



## MOMP and MIP DNA-loaded bacterial ghosts reduce the severity of lung lesions in mice after *Chlamydia psittaci* respiratory tract infection

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### ABSTRACT

*Chlamydia psittaci* is a well known zoonotic pathogen that can lead to severe respiratory disease in poultry, pet birds and humans. Development of an effective and safe vaccine would be the most effective way to control *C. psittaci* infection. In this study, we used bacterial ghosts (BGs) as a delivery vehicle to evaluate the protective effects of major outer membrane protein (MOMP) and macrophage infectivity potentiator (MIP) DNA vaccines in mice. We found that MOMP/MIP DNA-loaded BGs elicited a better immune response than a naked DNA vaccine, giving increased IgG titers, lymphocyte proliferation responses and higher levels of IFN- $\gamma$ . After challenge infection, MOMP/MIP DNA-loaded BGs-immunized mice showed lower chlamydial load and inflammation pathology in lung tissues. In addition, we found that MOMP and MIP co-immunization or a heterologous prime-boost strategy could induce stronger immune responses and better protective efficacy against *C. psittaci* infection. Together, the above results suggest that BGs can act as an effective delivery vehicle for *C. psittaci* DNA vaccines, and co-immunization or heterologous prime-boost strategy can enhance protective efficacy against infection, thereby providing an alternative strategy for the design of vaccines against *C. psittaci*.

### 1. Introduction

*Chlamydia psittaci* is a specialized intracellular gram-negative bacterial parasite and a well-recognized zoonotic pathogen that has the ability to cause respiratory disease in poultry and pet birds. Moreover, *C. psittaci* is also a human pathogen and can cause severe, fulminating respiratory disease, such as influenza, pneumonia or other atypical pneumonia, even resulting in death (Čechová et al., 2018). Antibiotic treatment is available, but vaccination is considered the most effective way to control *C. psittaci* infection.

An ideal vaccine against *Chlamydia* infection should induce both the cellular and humoral immune responses. The major outer membrane protein (MOMP) of *Chlamydia* has been the most widely used vaccine candidate protein (Khan et al., 2014; Rodrigues et al., 2018). Kollipara and his colleagues evaluated the efficacy of a *C. pecorum* MOMP-based multi-subunit vaccine, and found it could induce strong neutralizing

antibodies and lymphocyte proliferation responses in koalas (Kollipara et al., 2013). Zhang reported that oral immunization of BALB/c mice with an edible vaccine resulting from transgenic rice expressing MOMP stimulated high levels of IFN- $\alpha$ , IL-2 and IL-4, and induced protection against *C. psittaci* challenge (Zhang et al., 2013).

Macrophage infectivity potentiator (MIP), an abundant chlamydial lipoprotein, which is highly conserved among various *Chlamydia* species, is also widely used as a protective antigen (Humbert et al., 2015; Reimer et al., 2016). *Chlamydial* MIP protein shares some amino-acid sequence with the surface-exposed MIP protein from *Neisseria meningitidis*. Recent studies reported that recombinant *N. meningitidis* MIP protein could provide protection against meningococcal disease and may be considered a potential candidate protein for vaccination (Bielecka et al., 2015; Hung et al., 2011). In *Chlamydia*, Lu and colleagues also reported that intramuscular immunization with *C. muridarum* MIP protein could protect against chlamydial infection of the

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reproductive tract and reduce tubal inflammation in mice (Lu et al., 2013).

DNA vaccines are so-called third generation vaccines that can induce protective humoral and cellular immunity and have some clear advantages over traditional vaccines (Wang et al., 2013). However, one problem can be low uptake of naked plasmid DNA into cells and a consequently weak immune response. To address this issue, a number of delivery systems have been designed to improve the uptake efficiency of DNA vaccines by antigen presenting cells (APCs) (Hou et al., 2019; Prompetchara et al., 2019). Other strategies that can also promote long term immune responses and protect against various pathogens include co-immunization and heterologous prime-boost (He) immunization protocols (Chen et al., 2015; Zhang et al., 2017).

Bacterial ghosts (BGs) are novel non-living empty bacterial envelopes that lack cytoplasmic content, but still retain all the structural features and surface antigen components found in their living counterparts. The intact surface of BGs is easily recognized by professional APCs through pattern-recognition receptors, such that BGs are becoming widely used as new delivery systems of foreign proteins or plasmid DNA for vaccine development (Amro et al., 2014; Hu et al., 2019).

In this study, we examined the immunogenicity and protective efficacy of MOMP and MIP DNA-loaded BGs. We found that intramuscular immunization with DNA-loaded BGs could induce strong humoral and cellular immune responses, and enhance immune response against *Chlamydia psittaci* respiratory tract infection

## 2. Materials and Methods

### 2.1. Animals

Female BALB/c mice, 4–5 weeks old, were purchased from Hunan SJA Laboratory Animal Co. Ltd, and maintained under specific pathogen-free conditions at the Laboratory Animal Center of University of South China. The animal study was supervised by the Experimental Animal Ethics Committee of University of South China and carried out in accordance with the regulations and guidelines of this committee.

### 2.2. Production and analysis of *E. coli* JM109 BGs

*E. coli* JM109 BGs were produced using the Plackett-Burman randomization method as described previously (Amro et al., 2014). In brief, the biomass of a 24 h *E. coli* JM109 culture was collected and resuspended in sterile phosphate buffered saline (PBS: 3.2 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.5 mM KH<sub>2</sub>PO<sub>4</sub>, 1.3 mM KCl, 135 mM NaCl, pH 7.4), then adjusted to a final concentration to  $1 \times 10^6$  CFU/ml. One millilitre each of SDS (1.665 mg/ml), NaOH (0.0023 mol/l) and CaCO<sub>3</sub> (0.35 µg/ml) were added into 2 ml bacterial suspension and incubated for 2 h at 37 °C. After centrifugation at 4000 rpm for 15 min, the floccule in the supernatant was collected and washed twice with PBS. Then the cells were resuspended in 4 ml PBS and 1 ml 30% H<sub>2</sub>O<sub>2</sub> and incubated for 5 min at 37 °C. The cell pellets were collected by centrifugation at 10,000 rpm for 10 min. Finally, the cells were resuspended in 3 ml 60% ethanol and incubated at room temperature for 30 min with gentle vortexing. The cells (BGs) were then collected and washed three times with PBS. Then the final cell pellet was collected, resuspended in PBS and stored in concentration of 5 mg/ml at –20 °C until further use. The presence of DNA in BGs was tested by 1% agarose gel electrophoresis.

### 2.3. Loading recombinant DNA plasmids into BGs

Recombinant plasmids pcDNA3.1(+)-MOMP and pcDNA3.1(+)-MIP were constructed as follows: the MOMP or MIP gene was amplified by PCR. After restriction enzyme digestion, the products were linked to pcDNA3.1(+) vector by T4 ligase. Then the recombinant plasmids were loaded into BGs essentially as described (Paukner et al., 2005). Briefly,

100 µl recombinant plasmid DNA, 100 µl BGs suspension, 550 µl PBS and 250 µl CaCl<sub>2</sub> (0.1 mM) were mixed together, incubated for 12 h at 4 °C, heat shocked at 42 °C for 90 s, and then quickly placed in an ice bath for 2 min.

### 2.4. Transient transfection DNA-loaded BGs into RAW 264.7 macrophage cells

Recombinant plasmids pcDNA3.1(+)-MOMP and pcDNA3.1(+)-MIP were loaded separately into *E. coli* ghosts by the calcium chloride method. The murine macrophage cells (RAW 264.7) were used for investigating whether the recombinant plasmid DNA had been successfully loaded. RAW 264.7 cells were cultured in Rosewell Park Memorial Institute (RPMI) medium 1640 (Hyclone, Logan, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco BRL, Gaithersburg, USA) and 2 mmol/l L-glutamine (Sigma-Aldrich, Munich, Germany) at 37 °C in an incubator supplied with 5% CO<sub>2</sub>. The pcDNA3.1(+)-MOMP BGs or pcDNA3.1(+)-MIP BGs were added to the monolayer murine macrophage cell line (RAW 264.7) cultures to allow internalization over a 2 h period. Free BGs were then removed by washing the macrophages with PBS, and RPMI 1640 containing 10% fetal bovine serum was added. After incubation at 37 °C for 24 h, total cell proteins were extracted to test the expression of MOMP or MIP protein by western blotting using rabbit anti-Cps 6BC polyclonal antibody (our unpublished data) as primary antibody and HRP-conjugated goat anti-rabbit IgG (Proteintech, USA) as secondary antibody.

### 2.5. Preparation of recombinant MOMP and MIP

The full length *MOMP* and *MIP* genes were amplified by polymerase chain reaction from *C. psittaci* 6BC genomic DNA and cloned into pET30a plasmid with *Bam*H I and *Xho* I restriction sites. The forward primer for MOMP was 5'-CGCG▼GAT CCA TGA AAA AACTCTTGAA ATCGGCATT-3' and the reverse primer was 5'-CCGC▼TCGAGTTAGA ATCTGAATTGAGCATTCATG-3'. The forward primer for MIP was 5'-CGCG▼GAT CCATGAAAAACAATGGTATT-3' and the reverse primer was 5'-CCGC▼TCGAGTCATGAAGCTGTGTTTTTGTGTC-3'. After confirmed by DNA sequencing, the pET30a-MOMP or pET30a-MIP plasmid was transformed into *E. coli* BL21, and the His-tagged proteins were induced with isopropyl-β-D-thiogalactoside (IPTG). Then the fusion proteins were purified using Ni-nitrilotriacetic acid (NTA) beads (QIAGEN Inc., Germany) according to the manufacturer's instruction. The recombinant proteins were concentrated and endotoxins were removed with a polymyxin B cartridge (Sigma, USA).

### 2.6. Immunization procedure

Mice were divided into eight groups with 25 mice in each group. The first group was sham immunized with PBS as a blank control and the second group with empty BGs as negative control. The next four groups were each immunized intramuscularly with 50 µl PBS containing 40 µg pcDNA3.1(+)-MOMP, pcDNA3.1(+)-MIP, pcDNA3.1(+)-MOMP BGs or pcDNA3.1(+)-MIP BGs. The seventh group was co-immunized with 50 µl PBS containing 20 µg pcDNA3.1(+)-MOMP BGs and 20 µg of pcDNA3.1(+)-MIP BGs. All groups were immunized four times at two-week intervals (weeks 1, 3, 5, 7). The eighth group was the heterologous prime-boost immunization group (pcDNA3.1(+)-MOMP/MIP BGs He), which was immunized with 20 µg of pcDNA3.1(+)-MOMP BGs and 20 µg of pcDNA3.1(+)-MIP BGs on weeks 1, 3 and 5, and then immunized with 50 µg each of purified recombinant MOMP and MIP protein (Table 1) (Aleksseva et al., 2009). One week after the last immunization, five mice in each group were sacrificed, sera were collected to measure antibody titers, and spleen samples were collected to determine lymphocyte proliferation responses and IL-4, IFN-γ or IL-17A levels.

**Table 1**  
Immunization procedures.

Groups	PBS (ul)	BGs (μg)	pcDNA3.1(+)-MOMP (μg)	pcDNA3.1(+)-MIP (μg)	pcDNA3.1(+)-MOMP BGs (μg)	pcDNA3.1(+)-MIP BGs (μg)	recombinant MOMP/MIP (μg)
PBS (1)	100						
BG (2)		50					
pcDNA3.1(+)-MOMP (3)			40				
pcDNA3.1(+)-MIP (4)				40			
pcDNA3.1(+)-MOMP BGs (5)					40		
pcDNA3.1(+)-MIP BGs (6)						40	
pcDNA3.1(+)-MOMP/MIP BGs (7)					20	20	
pcDNA3.1(+)-MOMP/MIP BGs He (8)					20	20	50/50

## 2.7. Quantification of antibody levels by ELISA

To assess humoral immunity, MOMP-specific and MIP-specific IgG levels in serum were measured by indirect enzyme linked immunosorbent assay (ELISA). Briefly, 96-well plates were coated with 100 μl purified recombinant MOMP or MIP protein (10 μg/ml) in the sodium bicarbonate buffer (33.6 g/ml NaHCO<sub>3</sub> and 63.6 g/ml NaCO<sub>3</sub>, pH 9.5) at 4 °C overnight. For the mixed MOMP/MIP vaccination and the PBS or BG vaccination controls, we used mixed MOMP/MIP protein. Then, plates were washed with PBST (PBS containing 0.05% Tween-20) three times and blocked with 5% dry non fat skimmed milk at 37 °C for 1 h. After further washing, 100 μl of 1:64,000 dilutions of mice serum was added to each well and incubated at 37 °C for 1 h. After washing, 50 μl horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG was added to each well for 1 h at 37 °C. After washing again, 100 μl 3, 3', 5, 5'-tetramethylbenzidine substrate was added and incubated at 37 °C for 15 min. The reaction was then terminated with 100 μl/well stop solution (2 N H<sub>2</sub>SO<sub>4</sub>), and the absorbance was measured at 450 nm.

## 2.8. Splenocyte proliferation assay and cytokine measurement

Splenocytes were harvested from mice one week after the final immunization. Spleen cells were cultured in 24-well plates at a density of  $5 \times 10^6$  cells/well with or without stimulation *in vitro* with MOMP or MIP protein (10 μg/well). For the mixed MOMP/MIP vaccination and the PBS or BG controls, we used mixed MOMP/MIP protein as stimulant. After 72 h, supernatants were harvested and IL-4, IFN-γ and IL-17A levels were tested using a Ready-SET-Go! Kit (eBioscience, USA) according to the instructions provided by the manufacturer.

For the lymphocyte proliferation assay, a Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan) was used. Spleen cells were cultured in 48-well plates at a density of  $1 \times 10^4$  cells/well. After stimulation with MOMP or MIP protein (10 μg/well) for 44 h, 20 μl/well (5 mg/ml) CCK-8 was added to the cell suspension for 4 h and the absorbance at 450 nm (OD<sub>450</sub>) of each well was determined and expressed as a stimulation index (SI).

## 2.9. Challenge of mice with *C. psittaci*

One week after the final immunization, mice were challenged intranasally (i.n.) with  $1 \times 10^5$  IFUs of *C. psittaci* in 50 μl sucrose-phosphate-glutamic acid buffer (SPG, 218 mM sucrose, 3.76 mM KH<sub>2</sub>PO<sub>4</sub>, 7.1 mM K<sub>2</sub>HPO<sub>4</sub>, 4.9 mM glutamate, pH 7.2), under light anesthesia with isoflurane (Cai et al., 2016). Mice were weighed every day and sacrificed on day 4 or day 10 after infection. There were 10 mice in each group at each time point. Lung tissues were isolated, five being used for organism titration, and the other five for pathological evaluation.

## 2.10. Titration of live chlamydial organisms in mouse lung tissue

To quantitate live *C. psittaci* organisms, the whole lung tissue was homogenized, and the homogenates were titrated on HeLa cell monolayers as described previously (Chen et al., 2010; Cimolai et al., 1992). Briefly, serially diluted homogenate samples were inoculated into HeLa cell monolayers grown on coverslips in 24-well plates. After incubation for 30 h, coverslips were fixed with 4% paraformaldehyde, and permeabilized with 0.3% Triton X-100. After washing and blocking, the coverslips were inoculated with rabbit anti-*C. psittaci* 6BC antibody for 2 h at 37 °C. After washing again, the coverslips were incubated with Cy2-labeled (green fluorescence) goat anti-rabbit IgG and Hoechst 33,258 for 1 h at 37 °C. The inclusions were counted under a fluorescence microscope (Cai et al., 2016).

## 2.11. Evaluation of mouse lung tissue pathology

After being fixed, embedded and serially sectioned, the lung tissue sections were stained with hematoxylin and eosin (H&E) and assessed by a certified pathologist blinded to mouse treatment. The severity of inflammation and pathology was scored according to the pathology grading system using a numerical score ranging from 0 to 26 (Alekseeva et al., 2009; Chen et al., 2010). Scores were assigned to individual mice and the mean and SD was calculated for each group of animals.

## 2.12. Statistical analysis

ANOVA ([www.physics.csbsju.edu/stats/anova.html](http://www.physics.csbsju.edu/stats/anova.html)) was used to analyze the mouse IgG antibody levels, IFUs, the lung pathology score, and cytokine levels from multiple groups; LSD *t*-test (Microsoft Excel) was used to compare two given groups. All data were expressed as the mean and standard deviation (SD).  $P < 0.05$  was set as the criterion for statistical significance.

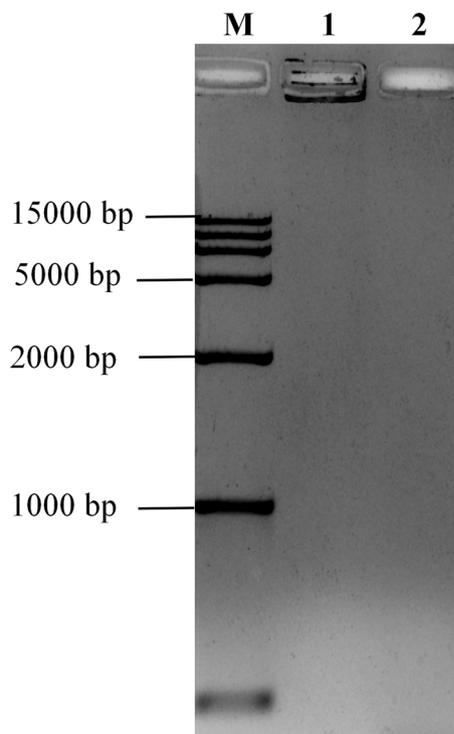
## 3. Results

### 3.1. Production of BGs

We prepared *E. coli* JM109 ghosts by the Plackett-Burman randomization method. As shown in Fig. 1, almost no DNA was detected by agarose gel electrophoresis in treated *E. coli* JM109 cells, compared to a non-treated control, consistent with successful preparation of DNA-free *E. coli* JM109.

### 3.2. Transient transfection of RAW264.7 macrophages by DNA-loaded BGs

Recombinant plasmids pcDNA3.1(+)-MOMP and pcDNA3.1(+)-MIP were loaded separately into *E. coli* ghosts by the calcium chloride method. To investigate whether the recombinant plasmid DNA had been successfully loaded, we used the BGs to transiently transfect RAW



**Fig. 1.** Testing for DNA in BGs prepared by the Plackett-Burman randomization method. 1% agarose gel electrophoresis of total DNA extracted from *E. coli* JM109 cells. (M) 10 kb DNA ladder. (1) DNA from untreated *E. coli* JM109 cells. (2) DNA from *E. coli* JM109 BGs.



**Fig. 2.** Expression of MOMP and MIP in RAW 264.7 cells after transfection with DNA-loaded BGs. BGs were used to deliver pcDNA3.1(+)-MOMP or pcDNA3.1(+)-MIP expression constructs into RAW 264.7 macrophage cells. The expression of MOMP or MIP protein was demonstrated by western blotting. The primary antibody was rabbit anti-Cps polyclonal antibody (our unpublished data) and the secondary antibody was HRP-conjugated goat anti-rabbit IgG. (1, 3) Proteins from RAW 264.7 cells treated with pcDNA3.1(+) BGs (negative control). (2) Proteins from RAW 264.7 cells incubated with pcDNA3.1(+)-MOMP BGs. (4) Proteins from RAW 264.7 cells incubated with pcDNA3.1(+)-MIP BGs.

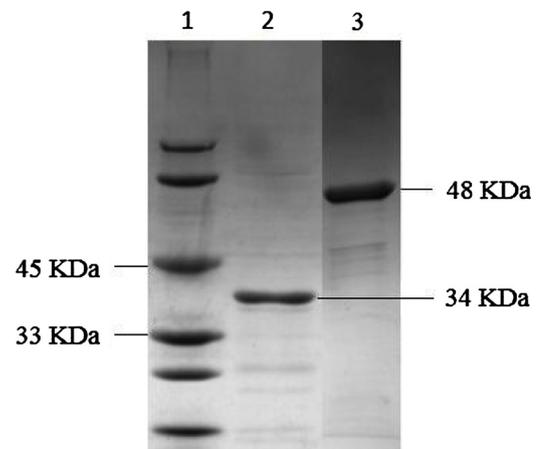
264.7 macrophages and then tested for the expression of MOMP or MIP protein by western blotting. As shown in Fig. 2, proteins of about 48 kDa or 34 kDa were expressed in RAW 264.7 cells following transfection with the MOMP or MIP construct, respectively. These bands correspond to the predicted size for each protein, which indicates successful loading of BGs with each DNA construct.

### 3.3. Expression of recombinant *C. Psittaci* MOMP and MIP

After constructed pET30a-MOMP or pET30a-MIP, the recombinant plasmid was transformed into *E. coli* BL21 and expressed as His-tagged proteins with a predicted size of 48 KDa (MOMP) or 34 KDa(MIP) and have a purity of > 95% after purification (Fig. 3).

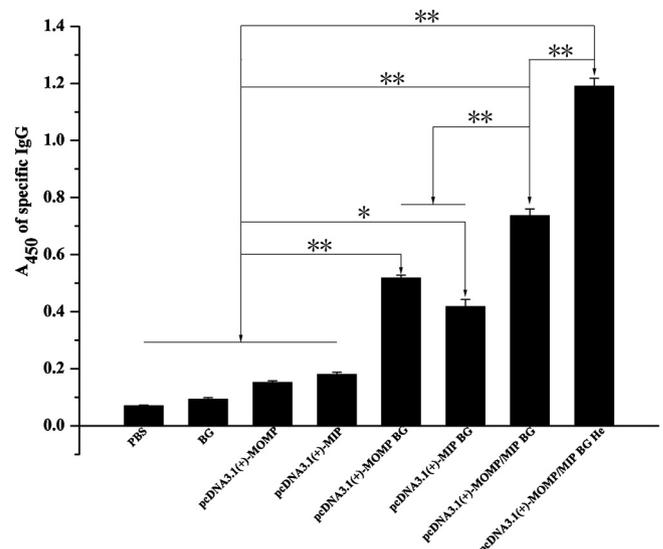
### 3.4. Immunization with DNA-loaded BGs induces strong anti-*C. psittaci* antibody responses

Mice were immunized with DNA-loaded BGs or various controls as described in the Methods. One week after the last immunization, serum

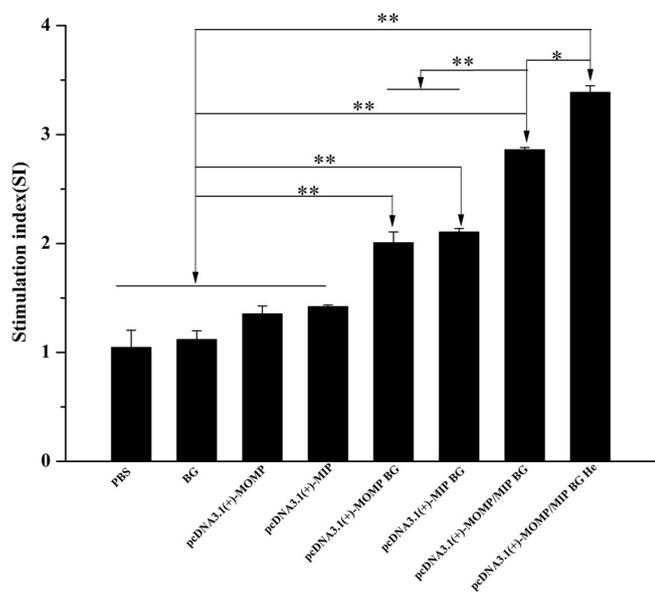


**Fig. 3.** Production of recombinant MOMP and MIP. The recombinant pET30a-MOMP or pET30a-MIP plasmid was transformed into *E. coli* BL21 and a His-tagged protein was expressed with IPTG induction, then the fusion proteins were purified with Ni-nitrilotriacetic acid (NTA) beads.

was collected and levels of IgG against either MOMP or MIP were evaluated by ELISA. Immunization with DNA-loaded BGs induced significantly higher levels of antigen-specific IgG antibodies than were found in control mice, which had been sham immunized with PBS or empty BGs (Fig. 4). As a further control, mice were also immunized with naked pcDNA3.1(+)-MOMP or pcDNA3.1(+)-MIP DNA, but this resulted in a poor humoral immune response that was statistically indistinguishable from negative controls. Mice that were co-immunized with both MOMP and MIP BGs produced levels of specific IgG antibodies (against MOMP and MIP combined) that were higher than those in mice immunized with pcDNA3.1(+)-MOMP BGs or pcDNA3.1(+)-



**Fig. 4.** Specific antibody response after immunization with DNA-loaded BGs. Serum IgG against *C. psittaci* MOMP or MIP was assessed at one week after the last immunization. A total of 100 µl of 1:64,000 dilutions of serum was added in duplicate to each well. For the PBS/BGs controls, the mixed MOMP/MIP immunization group or the He immunization group (groups 1, 2, 7, 8), mixed purified recombinant MOMP/MIP protein were used as bait in the ELISA; for MOMP-only vaccination groups (groups 3, 5), purified recombinant MOMP alone was used as bait; while for MIP-only vaccination groups (groups 4, 6), purified recombinant MIP alone was used as bait. There were significant differences in serum specific antibodies among all DNA-loaded BGs-immunized mice and control or naked plasmid DNA-immunized groups. Data are represented as means ± SD of the results from five individual mouse sera in three independent experiments (\*  $P < 0.05$ , \*\*  $P < 0.01$ ; LSD  $t$ -test).



**Fig. 5.** Stimulation index of spleen cells from DNA-loaded BGs-immunized and control mice. One week after the final immunization, mice were euthanized and spleens were tested for T lymphocyte proliferation response by CCK-8. Spleen cells from the PBS/BGs controls, the mixed MOMP/MIP immunization group or the He immunization group (groups 1, 2, 7, 8) were stimulated with mixed purified recombinant MOMP/MIP protein; spleen cells from MOMP vaccination groups (groups 3, 5), were stimulated with purified recombinant MOMP alone; while spleen cells from MIP vaccination groups (groups 4, 6), were stimulated with purified recombinant MIP alone. There were significant differences in the proliferation response of spleen cells between all DNA-loaded BGs immunized mice and control or naked DNA-immunized groups. Data are represented as means ± SD of the results from five individual mouse splenocytes in three independent experiments (\*  $P < 0.05$ , \*\*  $P < 0.01$ ; LSD  $t$ -test).

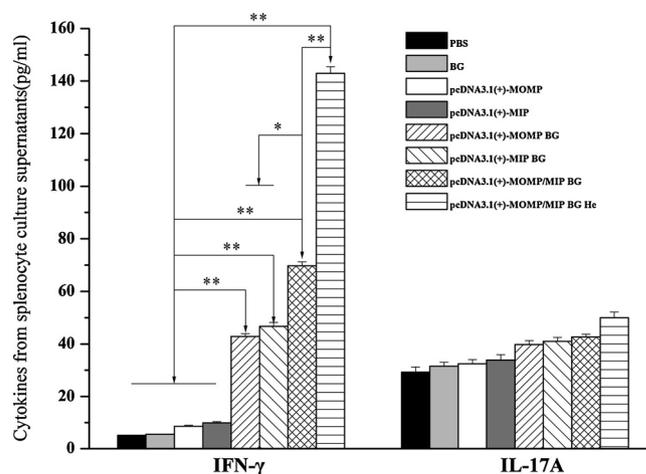
MIP BGs alone, as might be expected. Finally, a heterologous prime-boost (He) immunization protocol induced the highest combined levels of specific IgG antibodies.

**3.5. Immunization with DNA-loaded BGs induces strong anti-*C. psittaci* cellular immune responses**

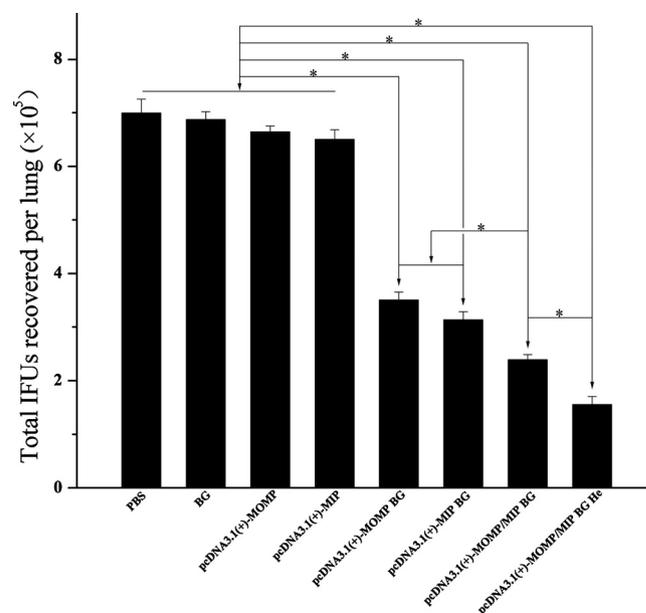
There was a strong T lymphocyte response in all DNA-loaded BGs-immunized groups (Fig. 5). Splenocytes taken from DNA-loaded BGs-immunized mice responded to restimulation with recombinant *C. psittaci* MOMP or MIP, while those from control mice or mice immunized with naked DNA failed to respond. When the splenocytes were measured for cytokine production, significantly higher levels of IFN- $\gamma$  were detected in those derived from DNA-loaded BGs-immunized mice (Fig. 6). Moreover, the T lymphocyte response and levels of IFN- $\gamma$  in co-immunization or He immunization groups were significantly higher than in mice immunized with a single type of DNA-loaded BGs. However, no difference in IL-17A production was found among the various groups, while the levels of IL-4 were below our ELISA detection sensitivity level (data not shown).

**3.6. Immunization with DNA-loaded BGs reduce bacterial load and the severity of lesions in mice lung after *C. psittaci* respiratory tract challenge infection**

After challenged with *C. psittaci*, all mice showed significant weight loss, but recovered on day 7 after infection regardless of the immunization reagents (data not shown). However, the number of live organisms recovered from lung homogenates of the DNA-loaded BGs-immunized mice was significantly lower than in control or naked DNA-immunized groups on day 4 after infection (Fig. 7). On day 10, almost



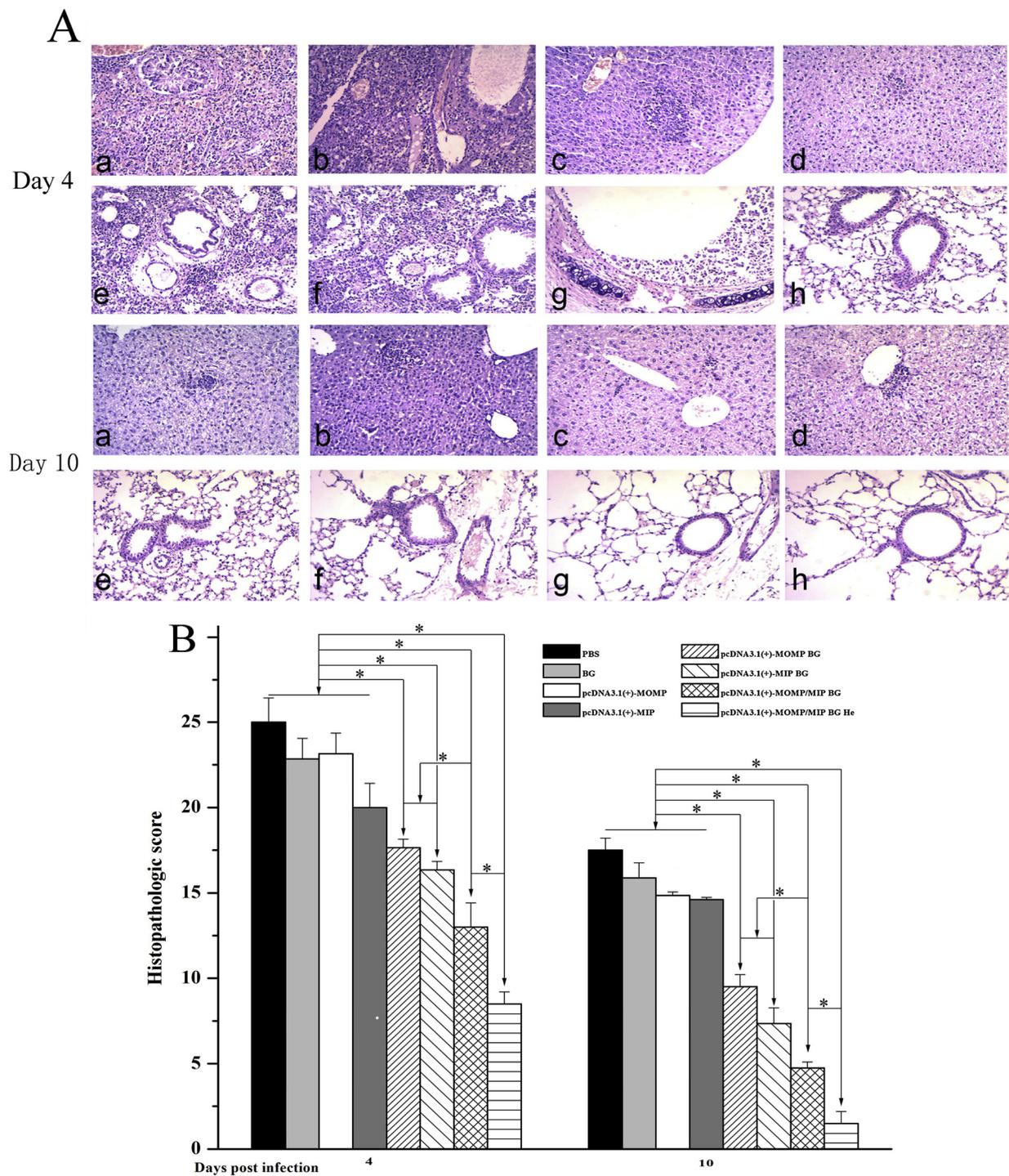
**Fig. 6.** Cytokine production in the spleen cells of DNA-loaded BGs-immunized and control mice. One week after the final immunization, mice were sacrificed and the levels of IL-4, IL-17 and IFN- $\gamma$  were tested in spleen cells by ELISA. Spleen cells from the PBS/BGs controls, the mixed MOMP/MIP immunization group or the He immunization group (groups 1, 2, 7, 8) were stimulated with mixed purified recombinant MOMP/MIP protein; spleen cells from MOMP vaccination groups (groups 3, 5), were stimulated with purified recombinant MOMP alone; while spleen cells from MIP vaccination groups (groups 4, 6), were stimulated with purified recombinant MIP alone. Data are represented as means ± SD of the results from three individual mouse spleens in three independent experiments (\*  $P < 0.05$ , \*\*  $P < 0.01$ ; LSD  $t$ -test).



**Fig. 7.** *C. psittaci* loading in the lungs after *C. psittaci* challenge. Mice were challenged with  $1 \times 10^5$  IFUs *C. psittaci* and sacrificed on day 4 or day 10 post-infection (only day 4 data are shown). The whole lung tissue was harvested and homogenized, and the homogenates were titrated to determine IFU values by indirect immunofluorescence. There were significant differences between DNA-loaded BGs-immunized mice and control or naked DNA-immunized groups. Data are represented as means ± SD of the results from five individual mouse lung tissues in three independent experiments (\*  $P < 0.05$ , \*\*  $P < 0.01$ ; LSD  $t$ -test).

no live organisms were detected in any mice (data not shown).

We further evaluated the inflammatory pathologies in mice lung tissue after challenge infection. On day 4 post infection, mice sham immunized with PBS or empty BGs showed severe inflammatory damage as indicated by alveoli hyperemia, exudate in the alveoli and



**Fig. 8.** Inflammatory pathology in mouse lung tissue after *C. psittaci* challenge infection. After sacrifice, lung tissues of PBS- (a), empty BGs- (b), pcDNA3.1(+)-MOMP- (c), pcDNA3.1(+)-MIP- (d), pcDNA3.1(+)-MOMP BGs- (e), pcDNA3.1(+)-MIP BGs- (f), pcDNA3.1(+)-MOMP/MIP BGs- (g), and pcDNA3.1(+)-MOMP/MIP BGs He-immunized (h) mice were subjected to histological section analyses under the microscope after H&E staining (A). The extent of lung tissue inflammation was semi-quantitatively scored as described in Materials and Methods (B). Data are represented as means ± SD (\*  $P < 0.05$ , \*\*  $P < 0.01$ ; LSD *t*-test).

infiltration with neutrophil and lymphocyte (Fig. 8A, upper panels). On day 10 post infection, some mice still developed severe inflammatory damage as showed interstitial pneumonia, which characterized by large numbers of inflammatory infiltrates of polymorphonuclear leukocytes, mononuclear cells, alveoli interstitial thickening, and hemorrhage. And some mice developed patchy pneumonia, as indicated that the alveoli cavity was filled with fibrinous exudate mixed with red blood cells, neutrophils and macrophages. (Fig. 8A, lower panels). In contrast, only a few DNA-loaded BGs-immunized mice developed interstitial

pneumonia or patchy pneumonia, with neutrophil and lymphocyte infiltration occurring in peribronchial tissue and visible exudate in some alveoli and bronchi on day 4 post infection; by day 10, these mice had almost completely recovered and returned to normal. We next semi-quantitatively analyzed the lung histopathology (Fig. 8B). The mice immunized with DNA-loaded BGs had a significantly lower histopathology score than these of control mice or mice immunized with naked DNA either on day 4 or day 10 after challenge infection. Moreover, co-immunized or He-immunized mice showed a much lower live

organism burden and reduced lung histopathology than mice immunized with pcDNA3.1(+)-MOMP BGs or pcDNA3.1(+)-MIP BGs alone.

#### 4. Discussion

Although DNA vaccines can induce humoral and cellular immunity, the weak immunogenicity, especially in humans, still limits their application (Xu et al., 2014). In the present study, we used *E. coli* BGs as a delivery system, and tested the protective immunity of a *C. psittaci* DNA vaccine encoding MOMP and MIP proteins against *C. psittaci* infection in a mouse model.

After constructing recombinant *E. coli* BGs loaded with *C. psittaci* MOMP and/or MIP DNA, we confirmed that DNA-loaded BGs could be taken up and the MOMP and MIP constructs efficiently expressed in murine macrophage RAW 264.7 cells which indicated that the DNA-loaded BGs could express corresponding proteins in host.

After immunized mice, compared with DNA-loaded BGs, animals immunized with naked pcDNA3.1(+)-MOMP or pcDNA3.1(+)-MIP DNA showed only weak humoral and cellular responses, generating little specific IgG, and inducing a limited lymphocyte proliferation response with low levels of IFN- $\gamma$  production. Kim also reported that naked DNA vaccines generally induce modest immune response (Kim et al., 2015). This may be because naked DNA is less likely to be taken up by APCs, and therefore fails to induce an effective immune response (Muhammad et al., 2012). In contrast, DNA-loaded BGs were able to activate antigen-specific T cells to trigger a strong immune response. Vaccine efficacy is defined not only by the type of antigen but also the adjuvant or delivery system used. Our results indicated that BGs could act as a potentially excellent vehicle for the enhancement of DNA vaccines, just as previous studies reported (Eko et al., 2008; Inic-Kanada et al., 2015).

In order to analyze whether the DNA-loaded BGs could protect against *C. psittaci* infection, mice were challenged intranasally with *C. psittaci* one week after the final immunization. In a respiratory model of experimentally induced acute *C. psittaci* infection, disease symptoms were apparent 2–3 days post-inoculation and lasted for about one week (Bode et al., 2012; Reinhold et al., 2012). Consequently, we chose days 4 and 10 post-inoculation to study the effect of vaccination.

On day 4 after challenge, the chlamydial load in lung tissue of DNA-loaded BGs-immunized mice was significantly decreased compared to controls. However, on day 10 after challenge, all mice – including control animals – were able to spontaneously clear *C. psittaci* lung infection, suggesting that even naïve mice have a sufficiently robust immune system to clear the pathogen. However, in DNA-loaded BGs-immunized groups, on day 4 and day 10 after challenge, the extent of lung inflammation was significantly reduced and recovered sooner than in PBS, empty BGs and naked DNA-immunized mice. This might be because the DNA-loaded BGs could induce both high levels of specific IgG antibodies and strong cellular immune responses including elevated levels of IFN- $\gamma$  and stimulation index of spleen cells in immunized mice. Antibodies can neutralize bacteria before they enter host cells, while CD4<sup>+</sup> T-cell-dependent and IFN- $\gamma$ -mediated Th1-type immunity has been identified as a major protective mechanism by which mice control chlamydial infection (Gondek et al., 2012; Ziklo et al., 2016). Recent investigations also indicated that IL-17 plays an important role in the control of intracellular pathogens (Alizadeh et al., 2019; Bai et al., 2009). Splenocytes from DNA-loaded BGs-immunized mice show increased T lymphocyte proliferation responses and IFN- $\gamma$  generation, but no effect on IL-17A. Scurlock also reported that IL-17 contributes to the generation of TH1 immunity but is not for normal resolution against *C. muridarum* infection (Scurlock et al., 2011). The reason maybe when IFN- $\gamma$ -mediated Th1-type immune response predominant, TH17-type immunity is unnecessary, or IFN- $\gamma$  will negatively regulate the TH17 response (Cruz et al., 2006).

The current study also showed that co-immunization with MOMP

and MIP DNA-loaded BGs or Heterologous prime-boost (He) immunization could induced stronger immune response and resulted in lower chlamydial load and inflammation pathology in the lungs of *C. psittaci*-challenged mice. This may be due to more B and T cell epitopes being provided by two proteins, and may produce some new conformational epitopes with greater diversity, which can induce better humoral and cellular immune responses (Wang et al., 2013). While He immunization involves different immunization systems, such as a primary immunization with a DNA vaccine followed by a boost with a recombinant protein, and has been proven to generate strong humoral and cellular immune responses (Bolhassani et al., 2015; Meng et al., 2013).

In summary, we have shown that BGs represent an effective delivery vehicle for DNA vaccines, MOMP and MIP DNA vaccine co-immunization and He immunization, giving enhanced protective efficacy against acute *C. psittaci* infection in mice. This should guide future development of vaccines against *C. psittaci*.

#### Declaration of Competing Interest

The authors have no conflicts of interest.

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