Vaccine 37 (2019) 6076–6084

Contents lists available at ScienceDirect

Vaccine

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

Enhanced protection against Q fever in BALB/c mice elicited by immunization of chloroform-methanol residue of *Coxiella burnetii* via intratracheal inoculation

Junxia Feng [a], Xueyuan Hu [a], Mengjiao Fu [a], Lupeng Dai [a], Yonghui Yu [a], Wenbo Luo [b], Zengming Zhao [c], Zhiyu Lu [a], Zongmin Du [a], Dongsheng Zhou [a], Bohai Wen [a], Jun Jiao [a,*], Xiaolu Xiong [a,*]

[a] State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, 20# Dong-Dia-Jie Street, Fengtai, Beijing 100071, China
[b] Anhui Medical University, Mei-Shan Road, Hefei, Anhui 230032, China
[c] Center for Disease Control and Prevention of Chinese People's Liberation Army, 20# Dong-Dia-Jie Street, Fengtai, Beijing 100071, China

ARTICLE INFO

Article history:
Received 28 March 2019
Received in revised form 7 August 2019
Accepted 19 August 2019
Available online 30 August 2019

Keywords:
Coxiella burnetii
Q fever
Intratracheal inoculation
Mucosal immunity

ABSTRACT

Human Q fever is recognized as a worldwide public health problem. It often occurs by inhalation of airborne aerosols contaminated with *Coxiella burnetii*, a gram-negative intracellular bacterium, mainly from domestic livestock. In this study, we analyzed the possibility to establish mucosal and systemic immunity against *C. burnetii* infection using a pulmonary delivery of chloroform-methanol residue of *C. burnetii* (CMR) vaccine. Mice were immunized by the intratracheal inoculation of CMR (IT-CMR) or the subcutaneous injection of CMR (SC-CMR), and the immunized mice were challenged with *C. burnetii* by the intratracheal route. The levels of IFN-γ, IL-12p70, IL-5, and IL-4 in the IT-CMR group in splenic T cells stimulated *ex vivo* were significantly higher than in the SC-CMR group. Significantly elevated sIgA to *C. burnetii* was detected in the bronchoalveolar lavage fluid of mice immunized by IT-CMR but not by SC-CMR, which might have contributed to the significant reduction in *C. burnetii* load and pathological lesions in the lungs of the mice after the challenge of *C. burnetii*. These results suggest that compared with SC-CMR in mice, IT-CMR was more efficient to elicit cellular and lung mucosal immune responses against aerosol infection of *C. burnetii*.

1. Introduction

Q fever is a worldwide zoonotic disease caused by *Coxiella burnetii*, a gram-negative intracellular bacterium. The typical manifestations of ruminant Q fever are abortion and infertility, and the infected ruminants represent a major resource of human infection. Human Q fever usually manifests as an acute flu-like and often self-limiting illness or less frequently as a chronic disease in the form of endocarditis or hepatitis [1]. Between 2007 and 2011, the largest outbreak of Q fever ever described occurred in the Netherlands, with >4000 reported human cases and at least 24 deaths [2], which showed that Q fever could become a major public health problem in the world.

A whole-cell formalin-inactivated Q fever vaccine (Q-vax®) has been used in immunization of abattoir and agricultural workers in Australia for more than twenty years. The possibility of adverse reactions in the persons with previous exposure to *C. burnetii* has prevented its use elsewhere [3]. A Q fever vaccine of chloroform-methanol residue of *C. burnetii* (CMR) developed by the US Army Research Institution was thought to be an efficacious alternative to Q-vax, as the efficacy of the CMR vaccine had been shown in mice, rodents, sheep, non-human primates, and human volunteers with clearly less adverse reactions [4–7]. However, the production of CMR from embryonic cells is difficult due to the complex procedures of culturing and purifying the organism. Recently, progress in the host-cell free cultivation of *C. burnetii* has facilitated the production of CMR [8–10].

Usually, subcutaneous injection is a convenient way to inoculate Q fever vaccines in animals and humans. In the natural *C. burnetii* infection in humans, the pathogen mainly enters the lung via respiratory route, and thus optimal protection against the respiration pathogen requires induction of specific immunity in the lung, where tissue-resident memory T cells and specific antibodies play important roles in the rapid recognition and clearance of the pathogen in the organ [11–13]. The vaccines administered via the lung show more comparable bio-availability and rapid onset time

* Corresponding authors.
E-mail addresses: jiaojun51920@sina.com (J. Jiao), xiongxiaolu624@sohu.com (X. Xiong).

https://doi.org/10.1016/j.vaccine.2019.08.041
0264-410X/

than alternative routes [14]. Pulmonary vaccination has been suggested as a mimic of natural infection to improve local immunity at the site of infection, and it can induce long-lasting systemic and mucosal immune responses against respiratory pathogens [15–18].

Although great efforts have been made on the development of safe and effective Q fever vaccines [10,19–22], little is known about the role of a specific mucosal immune response against *C. burnetii* infection in the lung. Recent studies on *C. burnetii* infection suggested that local immune defense mechanism in the lung, especially neutrophils and activated macrophages, have a pivotal role in the early control of *C. burnetii* aerosol infection [23,24]. Taken together, these findings prompted us to explore whether the intratracheal inoculation of Q fever vaccine could rapidly and effectively induce a specific mucosal immune response in the lung and thereby improve protection against *C. burnetii* aerosol infection. In the present study, the CMR vaccine was chosen to immunize mice [7,25] by the intratracheal inoculation or the subcutaneous injection (due to its effectiveness and improved safety profiles in pre-clinical testing [4,5]), and the specific humoral and cellular immune responses following vaccination, as well as the bacterial burden and pathological lesion after *C. burnetii* challenge, were analyzed in the immunized mice. We found that the intratracheal inoculation of CMR induced higher levels of *C. burnetii*-specific IgA responses in the lung and *ex vivo* splenic T cell responses and lower levels of bacterial burden and pathological lesion in lung after *C. burnetii* aerosol infection compared with the subcutaneous injection of CMR.

2. Materials and methods

2.1. Cultivation of C. burnetii and preparation of CMR

C. burnetii Xinqiao strain (phase I) and Grita strain (phase II) were cultivated in the acidified citrate cysteine medium-2 (ACCM-2) for two passages as described previously [9]. The phases of *C. burnetii* were confirmed by SDS-PAGE analysis of extracted LPS (Fig. S1) as described previously [26]. The purified *C. burnetii* organisms of Xinqiao strain [27] were inactivated with formalin and extracted three times with chloroform-methanol (4:1) to obtain the CMR fraction according to the procedures described previously [10].

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2019.08.041.

2.2. Animals

Female BALB/c mice (6–8-week of age) were purchased from Vital River Laboratories (Beijing, China). All infectious experiments were conducted in the animal biosafety level-3 laboratory (ABSL-3) and carried out according to the guidelines of the authors' institution. All of the animal experiments were performed with the permission of Institute of Animal Care and Use Committee (IACUC) at the Academy of Military Medical Sciences (AMMS), and all efforts were made to minimize mice suffering. The ethical approval number was IACUC of AMMS-13-2017-017.

2.3. Animal immunizations

BALB/c mice were randomly divided into four groups (28 mice per group). Mice [19,28] were immunized three times at 3-week intervals (Fig. 1A) with a mixture of 20 μg of CMR [20] and 20 μg of CpG (CpG oligodeoxynucleotide, Invitrogen, MA), a promising mucosal adjuvant [29–32], in PBS by the intratracheal inoculation (IT-CMR) or by the subcutaneous injection (SC-CMR). Mice immunized with 20 μg of CpG in PBS by the intratracheal inoculation (IT-CpG) or by the subcutaneous injection (SC-CpG) served as negative controls. For the intratracheal inoculation, mice were anesthetized by the intraperitoneal injection of 100 mg/kg body weight pentobarbital sodium and then supported by a nylon band under its upper incisors and restrained on a slanted board (60 °C from the horizontal direction). The tracheal opening was visualized by inserting a laryngoscope (Huironghe Company, Beijing, China); the Micro Sprayer (Huironghe Company, Beijing, China) (Fig. 1B), which is capable of ejecting liquid particles with a particle size of 5.02 ± 0.35 μm, was inserted 25 mm from the larynx (near the tracheal bifurcation) of the mouse. At each immunization, 50 μl of CMR solutions was aerosolized into the lungs by depressing the Micro-Sprayer plunger with a constant force. For the subcutaneous injection, 100 μl of the inoculum was subcutaneously injected into the inner thigh of each mouse.

2.4. Determination of specific antibodies by ELISA

Four mice per group were sacrificed 21 days after each immunization; the serum and bronchoalveolar lavage fluid (BAL) from each mouse were collected. The titers of immunoglobulin (Ig)Gs and secretory IgA (sIgA) to phase I or II antigen of *C. burnetii* were determined in each serum and BAL sample by enzyme-linked immunosorbent assay (ELISA). Briefly, 96 well plates (Nunc, Shanghai, China) were coated overnight with 1×10^8 genome equivalents (GE) of the inactivated *C. burnetii* phase I or II whole cell antigen (phase I or II antigen) in 100 μl carbonate buffer and then blocked with 1% bovine serum albumin (BSA) at 37 °C for 2 h. Each serum was 2-fold serially diluted from 1:100 to 1:102,400, and each BAL sample was 2-fold serially diluted from 1:1 to 1:2,048. One hundred microliters of the diluted sera or BAL was added to each well of the plates and incubated with phase I or II antigen at 37 °C for 45 min. After that, the plates were washed with phosphate-buffered saline containing 0.1% Tween-20 (PBST) for five times and then incubated with 100 μl (1:10,000 dilution) of sheep anti-mouse IgG or IgA secondary antibodies conjugated with horseradish peroxidase (Abcam, Cambridge, MA) at 37 °C for 45 min. After five washes, the antibody titers to phase I or II antigen were detected by a TMB substrate kit. Absorbance at 450 nm was analyzed by a UVM 340 microplate reader (Asys Hitech GmbH, Eugendorf, Austria).

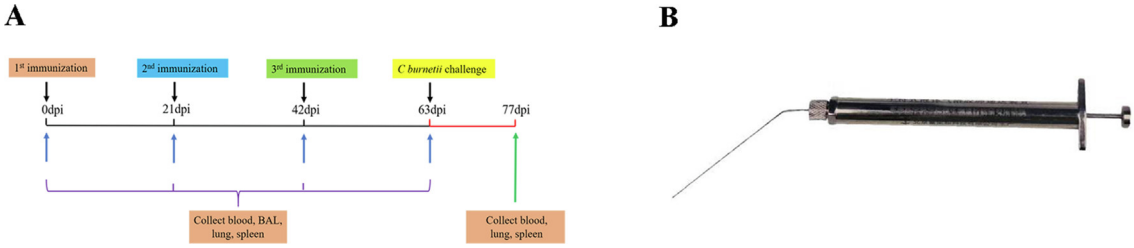

Fig. 1. The study design (A) and the Micro Sprayer (B) used for intratracheal inoculation. BAL, bronchoalveolar lavages; dpi, days post-primary immunization.

2.5. Proliferation and cytokine production of T cells

Four mice per group were sacrificed at each of the time points described above, and their spleens were collected individually. T cells with high purity (>97%) were isolated from the suspensions of each spleen and plated into 24 well plates (Corning, Corning, NY) in RPMI 1640 medium (Gibco, Grand Island, NY) supplemented with 10% FCS (Hyclone, Logan, UT) at the concentration of 5×10^6 cells/mL per well as previously described [20]. Then 20 μg of CMR, 2.5 μg of ConA (positive control), or cell culture medium only (negative control) was added to each well and incubated with T cells at 37 °C and 5% CO_2 for 48 h. After that, 200 μl of the supernatant from each well was collected for detection of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, interleukin (IL)-4, IL-5, IL-6, and IL-12p70 using Essential Th1/Th2 Cytokine 6-Plex Mouse Panel kits (eBioscience, San Diego, CA). Finally, the proliferation of T cells in the wells was detected by Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan) as described previously[20].

2.6. Protective efficacy of intratracheal inoculation of CMR

On day 63 after the primary immunization, six or seven mice per group were challenged with a sublethal dose of living organisms (*C. burnetii* Xinqiao strain, 1×10^8 GE per mouse) [28] in liquid aerosol via intratracheal inoculation described above. Fourteen days after the challenge, mice were sacrificed. The sera from each group were collected, and antibody responses against phase I or II antigen were determined by ELISA as described above. The body and spleen of each mouse were weighted, and *C. burnetii* genome copies in samples from each lung and the spleen were determined by a real-time quantitative polymerase chain reaction (qPCR) as described previously [33].

2.7. Histopathology

The lung and spleen tissues of mice were collected at different experimental time points. Each organ tissue was immediately fixed in formalin after being removed and then embedded in paraffin; the embedded tissue was sliced; and the slices on the slides were stained with hematoxylin-eosin (HE). The pathological alterations in the tissue slices were observed under light microscopy. The thickness of the alveolar wall was measured by Nano Measurer software (version 1.2) with the scale length of 100 μm as a standard.

2.8. Statistical analyses

All statistical analyses were performed using SAS statistical software (version 9.1, SAS Institute Inc., Cary, NC). The differences in *C. burnetii* loads, the ratio of spleen weight to body weight, the thicknesses of alveolar walls, and the levels of antibodies, cytokines, or T cell proliferation between two groups of mice were assayed using the two-way variance (ANOVA) procedure, followed by LSD and Student-Newman-Keuls test. Comparisons were considered significantly different if $P < 0.05$.

3. Results

3.1. Humoral immune responses induced by intratracheal inoculation of CMR

To compare the humoral immune responses between mice immunized by IT-CMR and SC-CMR, the titers of IgGs to *C. burnetii* phase I or II antigen were determined by ELISA in sera collected from mice 21 days after each immunization. As a result,

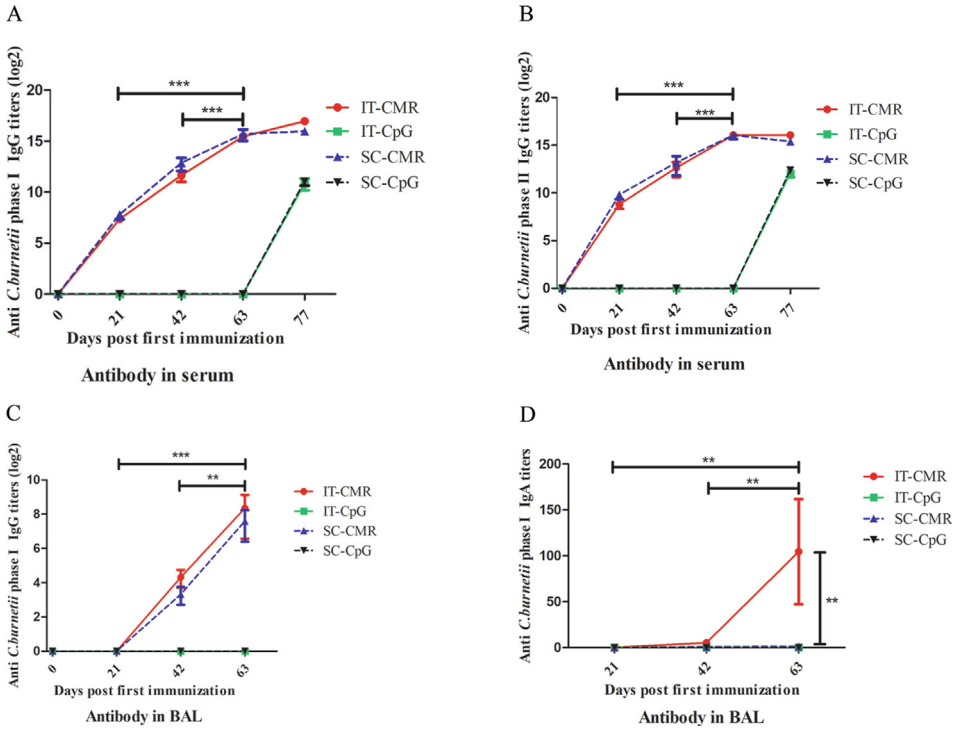

Fig. 2. Antibody responses to *C. burnetii* phase I or phase II antigen determined by ELISA. Sera were collected from four mice of each group immunized by IT-CMR, IT-CpG, SC-CMR, or SC-CpG on days 21, 42, and 63 after primary immunization and 14 days after challenge. Bronchoalveolar lavage fluid (BAL) was collected from four mice of each group on days 21, 42, and 63 after primary immunization. The reciprocal titers of IgGs to phase I antigen in mouse sera (A); the reciprocal titers of IgGs to phase II antigen in mouse sera (B); the reciprocal titers of IgGs to phase I antigen in mouse BALs (C); and the reciprocal titers of sIgA to phase I antigens of mouse BALs (D). The antibody titers are presented as mean with SEM of n = 4 samples per group. Data between groups were compared using the analysis of two-way variance (ANOVA) and then followed by Student-Newman-Keuls test. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

IgGs to phase I or II antigen in mice immunized by IT-CMR or SC-CMR were detected at low levels on day 21 after primary immunization and were significantly lower than on day 42, while IgGs to phase I or II antigen in mice immunized with CpG were undetectable (Fig. 2A and B). The level of the specific IgGs in sera of mice on day 63 after primary immunization by IT-CMR or SC-CMR was significantly higher than on day 42 ($P < 0.01$, Fig. 2A). On day 14 after challenge, the specific IgGs levels in sera of mice immunized by IT-CMR were higher than those of mice immunized by SC-CMR; however, the specific IgGs level in the sera of IT-CMR group was not significantly different from that of the SC-CMR group at each time point described above ($P > 0.05$, Fig. 2A and B).

3.2. Lung mucosal immune responses induced by intratracheal inoculation of CMR

To know whether the intratracheal inoculation of CMR could induce a specific mucosal immune response in the lungs of mice, the IgGs and sIgA to phase I antigen in the BAL of mice were determined by ELISA. The very low levels of specific IgGs were determined in BAL on day 21 after primary immunization by IT-CMR or SC-CMR (Fig. 2C). The level of specific IgGs in BAL on day 63 was significantly higher than that on day 21 after primary immunization by IT-CMR ($P < 0.05$), while the IgGs level of BAL on day 63 after primary immunization by SC-CMR was not significantly higher than on day 21 ($P > 0.05$, Fig. 2C). Meanwhile, the specific sIgA was determined as early as on day 21 after primary immunization by IT-CMR, which was increased on day 42 and significantly augmented on day 63 ($P < 0.05$ Fig. 2D). However, a very low level of specific sIgA was determined in the BAL from mice on day 42 or day 63 after primary immunization by SC-CMR, which was lower or significantly lower than in the corresponding BAL from mice immunized by IT-CMR (Fig. 2D).

3.3. Cellular immune response induced by intratracheal inoculation of CMR

To determine the specific cytokine responses and the T cell proliferation induced by IT-CMR or SC-CMR immunization, splenic T cells isolated from the immunized mice were stimulated *ex vivo* with CMR, and then the supernatant and cells were detected by Luminex assay and CCK-8 assay respectively. The level of each cytokine secreted by the T cells from mice on day 21 or day 42 after primary immunization by IT-CMR was not significantly different from that of mice immunized by SC-CMR (data not shown). However, on day 63 after primary immunization, the levels of IFN-γ, IL-12p70, IL-4, and IL-5 secreted by the T cells from mice immunized by IT-CMR were significantly higher than the respective levels from mice immunized by SC-CMR (Fig. 3A and C–E). The proliferation level of T cells of mice immunized by IT-CMR was modestly higher compared with that of SC-CMR-immunized mice ($P > 0.05$) (Fig. 3F).

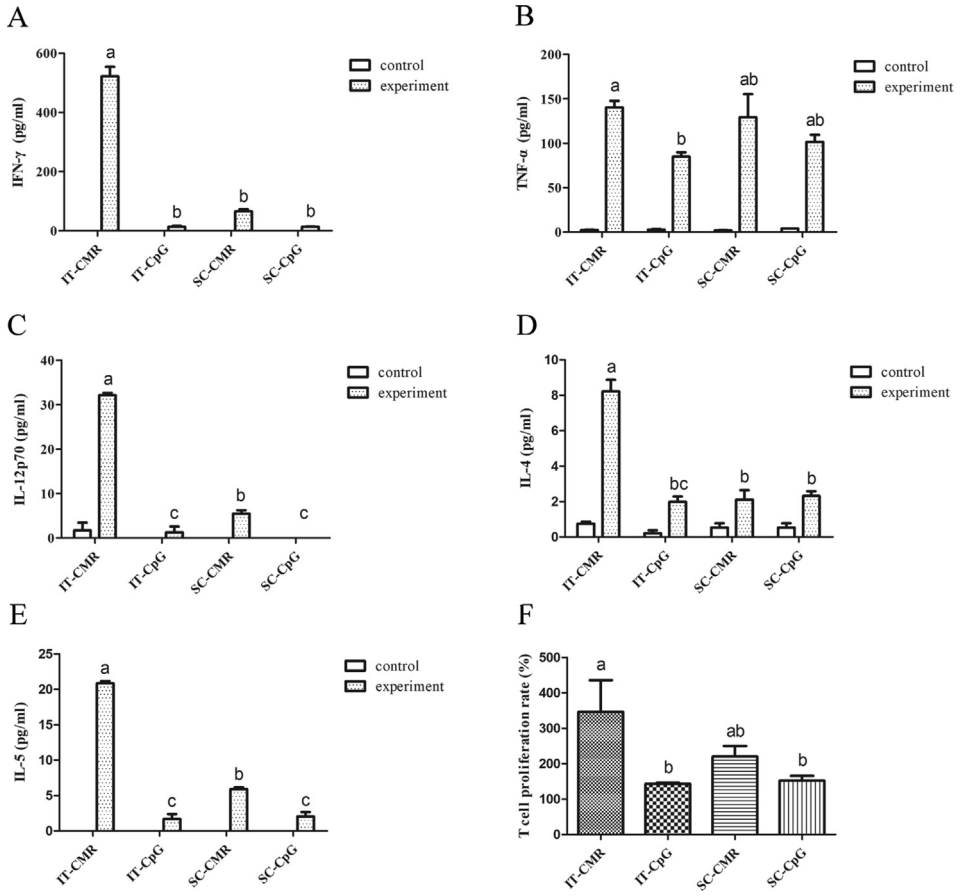

Fig. 3. The levels of cytokines and the proliferation of T cells. T cells were individually isolated from the spleens of four mice from each group on day 63 after primary immunization and stimulated with CMR for 48 h, and then the level of IFN-γ (A), TNF-α (B), IL-12p70 (C), IL-4 (D), or IL-5 (E) was determined in the supernatant of T cells. The proliferation level of T cells (F) was determined by CCK-8. Data are presented as mean with SEM of n = 4 samples per group. The bar represents the SEM of four samples. Significant differences between groups were found by two-way variance (ANOVA), then followed by Student-Newman-Keuls test. Significant differences ($P < 0.05$) between the groups are labeled with different letters (a, b or c), but there is no significant difference ($P > 0.05$) if two bars share a letter.

3.4. Intratracheal inoculation of CMR caused slight pathological lesions in lungs

To evaluate the safety of the intratracheal inoculation of CMR, three mice per group were sacrificed on days 21, 42, and 63 after primary immunization, and the pathological alterations in their lungs and spleens were examined. In the lungs of mice immunized by IT-CMR or SC-CMR, the alveolar walls and inflammatory cell infiltration on day 63 after primary immunization were slightly thicker or more severe than on day 21 and day 42 (Fig. 4 and S2); there was no significant difference in the thickness of the alveolar walls between mice immunized by IT-CMR and SC-CMR. The red and white pith in the spleens of mice immunized by IT-CMR or SC-CMR were evenly distributed, and the morphology of leukocytes in the white pith was normal (data not shown).

3.5. Immunoprotection against C. burnetii induced by intratracheal inoculation of CMR

The protective efficacy of the intratracheal inoculation of CMR was evaluated by analyzing the bacterial burdens and pathological changes in the spleens and lungs as well as the splenomegaly of mice immunized by IT-CMR or IT-CpG on day 14 after challenge with *C. burnetii*. For splenomegaly, mice immunized by IT-CMR were significantly slighter than IT-CpG-immunized mice (Fig. 5A and B). Moreover, the *C. burnetii* genome copies in the lungs and spleens of mice immunized by IT-CMR were in a significantly lower number than in mice immunized by IT-CpG (Fig. 5C and D). In addition, *C. burnetii* genome copies in the spleens of mice immunized by SC-CMR were also in a significantly lower number than in mice immunized by SC-CpG, but the bacterial load in the lungs of mice immunized by SC-CMR was not markedly lower than that of the control mice, and it was higher than that of mice immunized by IT-CMR (Fig. 5C). After challenge with *C. burnetii*, epithelial degeneration, inflammatory cell infiltration, and chronic interstitial inflammation were observed in the lungs of mice immunized by IT-CMR or SC-CMR. However, the pathological lesions in the lungs of mice immunized by IT-CMR were markedly slighter than those of mice immunized by SC-CMR (Fig. 6), and the alveolar walls in the lungs of mice immunized by IT-CMR were significantly thinner than those of mice immunized by SC-CMR (Fig. S2). The red pulp hyperplasia of the spleens in mice immunized by IT-CMR or

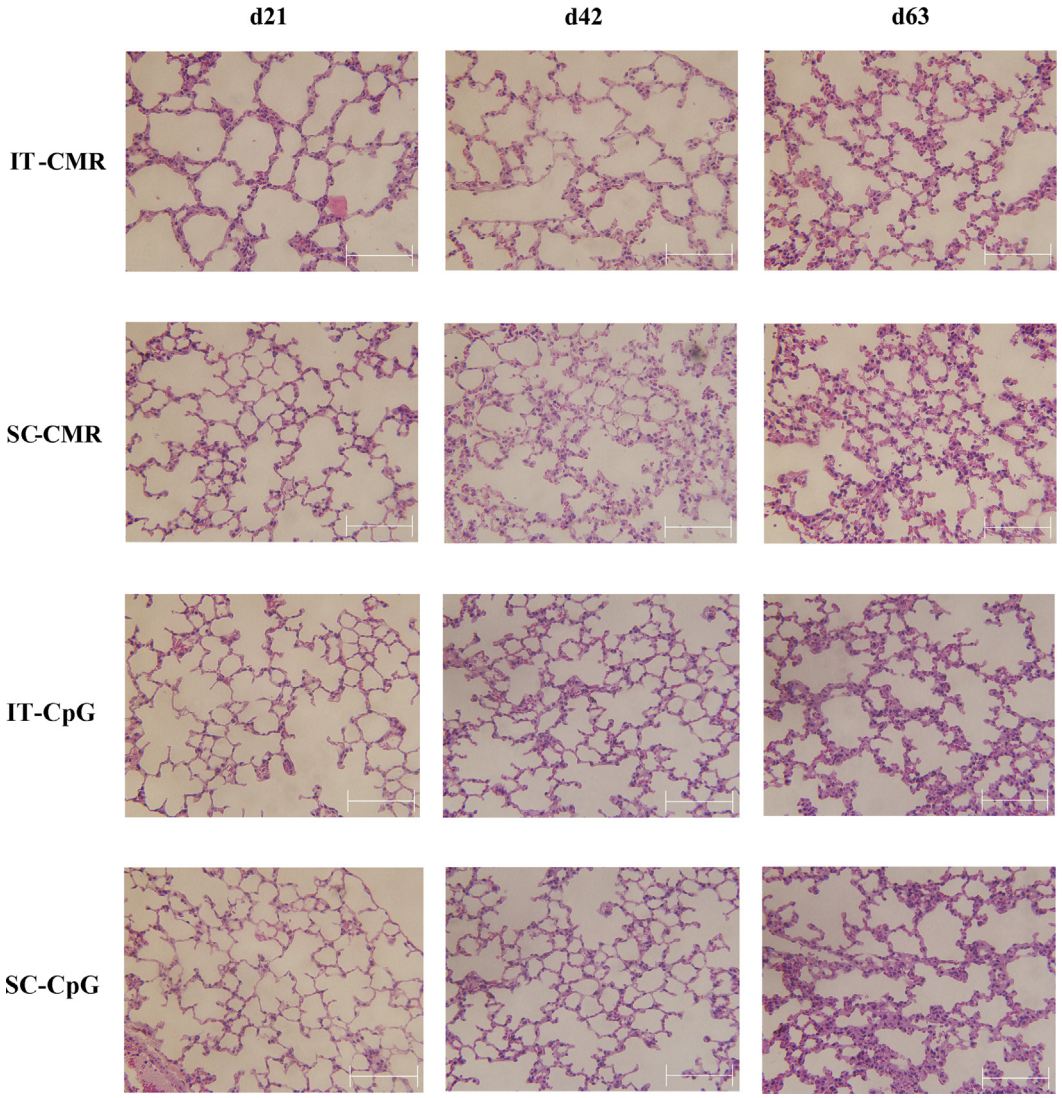

Fig. 4. Pathological changes of mice after immunization. Twenty-one days after each immunization, three mice per group were sacrificed, and their lungs were treated by conventional histopathological methods. The pathological alterations of lungs were observed in the HE-stained sections under light microscopy. On day 63, slightly thickened alveolar walls were observed in the lungs of mice in each group (original magnifications 400, bar = 100 μm).

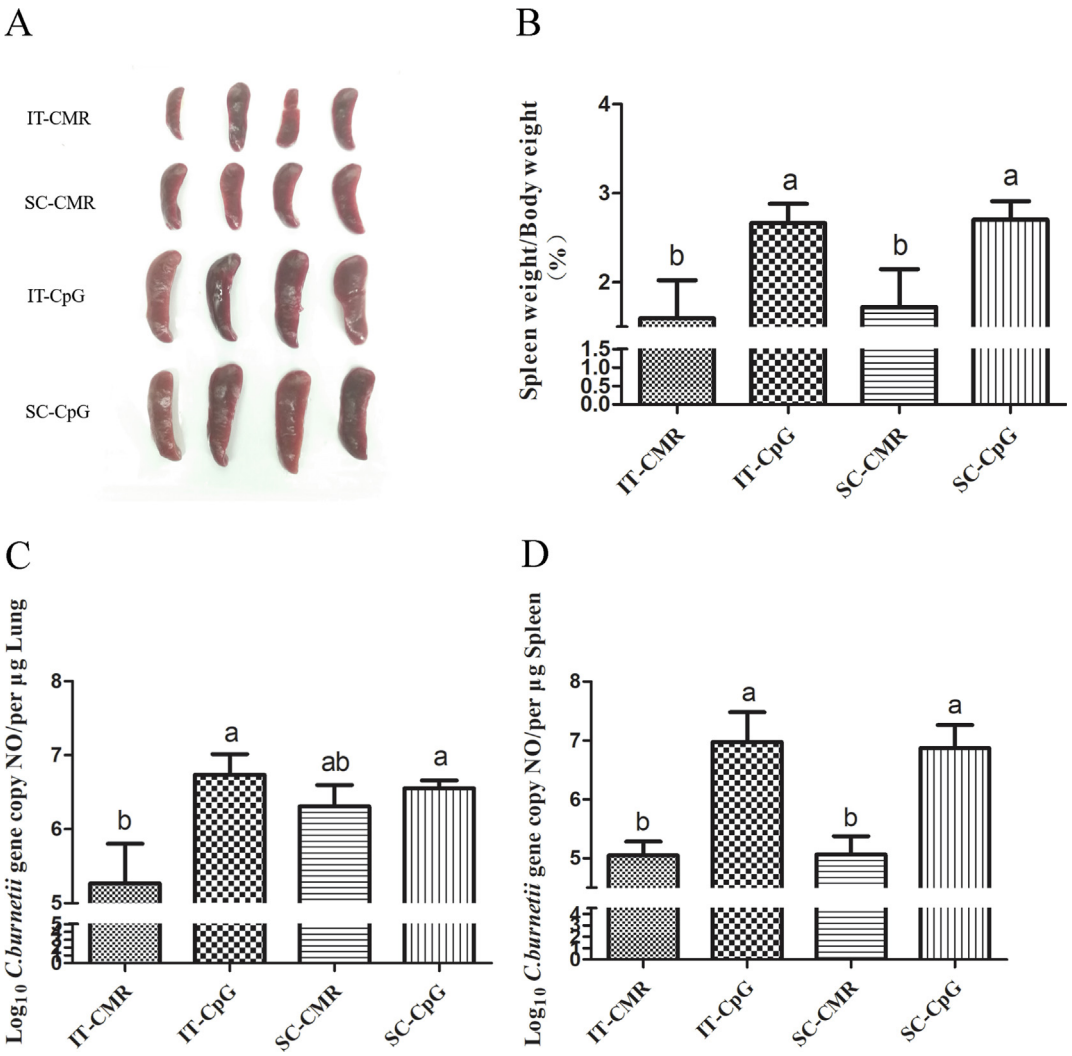

Fig. 5. Protection against *C. burnetii* induced by intratracheal inoculation of CMR. Mice immunized with CpG or CMR by the intratracheal inoculation or the subcutaneous injection were challenged with 1×10^8 GE *C. burnetii* (Xinqiao strain, phase I). Fourteen days after challenge, the spleens were photographed (A), and the ratio of spleen weight to body weight of each mouse was calculated (B). The *C. burnetii* genome copies of the lung (C) and spleen (D) were determined by qPCR analysis. The data are presented as the mean of n = 6 or 7 mice per group, and the standard error was indicated by the error bar. Data comparisons were performed with two-way variance (ANOVA) and then followed by Student-Newman-Keuls test. Significant differences ($P < 0.05$) between the groups are labeled with different letters (a, b or c), but there is no significant difference ($P > 0.05$) if two bars share a letter.

SC-CMR was slighter than that in corresponding control mice (Fig. S3).

4. Discussion

The disease of Q fever typically occurs following inhalation of contaminated aerosols, resulting in an initial pulmonary infection. To mimic natural infection of *C. burnetii* and study the lung response against this infection, several animal models of *C. burnetii* infection have been developed by using the whole-body aerosol inhalation exposure system [23,34,35]. Following aerosol exposure, *C. burnetii* enters the lungs via the intratracheal route, initially replicating within the lungs, and then spreads through the blood to other organs such as the livers and spleens [34]. In the present study, mice were infected with *C. burnetii* via the intratracheal inoculation using a Micro Sprayer Aerolizer. Fourteen days after challenge with *C. burnetii* by the intratracheal inoculation, a large amount of *C. burnetii* and severe pathological lesions were found in the lungs of mice immunized with CpG, suggesting the mice were acutely infected by *C. burnetii* via the intratracheal route.

Earlier studies have reported that the formalin-inactivated *C. burnetii* phase I vaccine could induce B cells to produce protective IgGs [36]. In the present study, the level of IgGs to *C. burnetii* phase I or II antigen in mice immunized by IT-CMR was comparable to that in mice immunized by SC-CMR, demonstrating that IT-CMR immunization was efficient to induce a specific humoral immune response. Many studies have proven that T-cell-mediated immunity is critical for the clearance of intracellular *C. burnetii* [19,20,22], since IFN-γ and TNF-α secreted by antigen-specific T cells can directly induce the production of nitric oxide and reactive oxygen species (ROS) in host cells to exert their toxic effects on phagocytosed bacteria. In the present study, after *ex vivo* stimulation with CMR, the splenic T cells from mice immunized by IT-CMR produced significantly higher levels of Th1 cytokines (IFN-γ and IL-12p70) and Th2 cytokines (IL-4 and IL-5), as well as a moderately higher level of T-cell proliferation than those of splenic T cells from mice immunized by SC-CMR. Th1 and Th2 cells are the main subsets of CD4 helper T cells and regulate cellular and humoral immunity, respectively, and therefore our results indicated that the IT-CMR vaccination was more effective in inducing the specific T cell responses compared with the SC-CMR vaccination.

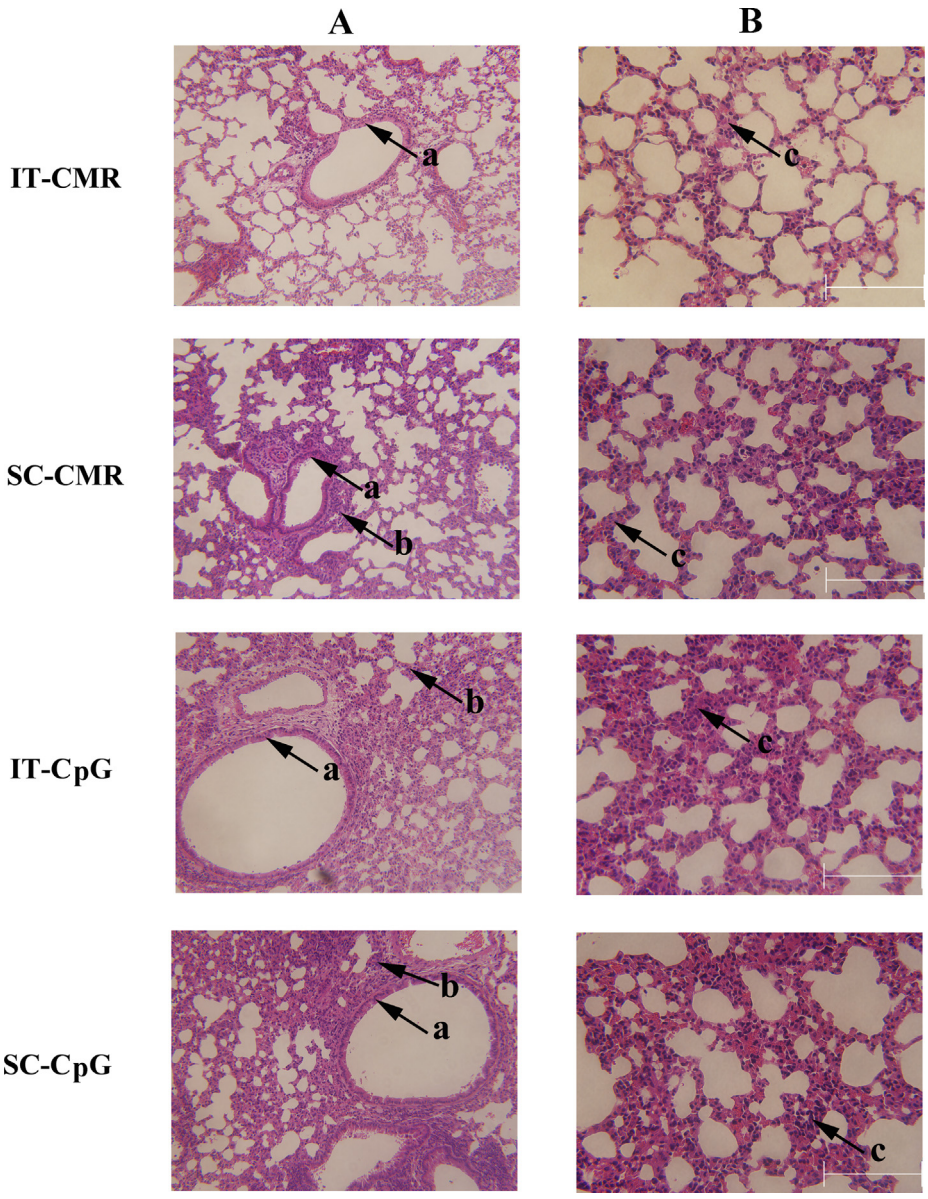

Fig. 6. Pathological lesions in the lungs of immunized mice after challenge. Fourteen days after challenge with *C. burnetii*, 6 or 7 mice per group were sacrificed, and the lungs and spleens of mice were fixed in formalin. The pathological alterations of lungs were observed in the HE-stained sections under light microscopy. Epithelial degeneration (arrow a), inflammatory cell infiltration (arrow b), and chronic interstitial inflammation (arrow c) were observed in the lungs of mice in the four groups (original magnifications 200 (A) or 400 (B), bar = 100 μm).

At the mucosal epithelium, sIgA is the predominant Ig isotype and exerts superior neutralization activity for its extracellular immune exclusion effect and pIgR-mediated cytosolic Fc receptor-participated intracellular pathogen neutralization activity. The importance of mucosal IgA during pathogen infection and its ability to neutralize virus in infected epithelial cells has previously been proven [37,38]. Furthermore, *C. burnetii*-specific IgA was detectable in both acute and chronic Q fever patients [39] as well as in vaccinators following Q fever vaccination [40]. In this study, a significantly higher level of sIgA was determined in the BAL of mice immunized by IT-CMR but not by SC-CMR, indicating that the IT-CMR vaccination was superior to the SC-CMR vaccination in inducing an antigen-specific mucosal immune response.

In the present study, the protective efficiency induced by the IT-CMR vaccination was evaluated in mice. After challenging with a sublethal dose of *C. burnetii*, the bacterial burdens in the lungs of mice immunized by IT-CMR but not by SC-CMR were significantly lower than in mice immunized by IT-CpG or by SC-CpG. Moreover, the pathological lesions in the lungs of mice immunized by IT-CMR were markedly slighter than those of mice immunized by SC-CMR. These data indicate that IT-CMR vaccination may improve bacterial clearance in the lung compared to SC-CMR vaccination, although alterations in the overall protective efficacy have not been fully elucidated. The IT-CMR vaccination might improve lung bacterial clearance via inducing specific mucosal immune response to produce a high level of sIgA, which might play a crucial role in the inhibition of primary intratracheal infection of *C. burnetii*. Conversely, systemic and mucosal IgG responses and bacterial burdens in the spleen were not significantly different between IT-CMR and SC-CMR, suggesting that the SC-CMR vaccination was as effective as the IT-CMR vaccine in regards to control of bacterial dissemination. Further experiments will be needed to test whether the

IT-CMR vaccination can completely prevent *C. burnetii* dissemination from the lung to other organs.

The CMR vaccine was reported to contain some components responsible for adverse reactions in human volunteers when administrated at a high dose (150 μg or more) [4]. Therefore, the immunization safety of IT-CMR still needs to be carefully evaluated. In the present study, a slight pathological change was found in the lungs of mice immunized by IT-CMR, which was not as severe as that found in mice immunized by SC-CMR, indicating that 20 μg CMR per mouse was a proper dose for the intratracheal inoculation of mice.

5. Conclusions

In the present study, compared with the SC-CMR vaccination, the IT-CMR vaccination induced significantly higher levels of *C. burnetii*-specific lung IgA response and *ex vivo* splenic T cell response, which might contribute to the reduced *C. burnetii* load and alleviate the pathological lesion in the lungs of mice immunized by IT-CMR after *C. burnetii* aerosol challenge. This suggests that, compared to the SC-CMR vaccination, the IT-CMR vaccination is more efficient to elicit the specific cellular immune response and lung mucosal immune response against *C. burnetii* aerosol infection in mice. We report the first evaluation using a mouse model of the protective ability and immunization safety of IT-CMR. Our study will enrich our understanding on mucosal immune response against *C. burnetii* infection, and also provide a potential route for intratracheal vaccine delivery against other intracellular bacteria transmitted by respiratory aerosols.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by grants from SKLPBS1802, National Natural Science Foundation of China (31970178) and the Beijing Nova Program (Z181100006218116).

References

[1] Maurin M, Raoult D. Q fever. Clin Microbiol Rev 1999;12:518–53.
[2] van Loenhout JA, Paget WJ, Vercoulen JH, Wijkmans CJ, Hautvast JL, van der Velden K. Assessing the long-term health impact of Q-fever in the Netherlands: a prospective cohort study started in 2007 on the largest documented Q-fever outbreak to date. BMC Infect Dis 2012;12:280.
[3] Hendrix LR, Chen C. Antigenic analysis for vaccines and diagnostics. Adv Exp Med Biol 2012;984:299–328.
[4] Fries LF, Waag DM, Williams JC. Safety and immunogenicity in human volunteers of a chloroform-methanol residue vaccine for Q fever. Infect Immun 1993;61:1251–8.
[5] Waag DM, England MJ, Bolt CR, Williams JC. Low-dose priming before vaccination with the phase I chloroform-methanol residue vaccine against Q fever enhances humoral and cellular immune responses to *Coxiella burnetii*. Clin Vaccine Immunol: CVI 2008;15:1505–12.
[6] Waag DM, Byrne WR, Estep J, Gibbs P, Pitt ML, Banfield CM. Evaluation of cynomolgus (Macaca fascicularis) and rhesus (Macaca mulatta) monkeys as experimental models of acute Q fever after aerosol exposure to phase-I *Coxiella burnetii*. Lab Anim Sci 1999;49:634–8.
[7] Waag DM, England MJ, Pitt ML. Comparative efficacy of a *Coxiella burnetii* chloroform:methanol residue (CMR) vaccine and a licensed cellular vaccine (Q-Vax) in rodents challenged by aerosol. Vaccine 1997;15:1779–83.
[8] Omsland A, Cockrell DC, Howe D, Fischer ER, Virtaneva K, Sturdevant DE, et al. Host cell-free growth of the Q fever bacterium *Coxiella burnetii*. PNAS 2009;106:4430–4.
[9] Omsland A, Beare PA, Hill J, Cockrell DC, Howe D, Hansen B, et al. Isolation from animal tissue and genetic transformation of *Coxiella burnetii* are facilitated by an improved axenic growth medium. Appl Environ Microbiol 2011;77:3720–5.
[10] Gong W, Wang P, Xiong X, Jiao J, Yang X, Wen B. Chloroform-methanol residue of *Coxiella burnetii* markedly potentiated the specific immunoprotection elicited by a recombinant protein fragment rOmpB-4 derived from outer membrane protein B of Rickettsia rickettsii in C3H/HeN mice. PLoS ONE 2015;10:e0124664.
[11] Park CO, Kupper TS. The emerging role of resident memory T cells in protective immunity and inflammatory disease. Nat Med 2015;21:688–97.
[12] Mueller SN, Mackay LK. Tissue-resident memory T cells: local specialists in immune defence. Nat Rev Immunol 2016;16:79–89.
[13] Chiu C, Openshaw PJ. Antiviral B cell and T cell immunity in the lungs. Nat Immunol 2015;16:18–26.
[14] Lee J, Lee C, Kim TH, Chi SC, Moon HR, Oh KT, et al. Pulmonary administered palmitic-acid modified exendin-4 peptide prolongs hypoglycemia in type 2 diabetic db/db mice. Regul Pept 2012;177:68–72.
[15] Perdomo C, Zedler U, Kuhl AA, Lozza L, Saikali P, Sander LE, et al. Mucosal BCG vaccination induces protective lung-resident memory T cell populations against tuberculosis. mBio 2016;7.
[16] Wu M, Zhao H, Li M, Yue Y, Xiong S, Xu W. Intranasal vaccination with mannosylated chitosan formulated DNA vaccine enables robust IgA and cellular response induction in the lungs of mice and improves protection against pulmonary mycobacterial challenge. Front Cell Infect Microbiol 2017;7:445.
[17] Timothy AA, Tokanovic A, Snibson KJ, Edwards SJ, Pearse MJ, Scheerlinck JP, et al. ISCOMATRIX adjuvant reduces mucosal tolerance for effective pulmonary vaccination against influenza. Human Vacc Immunother 2015;11:377–85.
[18] Florido M, Muflihah H, Lin LCW, Xia Y, Sierro F, Palendira M, et al. Pulmonary immunization with a recombinant influenza A virus vaccine induces lung-resident CD4(+) memory T cells that are associated with protection against tuberculosis. Mucosal Immunol 2018;11:1743–52.
[19] Chen C, Dow C, Wang P, Sidney J, Read A, Harmsen A, et al. Identification of CD4+ T cell epitopes in *C. burnetii* antigens targeted by antibody responses. PLoS ONE 2011;6:e17712.
[20] Xiong X, Qi Y, Jiao J, Gong W, Duan C, Wen B. Exploratory study on Th1 epitope-induced protective immunity against *Coxiella burnetii* infection. PLoS ONE 2014;9:e87206.
[21] Peng Y, Schoenlaub L, Elliott A, Mitchell WJ, Zhang G. Characterization of a lipopolysaccharide-targeted monoclonal antibody and its variable fragments as candidates for prophylaxis against the obligate intracellular bacterial pathogen *Coxiella burnetii*. Infect Immun 2014;82:4530–41.
[22] Xiong X, Jiao J, Gregory AE, Wang P, Bi Y, Wang X, et al. Identification of *Coxiella burnetii* CD8+ T-cell epitopes and delivery by attenuated listeria monocytogenes as a vaccine vector in a C57BL/6 mouse model. J Infect Dis 2017;215:1580–9.
[23] Elliott A, Peng Y, Zhang G. *Coxiella burnetii* interaction with neutrophils and macrophages in vitro and in SCID mice following aerosol infection. Infect Immun 2013;81:4604–14.
[24] Elliott A, Schoenlaub L, Freches D, Mitchell W, Zhang G. Neutrophils play an important role in protective immunity against *Coxiella burnetii* infection. Infect Immun 2015;83:3104–13.
[25] Williams JC, Damrow TA, Waag DM, Amano K. Characterization of a phase I *Coxiella burnetii* chloroform-methanol residue vaccine that induces active immunity against Q fever in C57BL/10 ScN mice. Infect Immun 1986;51:851–8.
[26] Fischer W, Koch HU, Haas R. Improved preparation of lipoteichoic acids. Eur J Biochem 1983;133:523–30.
[27] Wen BH, Yu SR, Yu GQ, Li QJ, Zhang X. Analysis of proteins and lipopolysaccharides from Chinese isolates of *Coxiella burnetii* with monoclonal antibodies. Acta Virol 1991;35:538–44.
[28] Wei Y, Wang X, Xiong X, Wen B. *Coxiella burnetii* antigen-stimulated dendritic cells mediated protection against *Coxiella burnetii* in BALB/c mice. J Infect Dis 2011;203:283–91.
[29] Bode C, Zhao G, Steinhagen F, Kinjo T, Klinman DM. CpG DNA as a vaccine adjuvant. Expert Rev Vacc 2011;10:499–511.
[30] Iho S, Maeyama J, Suzuki F. CpG oligodeoxynucleotides as mucosal adjuvants. Human Vacc Immunother 2015;11:755–60.
[31] Scheiermann J, Klinman DM. Clinical evaluation of CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases and cancer. Vaccine 2014;32:6377–89.
[32] Fukuyama Y, Ikeda Y, Ohori J, Sugita G, Aso K, Fujihashi K, et al. A molecular mucosal adjuvant to enhance immunity against pneumococcal infection in the elderly. Immune Netw 2015;15:9–15.
[33] Xiong X, Wang X, Wen B, Graves S, Stenos J. Potential serodiagnostic markers for Q fever identified in *Coxiella burnetii* by immunoproteomic and protein microarray approaches. BMC Microbiol 2012;12:35.
[34] Stein A, Louveau C, Lepidi H, Ricci F, Baylac P, Davoust B, et al. Q fever pneumonia: virulence of *Coxiella burnetii* pathovars in a murine model of aerosol infection. Infect Immun 2005;73:2469–77.
[35] Melenotte C, Lepidi H, Nappez C, Bechah Y, Audoly G, Terras J, et al. Mouse model of *Coxiella burnetii* aerosolization. Infect Immun 2016;84:2116–23.
[36] Zhang G, Peng Y, Schoenlaub L, Elliott A, Mitchell W, Zhang Y. Formalin-inactivated *Coxiella burnetii* phase I vaccine-induced protection depends on B cells to produce protective IgM and IgG. Infect Immun 2013;81:2112–22.

[37] Neuhaus V, Chichester JA, Ebensen T, Schwarz K, Hartman CE, Shoji Y, et al. A new adjuvanted nanoparticle-based H1N1 influenza vaccine induced antigen-specific local mucosal and systemic immune responses after administration into the lung. Vaccine 2014;32:3216–22.
[38] Ashtekar AR, Katz J, Xu Q, Michalek SM. A mucosal subunit vaccine protects against lethal respiratory infection with Francisella tularensis LVS. PLoS ONE 2012;7:e50460.
[39] Camacho MT, Outschoorn I, Echevarria C, Kovacova E, Yebra M, Mate I, et al. Distribution of IgA subclass response to *Coxiella burnetii* in patients with acute and chronic Q fever. Clin Immunol Immunopathol 1998;88:80–3.
[40] Camacho MT, Outschoorn I, Kovacova E, Tellez A. Distribution of immunoglobulin G (IgG) and A (IgA) subclasses following Q fever vaccination with soluble phase I *Coxiella burnetii* extract. Vaccine 2000;18:1773–7.