

Plasmid-encoded Pgp3 protein enhances the infectivity of *Chlamydia trachomatis* serovar L2 in mice

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ABSTRACT

Objective: Chlamydial infection can cause a variety of serious diseases, including trachoma, tubal infertility, and rectal lymphogranuloma venereum (LGV). However, the underlying pathogenic mechanisms remain incompletely understood. *Chlamydia trachomatis* (*C. trachomatis*) serovar L2 was among the first chlamydial strains to be successfully transformed and has been widely used to investigate the functions of chlamydial proteins, particularly plasmid-encoded proteins. Previous studies of serovar L2 have largely been limited to in vitro experiments, and the roles of plasmid-encoded proteins during in vivo infection remain poorly understood. This study aims to evaluate the infectivity in mice of L2 mutants carrying deletions or premature stop codon mutations in different plasmid genes.

Methods: Female C3H/HeJ and CBA/J mice received a subcutaneous injection of medroxyprogesterone acetate 5 days before infection and were subsequently inoculated intravaginally or intrauterinely with 1.0×10^6 inclusion-forming units (IFUs) of various L2 strains. These included wild-type L2 (L2wt), plasmid-free L2 (L2R), green fluorescent protein (GFP)-expressing L2 (L2GFP), plasmid-encoded glycoprotein (Pgp)-related deletion mutants (L2 Δ Pgp3, L2 Δ Pgp4, L2 Δ Pgp5, and L2 Δ Pgp7), and premature stop-codon mutants (L2Pgp3Y9S, L2Pgp4K13S, L2Pgp5L9S, L2Pgp7L11S, and L2Pgp8E11S). Vaginal swabs were collected at regular intervals after infection, and viable chlamydial shedding was monitored by immunofluorescence-based titration. The infectivity of each mutant was compared with that of the L2wt or L2GFP control group. For in vitro experiments, HeLa cells infected with the above strains were harvested at 30 or 48 h post-infection. Immunofluorescence staining and Western blotting (WB) were performed to assess the expression levels of Pgp3 and glycogen synthase A (GlgA), respectively.

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Results: Compared with L2wt, the plasmid-free strain showed significantly reduced chlamydial shedding after intrauterine infection ($P<0.05$), whereas no such difference was observed after intravaginal infection, suggesting that the L2 plasmid plays a more important role in upper genital tract infection than in lower genital tract infection. Deletion of either Pgp3 (L2 Δ Pgp3) or Pgp4 (L2 Δ Pgp4) resulted in significantly reduced vaginal shedding of viable chlamydia after intrauterine inoculation ($P<0.05$). Mice infected with the Pgp3 premature stop codon mutant L2Pgp3Y9S exhibited reduced shedding ($P<0.05$), whereas this phenotype was not observed with the Pgp4 mutant L2Pgp4K13S. Immunofluorescence staining and WB showed that L2Pgp4K13S still expressed a detectable amount of Pgp3, although markedly less than L2wt, and this residual expression may have been sufficient to maintain infectivity. WB further confirmed that expression of the *C. trachomatis* glycogen synthase GlgA depends on Pgp4 rather than Pgp3. The infectivity of the Pgp4-deficient strain L2Pgp4K13S was comparable to that of L2wt, suggesting that GlgA may not be required for L2 infection of the murine upper genital tract.

Conclusion: Pgp3 rather than Pgp4, other plasmid-encoded genes, or the plasmid-regulated GlgA gene, plays a critical role in the infectivity of *C. trachomatis* serovar L2 in mice. In addition, even very low levels of Pgp3 expression may be sufficient to maintain L2 infectivity in mice.

KEY WORDS

Chlamydia trachomatis; serovar L2; transformation; plasmid; Pgp3; mouse model; infectivity

质粒编码的Pgp3蛋白增强沙眼衣原体L2血清型在小鼠体内的感染能力

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[摘要] **目的:** 衣原体感染可引起多种严重疾病, 包括沙眼、输卵管性不孕和直肠性病性淋巴肉芽肿(lymphogranuloma venereum, LGV), 但其致病机制尚不清楚。沙眼衣原体(*Chlamydia trachomatis*, *C. trachomatis*)L2血清型是最早成功转化的菌株之一, 被广泛用于研究衣原体蛋白质(尤其是质粒编码蛋白质)的功能。既往对L2血清型的研究多局限于体外, 对质粒编码蛋白在体内感染中的作用认识有限。本研究旨在评估不同质粒基因缺失或提前终止密码子突变的L2突变株在小鼠中的感染性。**方法:** 雌性C3H/HeJ和CBA/J小鼠在感染前5 d皮下注射醋酸甲羟孕酮, 随后经阴道或宫腔内接种 1.0×10^6 内含物形成单位(inclusion-forming units, IFUs)的多种L2菌株, 包括野生型L2(wild type L2, L2wt)、无质粒株L2(plasmid-free L2, L2R)、表达绿色荧光蛋白(green fluorescent protein, GFP)的L2(L2GFP)、质粒编码糖蛋白(plasmid-encoded glycoprotein, Pgp)相关的缺失突变株(包括L2 Δ Pgp3、L2 Δ Pgp4、L2 Δ Pgp5、L2 Δ Pgp7)及提前终止密码子突变株(包括L2Pgp3Y9S、L2Pgp4K13S、L2Pgp5L9S、L2Pgp7L11S、L2Pgp8E11S)。感染后定期采集阴道拭子, 通过免疫荧光滴定法监测活菌脱落情况, 并将各突变株的感染性与L2wt或L2GFP对照组进行比较。体外实验中, 感染上述菌株的HeLa细胞于感染后30 h或48 h收获, 分别进行免疫荧光染色和蛋白质印迹法(Western blotting, WB)检测Pgp3及糖原合成酶A(glycogen synthase A, GlgA)的蛋白质表达水平。**结果:** 与L2wt相比, 宫腔感染后质粒缺失株的衣原体脱落量显著减少($P<0.05$), 而阴道感染后无此差异, 提示L2质粒在上生殖道感染中的作用较下生殖道更重要。Pgp3(L2 Δ Pgp3)或Pgp4(L2 Δ Pgp4)缺失均导致宫腔接种后阴道活菌脱

落显著降低($P<0.05$)。携带 Pgp3 提前终止密码子突变的 L2Pgp3Y9S 感染小鼠的脱落量减少($P<0.05$)，而 Pgp4 突变株 L2Pgp4K13S 则未见此现象。免疫荧光和 WB 结果显示 L2Pgp4K13S 仍表达一定量的 Pgp3(虽较 L2wt 明显减少)，该微量表达可能足以维持其感染性。WB 结果还证实 *C. trachomatis* 糖原合酶 GlgA 的表达依赖 Pgp4 而非 Pgp3；Pgp4 缺陷株 L2Pgp4K13S 感染性与 L2wt 相当，提示 GlgA 可能不参与 L2 在小鼠上生殖道的感染。**结论：**Pgp3，而非 Pgp4、其他质粒编码基因或质粒调控的 *GlgA* 基因，对 *C. trachomatis* L2 在小鼠体内的感染发挥关键作用。此外，即便极低水平的 Pgp3 表达可能也足以维持 L2 在小鼠中的感染性。

[关键词] 沙眼衣原体；L2 血清型；转化；质粒；Pgp3；小鼠模型；感染性

Chlamydia trachomatis (*C. trachomatis*) is an obligate intracellular pathogen that causes chlamydia, a common sexually transmitted infection (STI) in humans^[1-3]. So far, 3 biovars of *C. trachomatis* have been identified. The ocular strains of trachoma biovar (serovars A to C), which are major causes of blindness worldwide, are particularly prevalent in Africa^[4]. The genital strains of trachoma biovar (serovars D to K) are the leading cause of pelvic inflammatory disease (PID), ectopic pregnancy (EP), and tubal factor infertility (TFI) in women^[5]. The third biovar includes serovars L1 to L3, which cause lymphogranuloma venereum (LGV)^[6]. Since 2003, outbreaks of LGV have emerged across Europe and other developed countries, particularly among HIV-positive men who have sex with men (MSM)^[7-11]. LGV strains can cause invasive infection and lead to severe clinical outcomes, including genital ulcer disease (GUD), lymphadenopathy, and proctocolitis^[6]. If not treated promptly, LGV-induced proctocolitis can progress to severe and systemic infection or develop into chronic colorectal fistulas and strictures^[6, 12].

Despite decades of intensive research and clinical trials, a vaccine for human *C. trachomatis* has not been approved, largely because the pathogenesis of chlamydial infection is still not fully understood^[13-15]. Historically, a significant obstacle was the lack of a convenient genetic tool for manipulating individual *C. trachomatis* genes. A major breakthrough in chlamydial transformation was achieved by Wang, et al^[16]. They demonstrated that an exogenous shuttle vector derived from *C. trachomatis* L2 (pBR325::L2) could be introduced into plasmid-free *C. trachomatis* organisms using a calcium chloride-mediated transformation method. Using penicillin for selection,

stable chlamydial transformants were successfully maintained and propagated for multiple generations. This highly efficient and reproducible method has since been adopted by numerous laboratories, including our own^[17-19]. Since then, transformation has been successfully performed in several mouse- and human-related strains, such as serovar D^[18] and *Chlamydia muridarum* (*C. muridarum*) Nigg strain^[20]. This approach has enabled the characterization of several plasmid-encoded proteins^[20]. For instance, plasmid-encoded glycoprotein (Pgp) 3 of *C. muridarum* has been identified as a critical factor in the development of upper genital pathology in mice^[20], while Pgp1, Pgp2, and Pgp6 appear to be essential for plasmid maintenance^[17, 21].

Animal models have long served as useful tools for investigating the pathogenesis of *Chlamydia*. One of the mouse strains, *C. muridarum* Nigg strain, has been widely used due to its ability to establish infection in the mouse lower genital tract and subsequently induce upper genital tract pathology, such as hydrosalpinx. The *C. muridarum*-caused hydrosalpinx in mice is of particular research interest as it is believed to closely mimic tubal pathology observed clinically in women^[22]. Using this animal model, numerous virulence factors of *Chlamydia* have been identified, including chlamydial protease-like activity factor (CPAF)^[23], Pgp3^[20, 24], CT228^[25], TC0668^[26]. Furthermore, multiple host signaling pathways or inflammatory mediators have been shown to play critical roles in chlamydial infection, ascension and pathology development. These include interleukin (IL)-1 α ^[27], IL-6^[28], IL-12^[29], toll-like receptors (TLRs)^[30], type I interferons (IFNs)^[31], cyclic GMP-AMP synthase (cGAS), and stimulator of interferon gene (STING)^[32]. The influence of different mouse genetic backgrounds on

Chlamydia infectivity has also been studied using this model^[22].

The *C. trachomatis* L2 mouse infection model is also a valuable model for studying chlamydial pathogenesis. Following transcervical/intrauterine inoculation, the L2 strain can establish infection in the mouse genital tract and cause mild uterine dilation and inflammation^[33-34]. To study the roles of individual Pgp proteins in the *C. trachomatis* L2 strain, Song, et al^[21] and Gong, et al^[17] generated a series of L2 mutants by transforming plasmid-free L2 (L2R) organisms with chlamydial plasmid-based shuttle vectors carrying targeted deletion mutations or premature stop codon mutations in each of the 8 *Pgp* genes. Although the plasmid of the murine strain *C. muridarum* has been found to facilitate chlamydial infection in mice, the role of *C. trachomatis* L2 plasmid is still largely unknown^[20]. To investigate the contribution of the L2 plasmid in chlamydial infection in the upper and lower genital tracts, we intravaginally or intrauterinely inoculated mice with L2R, wild type L2 (L2wt), or L2 expressing green fluorescent protein (GFP) (L2GFP) strain. This study aims to investigate the infectivity of these L2 mutants in a mouse model, which can help elucidate the contributions of individual Pgp proteins to the pathogenesis of the L2 strain.

1 Materials and methods

1.1 Ethics statements

The study protocol was approved by the Ethics Committee of Central South University (approval number: XMSB-2024-0649, XMSB-2024-0449).

1.2 Cell culture and chlamydial infection

HeLa cells (human cervical epithelial carcinoma cells, catalog #CCL2) and L929 cells [mouse fibroblast cells, National Cancer Institute Tissue Culture Collection (NCTC) clone 929, catalog #CCL-1] were originally purchased from American Type Culture Collection (ATCC), and passaged in the authors' laboratory according to the manufacturers' instructions. The *C. trachomatis* L2 wild-type (L2/LGV-434/Bu,

L2wt) organism was also purchased from the ATCC and propagated in the authors' laboratory. The plasmid-free (pf) *C. trachomatis* [L2 (25667R), L2R] organism was kindly provided by Julius Schachter from the University of California at San Francisco^[35-36]. All the *C. trachomatis* organisms were amplified in HeLa cells, purified, aliquoted, and stored as described previously^[37]. For in vitro infection, cells were grown in 24-well plates or 6-well plates with coverslips containing Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Germany) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, USA) at 37 °C in an incubator supplied with 5% CO₂, and the chlamydial organisms were inoculated according to the methods as described elsewhere^[17].

1.3 Construction of recombinant plasmids for transformation

The method of constructing each individual open reading frame (ORF) deletion mutant was described previously^[17]. Briefly, to construct a plasmid carrying an ORF deletion mutation, 2 DNA fragments (to cover the entire chlamydial plasmid but with desired ORF deletion) were amplified by PCR with 2 sets of paired primers, and the wild-type plasmid pGFP::SW2, which was kindly provided by Dr. Ian Clark^[16], was used as the template. The AccuPrime Pfx SuperMix (Life Technologies, Grand Island) enzyme, which demonstrated high fidelity and high specificity, was used for the PCR amplification. The PCR conditions were listed as follows: An initial denaturation step at 95 °C for 3 min followed by 35 cycles of amplification at 95 °C for 15 s, 55 °C for 30 s, 68 °C for 1 min per kb, and a final extension step at 68 °C for 10 min. After amplification, the PCR products were purified by using the GeneJET PCR purification kit (Fisher Scientific, Pittsburgh) and digested with FastDigest DpnI (Fisher Scientific, Pittsburgh) to remove the methylated template plasmid DNA. After that, an In-Fusion HD cloning kit (Clontech Laboratories Inc., Mountain View) was used to fuse the desired PCR products to create a new chlamydial plasmid according to the manufacturer's instructions. The fusion products were

then transformed into Stellar competent cells (included in the Clontech In-Fusion HD cloning kit), and the transformants were selected for ampicillin resistance on a Luria-Bertani (LB) agar plate. The bacterial colonies with positive green fluorescence were selected and grown on a new LB agar plate (with ampicillin selection pressure). Then, the plasmids were extracted from the colonies and transformed into *Escherichia coli* K-12 ER2925 (Dam⁻Dcm⁻ strain; New England BioLabs, Ipswich) for amplification. The final plasmids were extracted, subjected to DNA sequencing analyses, and used for the following chlamydial transformation.

To construct the plasmids harboring premature stop codon mutations in plasmid ORFs of *C. trachomatis* L2, the PCR-based site-directed mutagenesis method was used as described previously^[17]. Briefly, the QuikChange II site-directed mutagenesis kit (Agilent Technologies, Santa Clara) was used, and procedures were followed according to the manufacturer's instructions. Initially, 2 fragments of chlamydial plasmids carrying the desired site-directed mutations were amplified by AccuPrime Pfx SuperMix. The PCR conditions were used as follows: an initial denaturation step at 95 °C for 30 s followed by 18 cycles of amplification at 95 °C for 30 s, 55 °C for 1 min, 68 °C for 1 min per kb, and a final extension step at 68 °C for 10 min. After amplification, the PCR products were purified by using the GeneJET PCR purification kit. All the following steps were the same as those for the generation of ORF deletion mutants described above. Sequences of the newly constructed plasmids were confirmed by Sanger sequencing and used for the following chlamydial transformation. A complete list of primer sequences is available in the study of Gong, et al^[17].

1.4 Transformation of plasmid-free *C. trachomatis* L2R organisms

A previous study^[17] has demonstrated that *Pgp3* and *Pgp4* ORFs of murine strain *C. muridarum* contribute to chlamydial replication and pathology in the mouse genital tract. To study the role of individual plasmid-encoded ORFs in the L2 strain, we intrauterinely infected C3H/HeJ mice with L2 mutants carrying

deletion mutations in *Pgp3*, *Pgp4*, *Pgp5*, or *Pgp7* ORFs, alongside L2R, L2wt, and L2GFP as controls. Stable L2 transformants lacking *Pgp1*, *Pgp2*, *Pgp6*, or *Pgp8* ORFs could not be obtained, as these ORFs are required for maintaining the plasmid^[17]. Although complete deletion of individual plasmid encoding ORFs (pORFs) can provide valuable insights into their functions, such an approach does not distinguish between the roles of the DNA sequences and those of the encoded protein products. Therefore, we evaluated the infectivity of L2 mutants carrying a premature stop codon in the ORFs of *Pgp3* (L2Pgp3Y9S), *Pgp4* (L2Pgp4K13S), *Pgp5* (L2Pgp5L9S), and *Pgp7* (L2Pgp7L11S) ORFs in C3H/HeJ mice following intrauterine infection. Although *Pgp8* ORF has been found to be essential for the maintenance of the chlamydial plasmid, the Pgp8 protein itself was dispensable, unlike Pgp1, Pgp2, and Pgp6 proteins. Therefore, the L2Pgp8E11S mutant was included in this experiment.

All L2 mutant strains used in this study were constructed in our previous work^[17]. Briefly, a series of L2 mutants were generated by transforming L2R organisms with chlamydial plasmid-based shuttle vectors carrying either targeted deletion mutations or premature stop codon mutations in each of the 8 plasmid-encoded ORFs. The deletion mutants included L2ΔPgp3, L2ΔPgp4, L2ΔPgp5, and L2ΔPgp7. The premature stop codon mutants included L2Pgp3Y9S, L2Pgp4K13S, L2Pgp5L9S, L2Pgp7L11S, and L2Pgp8E11S. The complete list of strains used in this study, along with their relevant characteristics and construction strategies, is summarized in Table 1.

The transformation of plasmid-free *C. trachomatis* L2 organisms was performed following the CaCl₂-based transformation protocol developed by Wang, et al^[16] and described elsewhere^[17,20], with minor modifications. Briefly, 10 μL of L2R organisms [1×10⁷ inclusion-forming units (IFUs)] and 10 μL of plasmid DNA (7 μg) were mixed in a total volume of 200 μL of CaCl₂ buffer and incubated at room temperature (RT) for 45 min. Then, the freshly trypsinized L929 cells (6×10⁶ cells) were re-suspended in 200 μL of CaCl₂ buffer, added to the elementary bodies (EBs)-plasmid mixture, and

incubated for 20 min at RT with occasional mixing. For each well, 70 μ L of the final mixture was plated onto a single well of a six-well plate together with 1.5 mL of pre-warmed DMEM plus 10% FBS. The cells were allowed to adhere to the plate by incubation at 37 °C with 5% CO₂ without cycloheximide or ampicillin selection for the first 24 h. The next day, the cell cultures were supplied with fresh selection medium containing cycloheximide (2 μ g/mL) and ampicillin (5 μ g/mL). The plate was incubated for an additional

24 h. The transformant colonies with positive green fluorescence were located under a fluorescence microscope, then picked and grown in a new cell culture plate under the selection pressure of ampicillin (20 μ g/mL). After 3 to 4 generations of passages with selection medium containing ampicillin (20 μ g/mL), *C. trachomatis* organisms were purified through a plaque cloning assay as previously described^[38]. Finally, the plaque-cloned organisms were amplified in HeLa cells for the following experiments.

Table 1 Strains of *C. trachomatis* and their characteristics and construction strategies

Strain	Genotype	Description	Construction method	Reference or source
L2wt	Wild type	Wild type <i>C. trachomatis</i> serovar L2	Purchased from ATCC	ATCC (L2/LGV-434/Bu)
L2R	pf	Mutant L2 strain lacking plasmid	Naturally occurring mutant	Carlson, et al ^[35]
L2GFP	<i>GFP</i> insertion	Engineered L2 strain with inserted <i>GFP</i> gene in wild-type plasmid backbone	Transformation of L2R with a shuttle vector carrying wild-type L2 plasmid inserted with <i>GFP</i> gene	Gong, et al ^[17]
L2 Δ Pgp3	<i>Pgp3</i> ORF deletion	L2 mutant with <i>Pgp3</i> ORF deletion	A shuttle vector carrying mutant L2 plasmid was made by In-Fusion cloning, then transformed to L2R.	Gong, et al ^[17]
L2 Δ Pgp4	<i>Pgp4</i> ORF deletion	L2 mutant with <i>Pgp4</i> ORF deletion	Same as L2 Δ Pgp3	Gong, et al ^[17]
L2 Δ Pgp5	<i>Pgp5</i> ORF deletion	L2 mutant with <i>Pgp5</i> ORF deletion	Same as L2 Δ Pgp3	Gong, et al ^[17]
L2 Δ Pgp7	<i>Pgp7</i> ORF deletion	L2 mutant with <i>Pgp7</i> ORF deletion	Same as L2 Δ Pgp3	Gong, et al ^[17]
L2Pgp3Y9S	<i>Pgp3</i> premature stop codon	L2 mutant with a stop codon substitution at the 9th codon of the <i>Pgp3</i> ORF	A shuttle vector carrying mutant L2 plasmid was made by quick-change cloning, then transformed to L2R	Gong, et al ^[17]
L2Pgp4K13S	<i>Pgp4</i> premature stop codon	L2 mutant with a stop codon substitution at the 13th codon of the <i>Pgp4</i> ORF	Same as L2Pgp3Y9S	Gong, et al ^[17]
L2Pgp5L9S	<i>Pgp5</i> premature stop codon	L2 mutant with a stop codon substitution at the 9th codon of the <i>Pgp5</i> ORF	Same as L2Pgp3Y9S	Gong, et al ^[17]
L2Pgp7L11S	<i>Pgp7</i> premature stop codon	L2 mutant with a stop codon substitution at the 11th codon of the <i>Pgp7</i> ORF	Same as L2Pgp3Y9S	Gong, et al ^[17]
L2Pgp8E11S	<i>Pgp8</i> premature stop codon	L2 mutant with a stop codon substitution at the 11th codon of the <i>Pgp8</i> ORF	Same as L2Pgp3Y9S	Gong, et al ^[17]

Stable L2 transformants lacking *Pgp1*, *Pgp2*, *Pgp6*, or *Pgp8* ORFs could not be obtained, therefore not included in this study. *C. trachomatis*: *Chlamydia trachomatis*; ATCC: American Type Culture Collection; pf: Plasmid-free; *GFP*: Green fluorescent protein; ORF: Open reading frame; *Pgp*: Plasmid-encoded glycoprotein.

1.5 *C. trachomatis* L2 strain infection in mice

It has been reported that the genetic background of a mouse strain also influences susceptibility to chlamydial infection^[22]. To systematically evaluate the infectivity of different L2 mutants *in vivo*, we performed intrauterine and intravaginal infection experiments in 2 mouse strains, C3H/HeJ and CBA/J.

For the intravaginal infection experiment in C3H/HeJ mice, animals were divided into 3 groups ($n=5$ per group) and inoculated with L2R, L2wt, or L2GFP, respectively.

For the intrauterine infection experiments in C3H/HeJ mice, 2 separate sets of mutants were evaluated ($n=5$ per group for each set): 1) Deletion mutants, including L2 Δ Pgp3, L2 Δ Pgp4, L2 Δ Pgp5, and L2 Δ Pgp7, alongside L2R, L2wt, and L2GFP as controls; 2) premature stop codon mutants, including L2Pgp3Y9S, L2Pgp4K13S, L2Pgp5L9S, L2Pgp7L11S, and L2Pgp8E11S, with the same controls.

To further confirm the effect of Pgp3 on chlamydial infection across different genetic backgrounds, we repeated the intrauterine infection experiments in CBA/J mice using L2R, L2wt, L2GFP, L2Pgp3Y9S, L2Pgp4K13S, and L2Pgp7L11S ($n=5$ per group).

Female C3H/HeJ (stock number, #000659) and CBA/J (stock number, #000656) mice were purchased from the Jackson Laboratory. Five days prior to infection, each mouse was injected subcutaneously with 2.5 mg medroxyprogesterone acetate (Depo-Provera; Pharmacia Upjohn, Kalamazoo). On the day of infection, mice were subjected to intravaginal or intrauterine inoculation with 1.0×10^6 IFUs of the corresponding chlamydial organisms in a volume of 30 μ L (intravaginal) or 10 μ L (intrauterine) of sucrose-phosphate-glutamate (SPG) buffer. For intrauterine inoculation, a midline abdominal incision was made under sterile conditions, and the inoculum was injected into each uterine horn via a 30-gauge needle. The incision was then closed with surgical sutures.

1.6 Live chlamydial organism shedding from the mouse lower genital tract

To monitor live organism shedding from the mouse lower genital tract, vaginal swabs were taken every 2 to

4 d for the first 3 weeks and weekly thereafter until negative shedding results were observed for 2 consecutive time points. Briefly, a small sterile polyester-tipped swab applicator (Fisher Scientific, #22-029-503) was gently inserted into the mouse vaginal vault and rotated several times against the vaginal wall. The swab was soaked in 0.5 mL of SPG buffer and vortexed with glass beads. The live chlamydial organisms in each swab were quantitated using an immunofluorescence assay as described previously^[20]. The calculated total number of IFUs per swab was converted into a log₁₀ value and presented as the mean and standard deviation.

1.7 Indirect immunofluorescence assay

The chlamydial EBs and Pgp3 expression in the *C. trachomatis*-infected cells were analyzed by an indirect immunofluorescence assay as described elsewhere^[39]. Briefly, the HeLa cells infected with *C. trachomatis* or mock-infected were fixed at 30 h post-infection with 2% paraformaldehyde dissolved in phosphate-buffered saline (PBS) for 30 min at RT, followed by permeabilization with 2% saponin (Sigma-Aldrich, St. Louis) for an additional 30 min. 1% bovine serum albumin (BSA) was used for blocking nonspecific binding. After blocking, the cell samples were incubated with primary and secondary antibodies, followed by chemical staining. The mouse anti-Pgp3 (clone #2H4) plus a goat anti-mouse IgG secondary antibody conjugated with Alexa Fluor 594 (red; Jackson ImmunoResearch, West Grove), and the rabbit anti-chlamydial organism antibody (R1L2)^[39] plus a goat anti-rabbit IgG secondary antibody conjugated with Alexa Fluor 488 (green; Jackson ImmunoResearch, West Grove) were used to visualize the Pgp3 protein and chlamydial EBs, respectively. Hoechst 33258 (blue; Sigma-Aldrich, St. Louis) was used to visualize DNA. The immunofluorescence images were acquired by using an Olympus AX-70 fluorescence microscope equipped with multiple filter sets and Simple PCI imaging software, as described previously^[39].

1.8 Western blotting

A Western blotting (WB) assay was carried out as

described elsewhere^[40-41]. Briefly, HeLa cells infected with different strains of *C. trachomatis* L2 organisms were harvested at 48 h post-infection and lysed in 8 mol/L urea supplemented with 325 U/mL of Benzonase nuclease (both urea and nuclease were from Sigma-Aldrich, USA). The samples were resolved in a sodium dodecylsulfate (SDS)-polyacrylamide gel after each lane was loaded with 2 µg of total proteins. The resolved protein bands were transferred onto nitrocellulose (NC) membrane for detection of the expression of various proteins. The bound primary antibodies were probed with a horseradish peroxidase (HRP) -conjugated secondary antibody (Jackson ImmunoResearch, USA) and visualized with an enhanced chemiluminescence (ECL) kit (Santa Cruz Biotechnology, USA). The following primary antibodies were used for WB assays in the current study: mouse polyclonal antibody (pAb) against Pgp3, mouse pAb against glycogen synthase A (GlgA), and mouse mAb (clone #MC22) against chlamydial major outer membrane protein (MOMP)^[40].

1.9 Statistical analysis

Statistical analyses were performed using SPSS 26.0 software. Continuous variables were tested for normality. Data with a normal distribution were presented as mean±standard. For comparisons between the 2 groups at each time point, the nonparametric Wilcoxon rank-sum test was used. All experiments were repeated at least twice independently, with representative results shown. A two-tailed $P < 0.05$ was considered statistically significant.

2 Results

2.1 L2 plasmid plays an important role in chlamydial infectivity in the upper genital tract but not in the lower genital tract in mice

Following intravaginal infection in C3H/HeJ mice, the shedding courses in the lower genital tract were similar among mice infected with all 3 L2 strains. Shedding remained detectable up to 28 d post-infection and became undetectable after 35 d post-infection. There was no significant difference in shedding between mice

inoculated with L2R and L2wt ($P > 0.05$). Mice inoculated with L2GFP showed increased shedding compared to those infected with L2R. The most pronounced difference in shedding between L2R- and L2GFP-infected mice was observed on days 10 and 14 post-infection (all $P < 0.05$, Figure 1). In contrast, after intrauterine inoculation, the shedding was significantly lower in L2R-infected mice than in L2wt- and L2GFP-infected mice ($P < 0.05$, Figure 1). After intrauterine infection, the shedding of the L2wt strain was detected for up to 42 d post-infection (Figure 1E), whereas shedding of the L2R strain was only detectable up to day 14 post-infection (Figure 1D).

2.2 Pgp3 and Pgp4 gene deletions led to significantly impaired infectivity in mice

The results (Figure 2) showed that deletion of either *Pgp3* or *Pgp4* ORF significantly attenuated the shedding of live organisms in the mice after intrauterine infection (both $P < 0.05$), whereas deletion of *Pgp5* or *Pgp7* ORF did not significantly affect shedding compared with that in L2GFP-infected mice (both $P > 0.05$). Shedding of live organisms from L2GFP-infected mice was detectable up to 56 d post-infection. In contrast, shedding from mice infected with L2Δ*Pgp3* or L2Δ*Pgp4* became undetectable after 35 d post-infection, with L2Δ*Pgp4* showing a marked decrease in live organism shedding as early as 7 d post-infection.

2.3 Premature stop codon mutation in Pgp3 but not Pgp4 significantly reduced the infectivity of the L2 strain in mice

The results (Figure 3) showed that L2Pgp3Y9S exhibited significantly reduced shedding compared with L2GFP ($P < 0.05$), consistent with the phenotype observed for L2Δ*Pgp3*. However, unlike L2Δ*Pgp4*, the L2Pgp4K13S displayed a shedding course similar to L2GFP. Consistent with the findings for L2Δ*Pgp5* and L2Δ*Pgp7*, neither L2Pgp5L9S nor L2Pgp7L11S showed significant differences in shedding compared with L2GFP (both $P > 0.05$). Similarly, L2Pgp8 appeared to be dispensable for L2 replication in vivo, as its shedding profile was similar to that of L2GFP.

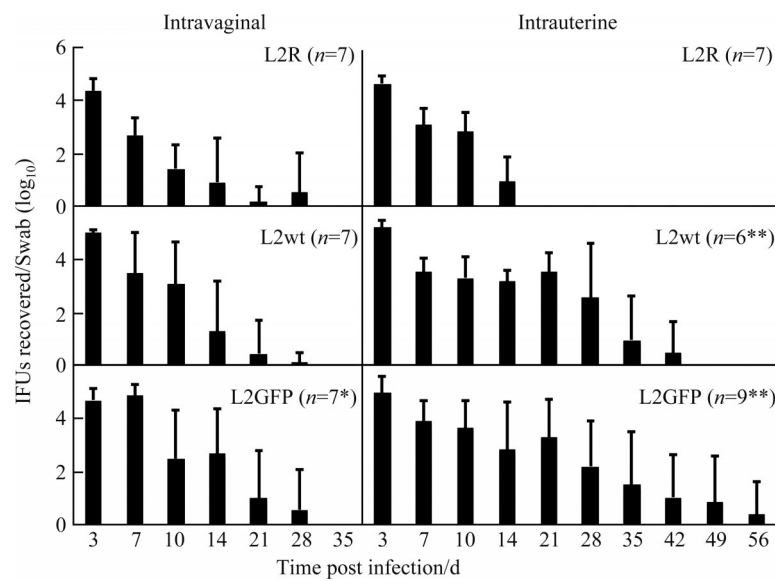


Figure 1 Shedding patterns of L2R, L2wt, or L2 transformant carrying a GFP plasmid after intravaginal or intrauterine infection in C3H/HeJ mice

C3H/HeJ mice were intravaginally infected with L2R, L2wt, and L2GFP, and intrauterinely infected with L2R, L2wt, and L2GFP for monitoring the live organism shedding from the lower genital tract. Compared with L2R, * $P<0.05$, ** $P<0.01$. L2R: Plasmid-free L2; L2wt: Wild type L2; GFP: Green fluorescent protein; L2GFP: L2 expressing GFP; IFUs: Inclusion-forming units; *C. trachomatis*: *Chlamydia trachomatis*.

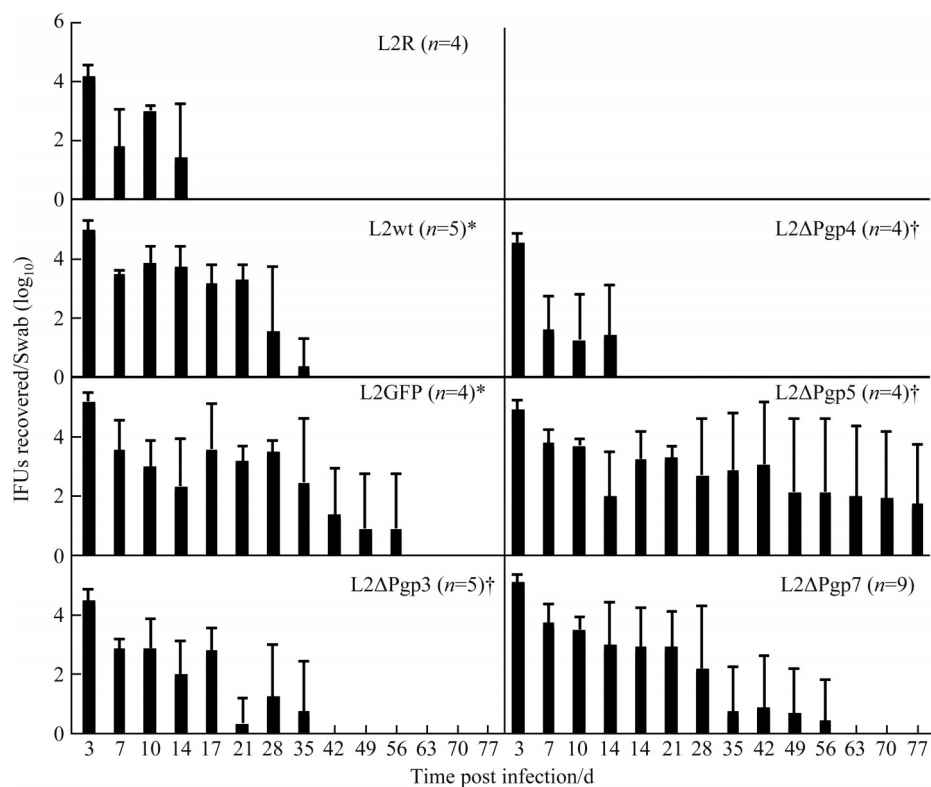


Figure 2 Shedding patterns of L2R, L2wt, L2GFP and L2 transformants carrying deletion mutations in individual *Pgp* ORFs following intrauterine infection in C3H/HeJ mice

C3H/HeJ mice were intrauterinely infected with L2R, L2wt, L2GFP, or L2 mutants harboring deletions in specific *Pgp* ORFs, including the *Pgp3* deletion (L2ΔPgp3), *Pgp4* deletion (L2ΔPgp4), *Pgp5* deletion (L2ΔPgp5), and *Pgp7* deletion (L2ΔPgp7). Compared with L2R, * $P<0.05$. Compared with L2GFP, † $P<0.05$. Pgp: Plasmid-encoded glycoprotein; ORFs: Open reading frames.

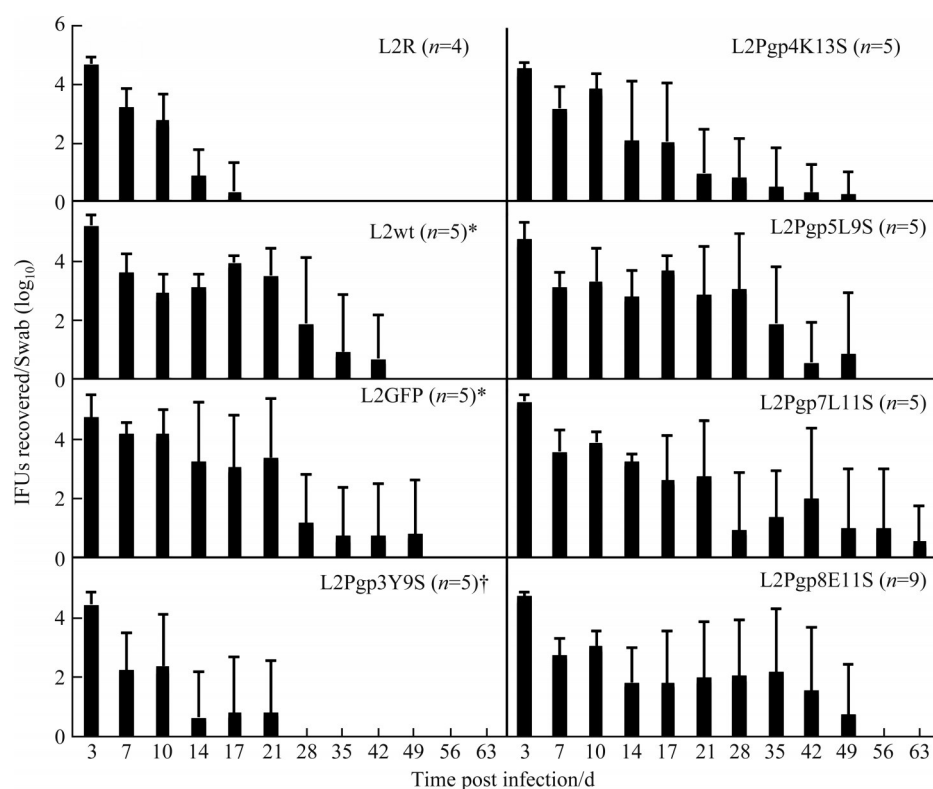


Figure 3 Shedding patterns of L2R, L2wt, L2GFP and L2 transformants carrying premature stop codon in different *Pgp* ORFs following intrauterine infection in C3H/HeJ mice

C3H/HeJ mice were intrauterinely infected with L2R, L2wt, L2GFP, or one of the pORF premature stop codon mutants, including L2Pgp3Y9S, L2Pgp4K13S, L2Pgp5L9S, L2Pgp7L11S, and L2Pgp8E11S. Compared with L2R, * $P < 0.05$. Compared with L2GFP, † $P < 0.05$. pORF: Plasmid encoding open reading frames.

2.4 Infectivity of L2Pgp3Y9S was significantly impaired in CBA/J mice

Consistent with the observations in C3H/HeJ mice, L2Pgp3Y9S ($P < 0.05$), but not L2Pgp4K13S or L2Pgp7L11S (both $P > 0.05$), exhibited significantly lower shedding compared with L2GFP in CBA/J mice (Figure 4). These findings suggest that the contribution of Pgp3 to L2 infectivity is not unique to a single mouse strain, but represents a general phenotype.

2.5 L2 mutant with premature stop codon in the Pgp4 ORF still exhibits minimal Pgp3 expression in vitro

In C3H/HeJ mice, we found that L2ΔPgp4, but not L2Pgp4K13S, showed reduced replication after intrauterine infection. To investigate the mechanism underlying this discrepancy, we analyzed the Pgp3 protein levels in HeLa cells infected with L2 mutants carrying premature stop codon in different plasmid-encoded ORFs. The immunofluorescence staining and WB analysis showed that no detectable Pgp3 expression

was observed in cells infected with L2R and L2Pgp3Y9S (Figure 5). In contrast, L2Pgp7L11S showed Pgp3 expression levels comparable to those of L2wt and L2GFP, indicating Pgp7 does not affect Pgp3 expression. Notably, Pgp3 remained detectable in HeLa cells infected with L2Pgp4K13S mutant, although its signal intensity was weaker than that observed in L2wt- and L2GFP-infected cells (Figure 5A). This phenotype was further confirmed by WB analysis (Figure 5B). These results suggest that the minimal expression of Pgp3 in L2Pgp4K13S-infected cells may be important in maintaining *C. trachomatis* infectivity of the L2 strain.

Pgp4 also acts as a positive regulator of chlamydial glycogen synthase GlgA expression. Indeed, we also observed that GlgA expression in L2Pgp4K13S-infected cells was significantly reduced compared with L2wt and L2GFP (Figure 5B). Since L2Pgp4K13S showed a shedding course similar to L2GFP, GlgA, whose expression was dependent on Pgp4, may not be involved in chlamydial infectivity.

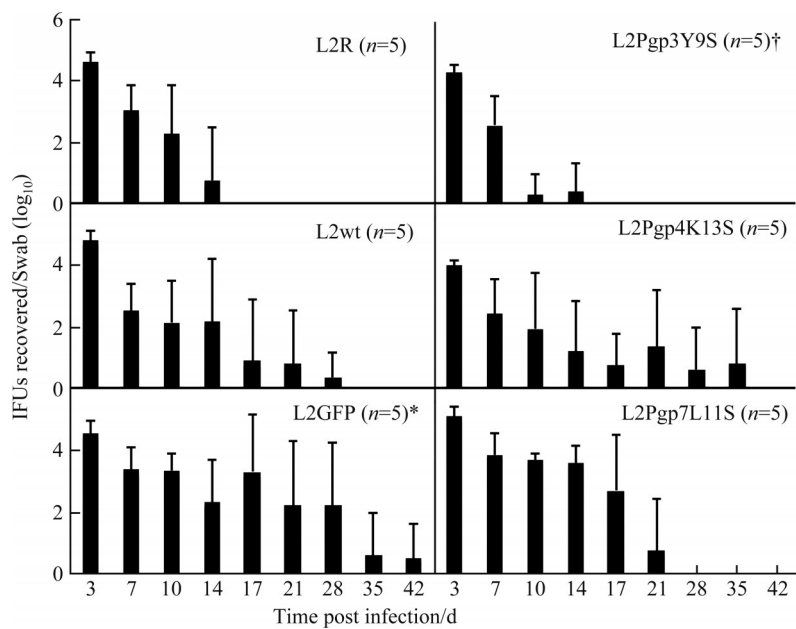


Figure 4 Shedding courses of L2R, L2wt, L2GFP and L2 transformants carrying premature stop codon in *Pgp3*, *Pgp4* or *Pgp7* ORFs following intrauterine infection in CBA/J mice
CBA/J mice were intrauterinely infected with L2R, L2wt, L2GFP, or one of the 3 pORF premature stop codon mutants, including L2Pgp3Y9S, L2Pgp4K13S, and L2Pgp7L11S. Compared with L2R, * $P<0.05$; compared with L2GFP, † $P<0.05$.

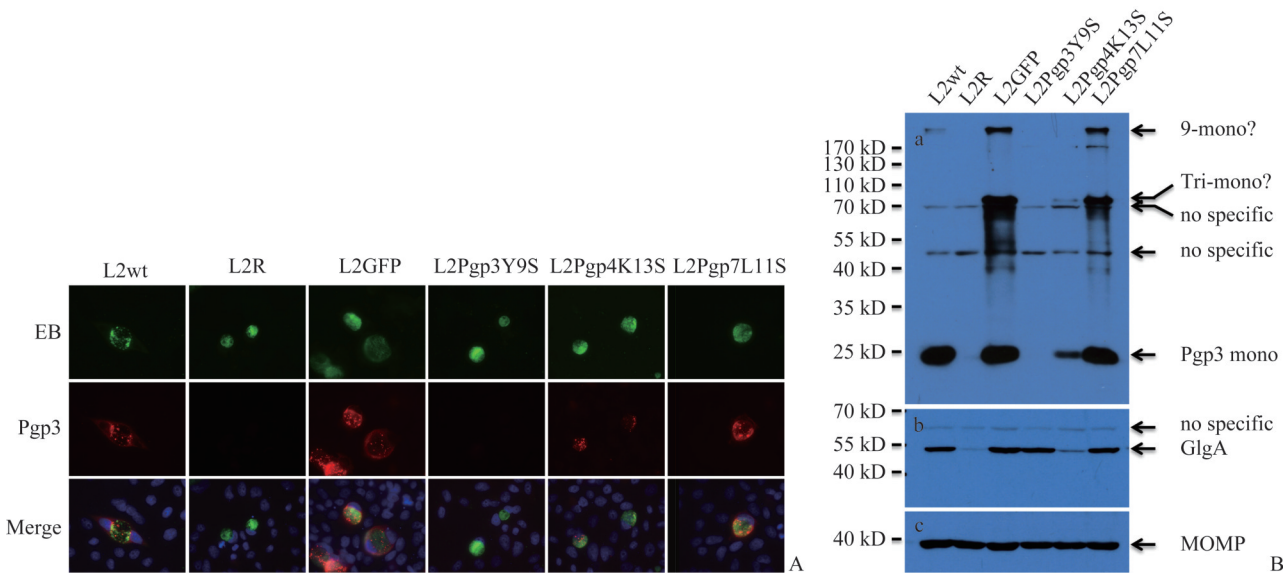


Figure 5 Protein levels of Pgp3 and GlgA in HeLa cells infected with L2wt, L2R or L2 mutants with premature stop codon in different plasmid ORFs
A: HeLa cells were infected with L2wt, L2R, L2GFP, or one of 3 L2 premature stop codon mutants (L2Pgp3Y9S, L2Pgp4K13S, and L2Pgp7L11S). The infected cells were stained with a rabbit anti-chlamydial organism antibody (green), a mouse anti-*C. trachomatis* Pgp3 antibody (red), and a DNA dye (blue) at a magnification of 100×. B: HeLa monolayer cells grown in 24-well plates were infected with L2wt, L2R, L2GFP, and different L2 premature stop codon mutants. 48 h post-infection, the cultures were harvested for WB to detect Pgp3, GlgA and MOMP as a loading control. WB: Western blotting; MOMP: Major outer membrane protein. EB: Elementary body; GlgA: Glycogen synthase A.

3 Discussion

C. muridarum mouse model has been widely used to study chlamydial pathogenesis and host immune responses in vivo^[20-22, 42]. A caveat of this model is that *C. muridarum* is a mouse pathogen with tropism specific to mice and does not cause obvious immunopathology in humans^[43]. Human-related chlamydial strains, such as serovar D and serovar L2, are unable to establish long-lasting infection or induce upper genital tract disease in mice after intravaginal infection^[23, 44]. Nevertheless, after intrauterine/transcervical inoculation, which bypasses the cervical barrier, these 2 human serovars can successfully establish infection in mice^[23, 44]. It has been reported that the chlamydial protease CPAF from serovar L2 promotes chlamydial survival in the mouse lower genital tract, but not in the upper genital tract^[23, 45]. In this study, we compared the contribution of the chlamydial plasmid during L2 infection after either intravaginal or intrauterine inoculation. Interestingly, after intravaginal infection, the plasmid-free strain L2R exhibited a shedding profile similar to that of L2wt. In contrast, after intrauterine infection, L2R showed significantly decreased shedding compared with L2wt. Previous studies^[20, 46] have demonstrated that the plasmid of murine strain *C. muridarum* plays a critical role during infection of the lower genital tract. After intravaginal infection, the plasmid-free organism (pf) showed a significantly shorter shedding course than wt and failed to induce upper genital pathology including hydrosalpinx^[20, 46]. These results suggest that the plasmid of *C. muridarum* may promote chlamydial survival in the lower genital tract and ascension during in vivo infection^[46]. Our study focuses on the plasmid of serovar L2. Our results indicate that the Pgp3 protein of human *C. trachomatis* serovar L2 may function differently from that of murine *C. muridarum* strain. The plasmid of serovar L2 may utilize Pgp3 to evade mucosal immunity in the upper genital tract, but not in the lower genital tract.

The advent of a highly efficient transformation technique has enabled researchers to introduce modified plasmids into live *Chlamydia* organisms^[1-18, 20-21]. With this method, characterization of different plasmid-encoded ORFs became possible^[17, 20-21]. To dissect the role of each individual gene in L2 plasmid, we used a

set of L2 mutants each carrying deletion mutations or premature stop codon mutations in individual plasmid ORFs. Although these mutants have been characterized in vitro^[17], their infectivity in animals has not been extensively studied. Results from this study showed that deleting either *Pgp3* or *Pgp4* led to significantly reduced infectivity in mice when inoculated via the intrauterine route. However, only the *Pgp3* premature stop codon mutant (L2Pgp3Y9S), but not the *Pgp4* premature stop codon mutant (L2Pgp4K13S) showed significantly reduced infectivity in mice compared with L2GFP. These results suggest that Pgp3 is a predominant virulence factor for *C. trachomatis* L2 organism infection in mice. Pgp4 has been identified as a positive regulator of Pgp3 expression and is required for Pgp3 secretion^[47]. WB data showed that the L2Pgp4K13S still expressed detectable levels of Pgp3, although at levels lower than those of L2wt. These results suggest that Pgp4 may indirectly facilitate L2 infection by upregulating Pgp3 expression and secretion. These data are consistent with previous reports showing that *C. muridarum* Pgp3 is a major virulence factor for chlamydial infection in mice^[20], whereas Pgp4 is believed to be a master transcriptional regulator for multiple chlamydial genes, including *Pgp3*^[21]. Liu, et al^[20] reported that a Pgp3-deficient *C. muridarum* strain showed significantly reduced shedding and induced significantly less severe hydrosalpinx and inflammatory infiltration in the mouse oviduct. The precise mechanism by which Pgp3 overcomes host defenses to promote chlamydial survival and ascension remains unclear. One proposed mechanism is that Pgp3 may neutralize the anti-chlamydial activity of host antimicrobial peptides, such as cathelicidin LL-37^[48]. Further mechanistic studies, both in vitro and in vivo, are warranted.

Chen, et al^[22] demonstrated that different mouse strains exhibit varying levels of susceptibility to chlamydial infection and upper genital tract pathology. Specifically, C3H/HeJ mice, which carry a TLR4 loss-of-function mutation on the BALB/c background, exhibit reduced inflammatory responses, whereas CBA/J mice harbor a different genetic background and have been reported to be more susceptible to chlamydial infection. To determine whether the observed effect of Pgp3 on chlamydial infectivity is not limited to a single

mouse strain but represents a general phenotype, we performed the intrauterine infection experiments in both C3H/HeJ and CBA/J mice. The consistent results obtained from two genetically distinct mouse strains strengthen the conclusion that Pgp3 protein indeed promotes L2 infectivity in vivo. This approach aligns with recommendations from previous studies emphasizing the importance of using multiple mouse strains to account for host genetic variability in chlamydial research^[49].

C. trachomatis GlgA has been identified as a *chlamydia*-secreted protein during chlamydial infection^[50]. GlgA is thought to play a crucial role in regulating chlamydial glycogen synthesis and utilization. Previous studies^[21, 50] in *C. muridarum* reported that GlgA expression is positively regulated by Pgp4, but negatively regulated by Pgp5. In addition, GlgA has been proposed to function as a virulence factor promoting mouse hydrosalpinx during *C. muridarum* infection^[51]. In the case of *C. trachomatis* serovar L2, deletion of *Pgp4* led to the loss of GlgA expression in vitro. However, the loss of GlgA expression caused by Pgp4 deficiency did not impair the infectivity of L2 in mice. Therefore, GlgA may not be important during L2 infection in the mouse upper genital tract. However, additional studies, including direct *GlgA* gene knockout and knock-in experiments in the L2 strain are needed to further verify this observation.

It should be noted that, not only mice, but also many other animals infected with L2wt do not develop obvious pathology in the upper genital tract. Only limited and transient inflammatory responses have been observed in the infected tissues^[52-54]. This observation is consistent with the human clinical diseases caused by serovar L2, which are characterized by severe inflammation in lymph nodes resulting in GUD, lymphadenopathy, and proctocolitis, rather than female reproductive tract sequelae. Recently, intragastric and intrarectal inoculations have been established and widely used^[55-59]. These methods would provide valuable opportunities to investigate host immune responses, pathogenesis, and immunogenicity during *C. trachomatis* infection in mice. Such approaches are more clinically relevant, as *C. trachomatis* serovar L2 is a predominant pathogen transmitted through anorectal routes among MSM populations, and therefore warrant

further investigation in future studies.

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