

Evaluating Potential Vaccine Antigens in both the *Chlamydia trachomatis* and *Chlamydia muridarum* Intravaginal Mouse Challenge Models

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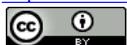
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Abstract

Identifying relevant animal challenge models adds to the complexity of human vaccine development. Murine challenge models have been the most utilized animal model for *Chlamydia trachomatis* vaccine development. The question arises as to whether the *C. trachomatis* or *C. muridarum* pre-clinical model is optimal. We compared *C. muridarum* and *C. trachomatis* intravaginal challenge models in a combined total of seventy-five studies evaluating potential vaccine candidates. In 100% (42/42) of *C. muridarum* studies, mice immunized with Chlamydia elementary bodies (EB) demonstrated a significant reduction in urogenital bacterial shedding as measured by qPCR ($p < 0.05$) compared to adjuvant-control-immunized mice. Significant reduction in urogenital shedding was observed for EB-immunized groups in only 82% (27/33) of *C. trachomatis* studies. We have evaluated proposed vaccine antigens in both models and observed immunization with Chlamydia major outer membrane protein (MOMP) vaccine formulations to be protective ($p < 0.05$) in both models, immunization with polymorphic membrane protein serovar D (PmpD) p73 passenger domain was protective only in the *C. trachomatis* model, and immunization with PmpD p82 translocator domain was not protective in either model. We also observed in both models that depletion of CD4⁺ T-cells in MOMP-immunized mice resulted in diminished protective immunity but animals were still able to reduce the infection level. In contrast, mice immunized with live EBs by intraperitoneal route did not require CD4⁺

T-cells to resolve urogenital infection from intravaginal challenge in either model. Overall, we have found the *C. muridarum* model to be a more robust, reliable, and reproducible model for vaccine antigen discovery.

Keywords

Chlamydia, Mouse Challenge Model, Intravaginal Challenge, Sexually Transmitted Diseases

1. Introduction

Chlamydia trachomatis is the most commonly reported disease in the United States and among the most prevalent sexually transmitted diseases. Greater than 1.6 million cases in the US and up to 131 million cases worldwide are reported annually [1] [2] [3]. Treatment is available but long-term damage to reproductive organs is common, as on average >70% of urogenital infections remain asymptomatic and are therefore largely undiagnosed [4] [5] [6]. A *Chlamydia trachomatis* vaccine would address a major global unmet medical need.

Several animal models have been developed to evaluate Chlamydia infections and potential vaccines. The most common is the mouse model in which two different species of Chlamydiae are used: *Chlamydia muridarum*, originally isolated from the lungs of reportedly normal albino Swiss mice [7] [8], and human-derived *Chlamydia trachomatis* strains. Neither mouse model completely mimics *C. trachomatis* infection in women.

C. muridarum administered intravaginally in mice readily ascends to the upper genital tract [9] [10]. The subsequent pathology of uterine horn dilatation, hydrosalpinx, and sterility is representative of that seen in some women with post-chlamydial infection sequelae [9] [11]. *C. trachomatis* ascension, though, is not consistent in mice. Studies have shown ascension is dependent on serovar [12] [13], mouse strain [14], or requires multiple daily inoculations intravaginally [15]. In contrast to *C. muridarum* infection, *C. trachomatis* urogenital infection in mice normally resolves with no pathology or obvious tissue damage [12] [15] [16] [17] similar to the majority of infections in women. Low to moderate numbers of infectious bacteria are shed in women and mice following *C. trachomatis* infection [16] [18]. In contrast, *C. muridarum* infection produces approximately 100-fold higher shedding of infectious bacteria in mice compared with *C. trachomatis* infection [12] [16] [19]. In women, *C. trachomatis* infection can become chronic and may persist for months [20] [21] [22]. In mice, chlamydial infection does not persist and usually resolves within 4 weeks, following either *C. trachomatis* or *C. muridarum* challenge [23]. Following *C. muridarum* infection, mice develop long lasting protective immunity [9] [16] which protects from reinfection. In contrast, reinfection can occur following *C. trachomatis* infection in women or mice [16] [24].

We evaluated potential vaccine antigens in both the *C. trachomatis* and *C.*

muridarum intravaginal mouse challenge models. We found the *C. muridarum* model to be a more robust, reliable, and reproducible model. A greater percentage of the *C. muridarum* studies, compared to the *C. trachomatis* studies, demonstrated significantly reduced vaginal shedding of Chlamydia in a live EB-immunized positive control group following intravaginal challenge with the homologous strain. We also observed that following *C. muridarum* challenge, more mice in the adjuvant-control groups were infected on day 7, as determined by qPCR, compared to mice challenged with *C. trachomatis* serovar D. This demonstrates the *C. muridarum* challenge model has a broader therapeutic window for the evaluation of vaccine candidates. This finding increases confidence in results obtained with potential vaccine candidates in the *C. muridarum* model, and therefore in the model's ability to differentiate between candidates. We also sought to evaluate the role of CD4⁺ T-cells in protection from primary challenge following native MOMP (nMOMP) or live EB-immunization in both the *C. muridarum* and *C. trachomatis* mouse challenge models with C57BL/6 mice. We observed that depletion of CD4⁺ T-cells in nMOMP-immunized mice resulted in diminished protective immunity but the animals were still able to reduce the infection level in both challenge models. In contrast, the live EB-immunized mice did not require CD4⁺ T-cells to resolve urogenital infection from intravaginal challenge in either model.

2. Methods

2.1. Ethics Statement

All animal experiments were approved by the Merck & Co., Inc., Kenilworth, NJ, USA, Institutional Animal Care and Use Committee (IACUC). All procedures were performed in accordance with our institution's IACUC guidelines in strict accordance with the recommendations in the Guide for Care and Use of Laboratory Animals of the National Institutes of Health.

Mice were housed in large mouse containers (n = 10 mice/box) with micro isolator lids, and the rooms were maintained with controlled humidity and temperature, and 12-hour light-dark cycles. All containers had nestlets and animals were provided standard chow (Purina 5001 rodent diet) and water ad libitum. The physical condition of the animals was monitored daily (7 days a week), and any health changes were noted.

At the end of the study, animals were euthanized using CO₂ inhalation (10% - 30% CO₂ in the air mixture inhaled per minute). No animals were found to be moribund throughout the study.

2.2. Cell Culture and Propagation of *Chlamydiae*

HeLa 229 (ATCC, Manassas, VA) cells were used for propagation of both Chlamydia strains. HeLa 229 cells were grown in Eagle's Minimal Essential Medium (EMEM, ATCC) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT), 50 µg/mL vancomycin (Sigma, St. Louis, MO), and

10 µg/mL gentamicin (Invitrogen, Carlsbad, CA). Host cells were seeded into tissue culture flasks at a cell density of 5×10^5 cells/mL and incubated overnight at 37°C in 5% CO₂ to achieve a confluent monolayer. Cell monolayers were treated with 45 µg/ml DEAE-Dextran Hydrochloride (Sigma) in Hank's Balanced Salt Solution (HBSS) for 10 minutes at 37°C in 5% CO₂ with slow rocking and then washed with HBSS. *Chlamydia trachomatis*, serovar D (strain UW-3/Cx) (ATCC) or *Chlamydia muridarum* (obtained from Dr. Bernard Arulanandam, University of Texas at San Antonio) diluted in sucrose-phosphate-glutamate (SPG; Boston BioProducts, Ashland, MA) buffer at an MOI 1:1 and were overlaid onto the monolayer and incubated for 2 hours at 37°C in 5% CO₂ with slow rocking. Cycloheximide (Sigma-Aldrich), at 2 µg/ml in media, was added to the flasks without removing the inoculum. Incubations continued at 37°C in 5% CO₂ without rocking for 44 - 48 hours. The Chlamydiae were harvested from the infected cells and purified by centrifugation through 30% MD-76[®]R (Mallickrodt, St. Louis, MO) and stored frozen at -80°C. Chlamydiae were either propagated and purified in-house or by Dr. Robert Suchland, University of Washington (Seattle, WA).

2.3. Protein Purification

C. muridarum or *C. trachomatis* serovar D native MOMP (nMOMP) was purified using a modified methodology from Pal *et al.* [25]. nMOMP was extracted from intact Chlamydia-infected HeLa cells using sonication. Initial steps used phosphate buffer with CHAPS (Calbiochem, La Jolla, CA) and 100 mM DTT (Acros Organics, Fair Lawn, NJ) and subsequently a phosphate buffer containing Zwittergent 3-14 (Calbiochem) with 100 mM DTT. nMOMP was further purified post-extraction with hydroxyapatite chromatography (Bio-Rad, Hercules, CA). The hydroxyapatite product was concentrated using tangential flow filtration (TFF), and then purified with anion exchange chromatography (Pall, Port Washington, NY). This process yields a product with purities of up to >99% by LC/MS. nMOMP was dialyzed into 10 mM 2-(N-morpholino) ethanesulfonic acid (MES) pH 7.3 (Sigma), 150 mM NaCl (Sigma-Aldrich), 0.1% Zwittergent 3-14 for use in animal immunization and in vitro studies. PmpD p73 passenger domain (amino acids 53 - 757), and PmpD p82 translocator domain (amino acids 758 - 1520) from *C. muridarum* or *C. trachomatis* serovar D gene sequences were synthesized by Genart (Thermo Fisher, Germany) and individually cloned into pET28a vectors (Novagen, San Diego, CA) using standard molecular biology techniques. Plasmids containing the PmpD genes were transformed into the Escherichia coli strain BL21 (DE3) (New England Biolabs, Ipswich, MA) with protein expression induced using the lac promoter for T7 RNA expression using isopropyl-β-D-thiogalactopyranoside (IPTG; Novagen). Cell pellets of E. coli expressing PmpD p73 or PmpD p82 were re-suspended in lysis buffer [50 mM Tris (Sigma-Aldrich), pH 8.0, 300 mM NaCl, and 1 × EDTA-free protease inhibitors (Roche, Indianapolis, IN)], and lysed with 3 passes through a microfluidi-

dizer (Microfluidics, Westwood, MA) @ 15,000 psi. The lysate was clarified by centrifugation ($\sim 19,000 \times g$) and the pellet (containing inclusion bodies) was solubilized in a urea buffer [8 M urea (Thermo Scientific, Rockford, IL), 10 mM MES pH 6.5, 150 mM NaCl, and 20 mM Imidazole (Sigma)] and clarified by ultracentrifugation ($\sim 160,000 \times g$). The solubilized fraction was purified by immobilized metal affinity chromatography (IMAC) with Ni NTA agarose (Qia-gen, Valencia, MA) and step eluted with 200 mM Imidazole. The IMAC product was refolded using extensive dialysis against 10 mM MES pH 7.3, 150 mM NaCl containing 0.1% Zwittergent 3-14 using a Pierce Slide-A-Lyzer cassette (Rockford, IL) with a 10 kDa molecular weight cut-off (MWCO) while step reducing the urea concentration to zero. The PmpD p73 and PmpD p82 in MES buffered saline containing 0.1% Zwittergent 3-14 was used in both in vitro and in vivo studies. The final product was sterile filtered and the concentration was determined by amino acid analysis. Purity of the final product was evaluated by SDS-PAGE (Invitrogen) and by in-solution enzymatic digestion with 1D/LC-MS-MS analysis demonstrating purity of both PmpD p73 and PmpD p82 at >99%.

2.4. Mouse Immunization and Challenge

Female C57BL/6 mice (Taconic Farms, Hudson, NY) were used at 6 to 8 weeks of age, and food and water were provided ad libitum. All animal procedures were in accordance with government and institutional guidelines for animal health and well-being, and were approved by the Merck & Co., Inc., Kenilworth, NJ, USA, Institutional Animal Care and Use Committee (IACUC).

As shown in **Figure 1**, animals were immunized by subcutaneous (s.c.) routes on days 0, 20 and 30 with nMOMP or PmpD in combination with an adjuvant containing IMO-2055 (IMO; Idera Pharmaceuticals, Cambridge, MA) and Montanide ISA 720 VG (SEPPIC Inc., Fairfield, NJ) at a ratio of 70:30 (v/v). Adjuvant-control

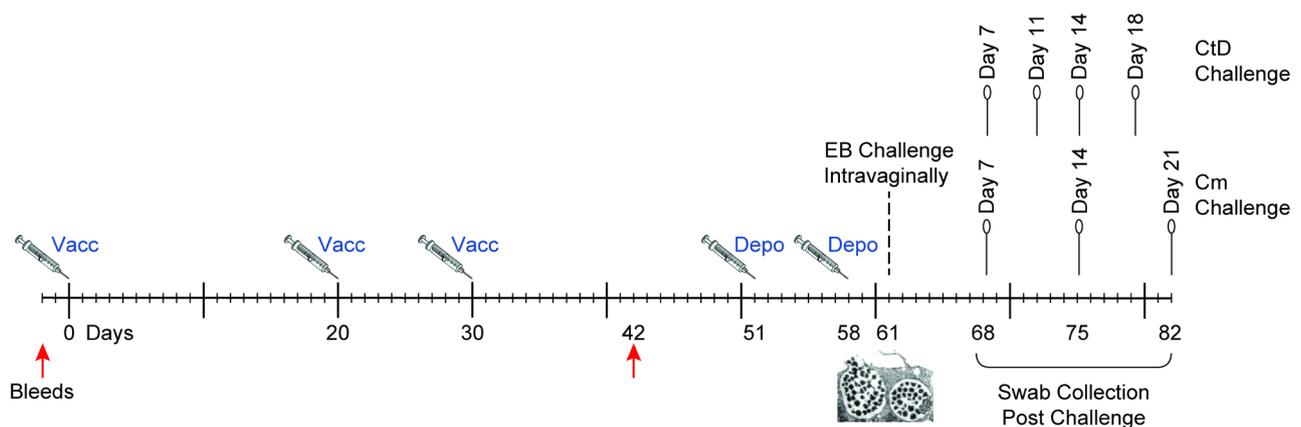


Figure 1. Immunization and challenge schedule. Blood was collected from the tail vein prior to the first immunization and two weeks following the final immunization. Immunizations with vaccines (Vacc) were performed on days 0, 20, and 30. Depo-Provera® (Depo) was administered 10 and 3 days before challenge. Mice were intravaginally challenged approximately 1 month following the final immunization. Swabs were collected on days 7, 11, 14, and 18 following *C. trachomatis* serovar D challenge (CtD challenge) or on days 7, 14, and 21 following *C. muridarum* challenge (Cm challenge).

groups were administered with a combination of IMO and Montanide ISA 720 VG only at the same concentration used for the vaccine antigen groups. Live EB groups were immunized either at day 0 with 500 EB in SPG per mouse by intranasal (i.n.) route or on days 0, 20, and 30 with 1×10^6 EB in SPG per mouse by intraperitoneal (i.p.) route.

Prior to the first immunization and two weeks following the final immunization, tail bleeds were performed with blood collected in BD Microtainer[®] Serum Separator Tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). Blood samples were centrifuged at 6000 rpm for 5 min and serum was transferred to a microcentrifuge tube.

At approximately 2 weeks following the final immunization, progesterone (medroxy-progesterone acetate, Depo-Provera[®]; Pharmacia & Upjohn Co., NY, NY) was administered (2.5 mg/dose) by s.c. route at 10 and 3 days before challenge. Mice were anesthetized prior to challenge only with a mixture of 85 mg/kg ketamine (Ketaset; Fort Dodge Animal Health, Fort Dodge, IA) and 5 mg/kg xylazine (AnaSed[®] Injection; Henry Schein Animal Health, Dublin, OH) by i.p. route. Mice were challenged intravaginally (approximately 1 month following the final immunization) by direct instillation of 10 μ L of SPG containing 1×10^5 *C. trachomatis* serovar D EBs or 1×10^4 *C. muridarum* EBs. The vaginal vault and ectocervix were swabbed using a microfiber swab (Puritan, Guilford, ME) on days 7, 11, 14, and 18 following *C. trachomatis* serovar D challenge or on days 7, 14, and 21 following *C. muridarum* challenge. For the in vivo depletion studies, the vaginal vault and ectocervix were swabbed on days 7, 11, 14, 18, 20, and 25 following *C. trachomatis* serovar D challenge or on days 7, 14, 17, 21, 24, and 28 following *C. muridarum* challenge.

Swabs were placed into a 1.5-mL tube containing 2 sterile 5 mm diameter glass beads (Corning, Corning, NY) and 300 μ L of Bartels[®] Chlamydia isolation medium (Trinity Biotech USA, Jamestown, NY) on ice. Bacteria were eluted from the swabs and separated from cells by vortexing for 60 seconds. Eluted cells/bacteria (100 μ L) were plated into a 96-well processing cartridge (Roche) containing 100 μ L of PBS and stored at -80°C until DNA extraction.

2.5. Real-Time Quantitative Polymerase Chain Reaction and Statistical Analysis

For initial studies, DNA from genital swab samples (~ 100 μ L) was extracted using the Roche High Pure PCR template kit (Roche) according to the manufacturer's instructions, with the exception that DNA was eluted from the column using 2 batch elutions of 30 μ L volume each. For subsequent studies DNA was extracted using the MagNA Pure 96 DNA and Viral NA small volume kit (Roche) on the MagNA pure 96 System (Roche) according to the manufacturer's instructions.

Real-time quantitative polymerase chain reaction (RT-qPCR) was performed to confirm the presence of infection according to procedures defined in Wooters *et al.* [26]. Briefly, the oligonucleotide primer set was designed for detection of

all species of Chlamydiae. The sense primer, 16S DIR 5'-CGCCTGAGGAGTACACTCGC-3', and anti-sense primer, 16S Rev 5'-CCAACACCTCACGGCAGAG-3', were designed to amplify a 208-bp fragment of the Chlamydial 16S ribosomal subunit gene, conserved across Chlamydia strains and serovars. Primers were obtained from Sigma Genosys (The Woodlands, TX), and the probe, 16S Fam-5'-CACAAAGCAGTGGAGCATGTGGTTTAA-3' Tamra, was synthesized by Applied Biosystems, (Foster City, CA).

The 50 μ L reaction mixtures consisted of 1 \times QuantiTect Multiplex PCR master mix without ROX (Qiagen), 100 nmol/L 16S probe, 200 nmol/L primer 16S DIR, 400 nmol/L primer 16S Rev, 30 nmol/L ROX reference dye (Stratagene, LaJolla, CA), and 5 μ L of sample DNA. Non-template controls consisting of the reaction master mix, primers, and probe, but no DNA, were included in each assay run. Reaction conditions were set as follows: 1 cycle at 95°C for 15 min, followed by 40 cycles at 94°C for 1 min and at 60°C for 1 min. Thermal cycling, fluorescent data collection, and data analysis were performed using the Agilent Mx3005P system (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. The level of urogenital bacterial shedding exhibited by each mouse was summarized by the area under the response-time curve (AUC) formed by the Log₁₀ copy number and the day post challenge. Differences in the level of bacterial shedding between groups within each study were assessed on the AUC responses using Wilcoxon's Rank Sum Test. Differences between the *C. muridarum* and *C. trachomatis* models in the proportion of studies achieving a specified threshold were assessed using the two-sided Fisher's Exact Test. Differences between the CD4⁺ T-cell depleted and non-CD4⁺ T-cell depleted groups within the *C. muridarum* and *C. trachomatis* models in the proportion of mice resolving Chlamydial urogenital infection by day 25 were assessed using the one-sided Fisher's Exact test, given the expectation that fewer mice would resolve their infection in the CD4⁺ T-cell depleted group than in the non-CD4⁺ T-cell depleted group.

2.6. Detection of Serum Antibody Levels by ELISA

Serum was analyzed by an enzyme-linked immunosorbent assay (ELISA). Nunc™ C96 Maxisorp Immunoplates (Thermo Scientific) were coated with 50 μ L of 0.5 μ g/ml *C. trachomatis* Serovar D or *C. muridarum* nMOMP in PBS and refrigerated overnight. The plates were washed three times with 0.05% Tween-20 (Fisher Scientific) in PBS (PBS-T). The wells were blocked with 5% heat-inactivated FBS (HyClone) in PBS (5% FBS-PBS) at 200 μ L/well for 1 hour at room temperature and washed three times with PBS-T. Serum was diluted 1:500 in 5% FBS-PBS and then serially diluted five-fold in 5% FBS-PBS. Diluted sera were added to the plate, incubated for 2 hours at room temperature and were washed three times with PBS-T. HRP-conjugated goat anti-mouse IgG, Fc γ fragment specific (Jackson Immuno Research Laboratories, West Grove, PA) was diluted 1:6000 in 5% FBS-PBS. The diluted secondary antibody was added at 100 μ L/well, incubated

for 1 hour at room temperature and was washed three times with PBS-T followed by three times with PBS. Room temperature BD Opt EIA™ TMB Substrate Reagent Set (BD Biosciences, San Jose, CA) was mixed and filtered through a 0.22 µm CA filter unit (Corning Life Sciences, Tewksbury, MA), and 100 µL was added to each well and incubated for 10 min at room temperature. The reaction was stopped with 100 µL/well of 2 M H₂SO₄ (Fisher Scientific, Waltham, MA). The optical density (OD) was read at 450 nm on a SpectraMax® M5 plate reader (Molecular Devices, Sunnyvale, CA). The cutoff OD for each post-immunization serum was calculated as two times of the OD₄₅₀ of the corresponding pre-immunization serum. ELISA titers were determined by linearly interpolating between the sequential log dilutions that bracket the cutoff OD, where the dependent variable is the OD response and the independent variable is the log serum dilution. The resulting dilution is then back transformed to obtain the reported titer. The reported titer is the estimated dilution of serum that results in a response equivalent to the cutoff OD.

2.7. In Vivo Depletion

The anti-CD4 hybridoma GK1.5 (ATCC) was grown in Iscove's Modified Dulbecco's Medium (ATCC) supplemented with 10% heat-inactivated FBS (HyClone). The antibody was purified from spent media using a column packed with Protein G Sepharose Fast Flow resin (GE Healthcare, Chicago, IL) and then buffer exchanged into PBS, pH 7.4. Based on previous work [27] [28] [29], in vivo depletion of CD4+ T-cells was accomplished by injecting mice i.p. with 0.375 mg of anti-CD4 monoclonal antibody for 3 consecutive days prior to challenge, followed by injections every third day with the final injection being administered on day 23 post-challenge for the *C. muridarum* model and day 26 post-challenge for the *C. trachomatis* model. Mice were injected starting 6 days prior to intravaginal challenge. Therefore, mice were injected with antibody on days -6, -5, -4, -1, 2, 5, 8, 11, 14, 17, 20, and 23 for the *C. muridarum* model and additionally on day 26 for the *C. trachomatis* model.

In these studies, an additional 18 mice were immunized with the adjuvant-control for each model. Half of the mice in this group were left untreated (immune competent) while the remainder were treated with anti-CD4 antibody (CD4+ T-cell depleted). Three mice were sacrificed from each group and spleens removed at three time points: prior to, during the course of the infection, and on the day of the last vaginal swab sample. Depletion of CD4+ T-cells in the spleens of anti-CD4 treated mice was confirmed on days -1, 13 and 25 for the *C. muridarum* model and days -1, 14 and 28 for the *C. trachomatis* model by fluorescent-activated cell sorting (FACS) analysis after staining splenocytes with R-phycoerythrin (PE)-conjugated anti-mouse CD4 antibody (Invitrogen), PE-Cy™ 5-conjugated anti-mouse CD8a antibody (BD Pharmingen, San Diego, CA), and Alexa Fluor 488-conjugated anti-mouse CD3e antibody (BD Pharmingen).

3. Results

3.1. Reproducibility in the *C. muridarum* and *C. trachomatis* Intravaginal Challenge Models

Mice were immunized, by either intranasal or intraperitoneal route, with *C. muridarum* (42 studies) or *C. trachomatis* (33 studies) live EBs and then challenged intravaginally with the homologous Chlamydia strain. We found that in 100% (42/42) of the *C. muridarum* intravaginal challenge studies the EB-immunized mice showed a significant reduction in vaginal bacterial shedding assessed by qPCR ($p < 0.05$) compared to the adjuvant-control-immunized mice (Figure 2(A)). By comparison, in the *C. trachomatis* model we observed a significant ($p < 0.05$) reduction in vaginal shedding in the EB-immunized mice in 82% (27/33) of the studies. The proportional difference between *C. muridarum* and *C. trachomatis* intravaginal challenge studies showing a significant reduction in vaginal bacterial shedding of EB-immunized mice is statistically significant ($p = 0.0055$).

Within many of the studies described above, additional groups of mice were immunized with native MOMP (nMOMP) purified from either *C. muridarum* or *C. trachomatis* bacteria. Mice immunized with *C. muridarum* or *C. trachomatis* nMOMP, as well as EB-immunized mice, had high IgG titers to the homologous strain of nMOMP (Figure 3).

When evaluating these studies, we found that in 100% (18/18) of the *C. muridarum* intravaginal challenge studies the nMOMP-immunized mice demonstrated

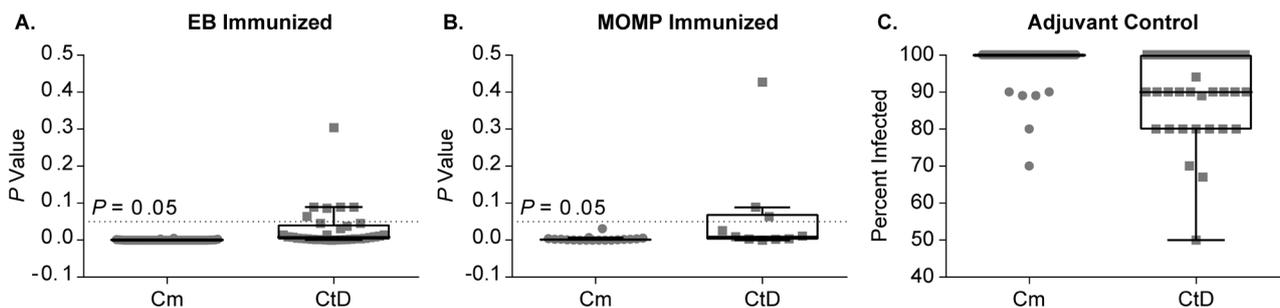


Figure 2. Comparison of intravaginal mouse challenge models for significant reduction in shedding following vaccination. (A) EB-immunized positive control group: mice were vaginally swabbed on days ~7, 14, and 21 in the *C. muridarum* studies ($n = 42$ groups of 10 mice) or ~7, 11, 14, and 18 in the *C. trachomatis* studies ($n = 33$ groups of 10 mice). (B) 10 μg MOMP-immunized test group: mice were vaginally swabbed on days ~7, 14, and 21 in the *C. muridarum* studies ($n = 18$ groups of 10 mice) or ~7, 11, 14, and 18 in the *C. trachomatis* studies ($n = 10$ groups of 10 mice). Area under the curve (AUC) for individual mice was determined and analyzed by Wilcoxon Rank Sum Test (compared to adjuvant-control group). Resulting p-values from each study comparing the EB-immunized control group to the adjuvant-control group are shown in panels (A) and (B). Data plotted with Box and Whisker Plots (Tukey), boxes = medians with interquartile ranges (IQR), whiskers = 1.5 times the IQR distances. For the EB-immunized and MOMP-immunized groups, the difference between *C. muridarum* and *C. trachomatis* in the proportion of intravaginal challenge studies showing a significant reduction in vaginal bacterial shedding (*i.e.*, $p < 0.05$) was statistically significant ($p = 0.0055$ and $p = 0.0366$, respectively). (C) Adjuvant-control group: mice were vaginally swabbed on day 7 in the *C. muridarum* studies ($n = 42$ groups of 10 mice) and in the *C. trachomatis* studies ($n = 33$ groups of 10 mice) and infection for individual mice was determined by qPCR. The percent of infected mice in the adjuvant-control group from each study is shown in panel (C). Data plotted with Box and Whisker Plots (Tukey), boxes = medians with interquartile ranges (IQR), whiskers = 1.5 times the IQR distances. The difference between *C. muridarum* and *C. trachomatis* in the proportion of intravaginal challenge studies having 100% of mice in the adjuvant-control group infected on day 7 was highly statistically significant, $p < 0.0001$.

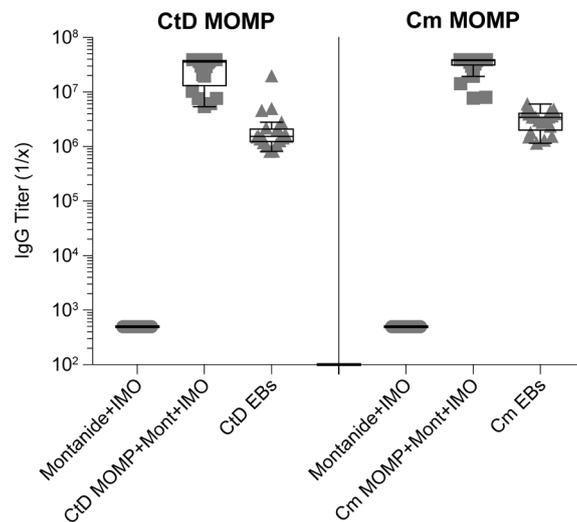


Figure 3. Anti-MOMP IgG Titers of mice vaccinated with MOMP or EBs. IgG titers to *C. trachomatis* serovar D MOMP or *C. muridarum* MOMP for *C. trachomatis* serovar D vaccinated groups or *C. muridarum* vaccinated groups were plotted with Box and Whisker Plots (Tukey), boxes = medians with interquartile ranges (IQR), whiskers = 1.5 times the IQR distances.

a significant reduction ($p < 0.05$) in vaginal bacterial shedding by qPCR compared to the adjuvant-control-immunized mice (**Figure 2(B)**). In the *C. trachomatis* model, 70% (7/10) of the studies showed a significant reduction ($p < 0.05$) in vaginal bacterial shedding in nMOMP-immunized mice. Two of these ten studies contained EB-immunized positive control groups that were not significantly different ($p > 0.05$) than the adjuvant-control group when comparing the vaginal bacterial shedding. If these studies were excluded, then 75% (6/8) of the *C. trachomatis* studies demonstrated a significant reduction of bacterial shedding following nMOMP immunization. The difference between *C. muridarum* and *C. trachomatis* in the proportion of intravaginal challenge studies showing a significant reduction in vaginal bacterial shedding in nMOMP-immunized mice borders on statistical significance ($p = 0.0366$ when all *C. trachomatis* studies are included in the comparison, and $p = 0.0862$ when the aforementioned two studies are excluded).

In initial studies we collected vaginal swab samples on both day 4 and day 7 post-challenge. We found that detectable shedding peaked on day 7 and in subsequent studies we have used day 7 as our baseline for infection level assessment. We found that 86% (36/42) of the *C. muridarum* intravaginal studies resulted in all the mice in the adjuvant-control group infected on day 7 as determined by qPCR assessment of vaginal swab samples. In contrast, in the *C. trachomatis* intravaginal studies only 36% (12/33) had all the mice in the adjuvant-control group infected on day 7 (**Figure 2(C)**). These combined observations support our observation that the *C. muridarum* challenge model is more reliable, robust, and reproducible to evaluate efficacy of vaccine targets. The difference between *C. muridarum* and *C. trachomatis* in the proportion of intravaginal challenge

studies having 100% of mice in the adjuvant-control group infected on day 7 was highly significant, $p < 0.0001$.

3.2. Evaluation of Potential Vaccine Candidates in the *C. muridarum* and *C. trachomatis* Intravaginal Challenge Models

The Chlamydia major outer membrane protein (MOMP) is a structurally and immunologically dominant protein that is surface exposed and constitutes over 60% of the total outer membrane protein mass [30] [31]. Mice vaccinated with native MOMP (nMOMP) elicit a protective immune response against both intravaginal and intrabursal challenge with *C. muridarum* [27] [32] [33] [34] [35]. *C. trachomatis* and *C. muridarum* purified nMOMP were evaluated as potential vaccine candidates and observed to be protective ($p < 0.05$) in both the *C. trachomatis* (Figure 4(A)) and *C. muridarum* (Figure 4(B)) intravaginal challenge models, respectively. A titration of nMOMP indicated that vaccination with as little as 1 μg nMOMP plus Montanide and IMO-2055 significantly (*C. trachomatis* $p = 0.023$ and *C. muridarum* $p = 0.011$) reduced vaginal bacterial shedding in both models and the reduction in shedding was comparable to the 5 μg nMOMP dose (*C. trachomatis* $p = 0.019$ and *C. muridarum* $p = 0.017$). In the *C. muridarum* model, the shedding level observed following challenge of mice immunized with the 10 μg nMOMP decreased sharply through day 14. By day 21 the level of shedding was almost equivalent to the positive control live EB-immunized group and a significant ($p = 0.0003$) reduction of Chlamydial shedding compared to the adjuvant-control group was observed. In the *C. trachomatis* model, while all three nMOMP arms showed a reduction in shedding relative to the adjuvant-control group at each sample time point, the reduction was not dose-dependent and only bordered on statistical significance within each of the nMOMP arms. This could be in part due to the overall lower bacterial shedding in the *C. trachomatis* model compared to the *C. muridarum* model, where there is a greater range between the maximal shedding level and limit of detection.

C. trachomatis polymorphic membrane protein D (PmpD) has been identified as a virulence factor involved in early host-cell interactions [36]. It is a surface exposed, *C. trachomatis* pan-neutralizing target [37]. Paes *et al.* reported that a 65 kDa PmpD passenger domain fragment used to immunize C57BL/6 mice in the presence or absence of a second-generation lipid adjuvant (SLA) significantly enhanced resistance to *C. trachomatis* infection and reduced bacterial load [38]. The PmpD p73 passenger domain (PmpD p73) and the p82 translocator domain (PmpD p82) were evaluated as potential vaccine candidates in both intravaginal challenge models. Immunization with 10 μg PmpD p73 plus Montanide and IMO-2055 resulted in significantly ($p = 0.026$) reduced shedding in the *C. trachomatis* model compared to the adjuvant-control group (Figure 5(A)), but not in the *C. muridarum* model ($p = 0.571$) (Figure 5(B)). Immunization with PmpD p82 did not induce significant reduction of shedding in either model.

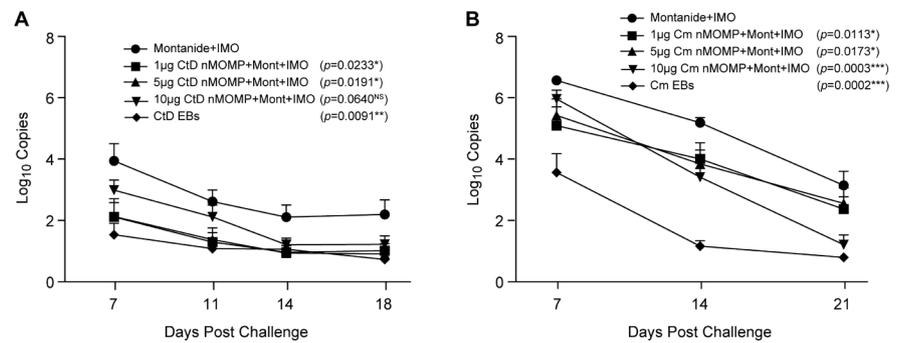


Figure 4. Shedding time course of mice vaccinated with MOMP or EBs following challenge with Chlamydia. (A) *C. trachomatis* serovar D vaccinated and challenged groups. Mice were vaginally swabbed on days 7, 11, 14, and 18. (B) *C. muridarum* vaccinated and challenged groups. Mice were vaginally swabbed on days 7, 14, and 21. Data were plotted as average Log₁₀ copy number \pm standard error of the mean for 10 mice over the course of the infection. Area under the curve (AUC) for individual mice were determined and analyzed by Wilcoxon Rank Sum Test (compared to Montanide + IMO adjuvant-control group).

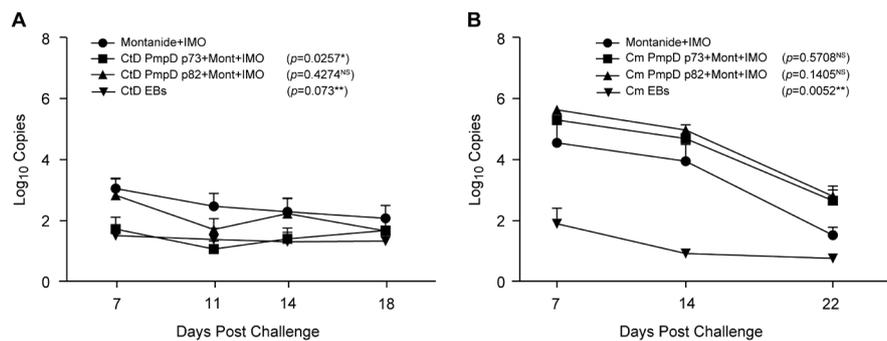


Figure 5. Shedding time course of mice vaccinated with PmpD constructs or EBs following challenge with Chlamydia. (A) *C. trachomatis* serovar D vaccinated and challenged groups. Mice were vaginally swabbed on days 7, 11, 14, and 18. (B) *C. muridarum* vaccinated and challenged groups. Mice were vaginally swabbed on days 7, 14, and 22. Data were plotted as average Log₁₀ copy number \pm standard error of the mean for 10 mice over the course of the infection. Area under the curve (AUC) for individual mice were determined and analyzed by Wilcoxon Rank Sum Test (compared to Montanide + IMO adjuvant-control group).

3.3. Role of CD4+ T-Cells in the Resolution of Primary Infection Following Vaccine Induced Immunity

CD4+ T-cells have been shown to play a major role in the resolution of primary Chlamydia infection and reinfection [10] [12] [16] [28] and in vaccine-induced protection [27]. To compare the importance of CD4+ T-cells in resolution of primary infection following vaccine-induced immunity between the *C. trachomatis* and *C. muridarum* intravaginal challenge models, we immunized C57BL/6 mice with either nMOMP plus Montanide and IMO-2055, live EBs, or Montanide plus IMO-2055 alone. Beginning 6 days prior to challenge CD4+ T-cells were depleted by infusing the mice with anti-CD4 antibody. Depletion of CD4+ T-cells was confirmed by fluorescence-activated cell sorting (FACS). Mice re-

ceived anti-CD4 antibody on days 6, 5, and 4 prior to challenge followed by additional injection every third day to ensure the depletion lasted for the duration of the infection (3 to 4 weeks). Mice not treated with anti-CD4 antibody maintained normal levels of CD4+ T-cells. Using the schedule of antibody injections described, depletion of CD4+ T-cells was effectively maintained at >95% reduction throughout the course of study (data not shown).

In the *C. trachomatis* model, mice immunized with adjuvant-control and then CD4+ T-cell depleted, exhibited a half-log increase in vaginal bacterial shedding compared to the non-CD4+ T-cell depleted adjuvant-control group (**Figure 6(A)**) on day 7 post-challenge. The CD4+ T-cell depleted group maintained a higher level of bacterial shedding throughout the course of the study with only 10% of the mice resolving Chlamydial urogenital infection by day 25 compared to 30% in the non-CD4+ T-cell depleted group [$p = 0.2910$] (**Table 1**).

On day 7 post-challenge, the live EB-immunized non-CD4+ T-cell depleted group and live EB-immunized CD4+ T-cell depleted group exhibited a 2 and 3-log reduction in shedding, respectively, compared to the corresponding adjuvant-control-immunized group (**Figure 6(A)**). Ninety percent of the mice in both the live EB-immunized non-CD4+ T-cell depleted and live EB-immunized CD4+ T-cell depleted groups were able to clear the urogenital infection by day 25 [$p = 0.7632$] (**Table 1**). On day 7 post-challenge, the nMOMP-immunized non-CD4+ T-cell depleted and nMOMP-immunized CD4+ T-cell depleted groups exhibited a 2 and 1.5-log reduction in shedding, respectively, in relation to their corresponding adjuvant-control group (**Figure 6(A)**). The nMOMP-immunized non-CD4+ T-cell depleted group and the nMOMP-immunized CD4+ T-cell depleted group maintained their same levels of urogenital bacterial shedding

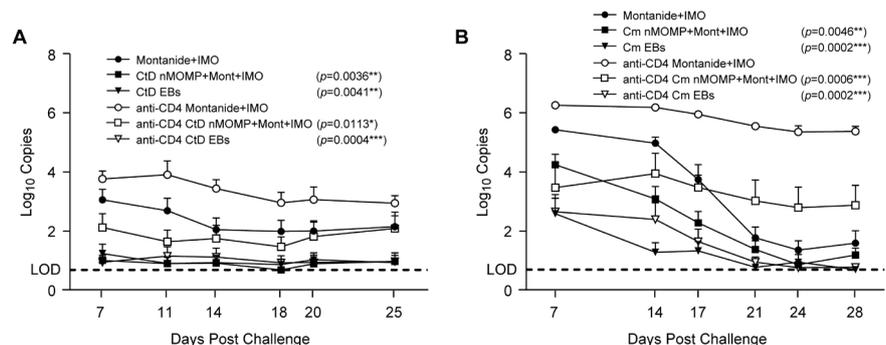


Figure 6. CD4+ T-cells involved in protection of MOMP vaccinated but not EB vaccinated mice. (A) Mice were vaccinated with adjuvant-control, *C. trachomatis* nMOMP, or *C. trachomatis* serovar D EBs and then were either left untreated (solid symbols) or treated with anti-CD4 antibody (open symbols) prior to and during the course of infection with *C. trachomatis* serovar D EBs. (B) Mice were vaccinated with adjuvant-control, *C. muridarum* nMOMP, or *C. muridarum* EBs and then were either depleted of CD4+ T-cells (open symbols) or non-CD4+ T-cell depleted (solid symbols) prior to and during the course of infection with *C. muridarum* EBs. Data were plotted as average Log₁₀ copy number \pm standard error of the mean for 10 mice over the course of the infection. Area under the curve (AUC) for individual mice were determined and analyzed by Wilcoxon Rank Sum Test (compared to Montanide + IMO adjuvant-control group).

Table 1. Percent of mice per group with detectable urogenital bacterial shedding following *C. trachomatis* intravaginal challenge (n = 10 mice per study group).

Study Group	Day 7	Day 11	Day 14	Day 18	Day 20	Day 25
Montanide + IMO	90	80	70	70	70	70
CtD MOMP + Mont + IMO	40	10	10	0	20	10
CtD EBs	30	10	10	10	10	10
anti-CD4 Montanide + IMO	100	90	100	90	80	90
anti-CD4 CtD MOMP + Mont + IMO	60	50	40	40	40	50
anti-CD4 CtD EBs	30	20	20	10	10	10

throughout the course of the study (**Figure 6(A)**). Similar to the EB-immunized non-CD4+ T-cell depleted group, 90% of the nMOMP-immunized non-CD4+ T-cell depleted mice resolved the urogenital infection by day 25 (**Table 1**). In contrast, the nMOMP-immunized CD4+ T-cell depleted group maintained higher levels of urogenital Chlamydial shedding throughout the course of the study compared with nMOMP-immunized or live EB-immunized groups without depletion, and the live EB-immunized CD4+ T-cell depleted group (**Figure 6(A)**). Only 50% of the nMOMP-immunized CD4+ T-cell depleted mice resolved the Chlamydial infection by day 25 compared to 90% in the nMOMP-immunized non-CD4+ T-cell depleted group [$p = 0.0704$] (**Table 1**).

In the *C. muridarum* model, C57BL/6 mice immunized with adjuvant and CD4+ T-cell depleted, exhibited a 1-log increase in shedding compared to the non-depleted adjuvant-control group (**Figure 6(B)**) on day 7 post-challenge. The CD4+ T-cell depleted group maintained a higher level of shedding throughout the course of study with none of the mice resolving the infection by day 28 compared to 60% in the non-CD4+ T-cell depleted adjuvant-control group [$p = 0.0054$] (**Table 2**).

On day 7 post-challenge, the live EB-immunized non-CD4+ depleted group and the live EB-immunized CD4+ T-cell depleted group exhibited a 3 and 3.5-log reduction in shedding, respectively, in relation to their corresponding adjuvant-control group (**Figure 6(B)**). All the live EB-immunized non-CD4+ T-cell depleted mice and 90% of the live EB-immunized CD4+ T-cell depleted mice were able to clear the infection by day 28 [$p = 0.5000$] (**Table 2**). On day 7 post-challenge, the nMOMP-immunized non-CD4+ T-cell depleted and the nMOMP-immunized CD4+ T-cell depleted groups exhibited a 1 and 3-log reduction in shedding, respectively, in relation to their corresponding adjuvant-control group (**Figure 6(B)**). The nMOMP-immunized CD4+ T-cell depleted group maintained higher levels of Chlamydia urogenital shedding throughout the course of the study compared with nMOMP-immunized non-CD4+ T-cell depleted group, live EB-immunized non-CD4+ T-cell depleted group, and EB-immunized CD4+ T-cell depleted group (**Figure 6(B)**). Only 40% of the nMOMP-immunized CD4+ T-cell depleted mice resolved the Chlamydial infection by day 28 compared to 60% in the nMOMP-immunized non-CD4+ T-cell depleted group [$p = 0.3281$] (**Table 2**).

Table 2. Percent of mice per group with detectable urogenital bacterial shedding following *C. muridarum* intravaginal challenge (n = 10 mice per study group).

Study Group	Day 7	Day 14	Day 17	Day 21	Day 24	Day 28
Montanide + IMO	100	100	100	60	40	40
Cm MOMP + Mont + IMO	100	90	80	40	30	40
Cm EBs	80	30	40	20	10	0
anti-CD4 Montanide + IMO	100	100	100	100	100	100
anti-CD4 Cm MOMP + Mont + IMO	80	90	70	60	60	60
anti-CD4 Cm EBs	60	70	40	30	10	10

4. Discussion

We have extensively evaluated the *C. muridarum* intravaginal challenge model and demonstrated that it is more reliable, robust, and reproducible compared to the *C. trachomatis* serovar D intravaginal challenge model. In the 42 *C. muridarum* studies presented, 86% of the mice within the adjuvant-control-immunized group were successfully infected compared to only 36% of mice in a similar group in the 33 *C. trachomatis* challenge studies. Further, live EB immunization resulted in a significant reduction of Chlamydial urogenital shedding by qPCR as compared to the adjuvant-control-immunized mice in a greater fraction of the *C. muridarum* studies. Lastly, the *C. muridarum* model was more reproducible as shown by more consistent protection observed using a native MOMP (nMOMP) vaccine candidate. The low virulence of *C. trachomatis* in mice may contribute to the variability observed in the *C. trachomatis* intravaginal challenge model.

Our results suggest that candidate vaccine antigens may not perform equally in the two models. For example, our studies confirmed that nMOMP is a protective antigen in the *C. muridarum* intravaginal challenge model, a finding that is similar to other published studies [27] [35], as well as in the *C. trachomatis* intravaginal challenge model. However, immunization with the PmpD p73 passenger domain, while protective in the *C. trachomatis* serovar D intravaginal challenge model (similar to the publication by Paes *et al.* [38]), was not protective in the *C. muridarum* model. This may warrant additional investigation to understand how differences in outcome between the models may relate to potential future success of a vaccine candidate.

Numerous studies in mice have shown the importance of CD4+ T-cells in the resolution of Chlamydia genital tract infection [10] [12] [16] [27] [28]. To further characterize our observations in the *C. muridarum* and *C. trachomatis* serovar D models in C57BL/6 mice, we evaluated protection following nMOMP or live EB immunization and CD4+ T-cell depletion. CD4+ T-cells were required for resolution of primary intravaginal infection in adjuvant-control-immunized mice in both the *C. muridarum* and *C. trachomatis* models. While resolution of infection for nMOMP-immunized mice appeared to be CD4+ T-cell dependent, the initial reduction in bacterial shedding level observed following CD4+ T-cell

depletion in these and other studies [27] [34] [39] [40] suggests a possible role for antibody or other factors. Live EB-immunized mice did not require CD4+ T-cells to resolve urogenital infection following intravaginal challenge in either the *C. muridarum* or the *C. trachomatis* model. Similar to our findings in mice immunized with live EBs by the intraperitoneal route, Nogueira *et al.* [41] observed that C57BL/6 mice allowed to clear a primary *C. trachomatis* serovar L2 infection following intranasal challenge before CD4+ T-cell depletion, were protected against infection following a secondary intravaginal challenge. Protective immunity against genital *C. trachomatis* infection following the intranasal challenge was not dependent on antibody response but was mediated by both CD4+ and CD8+ T-cells; however, either CD4+ or CD8+ T-cells were sufficient for protection [41]. Additional characterization of the immune responses associated with protection in mice immunized with live EBs by intraperitoneal injection would be of benefit; in particular, CD8+ T-cell and B-cell depletion individually or in combination with CD4+ T-cell depletion. The data in these studies provide value in understanding protection generated by vaccine candidates and may assist in future formulation consideration to drive effective immune response. The relative importance of CD4+ T-cells and antibody may differ between vaccine antigens and formulations, as these studies suggest.

Both the *C. muridarum* and *C. trachomatis* mouse models have value for evaluating human vaccine candidates. Neither model completely mimics Chlamydia infection in women, but each model provides some insight into how a specific vaccine could be protective in humans. The *C. muridarum* model mimics the acute phases of human infection [23] [42] and can be used to study pathogenesis of Chlamydia upper genital tract infection [11]. In contrast, the *C. trachomatis* model in many ways parallels the course and outcome of infection observed in most women with an asymptomatic and self-limiting infection with low frequency of severe upper genital tract sequelae [43]. Neither model allows for development of chronic infection observed in humans [23] [44] although the *C. trachomatis* model does allow for the evaluation of recurrent infections which is a frequent occurrence in humans [16] [24]. In addition, *C. muridarum* and *C. trachomatis* strains differ in their responses to IFN- γ . In particular, *C. trachomatis* strains are more sensitive to IFN- γ mediated inhibition, both in vitro and in vivo, whereas *C. muridarum* is relatively resistant [45] [46]. This may suggest possible differences in their response to other cytokines [31] [47] [48]. Therefore, the *C. trachomatis* model could be more clinically relevant when investigating cytokine profiles during the course of infection [48]. However, *C. trachomatis* infection in mice resolves in the absence of adaptive immunity while *C. muridarum* infection does not [16] [49]. Therefore, the *C. muridarum* model may be better for defining protective adaptive immune responses following natural infection or vaccination. When humanized mice are used, such as HLA-DR4 [50] or BLT mice [51], the *C. trachomatis* challenge model may be more advantageous in terms of evaluating human vaccine candidates since both the MHC-II antigen and the vaccine candidates are of human origin. Ultimately, both models

provide some insight into how a vaccine could be protective in humans. Our data and experience demonstrate both the *C. muridarum* and *C. trachomatis* models provide value during the pre-clinical assessment of Chlamydia vaccine candidates intended for human use.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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