was markedly lower (P<0.05) than that in the spleen but significantly higher (P<0.05) than that in the brain (Figure 3).

2.3 T-cell activation induced by antigen-activated BMDCs

$CD4^+$ or $CD8^+$ T cells isolated from the spleen of mice receiving BMDCs pulsed with various antigens were co-cultured with the cognate antigen-pulsed BMDCs, after which the expression of the early activation marker CD69 in both types of T cell was analyzed (Figure 4). The average percentage of CD69-positive cells in both $CD4^+$ and $CD8^+$ T cells from mice receiving BMDCs pulsed with WCA, OmpB-p1, OmpB-p2, OmpB-p3, or OmpB-p4 was 200% greater than that of mice receiving mock-pulsed BMDCs (P<0.05). The average percentage in both types of T cell from mice receiving OmpB-p1-pulsed BMDCs appeared to be lower, but was not significantly so (P>0.05), than in those from mice receiving BMDCs pulsed with WCA or one of the other OmpBs.

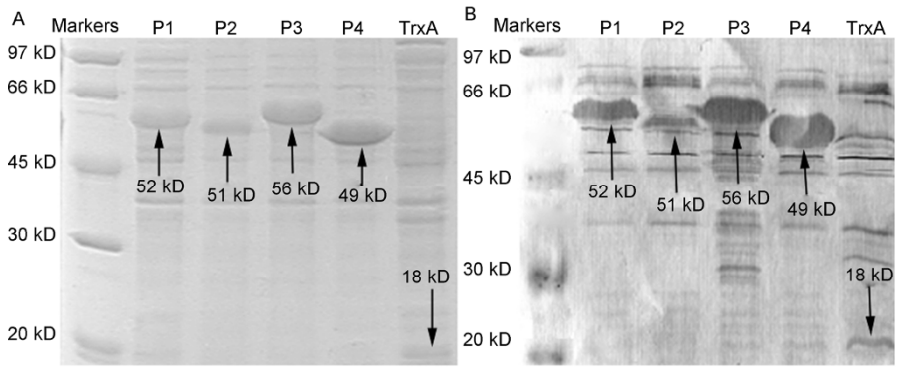

Figure 1 Analysis of the purified recombinant OmpBs by SDS-PAGE (A) and immunoblotting assay (B). The four amplified gene fragments (*ompB-1*, *ompB-2*, *ompB-3*, and *ompB-4*) were expressed in *Esherichia coli* BL21 (DE3) and purified by affinity chromatography. The purified recombinant OmpBs were analyzed by immunoblotting with sera from C3H/HeN mice infected with *R. heilongjiangensis* on day 21 post-infection. Lane 1, standard protein markers; lanes 2–5, OmpB-p1 (P1), OmpB-p2 (P2), OmpB-p3 (P3), OmpB-p4 (P4), which strongly reacted with sera obtained from mice experimentally infected with *R. heilongjiangensis* on day 21 post-infection (B); lane 6, purified TrxA, a fusion tag expressed by the pET-32a(+) vector.

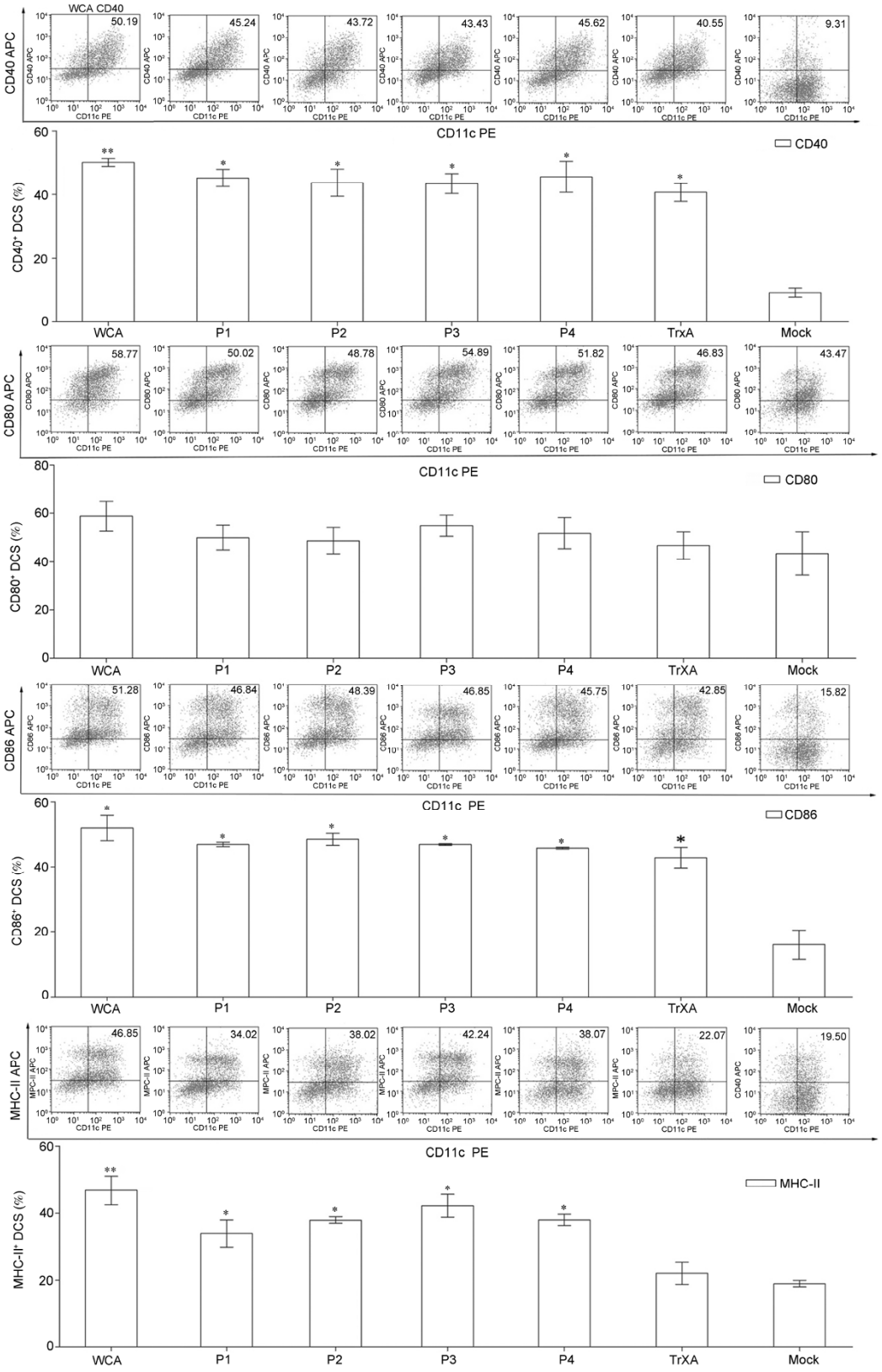

Figure 2 Expression of MHC class II molecules and surface molecules (CD40, CD80, and CD86) in BMDCs after antigen stimulation. After 24 h stimulation with WCA, OmpB-p1 (P1), OmpB-p2 (P2), OmpB-p3 (P3), OmpB-p4 (P4) Or TrxA, BMDCs were stained with monoclonal antibodies to CD40, CD80, CD86, or MHC class II molecules and the expression of these surface molecules was measured by flow cytometry. Antigen dilution buffer alone was used as a mock pulse. Data are representative of three independent experiments with similar results. The percentages of cells in the top right corners were analyzed by circling the double positive cells in BMDCs. $P<0.05$ is considered statistically significant and indicated by asterisks: *, $P<0.05$, **, $P<0.01$.

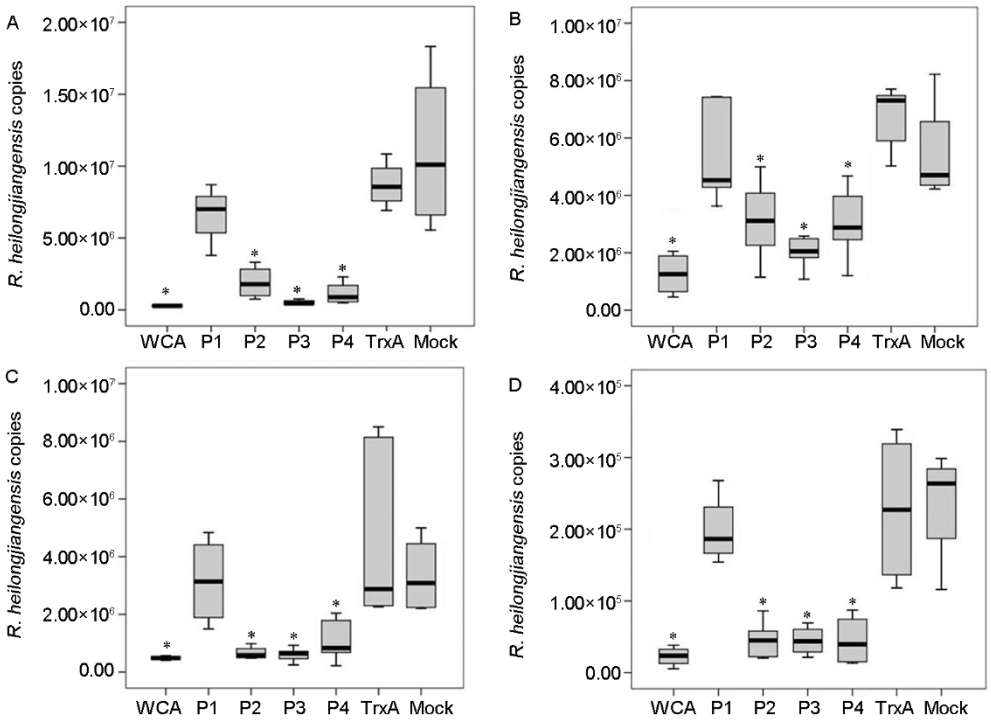

Figure 3 Estimation of Rickettsial load in recipient mice of antigen-pulsed BMDCs. Rickettsial load in the organs of C3H/HeN mice receiving BMDCs pulsed with WCA of *R. hielongjiangensis*, OmpB-p1 (P1), OmpB-p2 (P2), OmpB-p3 (P3), OmpB-p4 (P4), TrxA, or buffer (negative control, Ctrl) after challenge with *R. heilongjiangensis*. Rickettsial load in the spleen (A), lung (B), liver (C), and brain (D) of the mice. Median with 25% and 75% distribution represents the five mice per treatment group. $P<0.05$ is considered statistically significant and indicated by asterisks: *, $P<0.05$.

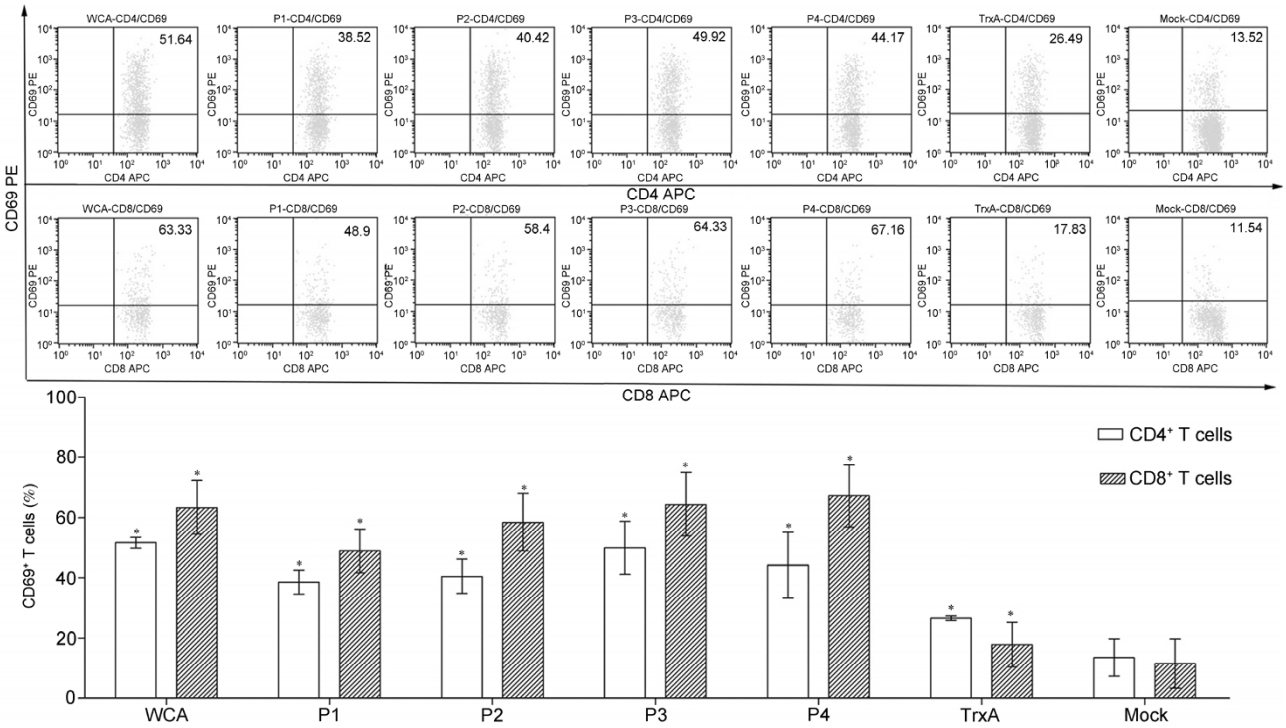

Figure 4 CD69 expression in T cells interacted with antigen-pulsed BMDCs. After co-culture with the cognate antigen-pulsed BMDCs, the percentages of CD69 expression in $CD4^+$or $CD8^+$ T cells from mice receiving BMDCs pulsed with each antigen (WCA, OmpB-p1 (P1), OmpB-p2 (P2), OmpB-p3 (P3), OmpB-p4 (P4), or TrxA) were determined using flow cytometry. In parallel, $CD4^+$or $CD8^+$ T cells from mice receiving mock-pulsed BMDCs were used as negative controls (Ctrl). The data are representative of three independent experiments with similar results. The percentages of cells in the top right corners were analyzed by circling the double positive cells in $CD4^+$ or $CD8^+$T cells. $P<0.05$ is considered statistically significant compared with the TrxA group, and is indicated by an asterisk.

2.4 Cytokine expression of T-cells induced by antigen-activated BMDCs

After interaction of $CD4^+$ or $CD8^+$ T cells with the cognate antigen-pulsed BMDCs, the expression of IFN-γ, TNF-α, or IL-4 in both types of T cell was determined (Figure 5). In flow cytometry analysis, the average percentage of TNF-α-positive cells of both $CD4^+$ and $CD8^+$ T cells from mice receiving BMDCs pulsed with WCA, or any of the OmpB proteins except OmpB-p1, was almost twice of mice receiving TrxA- or mock-pulsed BMDCs ($P<0.05$), while the average percentage of TNF-α-positive cells of $CD8^+$ T cells from mice receiving the OmpB-p1-pulsed BMDCs was only a third of mice receiving BMDCs pulsed with WCA or any of the other OmpBs ($P<0.05$). In addition, the average percentage of IFN-γ-positive $CD4^+$ or $CD8^+$ T cells from mice receiving BMDCs pulsed with WCA, or any of the OmpB proteins except OmpB-p1, was at least twice of mice receiving TrxA or mock-pulsed BMDCs ($P<0.05$), while the average percentage of IFN-γ-positive $CD4^+$ or $CD8^+$ T cells from mice receiving OmpB-p1-pulsed BMDCs was only a half of mice receiving BMDCs pulsed with WCA or any of the other OmpBs ($P<0.05$). Finally, the average percentage of IL-4-positive T cells of either type from mice receiving BMDCs pulsed with OmpB-p1, OmpB-p2, OmpB-p3, OmpB-p4, or TrxA was not significantly higher than that from mice receiving mock-pulsed BMDCs ($P>0.05$), whereas this average percentage in both types of T cell from mice receiving BMDCs pulsed with WCA was markedly higher ($P<0.05$) than that from mice receiving mock-pulsed BMDCs.

3 Discussion

In the SFG rickettsiae, two high-molecular-mass proteins, OmpA and OmpB, have been demonstrated to be the major immunodominant surface proteins and protective antigens [11,19,20]. Immunization with recombinant OmpA from *R. conorii* protected guinea pigs from experimental Rocky Mountain spotted fever and boutonneuse fever, respectively [21,22] and vaccination with native OmpB purified from *R. typhi* protected guinea pigs from experimental typhus [23,24]. OmpA and particularly OmpB, have been shown to play an important role in the humoral response against rickettsial infection in BALB/c mice [25]. Primary immunization of mice with *Mycobacterium vaccae* expressing the gene fragment of rickettsial *ompA* and subsequent booster administration with the recombinant protein encoded by the gene fragment offered partial protection against lethal challenge with *R. conorii* [26], which was found to be associated with T cell proliferation and IFN-γ secretion after the primary and booster immunization [13]. In addition, three synthetic peptides of OmpB from *R. conorii* elicited $CD8^+$ T cell proliferation and IFN-γ secretion as well as specific cytotoxic T lymphocyte (CTL) killing, suggesting that the cell-mediated immune response was efficiently induced by these epitope peptides [27].

DCs are key initiators and orchestrators of the immune response against rickettsial infections [8]. Rickettsial antigen-activated DCs can produce proinflammatory cytokines, initiate anti-rickettsial innate immunity, (namely expansion of the NK cell population and subsequent production of IFN-γ), and elicit an adaptive Th1-type response, which plays a critical role in antigen-specific protective immune responses against rickettsial pathogens [8]. This adaptive immune protection is associated with an increase in a number of known anti-rickettsial effector cells, specifically Th1-polarized $CD4^+$ and $CD8^+$ T cells [28].

Since rickettsial *ompB* gene is more than 4 kb long, which is difficult to completely express in prokaryotic cells, the *ompB* gene of *R. heilongjiangensis* was divided into four fragments to prepare four recombinant proteins, OmpB-p1, OmpB-p2, OmpB-p3, and OmpB-p4, in the present study. For evaluation of their immune efficacy, the four OmpB proteins were used *in vitro* to stimulate BMDCs isolated from naïve mice and the maturation and activation of the OmpB-pulsed BMDCs were evaluated by analysis of their surface molecule expression. Our results showed that the BMDCs pulsed by any of the OmpBs exhibited increased expression of surface molecules, including co-stimulatory molecules (CD40, CD80, and CD86), suggesting that all of the OmpBs have the ability to induce maturation and activation of naïve BMDCs, which were expected to have abilities to initiate primary adaptive immune responses [29]. Meanwhile, the expression of MHC-II on BMDCs pulsed by any of the OmpBs was significantly higher than that of BMDCs pulsed with TrxA, but the expression level of BMDCs pulsed by OmpB-p1 was lower than that BMDCs pulsed by each of the other three recombinant OmpBs. MHC-II on DCs functions mainly in the presentation of exogenous antigens to $CD4^+$T cells [29], by which an antigen-specific immune response can be elicited. This result therefore indicates that all of these OmpBs had the ability to elicit a specific adaptive immune response, but OmpB-p1 was weaker compared with the others.

To evaluate the protection (especially T cell-mediated immune protection) induced by these OmpBs, BMDCs pulsed with each OmpB were transferred to naïve mice instead of immunizing them directly with antigens. After challenge with *R. helongjiangensis*, the rickettsial burden in mice receiving BMDCs pulsed with different antigens were measured by qPCR at day 7 post-infection at which the highest level of rickettsial load was determined in *R. helongjaingensis*-infected mice in our previous studies [8]. As a result, mice receiving BMDCs pulsed with OmpB-p2, OmpB-p3, or OmpB-p4 exhibited significantly lower rickettsial load compared with mice receiving OmpB-p1-pulsed BMDCs. This result demonstrates that with the exception of OmpB-p1, these OmpBs were protective antigens due to the

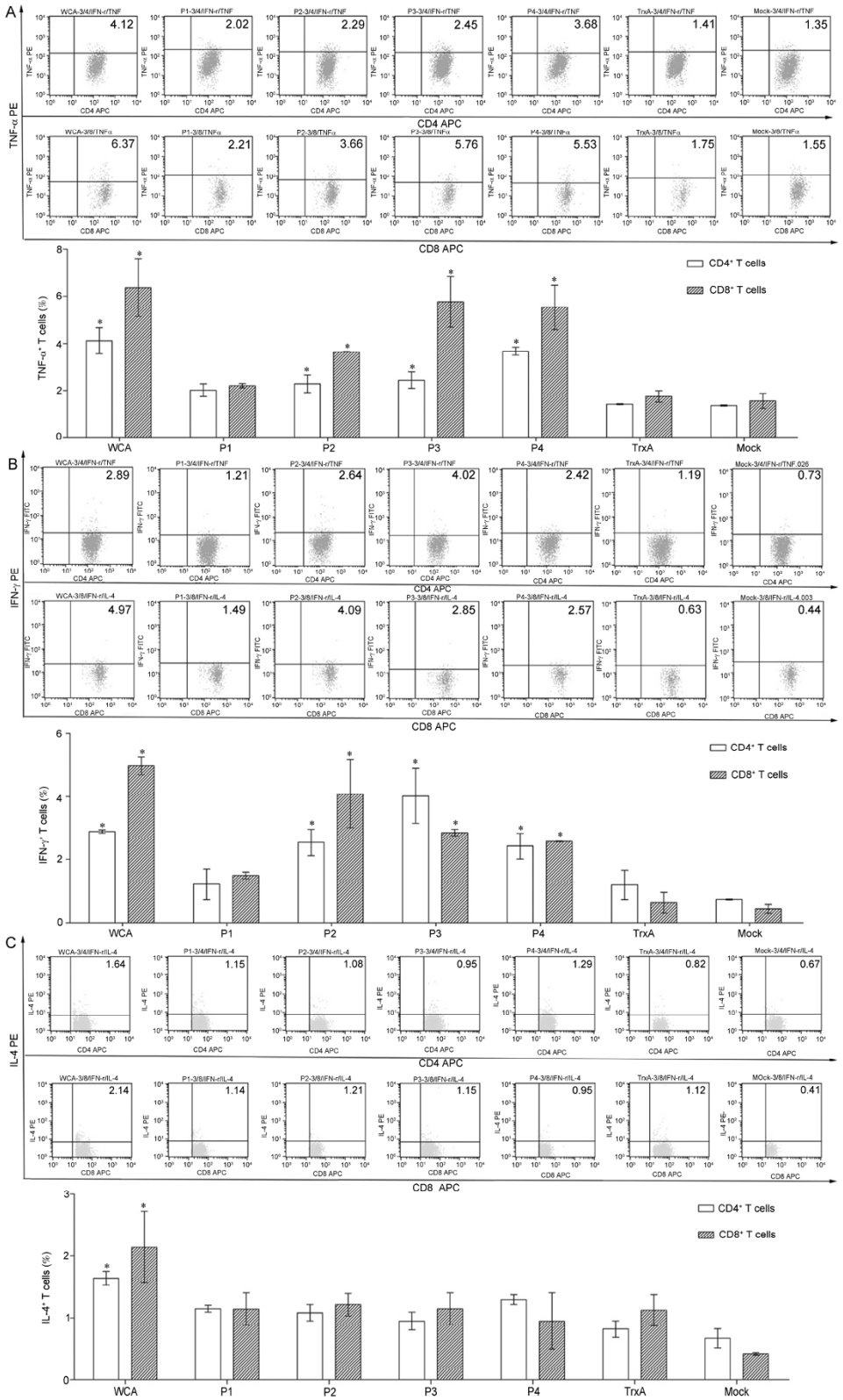

Figure 5 Expression of TNF-α, INF-γ, and IL-4 on $CD4^+$ or $CD8^+$ T cells induced with antigen-pulsed BMDCs. After co-culture with the cognate antigen-pulsed BMDCs, the percentage of TNF-α-, INF-γ-, and IL-4-positive $CD4^+$or $CD8^+$ T cells from mice receiving BMDCs pulsed with each antigen (WCA, OmpB-p1 (P1), OmpB-p2 (P2), OmpB-p3 (P3), OmpB-p4 (P4), or TrxA) was determined using flow cytometry, and is shown in figures A, B, and C. In parallel, $CD4^+$ and $CD8^+$ T cells from mice receiving mock-pulsed BMDCs were used as negative controls (Ctrl). The data are representative of three independent experiments with similar results. The percentages of cells in the top right corners were analyzed by circling the double positive cells in $CD4^+$ or $CD8^+$ T cells. $P<0.05$ is considered statistically significant compared with the TrxA group, and is indicated by an asterisk.

fact that BMDCs activated by them could confer the immune protection against *R. heilongjiangensis* infection.

To investigate the mechanism of adaptive immune defense against *R. heilongjiangensis*, both $CD4^+$ and $CD8^+$ T cells isolated from mice immunized with BMDCs pulsed with each OmpB were exposed to BMDCs loaded with cognate OmpB. After encountering the cognate antigen presented by BMDCs, the expression of surface molecules on the $CD4^+$ and $CD8^+$ T cells was assayed. Our results showed that the expression level of CD69, an early activation marker of T cells, on $CD4^+$ and $CD8^+$ T cells from mice receiving BMDCs pulsed with OmpB-p2, OmpB-p3 or OmpB-p4 was higher than that of mice receiving OmpB-p1-pulsed BMDCs. This result suggests that the protective OmpBs were more effective in their ability to activate T cells compared with the non-protective OmpB-p1.

Our results also showed that the expression level of TNF-α on $CD8^+$ T cells from mice receiving BMDCs pulsed with OmpB-p2, OmpB-p3, or OmpB-p4 was significantly higher than that of mice receiving OmpB-p1-pulsed BMDCs, while the expression level of IFN-γ on both $CD4^+$ and $CD8^+$ T cells of mice receiving BMDCs pulsed with OmpB-p2, OmpB-p3, or OmpB-p4 was significantly higher than that of mice receiving OmpB-p1-pulsed BMDCs. Laboratory studies have demonstrated that *IFN-γ* and *TNF-α* are both important host defense molecules against rickettsial infections and the protection against rickettsial pathogens is conferred by a Th1-oriented immune response. This in turn depends on the Th1-specific cytokine IFN-γ and the inflammatory cytokine TNF-α, because both cytokines have the ability to upregulate the bactericidal activity of host cells [28,29]. There is no distinct difference between OmpB-p1 and the other OmpBs, in construction and immunogenicity analysis of antigenic epitopes by computer (Figure S1), and thus protein misfolding may be a reason for incapacity of OmpB-p1 in inducing an efficient T cell immune response against *R. heilongjiangensis* infection. Additionally, no specific IgG antibodies against *R. heilongjiangensis* were detected in mice receiving BMDCs activated with WCA or each OmpB in the present study, which indicated that the specific antibodies did not participate in the protection against *R. heilongjiangensis* conferred by the antigen-pulsed BMDCs.

4 Conclusion

The *ompB* gene of *R. heilongjiangensis* was divided into four fragments for expression in *E. coli* cells, producing four recombinant OmpB proteins (OmpB-p1, OmpB-p2, OmpB-p3, and OmpB-p4). BMDCs activated by OmpB-p2, OmpB-p3, or OmpB-p4 (but notOmpB-p1) could mediate protective immunity against *R. heilongjiangensis* infection in mice. BMDCs stimulated by any protective OmpB had the ability to activate $CD4^+$ and $CD8^+$ T cells and drive their differentiation toward $CD4^+$ Th1 and $CD8^+$ Tcl cells, respectively, which produced greater amounts of TNF-α and, in particular, IFN-γ, to enhance rickettsicidal activity of the host cells. Therefore, OmpB-p2, OmpB-p3, and OmpB-p4 are protective antigens and potential candidates for the development of a subunit vaccine for FESF.

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Supporting Information

Figure S1 The gene encoding OmpB in *R. heilongjiangensis* was divided into four fragments to produce four recombinant OmpB proteins using Lasergene 8.1.3 software. Antigenic index, surface probability, and hydrophilicity of the protein are shown.

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