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ORIGINAL ARTICLE

Immunization with *Chlamydia psittaci* plasmid-encoded protein CPSIT_p7 induces partial protective immunity against chlamydia lung infection in mice

Yuan Tan[1,2,3,4] · Yumeng Li[1,2,3] · Yang Zhang[5] · Jian Yu[1,2,3] · Yating Wen[1,2,3] · Chuan Wang[1,2,3] · Man Xu[1,2,3] · Qian Chen[1,2,3] · Chunxue Lu[1,2,3] · Yimou Wu[1,2,3]

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Abstract

The present study evaluated the immune-protective efficacy of the *Chlamydia psittaci* (*C. psittaci*) plasmid protein CPSIT_p7 and analyzed the potential mechanisms of this protection. The current study used recombinant CPSIT_p7 protein with Freund's complete adjuvant and Freund's incomplete adjuvant to vaccinate BALB/c mice. Adjuvants alone or PBS formulated with the same adjuvants was used as negative controls. Mice were intranasally challenged with 10^5 inclusion-forming units (IFU) of *C. psittaci*. We found that CPSIT_p7 vaccination significantly decreased the mouse lung chlamydial load, interferon-γ (IFN-γ) level, and pathological injury. This protection correlated well with specific humoral and cellular immune responses against *C. psittaci*. In vitro or in vivo neutralization of *C. psittaci* with sera harvested from immunized mice did not reduce the number of recoverable *C. psittaci* in the infected lungs, but $CD4^+$ spleen cells collected from CPSIT_p7-immunized mice significantly decreased the chlamydial load via adoptive transfer to native mice. These results reveal that the protection conferred by CPSIT_p7 is dependent on $CD4^+$ T cells.

Keywords *C. psittaci* · Plasmid protein · CPSIT_p7 · Protective immunity · $CD4^+$ T cells

Introduction

Chlamydiae is an obligate intracellular of Gram-negative bacteria and has a biphasic development cycle. The elementary bodies (EBs) exhibit infectivity without metabolic activity, and the reticulate bodies (RBs) are metabolically active without infectivity. EBs infect nearby host cells and transform into RBs to complete one life cycle [1]. Bacteria of the genus *Chlamydiae* are composed of nine species, and four of these bacteria infect humans: *C. trachomatis*, *C. abortus*, *C. pneumoniae* and *C. psittaci*. *C. psittaci* has received less research attention than the other three species.

C. psittaci is a zoonotic pathogen that causes psittacosis or ornithosis, which results in appreciable economic damage to pet birds and poultry. *C. psittaci* also infects humans and leads to life-threatening atypical pneumonia via the spread of infection from birds to humans [2]. Psittacosis is a rare, but potentially severe, disease in humans. No or merely flu-like symptoms, such as headache, fever, listlessness, and dry cough, appear early in the infection. However, the infection may further affect other systems and produce a fatal outcome without timely and effective antibiotic therapy [3].

Chlamydia causes a wide range of infections, such as respiratory, genital, and ocular infections, which result in considerable public health threats. Several antibiotics effectively treat chlamydial infection, but the current medical opinion is that the

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✉ Yimou Wu
yimouwu@sina.com

[1] Institution of Pathogenic Biology, Medical College, University of South China, Hengyang 421001, China

[2] Hunan Province Cooperative Innovation Center for Molecular Target New Drug Study, University of South China, Hengyang 421001, China

[3] Hunan Provincial Key Laboratory for Special Pathogens Prevention and Control, University of South China, Hengyang 421001, China

[4] Dermatology, the First Hospital of Changsha City, Changsha 410000, China

[5] Department of Pathology, University of South China, Hengyang 421001, China

most effective strategy is prevention and protection of humans against chlamydial using an efficacious vaccination [4–6]. An effective vaccine should produce a persistent protective immune response via specific serotype and pathological immune stimulation. Unfortunately, no human vaccine has been developed despite significant advances and considerable efforts.

Plasmids may play a key role in chlamydial pathogenesis, and these structures widely exist in *Chlamydiae* species [7]. The plasmids of *C. trachomatis* and *C. muridarum* have been highly researched. Plasmid-deficient *C. trachomatis* and *C. muridarum* fail to induce severe pathology, which suggests that the plasmids are vital virulence factors in chlamydial infection [8–10]. Most *C. psittaci* strains also harbor a small highly conserved cryptic plasmid (~ 7.5 kb) [11, 12]. The plasmid of *C. psittaci* encodes eight open reading frames (ORFs) designated CPSIT_p1-8, and CPSIT_p7 encodes the protein Pgp3. Pgp3 is the only plasmid protein secreted into the host cell cytosol [13], and it is a major virulence factor for the induction of hydrosalpinx for *C. muridarum* [14]. Notably, human antibodies recognize Pgp3 as an important immunodominant antigen in *C. trachomatis* infection [15]. Immunization with Pgp3 protein induces protective immunity against *C. trachomatis* infection in a mouse model [16]. However, the role of CPSIT_p7 (Pgp3) in *C. psittaci* is not clear. Research demonstrated that the CPSIT_p8 (Pgp4) protein of *C. psittaci* induced significant protective immunity in BALB/c mice [17]. *Pgp4* regulates the expression of *pgp3*, but Pgp3 does not affect the function of Pgp4. Therefore, we hypothesized that CPSIT_p7 would exhibit protective effects against *C. psittaci* infection and may be a candidate vaccine for *C. psittaci.*

The present study investigated the efficacy of immunization with the plasmid-encoded protein of *C. psittaci* CPSIT_p7 (Pgp3) in protecting against *C. psittaci* infection in BALB/c mice using recombinant CPSIT_p7 proteins. The results demonstrated that three immunizations with recombinant CPSIT_p7 proteins induced strong immune responses and protection against *C. psittaci* infection. The protective effects of the CPSIT_p7-specific immune response relied on $CD4^+$ T lymphocytes.

Materials and methods

Cultivation of *C. psittaci*

C. psittaci 6BC EBs (a gift from G.M. Zhong, USA) were cultured on HeLa 229 monolayer cells, purified from infected cells using density gradient centrifugation, and stored at − 80 °C. The number of *C. psittaci* EBs was counted using indirect immunofluorescence (IFA) [17]. Serial dilutions of the infectious EBs were inoculated onto HeLa 229 monolayer cells after culturing for 36 h, and the infected cells were fixed and stained using an FITC-labeled monoclonal anti-Chlamydia LPS antibody (Meridian Diagnostics, Inc., Cincinnati, OH) and anti-mouse IgG (Sigma, St. Louis, MO, USA). The titer is expressed as inclusion-forming units/ml (IFU/ml).

Preparation of recombinant proteins CPSIT_p7

The open reading frames of *CPSIT_p7* gene containing 795 bp DNA fragments (GenBank:NC_015217) were amplified using polymerase chain reaction (PCR) from the genomic DNA of *C. psittaci* 6BC and inserted into the pET28a vector with CPSIT_p7 primer pairs (5′-CGC-GGATCC(BamHI)-ATGGGTAATTCTGGTTTTTAC-3′, 5′-CCC-AAGCTT(HindIII)-TTAACCATTTGTTTGTTGTTTT-3′) (Fig. 1a). The constructed DNA sequence was used to verify inclusion. The vectors were inserted into *Escherichia coli* BL21 strains and grown in Luria-Bertani medium (kanamycin 60 μg/ml). The his-tagged proteins were expressed from *E. coli* BL21 strains and induced using 0.6 M isopropyl-β-D-thiogalactoside (IPTG) for 4 h at 30 °C in LB medium (Fig. 1b). Bacterial suspensions were collected, washed with phosphate-buffered saline (PBS), and lysed using a lysis buffer (10 mM imidazole, 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 20% glycerol, and 1% Triton X-100) and lysozymes (final concentration, 2~4 g/ml). The resulting CPSIT_p7 proteins were purified using a Ni-NTA affinity resin (QIAGEN) (Fig. 1c). Fusion proteins were treated filtered through a polymyxin B cartridge (Sigma, St. Louis, MO, USA) to remove the endotoxins, and concentration was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

Western blotting

Purified recombinant proteins CPSIT_p7 were diluted (4:1) in 5× sample loading buffer and boiled for 10 min. Protein samples (10 μl) were separated using 12% SDS polyacrylamide gel (SDS-PAGE) electrophoresis. Separated proteins were blotted onto polyvinylidene fluoride (PVDF) membranes and blocked with Tris-buffered saline (TBS) containing 0.05% Tween 20 (Sigma, St. Louis, MO, USA) (TBST) and 5% skim milk at room temperature (RT) for 3 h [2]. The PVDF membrane was probed using an anti-His-tag mouse monoclonal antibody (Fig. 1f) (1:500 dilution in TBST containing 5% skim milk) or a pool of sera collected from CPSIT_p7 (Fig. 1e) or sera of control group mice (Fig. 1f). The membrane was washed with TBST and incubated with HRP-conjugated goat anti-mouse IgG (Abcam, UK) for 1 h at 37 °C. The color was developed using ECL Prime Western Blotting Detection Reagent (Auragene).

Mice and immunization

Three groups ($n = 3$) of 30 pathogen-free 6-week-old female BALB/c mice (Hunan SJA Laboratory Animal Co. Ltd.) were immunized three times at 2-week intervals via intraperitoneal

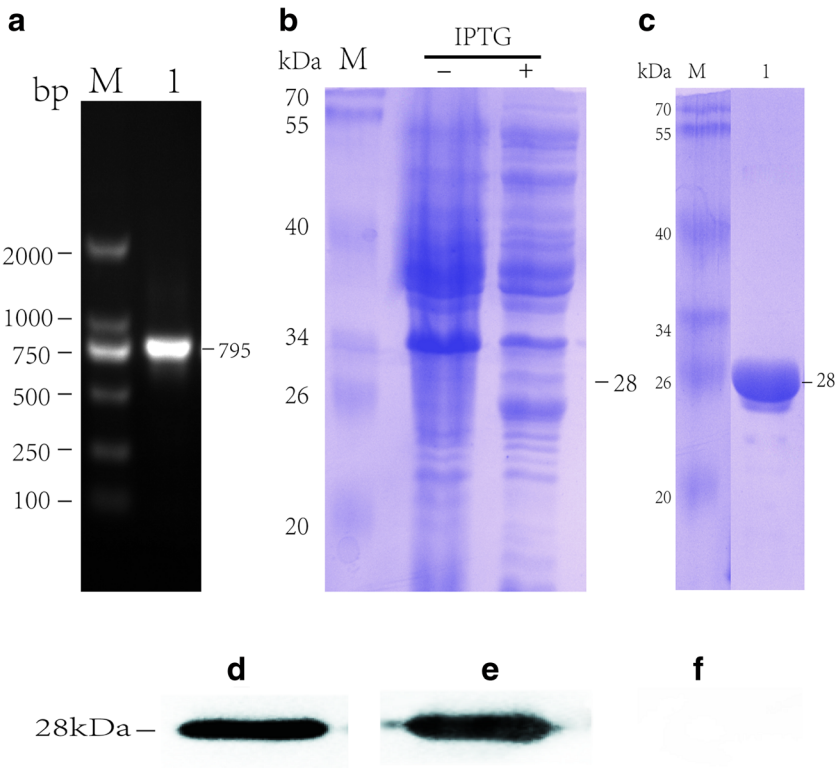

Fig. 1 Preparation of recombinant protein CPSIT_p7. **a** Amplification of the *CPSIT_p7* (795 bp) gene from *C. psittaci* 6BC genomic DNA. **b** Recombinant proteins CPSIT_p7 were expressed from *E.coli* BL21 strains containing a pET28a-CPSIT_p7 plasmid and induced with (+) or without (−) IPTG. **c** CPSIT_p7 proteins purified using Ni-NTA affinity resin. **d** Western blotting analysis was used to identify the CPSIT_p7 proteins and production of CPSIT_p7-specific antibodies in immunized-CPSIT_p7 mice. Ten microliters of purified CPSIT_p7 was loaded onto a 12% SDS-PAGE gel, and the gel was blotted onto a PVDF membrane using anti-His monoclonal antibody sera, **e** sera of BALB/c mice immunized with CPSIT_p7, and **f** sera of control group mice

injection (i.p.) with 40 μg purified recombinant CPSIT_p7 proteins diluted in PBS. PBS and Freund's adjuvant (FA, Sigma, St. Louis, MO, USA) were used as the negative control and blank control, respectively. The first immunization of CPSIT_p7 proteins was emulsified in an equivalent volume of Freund's complete adjuvant (FCA) in a volume of 0.1 ml and subsequently in Freund's incomplete adjuvant (FIA). Clinical manifestations and body weight were observed and recorded during immunization. Blood samples of the 30 mice in each group were harvested via tail bleeding 2 weeks after the first two immunizations, and 15 mice from each group were euthanized 2 weeks after the last immunization. Blood was harvested via retro-orbital bleeding, and spleens were removed using aseptic surgery. Serum samples were recovered via centrifugation (4000 rpm, 8 min) and stored at − 20 °C for the detection of antibody production and neutralization of *C. psittaci*. Twelve spleens from each group were homogenized in RPMI 1640 (Hyclone, Logan, Utah, USA) containing 10% fetal calf serum (FBS; Invitrogen, USA) for the detection of cytokine level and adoptive transfer experiments 2 weeks after the last immunization. All of the animal experiments complied with the University of South China Guide for the Use of Laboratory Animals.

Mouse challenge and culturing of *C. psittaci* from the lungs

Immunized and control mice were anesthetized with ether and infected intranasally (i.n.) with 30 μl of 5×10^5 IFUs *C. psittaci* in a sucrose-phosphate-glutamic acid (SPG) buffer 2 weeks after the last immunization to evaluate the protective efficiencies [18, 19]. All mice were weighed before and 3, 7, and 10 days after challenge. The remaining 15 mice in each group were euthanized, and ten infected lungs from each group were dissected and homogenized in Hanks' solution (Hyclone, Logan, Utah, USA) 10 days after infection, and one-half of the lung homogenates were re-suspended in SPG buffer for cytokine level determinations. The other homogenates were re-suspended in SPG buffer containing gentamicin (10 μg/ml), vancomycin (100 μg/ml), and streptomycin (100 μg/ml) for *C. psittaci* titer measurements. Lung homogenates from each mouse were centrifuged (20 min, 4000 rpm), and serial dilutions of the supernatants were inoculated onto HeLa 229 cell monolayers. The number of inclusion was counting using a fluorescence microscopy assay. The lungs of five mice from each group were fixed in 10% formaldehyde and embedded in paraffin for histopathological evaluation with hematoxylin-eosin (H&E) and an UltraSensitive™SP (Rabbit) IHC Kit (Maixin, China).

Splenic cell proliferation assay

Three spleens from each group were harvested, and splenic cells from each mouse were re-suspended in RPMI-1640 (1% antibiotics) to evaluate the CPSIT_p7-specific cellular immune response. Cells were diluted to 1×10^5 cells/ml and seeded onto culture plates. Each well received 10 μg/ml of puried-

CPSIT_p7 for re-stimulation, and the plate was incubated at 37 °C with 5% CO_2 for 48 h. Lymphocyte proliferative capacity was estimated using a Cell Counting Kit-8 (Dojindo, Japan).

Cytokine determinations from the spleen and lung

Splenic cells were cultured for 48 h and centrifuged (4 °C, 20 min, 4000 rpm). The supernatants were harvested for determination of cytokines. The supernatants of lung homogenates were collected from *C. psittaci*-infected mice after immunization and centrifuged as described above. Cytokine concentrations were measured using the Ready-SET-Go! Kits (eBioscience, USA). The limits of the IL-4, IL-6, IL-10, IL-2, IL-12, IL-17, and interferon-γ (IFN-γ) measurements were 500 pg/ml, 500 pg/ml, 4000 pg/ml, 200 pg/ml, 2000 pg/ml, 700 pg/ml, and 2000 pg/ml, respectively. Duplicate clarified supernatants were prepared according to the manufacturer's guidelines.

In vivo neutralization of mice

Three groups ($n = 3$) of five mice were infected intranasally with 5×10^5 IFUs of *C. psittaci*. The infected mice were injected with 200 μl of sera obtained from mice immunized with PBS, FA, or CPSIT_p7 24 h after infection. All mice were euthanized 10 days after sera injections, and *C. psittaci* titers were measured as described above.

In vitro neutralization of *C. psittaci*

Sera obtained from mice immunized with CPSIT_p7, FA, or PBS were mixed with 5×10^5 IFUs of *C. psittaci* and incubated for 1 h at RT. Three groups ($n = 3$) of five mice were treated intranasally with *C. psittaci*, which were pre-treated with immunized mice sera in vitro. All mice were euthanized 10 days after infection, and the *C. psittaci* titers were measured as described above.

Spleen cell negative selection and adoptive transfer

All spleen cells were collected individually from CPSIT_p7-, FA-, or PBS-immunized mice 2 weeks after the last immunization via lysis of the erythrocytes, washed twice with PBS, and re-suspended in PBS (pH 7.0). $CD4^+$ or $CD8^+$ T cells were depleted from the spleen cells using micro-beads coated with CD4 (L3T4) or CD8a (Ly-2) antibodies (Miltenyi Biotec, Germany) and a magnetic cell sorting system (Miltenyi Biotec, Germany) for the negative selective transfer. FACS analysis evaluated the efficiency of cell sorting after staining with anti-CD4-TC and anti-CD8-rPE antibodies. The remaining $CD4^+$ or $CD8^+$ cells were < 1%. Approximately 1×10^6 depleted cells were inoculated into the tail vein of native mice. Mice were infected with 5×10^5 IFUs of *C. psittaci* 24 h after transfer. Lung tissues of the mice were isolated and homogenized in SPG on day 10 post-infection. *C. psittaci* titers in lungs were measured as described above.

Statistical analysis

Statistical comparisons of the data between groups for *C. psittaci* burden and cytokine levels after infection were computed using SPSS 18.0 software and one-way analysis of variance (ANOVA) with the Student-Newman-Keuls test. Differences were considered statistically significant at $P < 0.05$.

Results

Evaluation of CPSIT_p7-specfic humoral and cellular immune responses

Sera obtained from immunized mice 2 weeks after the last immunization were assessed using Western blotting to investigate the ability of CPSIT_p7 proteins to induce the humoral immune response. No adverse reactions or weight changes were observed between CPSIT_p7-immunized and control groups during immunization (data not shown). Three immunizations with CPSIT_p7 proteins (Fig. 1e) induced the production of protein-specific antibodies, and the control sera did not react with CPSIT_p7 proteins (Fig. 1f). Splenic cells collected from PBS-, FA-, or CPSIT_p7-immunized mice were stimulated in vitro for 48 h with 10 μg/ml CPSIT_p7 proteins to determine cell proliferative responses and cytokine levels in spleen cell supernatants. The stimulation index (SI) of the CPSIT_p7-immunized group was significantly higher than the negative control groups (PBS- or FA-immunized mice) (Fig. 1s). Spleen cell supernatants were collected after stimulation with CPSIT_p7 proteins to determine the expressions of IL-2, IL-4, IL-6, or IFN-γ (Fig. 2) using ELISA. IL-4 and IL-10 levels were not significantly different between the CPSIT_p7-immunized and control mice, but IL-2 and IFN-γ levels of the CPSIT_p7-immunized group were significantly higher than control mice.

Effect of the protective immunization of the CPSIT_p7 proteins against *C. psittaci* infection

Mice were infected i.n. with *C. psittaci* and sacrificed 10 days after infection to examine whether the CPSIT_p7-specific immune response protected the mice against *C. psittaci* infection 2 weeks after the final immunization. Mice were weighed during the infection, and no significant differences between the CPSIT_p7-immunized and control groups were observed (data not shown). The protective efficacy of CPSIT_p7 was measured using the *C. psittaci* burden in the lungs of infected mice (Fig. 3). The CPSIT_p7-immunized mice exhibited a significantly lower chlamydial load compared to PBS- or FA-immunized mice.

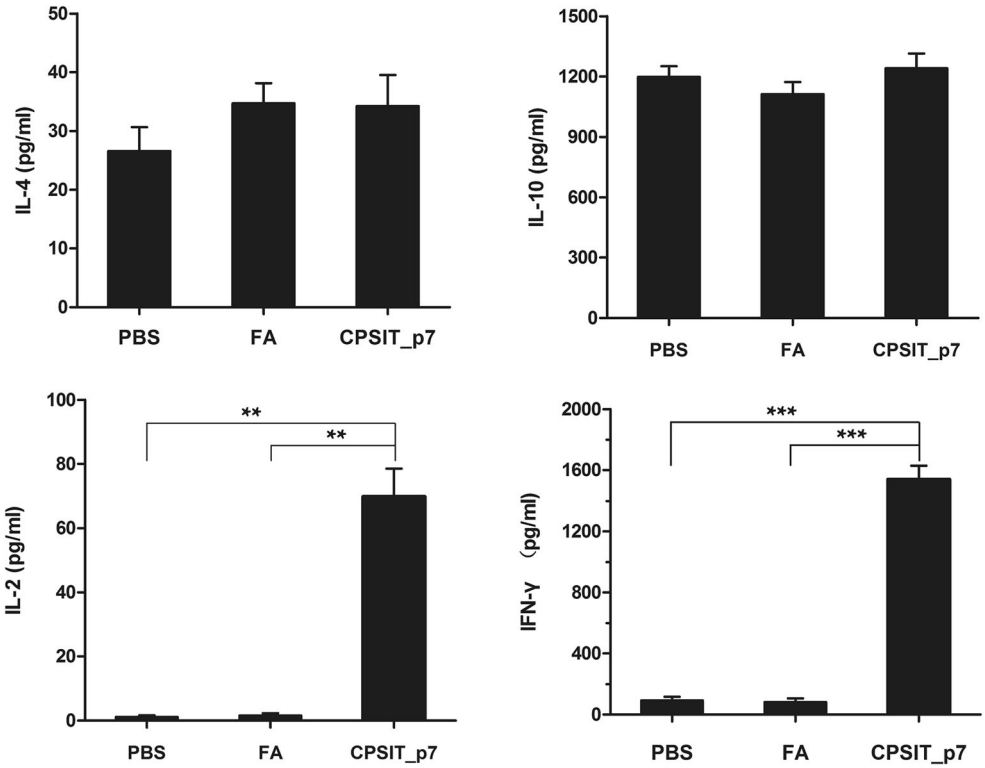

Fig. 2 Cytokine recall responses after immunization. The production of IL-2, IL-4, IL-10, and IFN-γ in splenic cell culture supernatants was detected using an ELISA kit according to the manufacturer's instructions. Means ± standard deviation of the data from 10 mouse lungs in three independent experiments. $^{**}P < 0.01$. $^{***}P < 0.001$

Serious pathological damages were observed in PBS-immunized (Fig. 4a(1)) and FA-immunized (Fig. 4a(2)) mice, and inflammatory infiltrates were significantly reduced in H&E-stained lung sections stained of *C. psittaci*-infected mice immunized with CPSIT_p7 proteins (Fig. 4a(3)). The alveolar structure was clearly recognized, and fewer pathological features were observed, such as alveolar interstitial thickening, alveolar hemorrhage, and inflammatory cell infiltration, compared to control animals (Fig. 4a). Streptavidin-peroxidase (S-P) immunohistochemistry revealed that CPSIT_p7-immunized mice (Fig. 4b(3)) exhibited a remarkably lower chlamydial load (brown granule) (black arrow) compared to control animals (Fig. 4b(1, 2)).

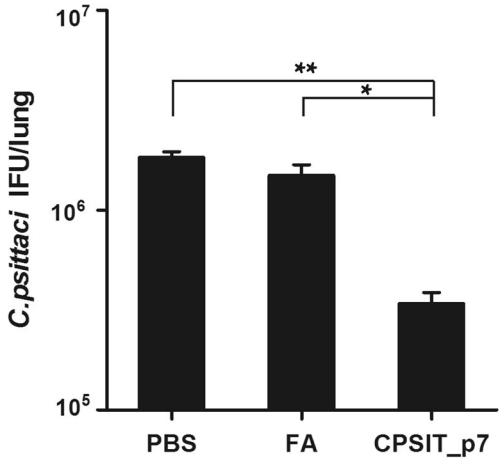

Fig. 3 The *C. psittaci* burden in the lungs of immunized mice after *C. psittaci* infection. Mice were infected with 5×10^5 IFUs of *C. psittaci* 2 weeks after the last immunization and sacrificed 10 days later. Lung homogenates were prepared and inoculated on HeLa cells, and the *C. psittaci* inclusions were counted using IFA. Means ± standard deviations of *C. psittaci* titers (IFU/lung) from 10 mice in three independent experiments are shown. $^{*}P = 0.028$. $^{**}P = 0.008$

Potential vaccine candidates must induce a protective immune response without stimulation of pathological immunity during infection. Therefore, we measured the production of IL-4, IL-6, IL-10, IL-12, IL-17, and IFN-γ in the lungs of immunized mice using ELISA (Fig. 5). The concentrations of IFN-γ in the lungs of CPSIT_p7-immunized mice were significantly lower than control mice, and there were no significant differences in the levels of other cytokines between the three groups.

Effect of serum neutralization and spleen cells of immunized mice on the outcome of *C. psittaci* infection

Immunization with CPSIT_p7 proteins induced specific humoral immune responses. Therefore, we further evaluated the capacity of these antibodies to protect against *C. psittaci* infection. Mice were intranasally infected with *C. psittaci,* and sera harvested from CPSIT_p7-, FA-, or PBS-immunized mice 2 weeks after the final immunization were intraperitoneally injected in infected mice. Mice were sacrificed 10 days after sera injection, and the *Chlamydiae* collected from lungs were measured. The *C. psittaci* burden was not significantly different between CPSIT_p7-treated immune sera and control sera (Fig. S2). Native mice were injected with *C. psittaci*-

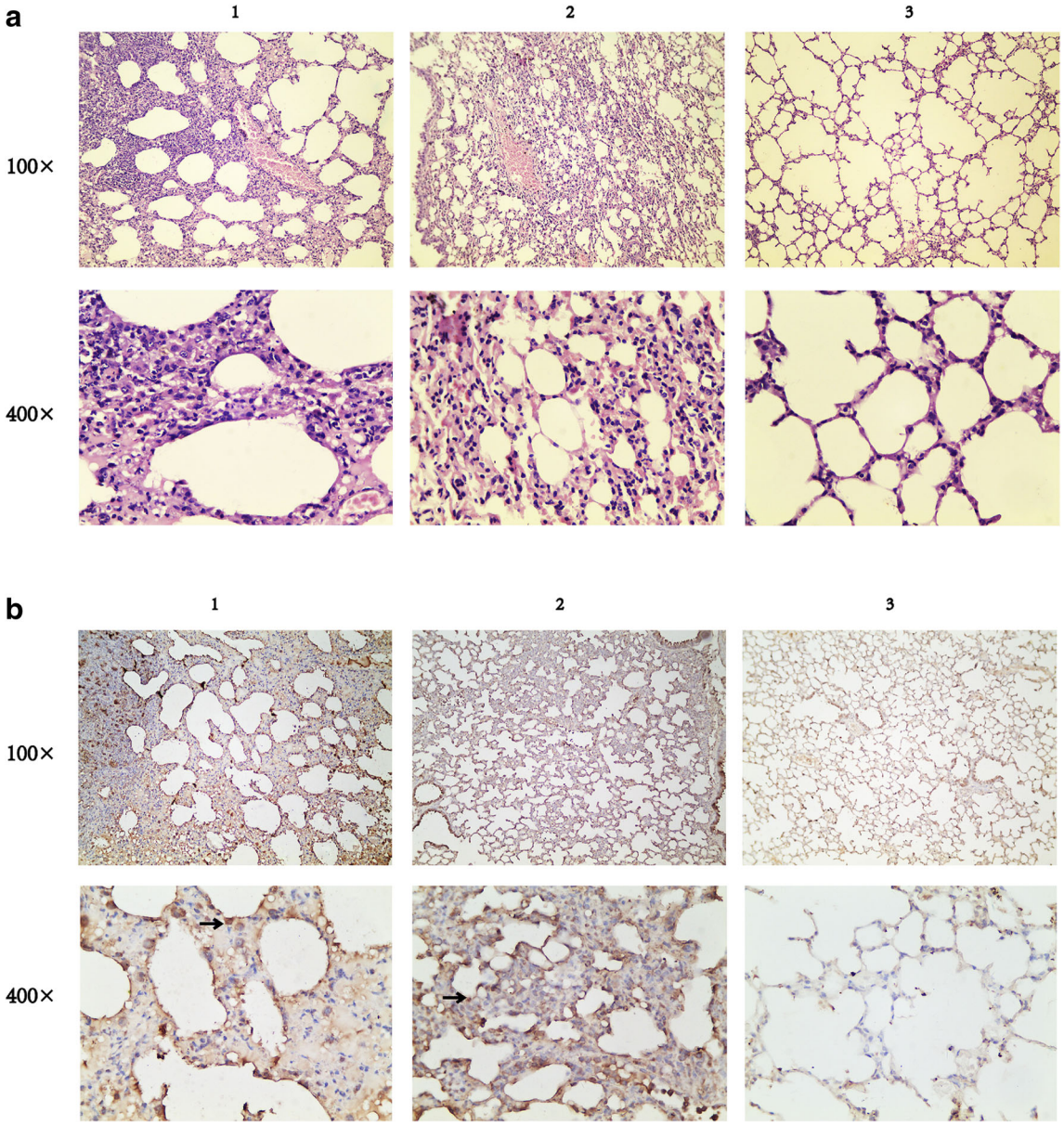

Fig. 4 Pathological lesion and *C. psittaci* burden of lung tissues from PBS-immunized (1), FA-immunized (2), or CPSIT_p7-immunized (3) mice after *C. psittaci* infection. **a** Ten days after *C. psittaci* infection, the lungs of immunized mice were stained with H&E. **b** The S-P immunohistochemistry kit with a rabbit anti-*C. psittaci* 6BC was the first antibody, and it was used to test *C. psittaci* inclusion. Brown granules (black arrow) express the *C. psittaci* inclusion

treated immune sera and sacrificed after 10 days to examine the in vitro neutralization of *C. psittaci*. Similarly, the chlamydial load of experimental group was not reduced compared with the control groups (Fig. S2). These results revealed that the sera of the CPSIT_p7-immunized mice did not contain antibodies to protect against *C. psittaci.*

The CPSIT_p7-specific antibodies did not exhibit neutralizing activity to *C. psittaci* infection. Therefore, we further investigated the protective efficiency of the CPSIT_p7-specific cellular immune responses. Spleen cells harvested from PBS-, FA- or CPSIT_p7-immunized mice were depleted of $CD4^+$ or $CD8^+$ cells and transferred to native mice. The native mice were infected with *C. psittaci* after 24 h and euthanized after 10 days. The adoptive transfer of specific spleen cells depleted of $CD4^+$ cells produced no significant difference in *C. psittaci* burden between the three groups. The adoptive transfer of CPSIT_p7-specific spleen cells depleted of $CD8^+$ cells significantly reduced the *C. psittaci* burden in the lungs of mice compared to control groups (Fig. 6).

Discussion

C. psittaci is an important zoonotic pathogen that infects humans and results in severe atypical pneumonia [2]. *C. psittaci*

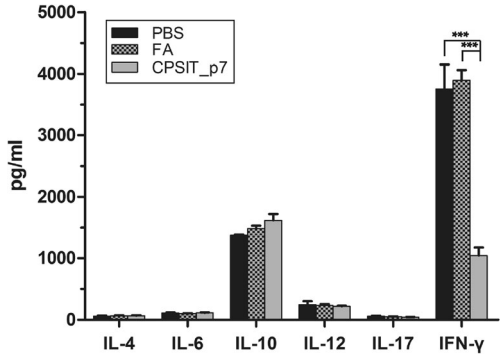

Fig. 5 Concentration of inflammatory cytokines in the lungs of immunized mice after *C. psittaci* infection. The supernatants of lung homogenates were tested using IL-4, IL-6, IL-10, IL-12, IL-17, or IFN-γ ELISA kits. Means ± standard deviations of the data from 10 mouse lungs in three independent experiments. ***$P < 0.001$

contains a highly conserved plasmid that is an essential virulence factor in chlamydial infection, similar to *C. trachomatis* and *C. muridarum*. The function of the plasmids of *C. trachomatis* and *C. muridarum* was the subject of considerable research, but little is known about the *C. psittaci* plasmid. Pgp3 is a plasmid-encoded protein of *chlamydia*. Analysis of the Smart BLAST database from NCBI demonstrated 70% sequence identity between CPSIT_p7 (*C. psittaci* plasmid-encode) and pORF5 (*C. trachomatis* plasmid-encode) and 71% between CPSIT_p7 and TCA04 (*C. muridarum* plasmid-encode), all of which encode plasmid protein Pgp3. The function

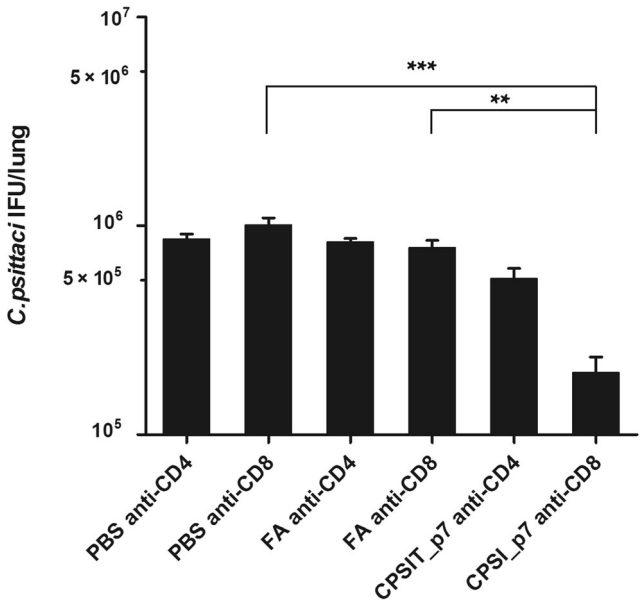

Fig. 6 *C. psittaci* burden in the lungs of mice after adoptive transfer. Spleen cells of groups of 10 mice were collected individually from PBS-, FA, or CPAIT-P7-immunized mice, depleted of $CD4^+$ or $CD8^+$ cells, and transferred to native mice. The native mice were infected with *C. psittaci* 24 h after transfer and euthanized 10 days later. Lung tissues of the mice were collected, and the *C. psittaci* titers were detected using IFA assay. **$P = 0.002$. ***$P < 0.001$

and immunological role of Pgp3 are well-defined in *C. trachomatis* and *C. muridarum*, but not in *C. psittaci*.

The current research evaluated the immune responses and protective efficacy of BALB/c mice immunized with CPSIT_p7 against *C. psittaci* infection to aid in the development of candidate vaccines for the prevention and control of *C. psittaci* infection. We examined whether immunization of BALB/c mice with CPSIT_p7 induced an immune response that was sufficient to protect against *C. psittaci* infection in mice.

The sera of immunized mice were used to detect specific antibodies using Western blotting, and the results confirmed that multiple immunization with CPSIT_p7 proteins induced the production of CPSIT_p7-specific antibodies in mice. We evaluated the cellular immune response of protein-immunized mice. Previous studies demonstrated that immunity against *chlamydia* infection was primarily dependent on the contribution of Th1 cells [20–23], and $CD4^+$ Th1-related IFN-γ played a key role in providing protective immunity against chlamydial infection [24, 25]. The levels of IFN-γ and IL-2 in CPSIT_p7-immunized mice were significantly increased in our experiments, which are $CD4^+$ Th1-related cytokines. We found that T-cell proliferative responses of mice immunized with CPSIT_p7 were significantly increased compared to control mice. These results indicated that the CPSIT_p7 proteins induced a strong $CD4^+$ T cell response.

The present study discovered that immunization with CPSIT_p7 induced a significant reduction in chlamydial burden and inflammatory infiltrates. Donati et al. demonstrated that immunization with Pgp3 DNA induced resistance to re-infection of *C. trachomatis* in mice [26]. Immunization with the pORF5 DNA vaccine induced protective immunity against genital *C. trachomatis* infection in a murine model [27]. Recombinant Pgp3 protein-immunized mice exhibited a significantly lower *C. muridarum* burden [16]. These results suggest that the role of Pgp3 of *C. psittaci* is similar to *C. trachomatis and C. muridarum*. The very low level of INF-γ in our experiments supports the decrease in inflammatory infiltrates in the lungs of CPSIT_p7-immunized mice. Earlier studies revealed that IFN-γ was responsible for the clearance of chlamydial infection in female mice [28], which supports CPSIT_p7 as a vaccine candidate to induce this immune response.

We designed in vitro and in vivo neutralization experiments and adoptive transfer experiments of $CD4^+/CD8^+$ T cells to further examine the potential mechanisms of CPSIT_p7 protection against *C. psittaci*. The number of recoverable *chlamydia* was not significantly different between CPSIT_p7-immunized sera and control sera for in vivo and in vitro neutralization experiments. These data suggest that the specific-CPSIT_p7 antibodies exert no protective effect against *C. psittaci* infection. Similar results were observed in previous experiments [16, 26]. These results demonstrated that CPSIT_p7-immunized mice elicited a powerful Th1 immune response, which suggests that the specific-CPSIT_p7

antibodies may assist Th1 immune responses by strengthening antigen uptake and presentation [29].

The adoptive transfer of CPSIT_p7-specific $CD4^+$ T cells significantly reduced the chlamydial load in the present experiment, but not the transfer of negatively selected $CD8^+$ T cells. Pgp3-specific (*C. muridarum* encoded) and MOMP-specific (outer membrane protein of *C. muridarum*) $CD4^+$ cells also significantly reduced the amount of recoverable *Chlamydiae* after infection [16, 30]. Our results support these findings. MOMP induced efficient protective immunity against *C. muridarum* genital infection in C3H/HeN [31–34]. $CD4^+$ T cells are important for protection against intracellular zoonotic bacterial pathogens, which are involved in the production of macrophage-activating factors and assist B cells to enhance antibody production [35–37]. In contrast, Morrison et al. found that $CD4^+$ T cells were not necessary to protect against murine *Chlamydia trachomatis* genital infection [38]. Some studies indicated that $CD8^+$ cells provided a very restricted or no protective effect, which is similar to our study results [39, 40]. However, the significance of the reduction of the chlamydial load in lungs is not certain, but the reduction of the chlamydial load reduced the transmission of chlamydial infection.

In conclusion, vaccination with CPSIT_p7 induced a strong Th1-type immunity response and elicited protective effects against *C. psittaci* infection. CPSIT_p7-specific $CD4^+$ T cells reduced the chlamydial load in the lungs of native mice after infection challenge. These results provide important directions for future studies of vaccine development against *C. psittaci* infection.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The Animal Welfare and Ethics Committee of the University of South China approved all animal procedures and treatments, which were performed in accordance with the regulations of the institution.

References

1. Abdelrahman YM, Belland RJ. The chlamydial developmental cycle. FEMS Microbiol Rev. 2005;29(5):949–59. https://doi.org/10.1016/j.femsre.2005.03.002.
2. Knittler MR, Sachse K. Chlamydia psittaci: update on an underestimated zoonotic agent. Pathog Dis. 2015;73(1):1–15. https://doi.org/10.1093/femspd/ftu007.
3. Gaede W, Reckling KF, Dresenkamp B, Kenklies S, Schubert E, Noack U, et al. Chlamydophila psittaci infections in humans during an outbreak of psittacosis from poultry in Germany. Zoonoses Public Health. 2008;55(4):184–8. https://doi.org/10.1111/j.1863-2378.2008.01108.x.
4. Longbottom D, Livingstone M. Vaccination against chlamydial infections of man and animals. Vet J. 2006;171(2):263–75. https://doi.org/10.1016/j.tvjl.2004.09.006.
5. Igietseme JU, Eko FO, Black CM. Chlamydia vaccines: recent developments and the role of adjuvants in future formulations. Expert Rev Vaccines. 2011;10(11):1585–96. https://doi.org/10.1586/erv.11.139.
6. Xu W, Liu J, Gong W, Chen J, Zhu S, Zhang L. Protective immunity against Chlamydia trachomatis genital infection induced by a vaccine based on the major outer membrane multi-epitope human papillomavirus major capsid protein L1. Vaccine. 2011;29(15):2672–8. https://doi.org/10.1016/j.vaccine.2010.12.132.
7. Pawlikowska-Warych M, Sliwa-Dominiak J, Deptula W. Chlamydial plasmids and bacteriophages. Acta Biochim Pol. 2015;62(1):1–6.
8. Sigar IM, Schripsema JH, Wang Y, Clarke IN, Cutcliffe LT, Seth-Smith HM, et al. Plasmid deficiency in urogenital isolates of Chlamydia trachomatis reduces infectivity and virulence in a mouse model. Pathog Dis. 2014;70(1):61–9. https://doi.org/10.1111/2049-632X.12086.
9. O'Connell CM, Ingalls RR, Andrews CW Jr, Scurlock AM, Darville T. Plasmid-deficient Chlamydia muridarum fail to induce immune pathology and protect against oviduct disease. J Immunol. 2007;179(6):4027–34.
10. Lei L, Chen J, Hou S, Ding Y, Yang Z, Zeng H, et al. Reduced live organism recovery and lack of hydrosalpinx in mice infected with plasmid-free Chlamydia muridarum. Infect Immun. 2014;82(3):983–92. https://doi.org/10.1128/IAI.01543-13.
11. Thomas NS, Lusher M, Storey CC, Clarke IN. Plasmid diversity in Chlamydia. Microbiology. 1997;143(Pt 6):1847–54. https://doi.org/10.1099/00221287-143-6-1847.
12. Palmer L, Falkow S. A common plasmid of Chlamydia trachomatis. Plasmid. 1986;16(1):52–62.
13. Li Z, Chen D, Zhong Y, Wang S, Zhong G. The chlamydial plasmid-encoded protein pgp3 is secreted into the cytosol of Chlamydia-infected cells. Infect Immun. 2008;76(8):3415–28. https://doi.org/10.1128/IAI.01377-07.
14. Liu Y, Huang Y, Yang Z, Sun Y, Gong S, Hou S, et al. Plasmid-encoded Pgp3 is a major virulence factor for Chlamydia muridarum to induce hydrosalpinx in mice. Infect Immun. 2014;82(12):5327–35. https://doi.org/10.1128/IAI.02576-14.
15. Chen D, Lei L, Lu C, Galaleldeen A, Hart PJ, Zhong G. Characterization of Pgp3, a Chlamydia trachomatis plasmid-encoded immunodominant antigen. J Bacteriol. 2010;192(22):6017–24. https://doi.org/10.1128/JB.00847-10.
16. Mosolygo T, Szabo AM, Balogh EP, Faludi I, Virok DP, Endresz V, et al. Protection promoted by pGP3 or pGP4 against Chlamydia muridarum is mediated by CD4(+) cells in C57BL/6N mice. Vaccine. 2014;32(40):5228–33. https://doi.org/10.1016/j.vaccine.2014.07.039.
17. Liang M, Wen Y, Ran O, Chen L, Wang C, Li L, et al. Protective immunity induced by recombinant protein CPSIT_p8 of Chlamydia psittaci. Appl Microbiol Biotechnol. 2016;100(14):6385–93. https://doi.org/10.1007/s00253-016-7494-8.
18. Dutow P, Fehlhaber B, Bode J, Laudeley R, Rheinheimer C, Glage S, et al. The complement C3a receptor is critical in defense against Chlamydia psittaci in mouse lung infection and required for antibody and optimal T cell response. J Infect Dis. 2014;209(8):1269–78. https://doi.org/10.1093/infdis/jit640.

19. Ran O, Liang M, Yu J, Yu M, Song Y, Yimou W. Recombinant protein CPSIT_0846 induces protective immunity against Chlamydia psittaci infection in BALB/c mice. Pathog Dis. 2017;75(3) https://doi.org/10.1093/femspd/ftx018.
20. Kimani J, Maclean IW, Bwayo JJ, MacDonald K, Oyugi J, Maitha GM, et al. Risk factors for Chlamydia trachomatis pelvic inflammatory disease among sex workers in Nairobi, Kenya. J Infect Dis. 1996;173(6):1437–44.
21. Holland MJ, Bailey RL, Conway DJ, Culley F, Miranpuri G, Byrne GI, et al. T helper type-1 (Th1)/Th2 profiles of peripheral blood mononuclear cells (PBMC); responses to antigens of Chlamydia trachomatis in subjects with severe trachomatous scarring. Clin Exp Immunol. 1996;105(3):429–35.
22. Faal N, Bailey RL, Sarr I, Joof H, Mabey DC, Holland MJ. Temporal cytokine gene expression patterns in subjects with trachoma identify distinct conjunctival responses associated with infection. Clin Exp Immunol. 2005;142(2):347–53. https://doi.org/10.1111/j.1365-2249.2005.02917.x.
23. Morrison RP, Caldwell HD. Immunity to murine chlamydial genital infection. Infect Immun. 2002;70(6):2741–51.
24. Yang X. Role of cytokines in Chlamydia trachomatis protective immunity and immunopathology. Curr Pharm Des. 2003;9(1):67–73.
25. Loomis WP, Starnbach MN. T cell responses to Chlamydia trachomatis. Curr Opin Microbiol. 2002;5(1):87–91.
26. Donati M, Sambri V, Comanducci M, Di Leo K, Storni E, Giacani L, et al. DNA immunization with pgp3 gene of Chlamydia trachomatis inhibits the spread of chlamydial infection from the lower to the upper genital tract in C3H/HeN mice. Vaccine. 2003;21(11–12):1089–93.
27. Li Z, Wang S, Wu Y, Zhong G, Chen D. Immunization with chlamydial plasmid protein pORF5 DNA vaccine induces protective immunity against genital chlamydial infection in mice. Sci China C Life Sci. 2008;51(11):973–80. https://doi.org/10.1007/s11427-008-0130-9.
28. Rank RG, Ramsey KH, Pack EA, Williams DM. Effect of gamma interferon on resolution of murine chlamydial genital infection. Infect Immun. 1992;60(10):4427–9.
29. Moore T, Ekworomadu CO, Eko FO, MacMillan L, Ramey K, Ananaba GA, et al. Fc receptor-mediated antibody regulation of T cell immunity against intracellular pathogens. J Infect Dis. 2003;188(4):617–24. https://doi.org/10.1086/377134.
30. Cunningham KA, Carey AJ, Timms P, Beagley KW. CD4+ T cells reduce the tissue burden of Chlamydia muridarum in male BALB/c mice. Vaccine. 2010;28(31):4861–3.
31. Pal S, Tatarenkova OV, de la Maza LM. A vaccine formulated with the major outer membrane protein can protect C3H/HeN, a highly susceptible strain of mice, from a Chlamydia muridarum genital challenge. Immunology. 2015;146(3):432–43. https://doi.org/10.1111/imm.12520.
32. Igietseme JU, Murdin A. Induction of protective immunity against Chlamydia trachomatis genital infection by a vaccine based on major outer membrane protein-lipophilic immune response-stimulating complexes. Infect Immun. 2000;68(12):6798–806.
33. Zhang D, Yang X, Berry J, Shen C, McClarty G, Brunham RC. DNA vaccination with the major outer-membrane protein gene induces acquired immunity to Chlamydia trachomatis (mouse pneumonitis) infection. J Infect Dis. 1997;176(4):1035–40.
34. Zhang DJ, Yang X, Shen C, Brunham RC. Characterization of immune responses following intramuscular DNA immunization with the MOMP gene of Chlamydia trachomatis mouse pneumonitis strain. Immunology. 1999;96(2):314–21.
35. Nandre RM, Jawale CV, Lee JH. Enhanced protective immune responses against Salmonella enteritidis infection by Salmonella secreting an Escherichia coli heat-labile enterotoxin B subunit protein. Comp Immunol Microbiol Infect Dis. 2013;36(5):537–48. https://doi.org/10.1016/j.cimid.2013.06.002.
36. Nandre RM, Lee JH. Generation of a safe Salmonella gallinarum vaccine candidate that secretes an adjuvant protein with immunogenicity and protective efficacy against fowl typhoid. Avian pathology : journal of the WVPA. 2014;43(2):164–71. https://doi.org/10.1080/03079457.2014.897682.
37. Nandre RM, Lee JH. Comparative evaluation of safety and efficacy of a live Salmonella gallinarum vaccine candidate secreting an adjuvant protein with SG9R in chickens. Vet Immunol Immunopathol. 2014;162(1–2):51–8. https://doi.org/10.1016/j.vetimm.2014.08.014.
38. Morrison SG, Farris CM, Sturdevant GL, Whitmire WM, Morrison RP. Murine Chlamydia trachomatis genital infection is unaltered by depletion of CD4+ T cells and diminished adaptive immunity. J Infect Dis. 2011;203(8):1120–8. https://doi.org/10.1093/infdis/jiq176.
39. Igietseme JU, Magee DM, Williams DM, Rank RG. Role for CD8+ T cells in antichlamydial immunity defined by Chlamydia-specific T-lymphocyte clones. Infect Immun. 1994;62(11):5195–7.
40. Su H, Caldwell HD. CD4+ T cells play a significant role in adoptive immunity to Chlamydia trachomatis infection of the mouse genital tract. Infect Immun. 1995;63(9):3302–8.