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Characterization of *Chlamydia muridarum* TC0668 Protein: Localization, Expression, and Inflammation-Inducing Effects on Host Cell

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Abstract

The objective of this study is to elucidate the basic biological properties and function of TC0668 in vitro. Laser confocal microscopy and immune-electron microscopy were used to detect localization of TC0668 in *Chlamydia*-infected human epithelial cells, while the expression phase was investigated by qRT-PCR and western blot analysis. Protein array technology was employed to evaluate differences in cytokine secretion between cells infected with *tc0668* single mutants and those infected with *tc0668* null mutants. We found that TC0668 is restricted to the chlamydial inclusion. Translation and transcription of TC0668 were detected at 4 h and peaked at 16 h during the life cycle of *Chlamydia* in vitro. The cytokines produced by tc0668 single mutant infected cultures compared with tc0668 null mutant group indicated that 36 cytokines were downregulated, while 10 were up-regulated significantly. *C. muridarum* bearing a single *tc0668* gene mutation have decreased urogenital pathogenicity that is explained by the effects of the mutation on the regulation of inflammation-related cytokine secretion.

Introduction

Chlamydia is an intracellular, parasitic, prokaryotic microorganism with a unique biphasic developmental cycle [1, 2]. Various pathological symptoms can occur in hosts colonized by different species of *Chlamydia* [3–5]. Human genitourinary system disease caused by *Chlamydia trachomatis* is one of the most common sexually transmitted diseases (STDs), with more than 100 million infections worldwide each year [6]. Without treatment, genitourinary *C. trachomatis* infection can result in serious complications including infertility, ectopic pregnancy, and cervical cancer [7]. *Chlamydia muridarum* is a separate species of the genus *Chlamydia*, but both *C. trachomatis* and *C. muridarum* are highly conserved in terms of their basic biology and their genomes. Thus, *C. muridarum* is used as a model of urogenital disease resulting from sexually transmitted *C. trachomatis* [8].

Chlamydia exists in two different forms during its growth cycle: the infectious elementary body (EB) and the reproductive form known as the reticulate body (RB) [9]. During the invasion of host cells, EBs are encapsulated by membranes to form inclusion bodies, then, converted into RBs during the early post-infection stages (0 to 8 h). During proliferation, RBs multiply by binary fission in the middle phase of the infection (8 to 16 h), during the late phase of infection (16 to 24 h), RBs develop into mature EBs released from the host cells to repeat the cycle [10].

Several studies have investigated the chromosomal gene *tc0668* of *C. muridarum*, which plays a role in genital tract inflammatory disease in mice [11]. The standard *C. muridarum* strain (CMG0) was serially sub-cultured under special conditions in vitro, and a resulting attenuated strain (CMG28, *tc0668* mutant) with markedly reduced pathogenicity was found to have a mutation in the *tc0668* gene [12]. Moreover, Shao et al. demonstrated that vaginal inoculation of mice with wild-type *C. muridarum* not only caused upper genital tract infection but also spread to and

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established stable colonization of the gastrointestinal tract [13]. The gene *tc0668* is approximately 1227 bp in length and encodes a highly conserved, hypothetical protein of 408 amino acids that contains a single domain of unknown function [1207 (DUF1207) motif, TC0668]. The TC0668 tertiary structure is homologous to that of the eukaryotic integrins fibronectin receptor (α5β1/αIIbβ3) and vitronectin receptor (α5β3), which are involved in numerous biological processes in eukaryotic cells [11].These observations further demonstrate the correlation between TC0668 and chlamydial pathogenicity, but the basic biological properties of TC0668 and the specific mechanisms that are involved have yet to be elucidated. In this study, we have investigated the localization, expression, and role of TC0668, providing a new idea for the treatment and prevention of diseases caused by *Chlamydia* infection.

Materials and Methods

Bacterial Strains, Cell Line and Other Materials

The *C. muridarum* Nigg II strains, C. *trachomatis* D strains, HeLa cells (ATCC, CCL-2.1, USA), vectors pGEX-4T-1 (Amersham Pharmacia Biotech Inc., Piscataway, NJ), and *E. coli* BL21/T7E strains were all preserved by the Institute of Pathogenic Biology at the University of South China. *C. muridarum tc0668* wild and mutant monoclonal strains were prepared by our research group during the early stage [14, 15].

Expression and Purification of Recombinant Proteins

The primers of tc0668 were synthesized by Sangon Biotech of Shanghai (F: ATGATGGAACCTTTACGTTTCGGT, R: CTAAAAGCCATAACTTAATCTAAAACCATAGT). The template DNA of C. muridarum was extracted with Tiangen bacterial genomic DNA extraction kit (Qiagen, Germany) according to the instructions. The PCR amplification products were purified included with the OMEGA PCR kit (Biotek, America). The DNA fragment was subcloned into the pGEX-4T-1 vector using restriction enzyme digestion and ligating reaction to generate the recombinant plasmid pGEX-4T-tc0668. The recombinant plasmid was transformed into *E. coli* BL21/T7E bacterial. After induction by IPTG (Isopropyl-β-D-Thiogalactoside), the bacterial cells were lysed by ultrasonic to release the target protein, and finally, the recombinant protein was purified by GST-Agarose Resin (G4510, MERCK). The purified recombinant protein fluid was screened by a dot enzyme-linked immunosorbent assay (ELISA), and antibody specificity was confirmed by an indirect immunofluorescence assay [16].

Production of Polyclonal Antibodies (Mouse-Derived and Rabbit-Derived)

The purified protein of TC0668 was injected into BALB/c mice and New Zealand white rabbits to produce polyclonal antibodies, according to the routine method (Experimental vaccine induces Th1-driven immune responses and resistance to Neisseria gonorrhoeae infection in a murine model.). With the aid of Freund's adjuvant, rabbits needed to undergo subcutaneous immunization three times (2 weeks apart) and 1 booster immunization (1 week after third time). The dose of antigen for each immunization was 1000 μg. A week later, rabbits' serum was separated by centrifugation and stored [17]. The preparation of polyclonal antibodies in mice is basically the same as that in rabbits. But, the dose of antigen for each immunization was 100 μg. All animals were treated according to the regulations of Chinese law and the local Ethical Committee. The antisera from the rabbits and mice were then collected and the antibody titer (> 1:64,000) was determined using an ELISA kit (Cloud-Clone, Inc. USA).

Co-localization of the TC0668 Protein

The localization of the TC0668 protein was analyzed using laser confocal microscopy with a Leica TCS SP5 SMD at 24 h post-infection using specific mouse antibodies (anti-HSP60, anti-PGP3 (primary antibodies)), and a goat anti-mouse IgG (secondary antibody) conjugated to Cy3 dye (red, Jackson Immuno-Research Laboratories) with co-staining with anti-rabbit TC0668 antibody (primary antibody) and goat anti-rabbit IgG (secondary antibody) conjugated to 488 dye (green; Jackson Immuno-Research Laboratories) mixed with DAPI dye (for nuclear staining).

Immuno-electron Microscopy (IEM)

After infected with *C. muridarum* Nigg II strains for 12 h and 24 h, HeLa cells were harvested with trypsin. Then, cells were fixed in fixative (4% paraformaldehyde, 0.1% glutaraldehyde, 0.06% picric acid, 0.1 M PBS), rinsed with PBS (phosphate-buffer saline solution, pH 7.6), dehydrated (50%, 70%, 85%, and 95% alcohol), and infiltrated (95% alcohol LR). The entire process was performed on ice and the cells were gently handled to maintain them in clumps as described previously [18]. Then, ultrathin (90–100 nm) thick sections of cells were prepared using a Lycao UC6 ultrathin microtome. The TC0668 rabbit-derived primary antibody and the 10 nm gold-labeled secondary antibody were incubated at room temperature for 2 h. After drying,

the cells were subject to immune-electron microscopy using an H-7650 transmission electron microscope.

Indirect Immunofluorescence Assay (IFA)

HeLa cells infected with *C. muridarum* at 4, 8, 12, 16, 20, and 24 h were fixed with 4% paraformaldehyde for 30 min at room temperature followed by permeabilization with 0.1% TritonX-100 for an additional 30 min. After washing and blocking, the cell samples were subjected to antibody and chemical staining. Notably, a specific rabbit anti-MoPn antibody was produced as described previously (Guangming et al. 1994). The purified Chlamyida *muridarum* EBs were used to infect BALB/c mice (4 weeks old) three times. After the final injection, spleens were removed aseptically, and a single-cell suspension was made. The monoclonal antibodies were isotyped with an Immunopure, monoclonal antibodies isotyping kit and purified with the Immunopure (A/G) immunoglobulin G (IgG) purification kit. The amount of TC0668 protein was measured at different time points post-infection using a specific rabbit anti-MoPn antibody that was used to detect expression in *Chlamydia* organisms in combination with a goat anti-rabbit IgG conjugated to 488 dye (green; Jackson Immuno-Research Laboratories); the cells were co-stained with specific mouse antibodies (TC0668, HSP60, and PGP3) and goat anti-mouse IgG conjugated to Cy3 dye (red; Jackson Immuno-Research Laboratories). Observation and imaging using a fluorescent inverted microscope was performed after the cells were incubated with the primary and secondary antibodies for 1 h and then washed with PBS.

Real-time Quantitative PCR

After infected with *C. muridarum* Nigg II strains at 4, 8, 12, 16, 20, and 24 h, cells were collected with TRizol reagent (Invitrogen, Carlsbad, CA, USA), RNA was extracted while a minuscule amount of DNA was removed. According to the instructions included in the RNA extraction kit, the primers of the *tc0668*, *pgp8,* and *16S rRNA* genes of *C. muridarum* Nigg II are shown in Table S1. For each gene, primers for conserved sequences were synthesized by Sangon Biotech of Shanghai and further tested using NCBI blast to ensure the specificity of these primers. The Real-time quantitative PCR was performed in a total volume of 25 µL, which included 1 µL of cDNA; 1 µL of forward primer, and 1 µL of reverse primer for target gene; 12.5 µL of 2 × superReal PreMix Plus; 9.5 µL deionized water was added to make a total volume of 25 µL. The PCR amplification program has a total of 40 cycles, cycling condition included pre-denaturation at 95 °C for 10 min; denaturation at 95 °C for 15 s; annealing at 60 °C for 1 min; extension at 60 °C for 1 min. The relative mRNA expression of *tc0668* was determined using *pgp8* as a control. The stable expression of *16S rRNA* was used as an internal reference to determine the relative expression of *C. muridarum tc0668* and *pgp8* mRNA. The Real-time Quantitative PCR data (slopes and y intercepts) of calibration curves for *tc0668*, *pgp8*, and *16S rRNA* are shown in supplementary Table 4. Analysis of relative gene expression data used the $2^{-\Delta\Delta CT}$ method, $2^{-\Delta\Delta T} = 2^{(\text{CT-target gene} - \text{CT-reference gene})}$.

Western Blotting

The whole protein of cells extracted by radioimmunoprecipitation assay (PIRA) buffer (Beyotime, China) was used for western blotting analysis according to the standard protocol. Equal amounts of protein were loaded and separated by 10% SDS-PAGE and transferred onto PVDF membranes. After locking with 5% skim milk for 2 h at room temperature, PVDF membranes were incubated overnight with our own primary antibodies. The primary antibody was probed using an HRP-conjugated goat anti-mouse IgG secondary antibody (Sigma-Aldrich) and visualized with using an enhanced chemiluminescence (ECL) kit (Santa Cruz Biotech, Santa Cruz, CA, USA). Expression of the chlamydial plasmid proteins PGP3 and HSP60 was detected using GAPDH as a control. Three times Western Blotting data of proteins are shown in supplementary Fig. 4. As there are almost no miscellaneous bands, the representative original images are shown in supplementary Fig. 5.

Protein Arrays

The *tc0668* single mutant and null mutant *C. muridarum* strains were used to infect the cells at an MOI of 1 after culture for 16–18 h. At 24-h post-infection, the cell supernatants and protein lysates from the two strains were collected. The 0.5 mL of supernatant was pipetted, then 200 µg of lysed total protein was added, and the total volume was brought up to 1.5 mL volume using Array Buffer 6 (R&D, Ary022B); the solution was then incubated with 106 detection antibodies on the membrane. The chemiluminescence reagent was incubated in the presence of the antibodies for 1 to 10 min and exposed for 2 to 5 min, and then, the cytokines were detected according to the instructions included with the antibody chip kit (R&D, Ary022B).

Analysis of Data

Quantitative data from qRT-PCR experiments are presented as mean ± SD of experiments repeated at least three times in triplicate. The data obtained from western blotting during three independent experiments are calculated as the relative expression using the *U*-test. Comparisons between groups of data were analyzed using analysis of variance and the results

of the experiments were analyzed using independent sample *t* tests. A *P* value of less than 0.05 was considered significant. GraphPad Prism software was used for the statistical analysis of the data.

Results

Localization of TC0668 During the Developmental Cycle of Chlamydia: Transmembrane Structural Protein

TC0668 is a pathogenic factor of rat chlamydia discovered by our research group earlier. Since there is no antibody product of TC0668 in the market, we first prepared rabbit and mouse polyclonal antibodies for the following experiments. To determine the localization of TC0668 protein during the chlamydial life cycle, the co-localization with specific *Chlamydia* markers were conducted using the IFA method. *Chlamydia* structural heat shock protein 60 (HSP60) is restricted to the chlamydial inclusion, and *Chlamydia* plasmid-encoded protein 3 (PGP3) is secreted into the inclusion lumen and the host cell cytosol [19–21]. After being infected for 24 h, it was observed that TC0668 (green) was surrounded by PGP3 (red) (Fig. 1A). The distribution range of the PGP3 signal extended outside the inclusion membrane. But in the HSP60 co-localizations, the TC0668 (green) and HSP60 (red) were completely overlapped (Fig. 1A). It was indicated that TC0668 was not secreted into the host cytosol.

To further analyze the localization of TC0668, we performed Immuno-electron Microscopy [18]. Cells were infected with *C. muridarum* Nigg II wild-type strains and collected by trypsin digestion at 12 and 24 h. At the mid-developmental stage (12 h post-infection), metabolically active chlamydial forms multiply within inclusion bodies as RBs. At 24 h post-infection, RBs have developed into mature EBs already. Interestingly, it was found that immunogold particles labeled TC0668 were predominantly distributed in the membrane of the RBs/EBs and were also located within the lumen of the inclusion body (Fig. 1B). Based on these results, it was suggested that the TC0668 protein is restricted to chlamydial inclusion and is predominantly associated with EBs and RBs. Next, the tertiary structure of TC0668 protein (containing 408 amino acids) was analyzed by I-TASSER (Fig. 2). This analysis further supported the above results. Amino acids 1 to 6 of TC0668 protein were located inside the membrane, 7 to 24 were within the transmembrane region, and 25 to 408 were located outside the membrane (Fig. 2A, B). Tied Mixture Hidden Markov Model (TMHMM) is a software which uses Hidden Markov Models to predict the transmembrane structure of proteins. We obtain the transmembrane spiral of TC0668 protein by TMHMM analysis. The abscissa axis represents the amino acid residue serial number corresponding to the submitted protein sequence, the value of the vertical axis is the probability at the inside of the membrane, outside the membrane, and the transmembrane helix region (TMHelix)of each amino acid. It was further validated that TC0668 is a structural transmembrane protein rather than a secretory protein.

Up-regulated Expression of TC0668 During the *C. muridarum* Growth Cycle

We had known that TC0668 is a structural protein, but we still need to study the expression changes during infection. To investigate the expression changes of TC0668 proteins during the C. muridarum growth cycle, we designed 6 gradient time points within 24 h of infection (4, 8, 12, 16, 20, 24 h). Through IFA, we began to observe the expression of TC0668 protein in cells infected with *C. muridarum* Nigg II strains at approximately 8 h post-infection (Fig. 3A). The expression of TC0668 protein showed an upward trend with the increase of infection time. Moreover, it can be clearly observed that the TC0668 protein is mainly localized inside inclusion bodies (Fig. 3A). Next, we further studied the changes of *TC0668* at the transcriptional level.

Previous studies have shown that *pgp8* is essential for plasmid maintenance and is stably maintained during the chlamydial development cycle [22]. Therefore, *pgp8* was used as a positive control in RT-PCR experiment. The highest mRNA expression level of *Tc0668* gene was at 16 h after infection (Fig. 3B). The ratio of *tc0668* to *pgp8* showed a gradual upward trend from 0 to 16 h and stabilized 16 h post-infection (Fig. 3C). The protein level of TC0668 could be visualized at 4 h post-infection and increased in intensity over time (Fig. 3D). TC0668 protein reached its peak at 16 h after infection, which was the stage of mass reproduction of chlamydial RBs.

From the early to the middle phase of the infection cycle, the expression of TC0668 changed rapidly, and this stage corresponded to the critical phase when intracellular bacteria must switch from EB to RB production. It could regulate the maturation of their vacuoles to create a replicative niche that can sustain efficient bacterial growth [1]. Thus, the role of TC0668 is closely correlated with *C. muridarum* growth and development.

Alignment of DUF1207 Domain-Containing Protein Sequences from TC0668 in Various Bacterial Species

The protein sequences of the TC0668 hypothetical protein that contains the DUF1207 motif were retrieved from the Swiss-Prot database and aligned using ClustalW (http://www.genome.jp/tools-bin/clustalw). The DUF1207 domain-containing proteins were derived from *Chlamydia*

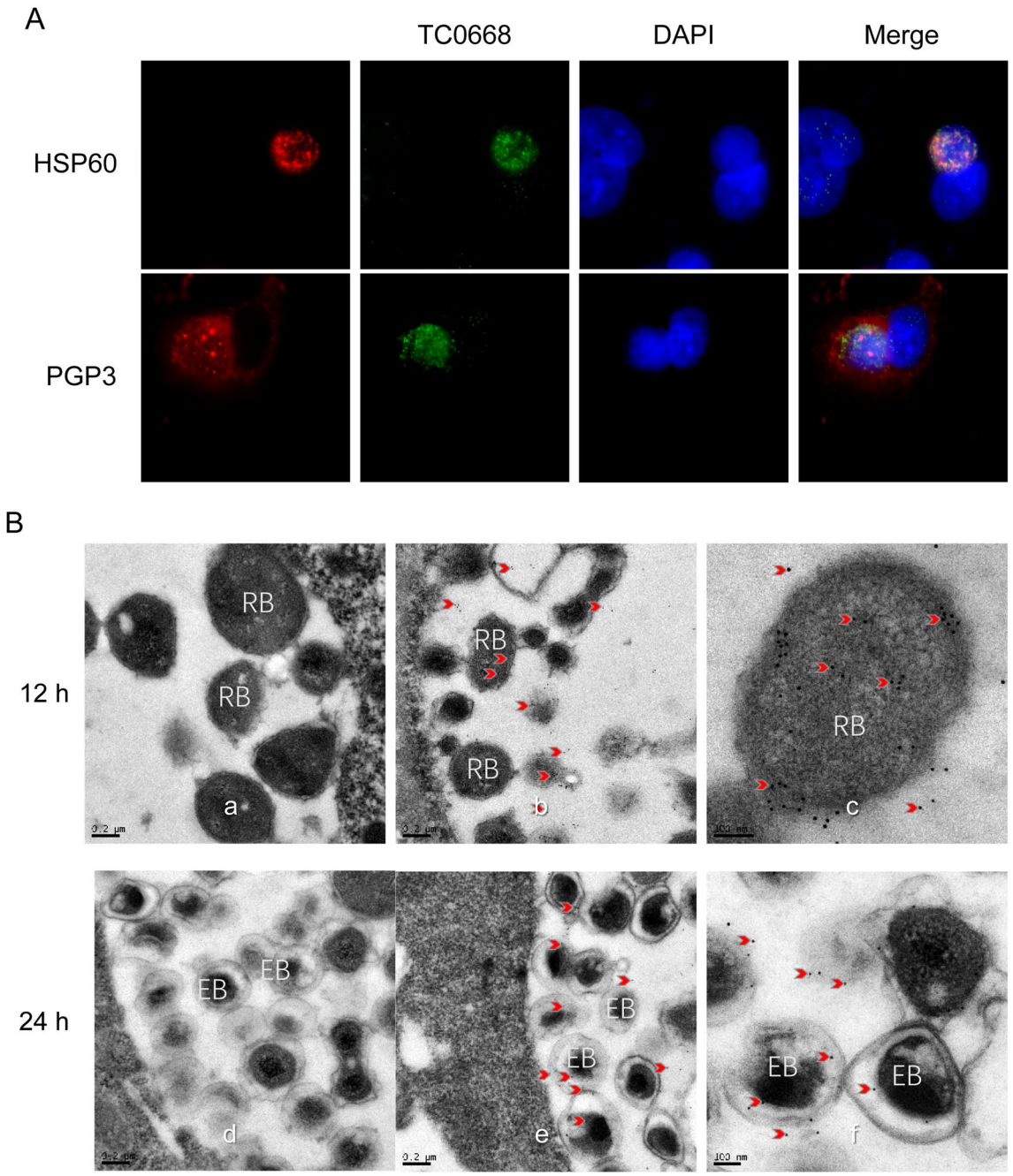

Fig. 1 Localization of TC0688. **A** Co-localization of TC0668 with HSP60 and PGP3. The localization of TC0668 was detected using a polyclonal antibody against TC0668 in combination with a 488-conjugated secondary antibody (*green fluorescence*). HSP60 and PGP3 were stained with a polyclonal antibody and visualized with a Cy3-conjugated secondary antibody (*red fluorescence*). DAPI was used for visualization of cell nuclei. 10×10 images. **B** Immunogold labeling of unicryl ultrathin sections of *C. muridarum*-infected HeLa cells at 12 and 24 h post-infection. Sections were labeled using a primary antibody against TC0668 and a secondary antibody conjugated with 10 nm gold particles. Red arrows indicate gold particles located within the chlamydial RBs/EBs and in the lumen of inclusion bodies. Untreated group (a, d): Sections were not labeled with TC0668 primary antibody. *Bar*, 0.2 um; Experimental group (b, e): *Bar*, 0.2 um; Experimental group (c, f): *Bar,* 100 nm (Color figure online)

muridarum (WP_010231168.1), *Chlamydia trachomatis* (WP_009871741.1), *Chlamydia psittaci* (WP_041460208.1), *Chlamydia pneumoniae* (WP_010883129.1), *Simkania negevensis* (WP_013943876.1), *Candidatus Protochlamydia amoebophila* (WP_039357161.1), *Criblamydia sequanensis* (WP_053331828.1), and *Oceanibaculum pacificum* (WP_084462656.1). Shading indicates areas where there is high conservation (red), and moderate conservation (blue

Fig. 2 Protein tertiary structure and homology analysis. **A** TMHMM posterior probabilities for TC0668. **B** Predicted tertiary model of the TC0668 protein. Blue: inside; Red: transmembrane; Magenta: outside. **C** Alignment of DUF1207 domain-containing protein sequences from various bacterial species. Shading indicates areas where there is high conservation (red) and moderate conservation (blue and pink). **D** Tertiary structure prediction and structural comparison of TC0668 and CT389 using the I-TASSER suite. Cyan: TC0668; red: CT389 (Color figure online)

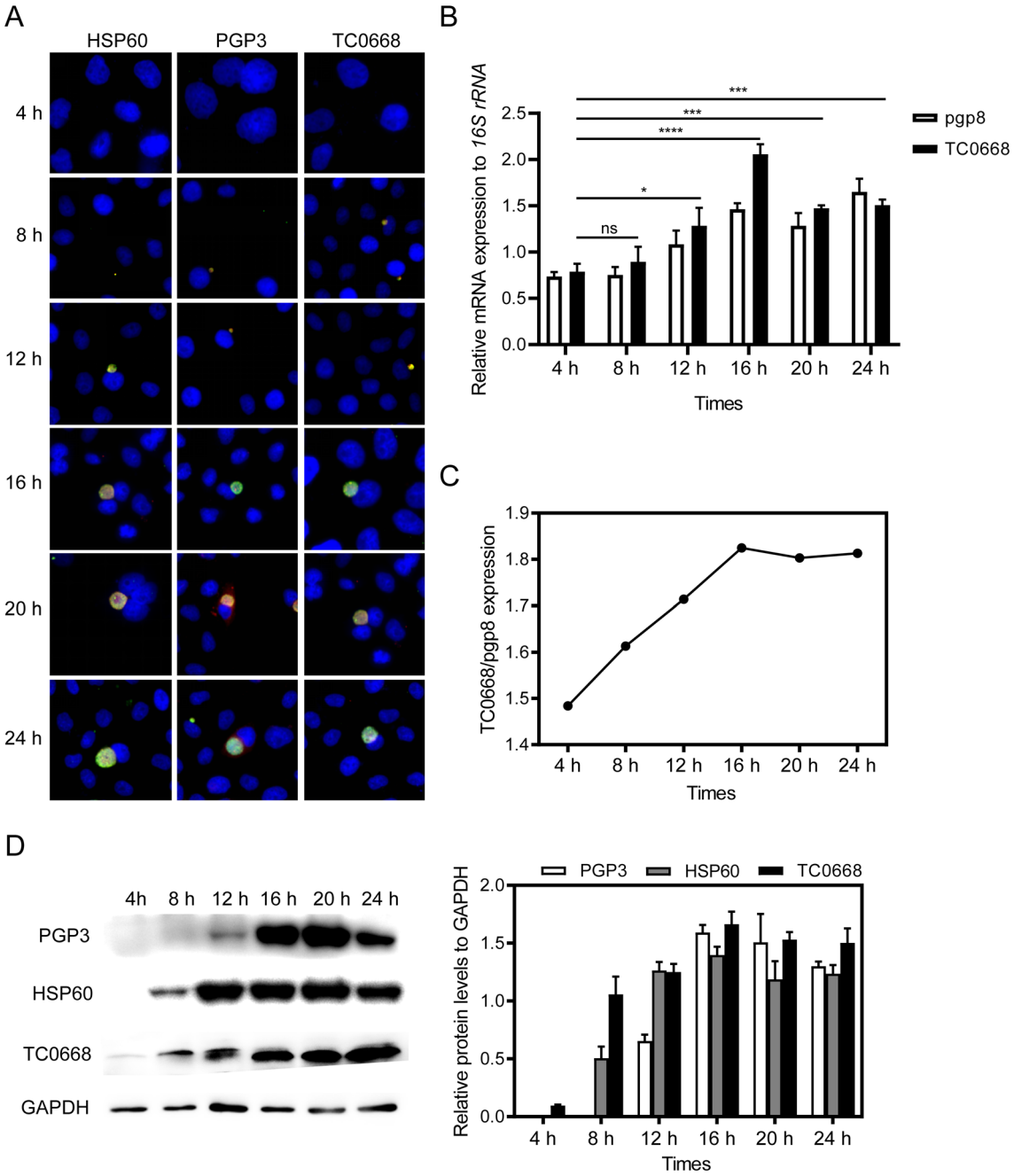

Fig. 3 Expression level of target proteins at different time points after infection of HeLa cells with C. *muridarum* Nigg II strains. **A** Expression of TC0668 in C. *muridarum* Nigg II strain-infected HeLa cells was observed using IF assay at different time points. Cells infected with *C. muridarum* organisms were immune-stained with a rabbit anti-MoPn antibody plus a goat anti-rabbit antibody conjugated to Alexa Fluor 488 (green), mouse anti-TC0668, anti-HSP60, anti-PGP3 antibodies plus a goat anti-mouse IgG conjugated to Cy3 (red), and DAPI (blue). The images were obtained using a ×100 objective lens. **B** Expression of TC0668 and pgp8 during the infectious cycle as determined using RT-qPCR. The *16S rRNA* gene which is expressed constitutively and used as an internal standard, while *pgp8* was used as a control. **C** The ratio between *tc0668* and *pgp8*, at each time point during the replicative cycle. **D** The amount of TC0668 protein was measured at different time points post-infection using WB. Anti-HSP60 and anti-PGP3 antibodies were used as controls, while anti-GAPDH was used as an internal control to estimate expression levels. Right bar plots were the Gray value analysis of WB (mean ± SEM). RT-PCR data were shown with mean ± SD. $^{*}P < 0.05$, $^{***}P < 0.001$, $^{****}P < 0.0001$ (Color figure online)

and pink, Fig. 2C), the conservation of pink is slightly higher than blue. The domain of unknown function 1207 motif is shared by chlamydial organisms. TC0668 shares 97% protein identity with *Chlamydia trachomatis* serovar D CT389 and TC0668 protein also contains a single DUF1207 motif. Thus, further evaluation of DUF1207-containing homologs may lead to a better understanding to combat highly prevalent urogenital *C. trachomatis* disease in humans [11, 12].

Tc0668 Single Mutants and tc0668 Null Mutants Induced Cytokines Production

C. muridarum TC0668 is a crucial virulence factor in the murine upper genital tract as evidenced by the fact that *tc0668* null mutants produce attenuated infections with decreased organismal loads that result in reduced inflammatory responses and hydrosalpinx development [11]. The gene *tc0668* encodes a highly conserved hypothetical protein that shares ~ 90% amino acid identity with CT389, its homolog from *C. trachomatis* serovar D [12]. Tertiary structure prediction and comparison of TC0668 and CT389 using the I-TASSER suite revealed that the tertiary structures of these two proteins were markedly similar (Fig. 2D). EBs from *C. trachomatis* serovar D wild-type strain, the *C. muridarum tc0668* single mutant strain, and the *C. muridarum tc0668* null mutant strain were subjected to SDS-PAGE using a polyclonal antibody to detect TC0668. A ~ 47-kDa band representing the CT389 and TC0668 protein was observed from *C. trachomatis* serovar D and *C. muridarum tc0668* null mutant strains, respectively (Fig. 4A). However, no trace of TC0668 was observed for the *C. muridarum tc0668* single mutant strain (Fig. 4B). The supernatants of cells infected with *tc0668* single mutant and *tc0668* null mutant strains were collected separately at 24 h after infection. The solution was then incubated with 106 detection antibodies, and then, the cytokines were detected with the antibody chip kit (Fig. 5). Comparison between the *tc0668* single mutant and *tc0668* null mutant strains showed that the expression of 36 cytokines was downregulated, while 10 cytokines were upregulated ($P < 0.05$) in the *tc0668* single mutant infected cultures (Tables S2, S3). We selected 11 cytokines associated with inflammation, as shown in Table 1 below, including the pro-inflammatory cytokines interleukin-1 and -6 (IL-1, 6), Th17 cytokine IL-17. It is clear from the results that *C. muridarum tc0668* mutants are attenuated in inducing secretion of inflammatory-related molecules.

Discussion

As a microorganism that depends on the host cell for survival, *Chlamydia* not only imports nutrients and exchanges metabolic intermediates with host cells, but also secretes virulence factors into host cells [23–25]. *Chlamydia* has a unique biphasic developmental cycle and exhibits distinct functions during its two forms of existence (EB/RB). EBs and RBs are characterized by profound differences in membrane composition and structure that play critical roles in the efficiency of host–pathogen interactions [9]. Differentiation of infectious EBs into metabolically active RBs is not yet fully understood but renders the chlamydial developmental cycle unique. Infectious EBs adhere to epithelial cells and induce cytoskeletal rearrangement by secreting effector molecules into host cells to promote invasion [26]. *Chlamydia* utilizes effector molecules to interfere directly or indirectly with intracellular processes. For example, effector proteins secreted into the host cell via the chlamydial type III secretion system (T3SS) during infection are used to reconstitute the inclusion membrane [23]. Recently, identification and characterization of TC0668 protein, a predicted chlamydial virulence factor, have become an area of intense investigation [11–13]. In the current study, IFA was employed in HeLa cells infected with *C. muridarum* Nigg II strains to demonstrate that TC0668 protein was not secreted into the

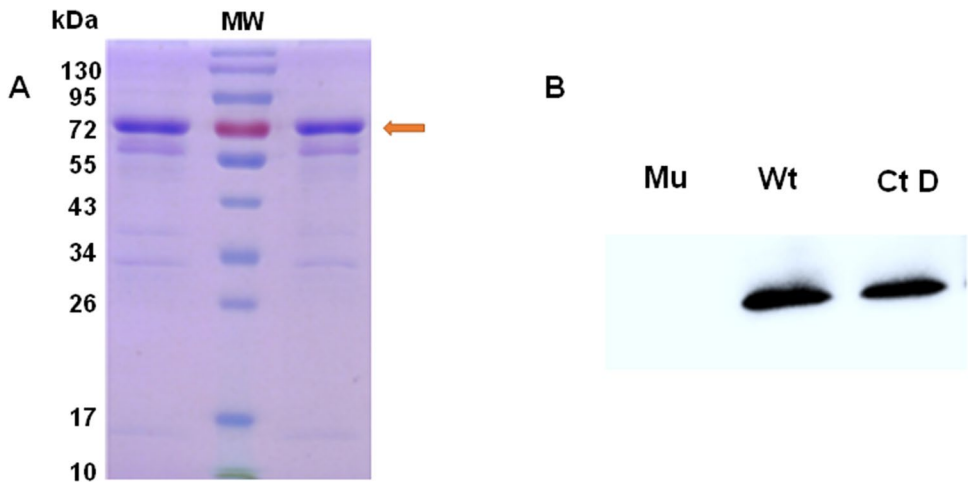

Fig. 4 Western blot detection of TC0668 protein in EBs from the *C. muridarum* tco668 null mutant, *tc0668* single mutant, and *C. trachomatis* serovar D (*Ct D*). **A** Purification of glutathione S-transferase (26 kDa) labeled TC0668 protein. **B** The TC0668 protein from *C. muridarum* Nigg II, *Ct D*, and *tc0668* mutant strains were detected using a mouse polyclonal anti-TC0668 antibody. 1: *tc0668* single mutant isogenic strain; 2: *tc0668* null mutant strain; 3: *Ct D* strain

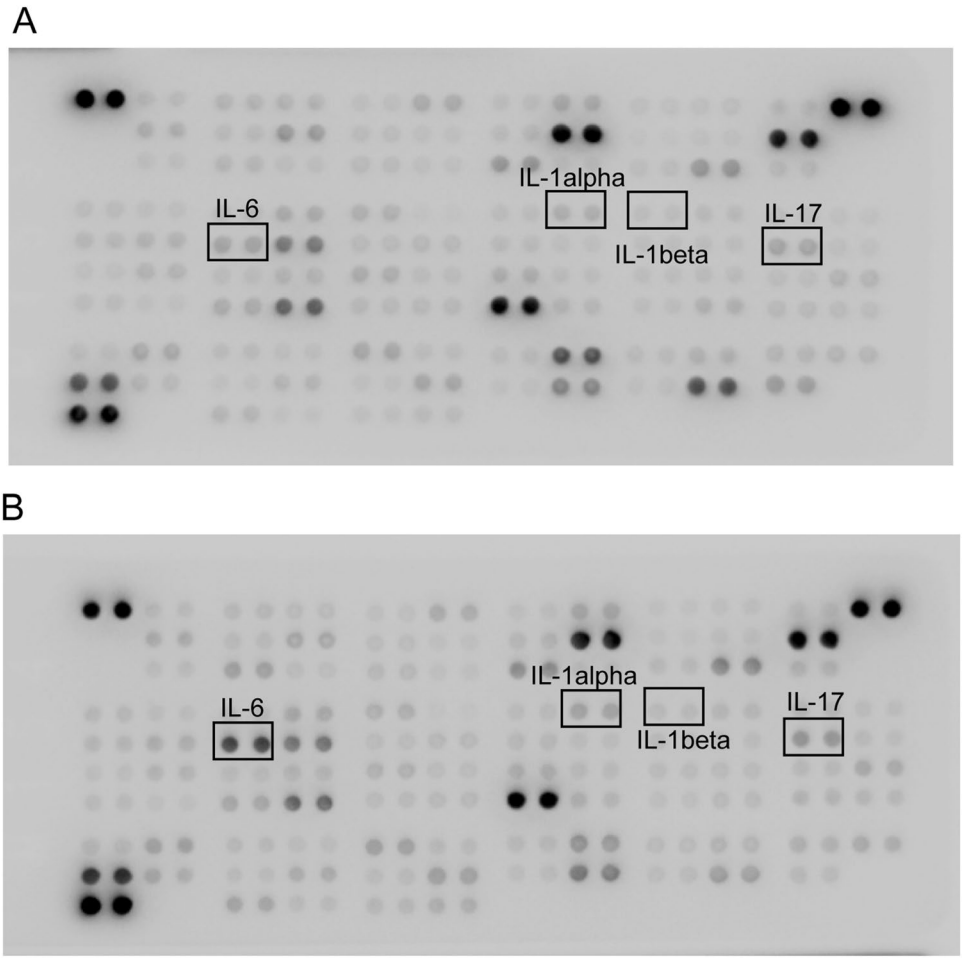

Fig. 5 Cytokines in supernatants of two different strains infected cultures. **A** Supernatant of the mutant strain. **B** Supernatant of wild-type strain

Table 1 Statistical analysis of 11 cytokines level produced by two different strains infected cells

[a]Cytokines	Cytokine levels		*P* value
	tc0668 null mutant group	*tc0668 single mutant group*	
Endoglin	19,028.6450 ± 30.7150	16,591.8100 ± 37.2800	0.000
G-CSF	16,333.9400 ± 22.6600	15,850.9150 ± 8.6450	< 0.01
GM-CSF	17,910.9600 ± 4.5000	17,212.8500 ± 62.6200	< 0.01
IL-1alpha	19,016.8650 ± 52.2750	17,331.6850 ± 81.9450	< 0.01
IL-1beta	16,253.7250 ± 25.5150	15,660.5600 ± 11.0600	< 0.01
IL-2	16,870.6500 ± 29.3100	15,786.9400 ± 0.0800	< 0.01
IL-5	17,470.3650 ± 7.5250	16,916.2800 ± 42.4100	< 0.01
IL-6	29,442.7750 ± 495.9450	18,881.5500 ± 253.3600	< 0.01
IL-15	16,108.0150 ± 40.2350	15,622.4800 ± 22.0700	< 0.01
IL-17A	20,148.9750 ± 59.3250	18,456.7000 ± 25.0100	< 0.01
VCAM-1	17,415.0800 ± 6.9500	15,911.4250 ± 2.8550	0.000

cytoplasm of HeLa cells. Co-localization of TC0668 and control proteins (HSP60and PGP3) using IFA showed that distribution of the TC0668 protein was markedly similar to that of HSP60, which was previously found to be restricted to the chlamydial inclusion [19, 27]. IEM further revealed that TCO668 immunogold particles were mainly present on the membranes of RBs or EBs at different stages of chlamydial development (at 12 and 24 h post-infection for RBs and EBs, respectively). Gold particles were also present in the lumen of inclusion bodies. Accordingly, excluding the possibility of non-specific binding by antibodies, TC0668 may be a protein that is not only located in EBs and RBs

but can also be secreted into the inclusion lumen and serves as an effector protein involved in pathogenesis. This finding could explain the mechanism of interaction of TC0668 with host cells and appears consistent with results indicating that TC0668 has a critical role in urogenital pathology induced by *C. muridarum* in a mouse model [11, 12]. If this hypothesis is not observed, it will be interesting to verify how TC0668 interacts with host cells and is involved in oviduct lesions. In either case, TC0668 was confirmed to be predominantly distributed within the membranes of EBs and RBs during the chlamydial developmental cycle, but it was not possible to determine whether TC0668 interacted with host cells directly as a secreted effector or indirectly as a component.

Gene transcription levels and protein expression of *C. muridarum* TC0668 during the replicative cycle of *C. muridarum* were investigated. To exclude interference due to chlamydial multiplication and variations in bacterial load, the number of *C. muridarum* 16S rRNA gene transcripts was used to reflect bacterial growth [28] and act as an optimal internal control during detection of the relative transcriptional expression of the *tc0668* gene in the replicative cycle. DNA replication, transcription, translation, and protein processing occurred in the early stage of the replicative cycle [29]. In the current study, TC0668 was detected in the early stage of infection (4 h post-infection) but peaked during a later stage. IFA confirmed that TC0668 could be detected at 8 h post-infection, while qRT-PCR and western blotting showed that a small amount of TC0668 could be detected at 4 h and reached a peak at 16 h after infection. Substances produced by microorganisms are associated with biological activities controlled by multiple mechanisms and are defined by protein synthesis and degradation, which vary with the physiological status of the bacteria during the growth/developmental cycle [9]. The *Chlamydia* growth cycle begins with the lag phase, which characterized by the differentiation of EBs into RBs. Genes expressed during primary differentiation (EB conversion into RBs) are mainly involved in establishing a system of nutrient acquisition and modifying inclusions to protect them from entering the endocytic pathway and undergoing lysosomal fusion [30, 31]. RBs have proliferative activity and experience exponential growth, which in *C. muridarum* is observed from 4 to 16 h after infection. During the exponential phase of chlamydial development, RBs proliferate by splitting and inclusion bodies continue to expand. Finally, the *Chlamydia* growth cycle enters stationary phase between 16 and 24 h post-infection, and this corresponds to the re-differentiation of RBs into EBs [30]. The EB membrane has a cholesterol content that is 8% higher than that of the RB and a high elasticity modulus, which makes the membrane stronger and is beneficial for penetration of host cells [32]. EBs require proteins for central and glucose catabolism, while RBs accumulate proteins required for ATP generation and nutrient transport in order to convert into EBs [33]. RBs synthesize proteins for multiplication and secrete them into the inclusion lumen. It can be speculated that the greatest expression of TC0668 at 16 h in the current study coincides with RB multiplication and chlamydial differentiation from RBs into EBs suggesting involvement in membrane reformation during re-differentiation of RBs into EBs, as well as the processes of chlamydial binary fission and inclusion body ripening. However, findings from the current study prevent a definitive conclusion being made due to variation between conditions and replicates in the absence of more specific time points.

Gene and amino acid sequence similarities of *C. muridarum* TC0668 and *C. trachomatis* CT389 were 84% and 92%, respectively, while similarities in the amounts of α-helices, random coils, and β-sheets were 17.4%, 60.78%, and 21.81%, respectively (Fig. S1-S3). In addition, the tertiary structures of TC0668 and CT389 were highly similar based on structural comparison using the I-TASSER suite. TC0668 is homologous to *C. trachomatis* CT389 within a region that includes a 360–380 residue section that is weakly homologous to a mammalian phosphatase signaling molecule [12]. CT389 is enriched in the outer membrane protein complex of *C. trachomatis* [34, 35]. The homology between TC0668 and CT389, confirmed by previous research, indicates that the TC0668 protein may also exist in the form of components of the outer membrane protein complex and may have a pathogenic role in host cells. Phosphatase activity in pathogens is related to the host inflammatory response [36]. After a pathogen invades the host, it can secrete phosphatase to alter the phosphorylation state in host signal transduction molecules, which regulates the synthesis and activity of inflammatory factors. This process, in turn, regulates the intensity of the inflammatory reaction in certain tissues or organs, and affects pathogenicity in the host [37, 38].

The function of *C. muridarum* TC0668 as a novel genetic factor involved in chlamydial pathogenicity was explored during the early stage of the chlamydial developmental cycle [12]. A starter population of *C. muridarum* (CMG0) was passaged in cultured cells for 28 generations. The resultant population, designated CMG28, had markedly low incidence and severity of upper genital tract pathology following intravaginal inoculation into mice compared with that of strain CMG0. Deep sequencing of strains CMG0 and CMG28 revealed novel protein variations in the hypothetical *tc0668* gene and a missense mutation in *tc0237***.** To determine the individual contribution of this variant to urogenital pathogenicity in mice lesions, Turner et al.[11] isolated and characterized the *tc0668* single and *tc0668* null mutants following intravaginal inoculation into mice. Compared with the *tc0668* single mutant, the *tc0668* null mutant was unable to elicit significant chronic inflammation in the

oviduct, failed to induce hydrosalpinx, and was attenuated in inducing secretion of inflammatory cytokines *in vivo*. This indicated that TC0668 was a key urogenital pathogenicity factor closely related to inflammation. Based on these previous findings, an effort was made to measure the cytokines secreted from cells infected with the *tc0668* single mutant and null mutant strains *in vitro*. Thirty-three cytokines were more highly expressed in cells infected with the *tc0668* single mutant than in cells infected with the null mutant strain, and 10 of these cytokines showed a significant difference ($P<0.05$) in expression. Conversely, 36 out of the 73 cytokines measured were significantly downregulated in cells infected with the *tc0668* single mutant compared with those infected with the null mutant. The downregulated cytokines included many immune/inflammation-related molecules, such as the pro-inflammatory cytokines IL-1, IL-2, IL-17, IL-6, Th1-promoting cytokine IL-12, and the chemokine MIP. These results suggest that the *tc0668* mutant strain is attenuated in inducing inflammation-associated cytokine secretion, congruent with the findings of previous *in vivo* studies [11]. Epithelial cells can recognize *Chlamydia* organisms, leading to the activation of the NF-κB, p38 MAP kinase, and JNK pathways and production of pro-inflammatory cytokines such as IL-1, IL-6, and the chemokine MIP, which has been linked to *Chlamydia*-induced pathology [39–41]. In contrast to the other molecules listed above, IL-12 is involved in the differentiation and activities of immune cells, and is especially important in the host defense against intracellular infection via its activation of cellular immunity to kill bacterial and infected cells [42]. During pathogen invasion, IL-17 can activate several signaling cascades, leading to the induction of chemokines. In addition, IL-17 recruits immune cells, such as monocytes and neutrophils, to the site of inflammation and promotes inflammation. Recent animal experiments have shown that IL-17 is also instrumental in defending against intracellular bacterial pathogens and viruses via innate immunity and adaptive immunity [43]. It is axiomatic that a potential *Chlamydia* vaccine ideally requires activation of protective immunity, stimulation of immune cells, and proper regulation of cytokine production. Appropriate levels of inflammation can promote immune protection, but conversely, overexpression of cytokines or chronic-persistent inflammation causes irreversible damage to the body. Thus, the question of whether TC0668 can be used for vaccine development requires further exploration. It is also necessary to consider that *Chlamydia* organisms used in in vivo studies are hybrids [11], and thus, the monoclonal strains used in in vitro studies are only partially useful in drawing conclusions. The specific ways in which cytokines induce inflammation remain unclear and may require further work in future studies.

Chen et al. compared live-organism recovery from genital tract tissues harvested from mice on day 14 after infection with CMG0 and CMG28 organisms (*tc0668* mutants) [12]. There were no significant differences between the *Chlamydia* loads in the genital tract tissues after inoculation with CMG0 and CMG28. However, significant differences were detected in some cytokines induced by the two strains in vitro in the present study. It is therefore possible that TC0668 plays a role in regulating the secretion of cytokines in host cells that leads to pathogenicity during *Chlamydia* growth and development. However, the mechanisms involved in *C. muridarum* TC0668-induced inflammatory responses that are regulated by cytokines have yet to be elucidated. Biochemical characterization of TC0668, including identification of sub-cellular localization and expression patterns during the developmental cycle, may shed new light on the potential role of TC0668 in chlamydial pathogenesis. It is thought that both intracellular replication and cell-to-cell spread significantly contribute to chlamydial pathogenicity. *Chlamydia* has evolved strategies for synthesizing proteins and contacting host cells, and these strategies may benefit chlamydial intracellular survival and enhance immunogenicity and corresponding pathogenicity [44].

Conclusion

This study investigated the basic biological characteristics of the novel virulence factor TC0668 and its ability to induce cytokine production. Findings from the study provide an important basis for further exploration of the pathogenic mechanisms of *C. muridarum.*

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Author Contributions NL and XL performed the experiments. NL and HC designed the project. CW, WX, and ST analyzed the data. NL, CS, EA, AM, and DA wrote the manuscript. All authors reviewed the manuscript and approved the final version for publication.

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Data Availability The data that support the findings of this study are available in the supplementary material of this article. All data generated or analyzed in the current study are included in this publication and are available on reasonable request.

Declarations

Conflict of interest The authors declare that they have no competing interests.

Ethical Approval This study was approved by the Ethics Committee of the University of South China (SYXK2021-002) and all animal treatments were carried out in accordance with the regulations of the institution.

Consent to Participate Not applicable.

Consent for Publication Not applicable.

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