

Transcription of seven genes in a model of interferon- γ -induced persistent *Chlamydia psittaci* infection

ZHIXI CHEN^{1,3*}, LILI CHEN^{1,3*}, CHUAN WANG^{1,3*}, JIAN YU¹, QINQIN BAI¹,
MINJUN YU¹, YIN SONG¹, YANQUN HU¹ and YIMOU WU^{1,3}

¹Institute of Pathogenic Biology, Medical College, University of South China, Hunan Provincial Key Laboratory for Special Pathogens Prevention and Control; ²Blood Transfusion Department, The First Affiliated Hospital of The University of South China; ³Hunan Cooperative Innovation Center for Molecular Target New Drug Study, University of South China, Hengyang, Hunan 421001, P.R. China

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Abstract. The obligate intracellular bacterium *Chlamydia psittaci* is the causative agent of psittacosis in birds and humans. The capability of this zoonotic pathogen to develop a persistent phase may serve a role in the chronicity of infections, in addition to the failure of antibiotic therapy or immunoprophylaxis. In the present study, a *C. psittaci* strain 6BC persistent infection cell model was induced using interferon (IFN)- γ , alterations in the infectivity and morphology of the pathogen were analyzed, and the transcript profile of seven selected genes was analyzed. Following treatment with IFN- γ , the infectivity of *C. psittaci* 6BC was decreased, the inclusion bodies appeared to be smaller, reticulate bodies were larger and the number of infectious elementary bodies was decreased compared with acute infection. In IFN- γ -induced persistently infected cells, the relative mRNA expression levels of the genes CPSIT-0208, CPSIT-0310, CPSIT-0846, CPSIT-0844 and CPSIT-0594 were upregulated at 2-48 h post-infection (p.i.). The genes CPSIT-0959 and CPSIT-0057 were downregulated at 2-36 h p.i. The results of the present study advanced the understanding of *C. psittaci* persistent infection and demonstrated a number of previously unknown alterations in chlamydial gene expression, which may provide novel targets to further analyze this particular host-pathogen interaction.

Introduction

Bacteria of the family Chlamydiaceae are obligate intracellular pathogens that cause a number of diseases in a wide range of animals, including humans. Among these bacteria, *Chlamydia psittaci*, the causative agent of human and avian psittacosis, is the most important zoonotic pathogen, which is frequently transmitted via aerosol from infected birds. The symptoms of human and avian psittacosis encompass clinically acute, protracted, chronic or subclinical disease, and may persist in the host for months or years, potentially leading to mortality (1-6). In humans, *C. psittaci* infection frequently causes acute symptoms; however, it may additionally lead to chronic inflammation and fibrosis, which requires the bacteria to be able to survive long-term *in vivo* (7).

Chlamydia exhibit a unique developmental cycle compared with other intracellular bacteria. Upon entry into eukaryotic cells, *Chlamydia* replicate within membrane-bound vacuoles, termed inclusions (8), and undergo a biphasic developmental cycle that alternates between infectious metabolically-inactive elementary bodies (EB) and noninfectious metabolically-active reticulate bodies (RB). In acute infections, the *Chlamydia* development cycle is 36-72 h. However, under adverse environmental conditions, including cytokine stimulation, antibiotic use, heat shock, nutritional deficiency, phage infection and viral co-infection, *Chlamydia* may enter a non-infectious viable state termed persistence, in order to evade host immune clearance (9-15). In this persistent state, *Chlamydia* is viable and metabolically active, although it exhibits an atypical development cycle, manifesting as the formation of small inclusions and aberrant bodies (AB), leading to increased RB which cannot produce infectious progeny EB. This persistent infection status of *Chlamydia* is reversible; when the inducing factors are removed, the RB to EB differentiation is restored and the development cycle completed.

Interferon (IFN)- γ is an important immune regulatory factor, which is able to stimulate the synthesis of indole-2,3-cyclooxygenase (IDO) (16-19). IDO is an important enzyme in the catabolism of tryptophan, and tryptophan depletion inhibits the growth of *Chlamydia*. Beatty *et al* (20,21) confirmed that increased concentrations of IFN- γ may

Correspondence to: Professor Yimou Wu, Institute of Pathogenic Biology, Medical College, University of South China, Hunan Provincial Key Laboratory for Special Pathogens Prevention and Control, 28 West Changsheng Road, Hengyang, Hunan 421001, P.R. China
E-mail: yimouwu@sina.com

*Contributed equally

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completely inhibit the growth of *C. trachomatis*; however, at low concentrations, *Chlamydia* will exhibit the atypical form of a non-infectious state with certain abnormal RB sub-cellular structures. Olivares-Zavaleta *et al.* (22) reported that *Chlamydophila pneumoniae* was able to inhibit the proliferation of activated T lymphocytes, thereby decreasing the production of IFN- γ and evading host immune clearance, leading to persistent infection.

Persistently-infectious *Chlamydia* is morphologically aberrant and exhibits an unusual transcription profile (23). It has been reported that the expression of a number of bacterial genes, including *omp1* and *ftsK*, is markedly decreased during persistence (12,24,25). This alteration in transcriptional activity is hypothesized to be a flexible means of adjusting the metabolic characteristics of the bacterium to support long-term infection of the host under varying circumstances, as observed in *Mycobacterium tuberculosis* and other organisms (26-28).

Numerous stress conditions, including iron deprivation, IFN- γ exposure and treatment with penicillin, have been investigated in the context of persistence in *C. trachomatis*, *C. pneumoniae* and *C. psittaci* (29-33). IFN- γ was used in the present study to induce the persistence of *C. psittaci* and to demonstrate its morphologically abnormal, persistent forms, and the altered expression of genes associated with cell division, energy metabolism and cell membrane structure formation.

Materials and methods

***C. psittaci* and cell culture.** Human cervical epithelial HeLa 229 cells (American Type Culture Collection, Manassas, VA, USA; CCL-2.1™) were used for culturing *Chlamydia*. HeLa 229 cells were maintained at 35°C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 2 mmol/l L-glutamine (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). *C. psittaci* 6BC (American Type Culture Collection; VR-125) was propagated in confluent HeLa 229 cell monolayers in complete growth medium with 10% FBS and 2 μ g/ml cycloheximide as described previously (34). Chlamydial EBs were harvested at 48 h post-infection (p.i.). Cells containing mature EBs were centrifuged at 1,500 \times g at 37°C for 10 min and washed twice with PBS (pH 7.4). Cell pellets were resuspended in sucrose-phosphate-glutamate acid buffer (0.2 mol/l sucrose, 3.8 mmol/l KH₂PO₄, 6.7 mmol/l Na₂HPO₄, 5 mmol/l L-glutamic acid; pH 7.4), disrupted by sonication and stored at -80°C until use. *C. psittaci* was used in all experiments at a multiplicity of infection (MOI) of 2. The MOI was determined using standard serial dilution methods (35) to count the number of chlamydial inclusion-forming units (IFU) in the HeLa 229 cells using 0.2% rabbit anti-*C. psittaci* antiserum (gift of G. Zhong, Departments of Microbiology and Immunology, University of Texas Health Science Center at San Antonio, San Antonio, USA).

Infection and induction of persistence. Human recombinant IFN- γ (R&D Systems, Inc., Minneapolis, MN, USA) was used to establish a state of *C. psittaci* persistence in HeLa 229

cells using protocols described below. HeLa 229 cells were trypsinized from stock cultures, plated onto 24- or 6-well cell culture plates (0.5 \times 10⁶ and 1.5 \times 10⁶ cells/well, respectively) and incubated overnight at 35°C in 5% CO₂. Sterile coverslips were placed into the 24-well cell culture plates prior to culture. Following 16 h of incubation, confluent HeLa 229 cell monolayers were infected with *C. psittaci* at an MOI of 2 for 2 h, and the medium was subsequently replaced with complete DMEM containing 10% FBS and 2 μ g/ml cycloheximide with different concentrations of IFN- γ (2.5, 5, 10, 25, 50 and 100 ng/ml). The infected cells were incubated at 35°C in 5% CO₂ for the indicated time periods (0, 12, 24, 36, 48 and 60 h).

Infectivity and reactivation analysis. Infectivity in the course of IFN- γ -induced persistence was determined by harvesting chlamydial organisms from infected monolayers by sonication at 48 h p.i. or the indicated time points (0, 12, 24, 36, 48 and 60 h p.i.), and reinoculating them onto fresh HeLa 229 monolayers. A total of 48 h after reinfection, the infected monolayers were fixed with 4% paraformaldehyde at room temperature for 30 min and stained as described below. The numbers of inclusion bodies were counted in 30 random high power fields using a fluorescent microscope at x400 magnification and calculated as IFUs/ml.

For the reactivation analysis experiments, HeLa 229 monolayers grown on coverslips were infected at an MOI of 2, and persistence was induced as described above. A total of 24 h p.i., the medium was replaced with complete growth medium without IFN- γ and supplemented with 100 μ g/ml tryptophan. Following incubation at indicated time points (12, 24 and 36 h), chlamydial organisms were harvested by sonication and inoculated onto fresh HeLa 229 cell monolayers using complete growth medium. A total of 48 h later, the monolayers were fixed with 4% paraformaldehyde at room temperature for 30 min, samples were stained as described below, and the infectious titer was estimated by counting 30 random fields using a fluorescent microscope at x400 magnification per coverslip.

Indirect immunofluorescence staining. During IFN- γ -induced persistence, infected cells were fixed with 4% paraformaldehyde for 30 min at 48 h p.i., permeabilized with 0.1% (v/v) Triton X-100 for 10 min, and blocked with DMEM containing 10% FBS at 37°C for 1 h. Subsequently, the samples were inoculated with rabbit anti-*C. psittaci* antiserum at 37°C for 1 h. Following washing twice with PBS, the infected monolayer cells were incubated with 0.5% Cy2-conjugated goat-anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; cat. no. 111-225-144) and 0.1% DAPI (Thermo Fisher Scientific, Inc.) at 37°C for 1 h. Images of inclusion-containing cells were obtained using a fluorescent microscope at x400 magnification.

Transmission electron microscopy. HeLa cells were plated in 6-well culture plates, and infections were performed as described above. The culture supernatant was replaced with 2.5% glutaraldehyde in phosphate buffer (0.1 mol/l; pH 7.4) at the indicated time points (24, 36 and 48 h) following infection. Following 2 h of fixation at 4°C, the cells were removed from the cell culture plates using a cell scraper, collected into

an Eppendorf tube and centrifuged at 1,500 x g for 5 min to remove dead and apoptotic cells at 4°C. The cell pellets were postfixed in 1% osmic acid at room temperature for 2-3 h. Following washing twice with PBS, samples were dehydrated in increasing concentrations of ethanol (50, 70 and 100%) and acetone (90 and 100%) prior to infiltration and embedding in a mixture of Spurr epoxide resin (EPON812, DDSA, MNA and DMP30) and pure acetone at room temperature for 2-3 h. Ultrathin sections (~70 nm) were stained with uranyl acetate and lead citrate at 30°C for 30 min prior to examination and imaging under a Hitachi HT-7100 transmission electron microscope (Hitachi, Ltd., Tokyo, Japan).

Preparation of RNA samples. The HeLa 229 monolayers were grown in 6-well cell culture plates at a density of 1.5×10^6 cells/well. A total of two independent rounds of infection experiments were performed, including acute and persistent *Chlamydia* infections. At 2, 6, 12, 24, 48 and 60 h p.i., infected cells were removed using TRIzol reagent (Tiagen Biotech Co., Ltd., Beijing, China), and total RNA was isolated through delamination in chloroform, precipitation in isopropyl alcohol and washing with 75% ethanol. Following centrifugation at 10,000 x g at 4°C for 10 min, the RNA sediments were dissolved into 50 μ l sterile diethyl pyrocarbonate-treated water, aliquoted, titrated and stored at -80°C until use.

Design of gene-specific primers. Primers used for RT-qPCR were designed using Primer Premier software (version 5.0; Premier Biosoft International, Palo Alto, CA, USA) and chromosomal DNA of *C. psittaci* 6BC was used as the template. The primers for the target genes are presented in Table I.

Reverse transcription-quantitative (RT-q)PCR analysis. Total RNA was reverse-transcribed into cDNA using a RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Measurement of mRNA expression levels using RT-qPCR was performed using a fluorescent quantitation PCR system (Thermo Fisher Scientific, Inc.), with the following temperature-time profile: Initial denaturation at 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 1 min. The melting curves of the amplification products were analyzed subsequently. Each reaction tube contained a reaction mixture of 1 μ l cDNA template, 0.5 μ l each primer (final concentration, 500 nmol/l), 15 μ l FS Universal SYBR-Green Master mix (Roche Diagnostics, Indianapolis, IN, USA), and 13 μ l deionized water. All RT-qPCR assays from two independent series of infections were performed in triplicate for each target gene, with samples collected from the persistence model at 2, 6, 12, 24, 36, 48 and 60 h p.i. The relative gene expression ratios of the target genes were calculated based on the real-time amplification efficiency and cycle threshold deviation of a given sample vs. the control (36) (acute infection), compared with a reference gene, the 16S ribosomal (r)RNA gene.

Statistical analysis. In order to compare the relative transcriptional profiles of the candidate genes in acute and persistent *C. psittaci* infection, statistical comparisons were made using one-way analysis of variance and Student's t-test. $P < 0.05$ was considered to indicate a statistically significant difference.

Data are expressed as the mean \pm standard deviation of at least three independent experiments and statistical analysis of the data was performed using SPSS version 18.0 software (SPSS, Inc., Chicago, IL, USA).

Results

IFN- γ treated cultures exhibit decreased *C. psittaci* infectivity. Compared with the untreated cultures ($41.0 \pm 1.0 \times 10^6$ IFU), following treatment with IFN- γ at concentrations of 2.5, 5, 10, 25, 50 and 100 ng/ml, the infectivity of *C. psittaci* was decreased to 91.0% ($37.3 \pm 0.85 \times 10^6$ IFU), 82.9% ($34.0 \pm 0.94 \times 10^6$ IFU), 74.1% ($30.4 \pm 1.08 \times 10^6$ IFU), 39.5% ($16.2 \pm 0.87 \times 10^6$ IFU), 10.0% ($4.1 \pm 0.62 \times 10^6$ IFU), and 3.7% ($1.5 \pm 0.47 \times 10^6$ IFU), respectively, which demonstrated that the infectivity of the IFN- γ -treated *C. psittaci* strain was decreased in a dose-dependent manner (Fig. 1). A concentration of 25 ng/ml IFN- γ was used in all subsequent trials.

Following treatment with 25 ng/ml IFN- γ , the IFU of *C. psittaci* was detected at 36, 48 and 60 h p.i. (Fig. 2). The results demonstrated that, in the absence and presence of IFN- γ in the HeLa cells, the numbers of inclusion bodies increased progressively with time, peaking at 48 h p.i. In addition, the infectivity of the IFN- γ -treated *C. psittaci* strain was significantly decreased compared with the untreated cultures at each time point ($P < 0.01$).

IFN- γ treated cultures of *C. psittaci* exhibit aberrant morphological characteristics. In the persistence model, chlamydial bodies exhibiting abnormal morphology were observed from 24 h p.i. onwards. Immunofluorescent images demonstrated that the inclusion bodies appeared to be smaller, and the RBs larger, compared with those observed in IFN- γ -untreated *C. psittaci* infection. At increased magnification, images of aberrant inclusions revealed characteristic inhomogeneities resulting from the enlarged bodies, which distinguished them from normal inclusion bodies (Fig. 3).

The morphological characteristics of *C. psittaci* 6BC were further evaluated at each time point by transmission electron microscopy. No clear morphological alterations were detected in chlamydial structures between untreated and IFN- γ -treated cells at 24 h p.i., and the two groups exhibited large inclusion bodies containing numerous EBs and few RBs. However, evident morphological differences were observed at 36 and 48 h p.i. (Fig. 4). Following treatment with IFN- γ , the inclusion bodies were smaller with enlarged RBs, and few or no EBs. In acute infection cultures, the inclusion bodies contained normal EBs and RBs; the EBs were electron opaque, spherical-oval shaped particles with little periplasmic space and were surrounded by an undulating cell membrane, while the RBs were round to oval in shape, with a typical electron translucent center and a cytoplasm condensed towards the periphery. In the IFN- γ -treated cultures, the inclusion bodies were small contained few chlamydial particles. The RBs (termed ABs) were larger compared with normal inclusion bodies, exhibited an electron-lucent cytoplasm with a loose network of filaments and multiple electron-dense foci.

Activity of IFN- γ -treated *C. psittaci* may be rescued. In order to assay the reversibility of the persistent state in the

Table I. Sequences of primers used for the reverse transcription-quantitative polymerase chain reaction.

Target gene	Primer sequence (5'-3')	Amplicon size, bp
16S rRNA	F: CATCTGCCAGACTTCTACGG R: ACACGCCCTTTACGCCCAAT	255
CPSIT_0844	F: TGTGCTAACTCCTTCGTCTTG R: CTCATCTCGAAGAGTTCCTGG	420
CPSIT_0594	F: GGCTCATGGTTACTGATGGAG R: TTGTGAGTGCTGTAAGTGAGG	113
CPSIT-0846	F: GGTGTGCAAGCTTCTCTTTTAG R: GAGCTTCTAGTTATGCCCAAATTG	37
CPSIT-0959	F: AGGCATTGGAACATCGGAG R: GACAGAGGAAAGGCGATACAC	368
CPSIT-0057	F: TGCAAGCTCAATCTCTACCG R: GCACAACCACATCCCATAAAG	95
CPSIT-0208	F: CCTTGCGAAGTTGAGTTTGTC R: GCAGTAAGATACAGTAGCCACG	433
CPSIT-0310	F: TTTTGATGCGGAGAGTATGGG R: TCGGTTCTGGTTCTTTAGTTC	259

rRNA, ribosomal RNA; F, forward; R, reverse.

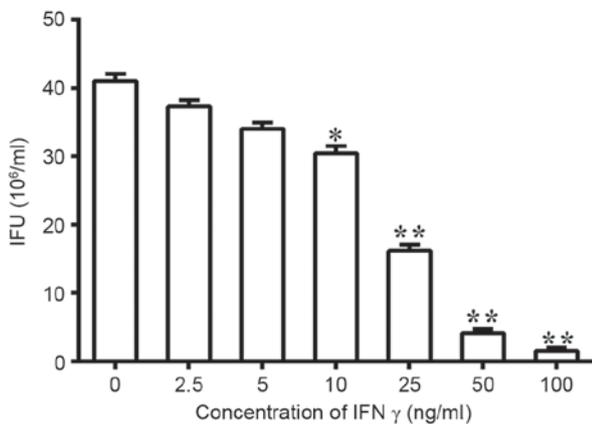


Figure 1. Infectivity of *Chlamydia psittaci* 6BC cultures with different concentrations of IFN- γ . The number of inclusion-forming units was determined from paraformaldehyde-fixed immunostained cell cultures at 48 h post-infection using fluorescence microscopy. The data represent the mean \pm standard deviation for three independent experiments. * P <0.05, ** P <0.01 vs. 0 ng/ml IFN- γ . IFU, inclusion-forming units; IFN- γ , interferon- γ .

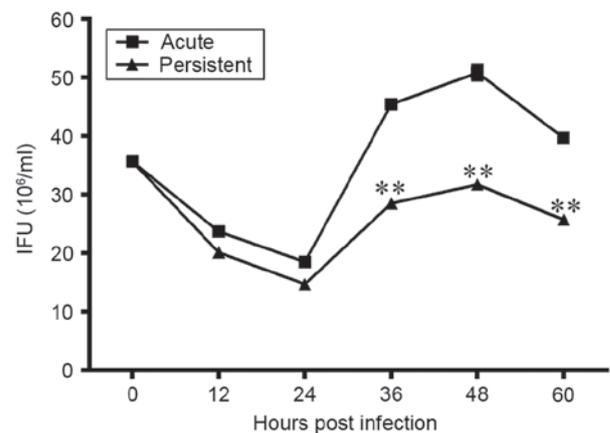


Figure 2. Infectivity of *Chlamydia psittaci* 6BC treated with 25 ng/ml IFN- γ at different time points. The persistent infection group was treated with 25 ng/ml IFN- γ . The acute and persistent infection groups exhibited a latency period; a rapid burst in IFU was observed in the acute infection group, although not in the persistent group. The data represent the mean \pm standard deviation for three independent experiments. ** P <0.01 vs. 0 h post-infection. IFU, inclusion-forming units; IFN- γ , interferon- γ .

IFN- γ -induced model, the capability of persisting chlamydial bodies to recover was examined using freshly infected host cells, following removal of the persistence inducer and tryptophan supplementation. The morphological features of *C. psittaci* were observed to be rescued in this model. The inclusion bodies were larger, and more EBs and fewer ABs and RBs were observed in the inclusion bodies by electron microscopy (Fig. 5).

In addition to the morphological data, IFUs were additionally compared to evaluate the infectivity of the organisms. Following IFN- γ removal and tryptophan supplementation, *C. psittaci* recovered and returned to an infectious state. At 48 h p.i., compared with the persistent infection, the recovery rate was 79.5% (Fig. 6).

IFN- γ induces modulation of *C. psittaci* gene expression.

RT-qPCR analysis was used to analyze the expression of seven chlamydial genes in IFN- γ -treated *C. psittaci* cultures at 2-60 h p.i. The 16S rRNA gene served as an internal standard. The results of the present study demonstrated that treatment with IFN- γ modulated the expression of specific *C. psittaci* genes (Fig. 7). From 2 h p.i. onward, the genes encoding the polymorphic outer membrane protein (CPSIT-0310) was upregulated in *C. psittaci* IFN- γ -induced persistence at 2-48 h p.i., although it was downregulated from 48 h p.i. onwards. Inclusion membrane protein (CPSIT-0594; incA) expression was upregulated at 2-48 h p.i., and downregulated at 60 h p.i. The gene encoding the large cysteine-rich

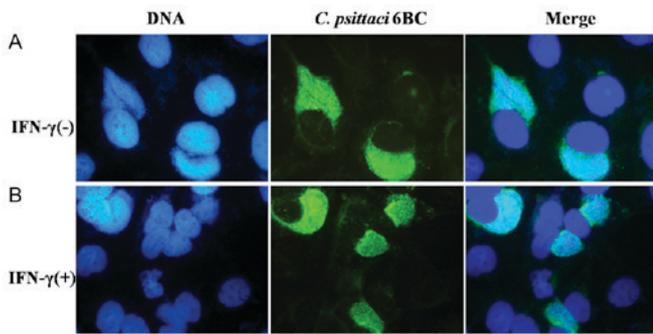


Figure 3. Indirect immunofluorescent staining of *Chlamydia psittaci* 6BC cells in acute and IFN- γ -induced persistent infection. Infected HeLa monolayers were incubated at 35°C with 5% CO₂ for 48 h, fixed with paraformaldehyde, stained and visualized using fluorescence microscopy (magnification, x1,000). (A) HeLa monolayer infected with *C. psittaci* 6BC without persistence inducer. (B) Persistent infection upon exposure to 25 ng/ml IFN- γ . Blue, nuclear; green, inclusions of *C. psittaci* 6BC. IFN- γ , interferon- γ .

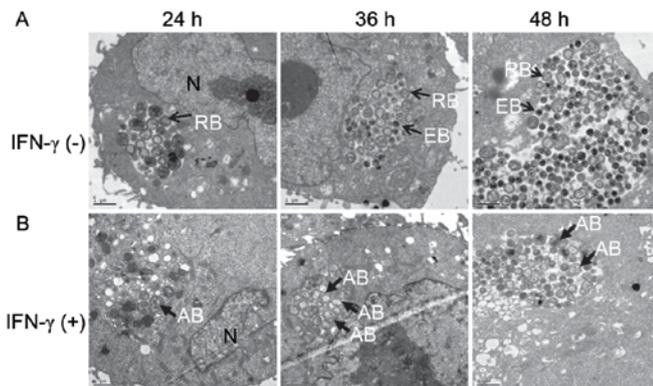


Figure 4. Transmission electron microscopy of *Chlamydia psittaci* 6BC inclusion bodies in acute and persistent infection. (A) Untreated *C. psittaci* 6BC-infected HeLa cells. The typical inclusion structure was observed. (B) IFN- γ -treated infected HeLa cells. The inclusions became small and contained expanded, low electron density Abs. Scale bar, 1 μ m. N, nucleus; EB, elementary body; RB, reticulate body; AB, aberrant body; IFN- γ , interferon- γ .

periplasmic protein (CPSIT-0208; omcB) was significantly upregulated in the persistence model at 2-36 h p.i., and downregulated from 48 h p.i. onwards. The expression of the gene encoding type III secretion protein CPSIT-0844 was upregulated at 2-48 h p.i., and downregulated at 60 h p.i., although CPSIT-0846 was upregulated at 2-24 h p.i. and downregulated from 36 h p.i. The genes encoding proteins with functions in cysteine desulfurase (CPSIT-0959) and a major outer membrane protein (CPSIT-0057; ompA) were downregulated in the IFN- γ -treated cultures from 2 h p.i. onwards.

Discussion

C. psittaci is a zoonotic pathogen which may cause persistent infection, leading to severe sequelae which are difficult to treat (23). However, the molecular mechanisms that evoke and maintain the persistence of *Chlamydia* remain poorly understood. In order to further investigate the potential underlying mechanisms, a state of *C. psittaci* persistence was established in HeLa cells using IFN- γ treatment in the present study.

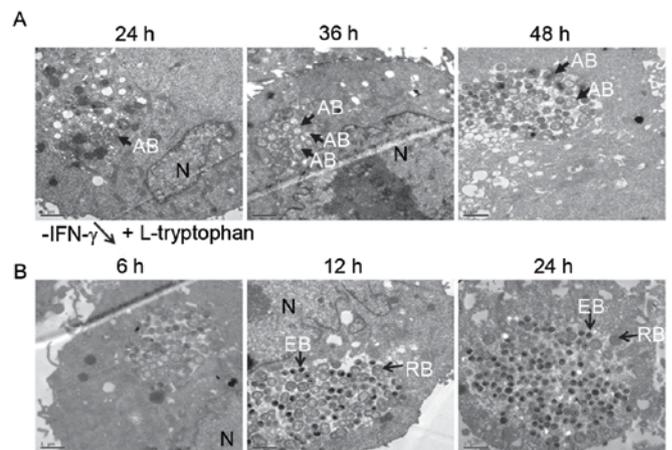


Figure 5. Reversibility of chlamydial morphological characteristics in persistent infection. The morphological characteristics of chlamydial inclusion bodies from the persistent infection and reactivation assay were observed under transmission electron microscopy, including the removal of the persistence inducer at 24 h p.i. and subsequent culture in complete growth medium with tryptophan supplementation for 6, 12 and 24 h. (A) IFN- γ -treated chlamydia-infected HeLa cells. (B) Cultures following removal of the persistence inducer at 24 h p.i. and subsequent culture in complete growth medium with tryptophan supplementation. Scale bar, 1 μ m. N, nucleus; EB, elementary body; RB, reticulate body; AB, aberrant body; IFN- γ , interferon- γ ; p.i., post-infection.

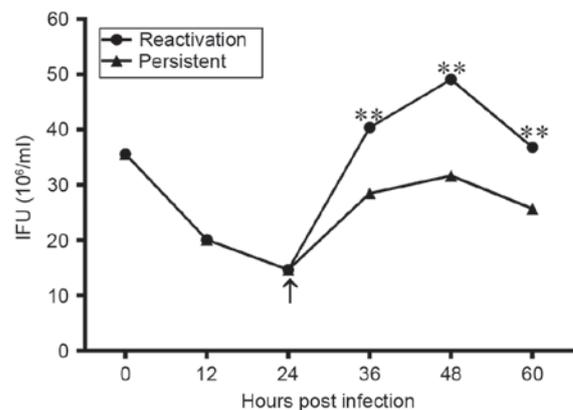


Figure 6. One-step growth curves of *Chlamydia psittaci* 6BC following removal of IFN- γ . The numbers of recoverable infectious chlamydial organisms (measured as IFU) from the IFN- γ (25 ng/ml) -induced persistent infection and reactivation assay, which included the removal of the persistence inducer at 24 h p.i. (arrow) and subsequent culture in complete growth medium with tryptophan supplementation. The data represent the mean \pm standard deviation for three independent experiments. **P<0.01 vs. 0 h p.i. IFN- γ , interferon- γ ; p.i., post-infection; IFU, inclusion-forming units.

Different concentrations of IFN- γ were used to treat *C. psittaci* 6BC, and it was observed that the infectivity of the IFN- γ -treated *C. psittaci* strain was significantly decreased in a dose-dependent manner. Previous studies have reported that high concentrations of IFN- γ may inhibit *Chlamydia* growth; however, an infected host will frequently produce sub-inhibitory concentrations of IFN- γ , which is insufficient to eliminate the bacteria and thereby induces persistent infection (20,21). In the present study, 25 ng/ml IFN- γ was able to significantly decrease the infectivity of *C. psittaci*; therefore, this concentration was used to induce the *C. psittaci* 6BC persistent infection model. Previous studies have used different

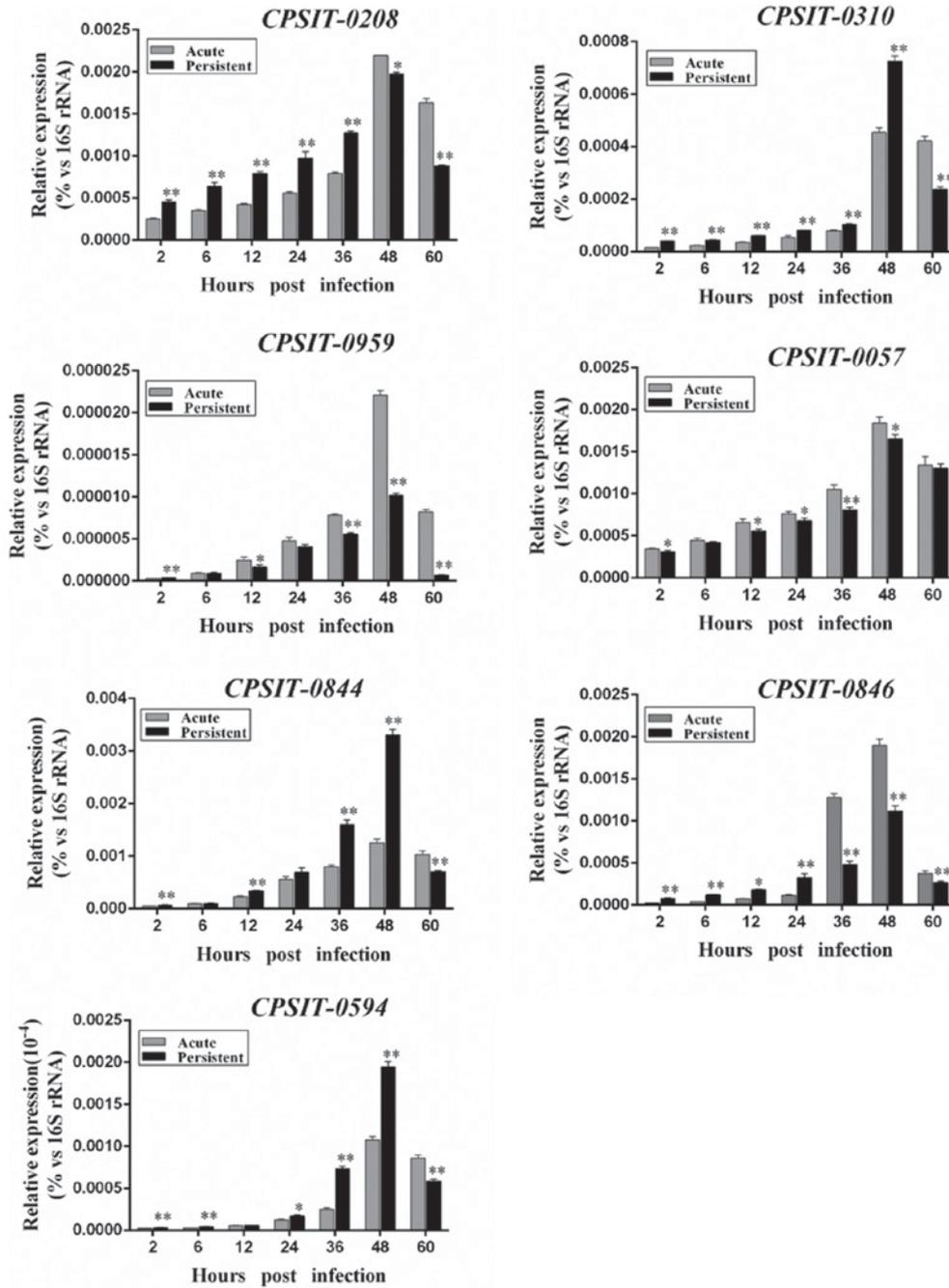


Figure 7. Relative mRNA expression levels of genes in IFN- γ -induced *C. psittaci* 6BC at different time points. *C. psittaci* 6BC was treated with 25 ng/ml IFN- γ to induce persistence and cultivated at different time points in three independent experiments. Relative mRNA expression was detected using the reverse transcription-quantitative polymerase chain reaction. The expression levels of target genes were normalized to 16S rRNA mRNA levels. The data represent the mean \pm standard deviation for the three independent experiments as calculated using the $2^{-\Delta\Delta C_t}$ method. * $P < 0.05$ and ** $P < 0.01$ vs. acute infection group at same time point. rRNA, ribosomal RNA; *C. psittaci*, *Chlamydia psittaci*; IFN- γ , interferon- γ .

concentrations; for example, Beatty *et al* (20) used 0.2 ng/ml IFN- γ to induce *C. trachomatis* serotype A persistent infection, Pantoja *et al* (37) used 25 U/ml to induce *C. pneumoniae* A-03 persistent infection, and Goellner *et al* (38) used 120 U/ml to induce *C. psittaci* DC15 persistent infection. This discordancy between previous experimental protocols may be associated with the differing sensitivity of the various *Chlamydia* species to IFN- γ .

Following treatment with IFN- γ , the morphological alterations of the inclusions of *C. psittaci* were less apparent at 24 h p.i.; however, at 36 and 48 h p.i., the number of abnormal

RB was increased, mature progeny EBs were decreased in number, and vacuolar alterations were visible in inclusion bodies, suggesting that IFN- γ exposure inhibits binary fission and RB to EB differentiation. The results of the present study are consistent with the results reported in *C. trachomatis* and *C. pneumoniae* persistent infections induced by IFN- γ (39,40). It was additionally observed that the infectivity and morphological changes were recoverable upon removal of IFN- γ and exogenous tryptophan supplementation. The results of the present study demonstrated that the persistent infection state of *C. psittaci* is reversible, and the growth and development

of the organism may return to normal when the stimulus is removed.

Although the microbiological and morphological features of *C. psittaci* were altered in persistent infection, the atypical organisms were able to proceed to replicate their genomes. However, the gene expression of *C. psittaci* during persistent infection was altered compared with active infection and varied in different persistent cultures. In the present study, following IFN- γ stimulation, alterations in chlamydial gene expression were observed from early time points (2-96 h). Among the seven genes analyzed, five were significantly upregulated and two were downregulated.

Three membrane protein genes, CPSIT-0208 (encoding large cysteine-rich periplasmic protein omcB) and CPSIT-0310 (encoding polymorphic outer membrane G family domain protein) were upregulated, and CPSIT-0057 (encoding major outer membrane protein ompA) was downregulated during the state of IFN- γ -induced persistent infection. Previous studies have reported that the expression of major outer membrane porin was upregulated for *C. pneumoniae* (strains IOL-207, A-03 and CM-1) (41-43), and downregulated for *C. trachomatis* serotype D and *C. psittaci* DC15 (20,23). The expression of omcB was not altered in *C. pneumoniae* IOL-207, was upregulated in *C. pneumoniae* CM-1, and was downregulated in *C. trachomatis* serotype D (20,39,43). In *C. psittaci* DC15, the expression of omcB and polymorphic outer membrane protein 91B was upregulated at 12 h p.i., and downregulated from 24 h p.i. (23). These previous results demonstrated that the diversity of membrane proteins might serve different roles in persistent infection in different species of bacteria.

Three inclusion membrane (Inc) proteins were upregulated in the present persistent infection model. CPSIT-0594 encodes protein IncA. CPSIT-0846 and CPSIT-0844 are two putative transmembrane head protein family proteins which are additionally located in the inclusion membrane, and have been predicted to be type III secretion system (T3SS) effector protein encoding genes (44). Inc proteins may be associated with the processes of inclusion formation, transportation to the nuclear space and evasion of early lysosomal fusion (45,46). T3SS effector proteins are injected into the host cell cytosol, inclusion lumen or chlamydial inclusion membranes via the T3SS, and they exert important roles in facilitating EB invasion, preparing chlamydial organisms for the dissemination of progeny EBs to new cells, and promoting the survival and replication of intracellular chlamydial organisms (47). Therefore, the upregulation of Inc or T3SS effector proteins may be a mechanism through which *C. psittaci* is able to modulate its survival under stress conditions.

CPSIT-0959, encoding a protein with functions in cysteine desulfurase activity, was significantly downregulated at the later stages of persistent infection. This protein is principally associated with metabolism in *Chlamydia*, and its downregulation may affect nutrient biosynthesis for *C. psittaci*, thereby disrupting the normal developmental cycle.

The present study advances the understanding of *C. psittaci* persistent infection and reveals a number of previously unknown alterations in chlamydial gene expression, which may provide novel targets to further analyze this particular host-pathogen interaction.

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