Immunization with chlamydial type III secretion antigens reduces vaginal shedding and prevents fallopian tube pathology following live C. muridarum challenge

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Chlamydia trachomatis infections in women are often asymptomatic and if left untreated can lead to significant late sequelae including pelvic inflammatory disease and tubal factor infertility. Vaccine development efforts over the past three decades have been unproductive and there is no vaccine approved for use in humans. The existence of serologically distinct strains or serovars of C. trachomatis mandates a vaccine that will provide protection against multiple serovars. Chlamydia spp. use a highly conserved type III secretion system (T3SS) composed of both structural and effector proteins which is an essential virulence factor for infection and intracellular replication. In this study we evaluated a novel fusion protein antigen (BD584) which consists of three T3SS proteins from C. trachomatis (CopB, CopD, and CT584) as a potential chlamydial vaccine candidate. Intranasal immunization with BD584 elicited serum neutralizing antibodies that inhibited C. trachomatis infection in vitro. Following intravaginal challenge with C. muridarum, immunized mice had a 95% reduction in chlamydial shedding from the vagina at the peak of infection and cleared the infection sooner than control mice. Immunization with BD584 also reduced the rate of hydrosalpinx by 87.5% compared to control mice. Together, these results suggest that highly conserved proteins of the chlamydial T3SS may represent good candidates for a Chlamydia vaccine.

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1. Introduction

Chlamydia trachomatis is the most common sexually transmitted bacterial infection in North America and in many developed countries around the world [1]. The World Health Organization has estimated that there are 113 million new chlamydial infections globally every year, with the highest incidence occurring in underdeveloped regions of the world where adequate control programs are significantly lacking [2]. Chlamydial infection can lead to acute inflammation characterized by redness, edema, and discharge, presenting as mucopurulent cervicitis in women and non-gonococcal urethritis in men [3]. It has been estimated that more than 70% of women and 50% of men experience subclinical, asymptomatic infections [3]. Since individuals with asymptomatic infections do not seek medical treatment, untreated infections in women can lead to upper genital tract (UGT) infection, pelvic inflammatory disease (PID), hydrosalpinx, and tubal infertility [3]. The high rate of asymptomatic infections, together with the severity of the infection-induced pathology, suggests that controlling C. trachomatis infections would benefit from a vaccine.

Early vaccine studies were initiated after the isolation of the etiologic agent of trachoma in 1957 [4]. Children were vaccinated with formalin-fixed chlamydial elementary bodies (EBs), which conferred only partial, serovar-specific, and short-lived immunity. Furthermore, when compared to their unvaccinated counterparts, a small number of vaccinated children had a greater risk of developing exacerbated disease following exposure to Chlamydia [4,5]. The same observations were made when similar experiments were carried out in non-human primates [6]. The development of desirable immune responses following vaccination with intact Chlamydia suggests the presence of deleterious antigen in EBs, highlighting the need for an effective and broadly protective subunit vaccine.
Chlamydial vaccine research efforts over the past three decades have led to the discovery of a large number of protective antigens [5,7,8]. Efforts have largely been focused on the major outer membrane protein (MOMP), and more recently on polymorphic membrane proteins (Pmps), both of which elicit antibody and cell-mediated protective immune responses. However, the use of MOMP as an antigen has been hampered by its complex structure and its allelic variation among different serovars of C. trachomatis. Antigenic variation seen with MOMP and Pmps suggests that a single antigen may not be sufficient in a Chlamydia vaccine, and that immunization with a multi-subunit vaccine consisting of novel antigens may represent a better strategy.

As a highly evolved obligate intracellular pathogen with a small genome size, chlamydia likely employs many virulence factors to manipulate the host cell environment ensuring successful infection. One essential virulence factor is the T3SS, which is required for cell invasion. Many of the T3SS proteins are surface exposed and as such can be targeted by neutralizing antibodies. For this reason, the use of T3SS components as antigens in the development of vaccines against other pathogenic bacteria has recently garnered attention. Antigens to the T3S tip proteins LcrV in Cystisine spp. and PcrV in Pseudomonas aeruginosa have been shown to block infection [9,10]. LcrV is a well-characterized Cystisine antigen and is currently being tested as a subunit vaccine against plague [11]. A broadly protective vaccine consisting of T3S translocator proteins IpAB and IpAD has been shown to be effective against Shigella [12,13]. There is much interest in IpAB and IpAD as vaccine candidates, because of their ability to induce cross-protective immunity in mouse models of Shigella infection. Since the T3S system proteins are highly conserved across some chlamydial species, these proteins may represent good candidates for use in a Chlamydia vaccine. Furthermore, antibodies towards the translocator proteins (CopB and CopD) have been identified in patients who have been infected with C. trachomatis [14].

We have previously shown that rabbits immunized with T3S proteins CopB, CopD, and Cpn0803 from Chlamydia pneumoniae were able to produce neutralizing antibodies that blocked infection in vitro by 98% [15]. We now show that immunization with a novel multi-component vaccine (BD584) consisting of the N-terminal 100 amino acids of C. trachomatis CopB, CopD, and full length CT584 reduced both bacterial shedding from the vagina and upper genital tract pathology following intra-vaginal challenge in mice.

2. Methods & materials

2.1. Hydrophilicity prediction

Amino acid sequences of CopB, CopD and CT584 were analyzed for transmembrane domains using an online prediction software, TMPred, which predicts transmembrane domains using an online prediction software, TMpred [4]. Amino acid sequences of CopB and CopD were analyzed in patients who have been infected with C. trachomatis [14].

2.2. Cloning

Genomic DNA from C. trachomatis serovar D was used as the source for all genes cloned in this study. Using Platinum pfx DNA amplification enzymes (Life Technologies), the following PCR products were generated (note: subprotocol describes amino acid number): CopB1–100 with a 5′ BamHI restriction site and 3′ EcoRI restriction, CopD1–100 with a 5′ EcoRI restriction site and a 3′ SalI restriction site, full length CT584 with a 5′ SalI restriction site and 3′ HindIII restriction site. PCR products were digested with their respective endonuclease (New England Biolabs) (Fig. 2). The multiple cloning site (MCS) 1 of pET-DUET was restriction digested with BamHI and HindIII (New England Biolabs). Restriction digested CopB1–100, CopD1–100, CT584, and pET-DUET were ligated in a 3:3:3:1 ratio using T4 DNA Ligase (Invitrogen) and transformed into NEB Turbo Cells (New England Biolabs). Prior to protein expression, all constructs were verified by Sanger sequencing at the MOBIX laboratory (McMaster University).

2.3. Protein expression and purification

The cloned construct in pET-DUET was transformed into Escherichia coli BL21(DE3) cells for protein production and purification. For protein expression, an overnight culture of bacteria was subcultured 1:50 into 6 L of pre-warmed LB in the presence of 100 µg/mL ampicillin. The bacteria were incubated at 37 °C while shaking at 250 RPM until an optical density of 0.600 at 600 nm was reached. Protein expression was induced by the addition of 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG) and remained shaking for 3 h at 37 °C. The bacteria were harvested via centrifugation at 10,000 g for 5 min using a Sorvall 5B centrifuge. The bacterial pellets were washed once with phosphate buffered saline (PBS) and then pelleted again before resuspending it in Nickel A buffer for protein purification (20 mM TRIS–HCl pH 7.4, 500 mM KCl, 0.03% LDAO, 10% glycerol, 10 mM imidazole). To release soluble protein from the bacterial cytoplasm, the bacteria were sonicated and then pelleted at 40,000 g for 45 min. The supernatant was loaded onto a 1 mL HiSTRAP column (GE Life Sciences) using an AKTA FPLC (GE Life Sciences). The bacteria were washed with approximately 20 column volumes of 100% Nickle A, 5% Nickle B (20 mM TRIS–HCl pH 7.4, 500 mM KCl, 0.03% LDAO, 10% glycerol, 300 mM imidazole), 10% Nickle B, and 15% Nickle B before eluting in 100% Nickle B. The protein was buffer exchanged into PBS using a desalting column and an AKTA FPLC. Purified protein was concentrated to 250 µL using a centrifugal filter (EMD-Millipore) and protein concentration was determined using a BioRad Protein DC Assay with protein standards.

2.4. Detection of serum antibodies towards BD584

SDS–PAGE and Western blot was used to detect the presence of BD584 specific serum antibodies in mice infected with C. muri- durum. Briefly, 2 µg of purified BD584 was resolved on a 10% gel using SDS–PAGE and then transferred to a nitrocellulose membrane. The membrane was blocked overnight at 4 °C in blocking buffer (5% skim milk powder in PBS-T). The blocking buffer was removed, and mouse serum was added to the membrane at a 1:100 dilution in blocking buffer and incubated overnight at 4 °C. The membrane was then washed twice with PBS-T, and a 1:5000 dilution of goat anti-mouse IgG HRP-conjugated secondary antibody (Sigma) in blocking buffer was added to the membrane for 1 h at room temperature. The membrane was then washed twice with PBS-T and developed using chemiluminescent detection reagents (Pierce).

2.5. Enzyme-linked immunosorbant assay (ELISA) for immunoglobulin detection

An indirect ELISA was used to detect the presence of antibodies against immunizing antigens. Briefly, cheek bleeds or vaginal washes were collected from the mice two weeks following the second immunization. The samples were pelleted in a microcentrifuge at 16,000 g, and the supernatant was collected. Serial dilutions of the supernatants, from either the vaginal washes or serum, were made from 1:25 to 1:3200. Approximately 250 ng of antigen were immobilized on nickel coated plates (Pierce) for 1 h at room temperature with shaking. The 96 well plate was then washed 1 time
with PBS + 0.1% Tween 20 (PBS-T). 200 μL of blocking solution (5% BSA in PBS-T) was added to each well and incubated at room temperature for 1 h. After removal of the blocking solution, 100 μL of the seral dilutions (serum or vaginal washes) were added to the wells and incubated for 1 h at room temperature while shaking. The seral dilutions were removed, and wells were washed with 200 μL of PBS-T and incubated for 5 min at room temperature with shaking, and repeated three times. After the final wash was removed, a 1:1000 dilution of goat anti-mouse HRP-conjugated secondary antibody (Sigma) was in made in 5% BSA + PBS-T, and 100 μL was added to each well and incubated at room temperature with shaking for 1 h. After repeating the previously described washing procedure, Ultra TMB (Pierce) was used to develop the ELISA as per the manufacture specifications. Briefly, 100 μL of Ultra TMB was added to each well, after a 30 min incubation at room temperature, 100 μL of stop solution (1 M H2SO4) was added to each well. The absorbance of each well was measured at 450 nm. The end point titer was the lowest dilution which produced an absorbance which was two standard deviations higher than control serum.

ELISA was used to analyze the BD584-specific IgG2c:IgG1 ratio to determine the polarization of the immune response elicited by intranasal immunization with BD584. After binding BD584 on nickel coated plates as described above, the protocols from the mouse IgG1 and IgG2c ELISA kits (elbioscence) were followed to determine the different antibody isotype concentrations in each sample. Standard curves were generated according to the manufacturer’s instructions. A paired t-test was used to determine statistical significance.

2.6. Intranasal immunization

Mice (n = 5 per group) were sedated with 5% isoflurane in 4 L/min oxygen. The mouse was held at a slight angle to position the nose towards the ground and then using a micropipette, the vaccine was delivered to the right nares while the mouse was waking from anesthesia. Vaccinated mice received 20 μg of antigen and 10 μg of CpG ODN1826 (5’ccatgacgttcctgacgtt with a phosphorothioate backbone) (Life Technologies) in 15 μL of PBS. Control mice received vehicle (PBS) alone. Immunizations were administered twice, at 6 weeks and 3 weeks prior to infection.

2.7. Chlamydia muridarum vaginal challenge

This animal study was approved by the Animal Research Ethics Board of McMaster University (AUP# 15-04-13). C. muridarum (ATCC VR-123) was grown in McCoy cells (ATCC CRL-1696) and harvested 48 h post infection. C. muridarum was aliquoted and stored in a sucrose–phosphate–glutamate (SPG) buffer at -80 °C. Eight week old female C57Bl/6 mice (Charles River) were used for all animal experiments. Mice were given 2.5 mg of medroxyprogesterone acetate (Depo-Provera, Pfizer) subcutaneously one week prior to infection to force the mice into the diestrus phase of the estrous cycle, thereby increasing the susceptibility to C. muridarum infection. The mice received 1 x 10^7 inclusion forming units (IFUs) of C. muridarum intravaginally. Non-infected controls received PBS only. Each group consisted of five mice. Fisher’s exact test was used to analyze the statistical difference in the presence of hydrosalpinx between the immunized and control groups.

2.8. Quantitation of vaginal shedding

Quantitative real-time PCR was used to quantify chlamydial shedding. Vaginal swabs (REF 516CS01, Copan, Italy) were collected from each mouse on days 1, 3, 5, 7, 10, 14 days post infection. Mouse vaginal swabs were placed in SK38 tubes containing ceramic beads (Berkin Technologies, Montigny, France) and stored at -20 °C until testing. Before nucleic acid extraction, 1 mL of lysis buffer (bioMerieux) was added to the swab. The tubes were vortexed for 5 min and left stand at room temperature for 15 min. The tubes were then spun at 14000g for 2 min. The supernatant was collected and then subsequently extracted on an easyMAG extractor using generic 2.01 protocol. Quantitative PCR was performed using a RotorGene Q instrument (Qiagen). A pair of primers and a FAM-BHQ probe were used to target the MOMP gene from C. muridarum. qRT-PCR was performed in a 20 μL reaction containing 1 x SensiFAST Probe Mix (Bioline), 0.4 μM forward primer, 0.4 μM reverse primer, 0.2 μM probe and 5 μL of template. The amplification profile consisted of 95 °C for 3 min, followed by 45 cycles of 95 °C for 10 s, and 55 °C for 40 s. Standards used for quantitation were from the MOMP gene, cloned into the pGEM-T easy vector system. Results were analyzed using an area under the curve analysis and Student’s t-test.

2.9. Serum neutralization of C. muridarum Infection

C. muridarum was propagated in McCoy cells using DMEM supplemented with 10% FBS, 1 μg/mL cyclohexamide, and 50 μg/mL gentamicin. Briefly, confluent shell vials of McCoy cells were infected by removing growth media and replacing it with supplemented DMEM containing C. muridarum at an MOI of 1.0 and serum from either vaccinated or control mice diluted 1:10. The shell vial was centrifuged for 1 h at 3000g at room temperature. After centrifugation, shell vials were incubated at 37 °C with 5% CO2 for 48 h. Chlamydial inclusions were stained with the Pathfinder Chlamydia detection reagent (BioRad) and visualized with multiple, random fields of view visualized. Percent reduction of infection was calculated compared to a control infection, and statistical significance was calculated using a Student’s t-test.

3. Results

3.1. Type III secretion proteins are highly conserved between C. trachomatis and C. muridarum

To determine whether the three components of the trivalent BD584 antigen are genetically conserved between Chlamydia spp., a bioinformatic analysis was performed to examine the amino acid identity of CopB1_1-100, CopD1_1-100, and CT5844F between C. trachomatis and C. muridarum. BLAST-P analysis revealed that CopB1_1-100, CopD1_1-100, and CT5844F were highly conserved between C. trachomatis and C. muridarum, with 86%, 78%, and 97% amino acid identity, respectively (Fig. 1A). We have previously shown that antibodies generated against N-terminal peptides of CopB and CopD were capable of neutralizing Chlamydia spp. [15,29]. Mice infected with C. muridarum produce serum antibodies against BD584 (Fig. 1B). Cloning of CopB1_1-100, CopD1_1-100, and CT5844F into pET-DUET1 generated an N-terminal polyhistidine-tagged fusion protein hereafter referred to as BD584 with a predicted molecular weight of approximately 47 kDa. Following purification via FPLC, BD584 was greater than 95% pure as analyzed by SDS–PAGE (Fig. 2).

3.2. Chlamydial T3S proteins elicit neutralizing antibodies

Two groups of mice (N = 5) were immunized twice intranasally with both BD584 and CpG adjuvant, or with PBS alone (control group). Following immunization, sera were tested for antibodies to BD584 by ELISA (Fig. 3A). Serum from immunized mice had anti BD584 antibodies with an end point titer of 1:3200 while serum from control mice had no detectable BD584 antibodies.
Conserved amino acid sequences of CopB1–100, CopD1–100, and CT584FL orthologs. (A) BLAST-P analysis was performed using the amino acid sequences of CopB1–100, CopD1–100, and CT584FL from *C. trachomatis* and *C. muridarum* orthologs. Alignment between *C. trachomatis* and *C. muridarum* orthologs revealed 86%, 78%, and 97% amino acid identity between CopB1–100, CopD1–100 and CT584FL, respectively. (B) Western blot analysis using serum from mice infected with *C. muridarum* (1), BD584 vaccinated (2), or pre-immune serum (3), demonstrates the presence of anti-BD584 antibodies in mice infected with *C. muridarum*.

**Fig. 2.** Oligonucleotide primers used to generate recombinant protein BD584. (A) BD584 was constructed by amplifying the genes for CopB, CopD and CT584 using genomic DNA from *C. trachomatis* serovar D using the primers indicated. PCR products were digested with their respective endonuclease (in bold), and then ligated into the MCS-1 of the pET-DUET1 vector. (B) Organization of the final trivalent antigen, termed BD584. (C) BD584 was expressed from pET-DUET in *E. coli* BL21 and purified by FPLC on a Ni-NTA agarose column, buffer exchanged into PBS and analyzed by SDS–PAGE. The positions of molecular weight markers are shown on the left. Note: (+) denotes forward primer, (−) denotes reverse primer, subscripts denotes amino acid number, FL = full length.

**Fig. 3.** Measurement of serum antibodies to BD584 in immunized mice. (A) Serum from vaccinated and control mice were tested by ELISA at serial twofold dilutions from 1:25 to 1:3200 for specific anti-BD584 antibodies as described in Section 2. Absorbance for the last three dilutions 1:800, 1:1600, and 1:3200 is shown. Histogram bars represent the mean absorbance for BD584 + CpG, BD584 alone, and PBS + SEMs. Cutoff value represents two times the mean of the antigen alone vaccinated group at a 1:3200 dilution. (B) BD584-specific serum IgG1 and IgG2c were determined using ELISA. The ratio of IgG2c:IgG1 is used to determine Th1:Th2 polarization and is indicated above the significance bar. Significance was set at \( p < 0.05 \) for all tests. * Indicates \( p < 0.5 \). ** Indicates \( p = 0.01–0.001 \). *** Indicates \( p = 0.001–0.0001 \). **** Indicates \( p < 0.0001 \).
The combination of intranasal immunization with BD584 and CpG induced a Th1-polarized immune response, as shown by the IgG2c: IgG1 ratio of 10:1 (Fig. 3B). To determine whether BD584 immunization elicited neutralizing antibodies, sera were pooled and tested using a neutralization assay. Pooled serum from the immunized mice reduced the number of inclusions by 77% while pooled control sera failed to neutralize *C. muridarum*. (Fig. 4)

### 3.3. Immunization with BD584 reduces bacterial shedding of *C. muridarum*

Mice were immunized then challenged vaginally to determine whether BD584 immunization reduced chlamydial shedding. Vaginal swabs were collected at various time points during the course of infection (days 1, 3, 5, 7, 10, 14, 32) and tested for the presence of *C. muridarum* by qPCR. On day 3 post-infection, vaccinated mice had an 85% (*p = 0.028*) reduction in bacterial shedding as compared to control mice (mean of 256 versus 1504 copies, respectively). At the peak of infection, days 5 (*p = 0.0023*) and 7 (*p = 0.0022*), a 95% reduction in bacterial shedding was observed in the vaccinated group (mean of 90 and 13 copies, on day 5 and 7) compared to the control group (mean of 1555 and 121 copies, on day 5 and 7) (Fig. 5A). Vaccinated mice also cleared the infection sooner than control mice as no *Chlamydia* was detected 32 days post infection in the vaccinated mice (Fig. 5A). Area under the curve (AUC) analysis was determined for shedding during the entire course of the infection (Fig. 5B) and clearly indicates the significant reduction in shedding in the immunized group compared to the control group (*p < 0.0001*).

### 3.4. Immunization with BD584 reduces *Chlamydia*-induced immunopathology

One of the primary goals for an effective Chlamydial vaccine is to reduce or prevent upper genital tract pathology following lower tract infection. To assess the ability of BD584 immunization to reduce *Chlamydia*-induced immunopathology of the ovarian ducts,
mice were vaccinated then challenged as previously described. Animals were sacrificed 35 days post infection to assess the development of hydrosalpinx. In the vaccinated group of five mice, only 1 out of 10 ovaries had hydrosalpinx, which was significantly lower ($p = 0.0055$) than that seen in the control group where 8 out of 10 ovaries had hydrosalpinx (Fig. 6).

4. Discussion

Despite decades of research on Chlamydia immunobiology, limited success has been achieved in vaccine development [6,16]. Given the success of vaccines derived from T3SS proteins in other bacterial species, we developed a novel trivalent antigen consisting of three T3S chlamydia proteins and evaluated this antigen in a C. muridarum mouse model of infection [9,10,12,17–20]. The highly conserved nature of T3SS proteins across different bacterial species highlights the essential nature of this virulence factor and suggests that immunization with T3S antigens could produce an effective pan-serovar Chlamydia vaccine [21–23]. We show here for the first time that T3SS antigens from Chlamydia represent a good vaccine candidate. Intranasal immunization of mice with BD584 antigen plus CpG adjuvant elicited serum antibodies against BD584 which were capable of neutralizing Chlamydia infection in vitro. Mice vaccinated with BD584 and challenged with C. muridarum had a reduction in both bacterial shedding ($p < 0.05$) and Chlamydia-induced fallopian tube pathology ($p < 0.05$). Together, this data suggests that BD584 represents a good chlamydial vaccine candidate.

Since there are over fifteen serovars of C. trachomatis, an ideal vaccine should provide cross-serovar protection. The T3SS is structurally conserved across many Gram-negative bacteria and is an essential virulence factor. Chlamydia spp. are obligate intracellular pathogens and use a T3SS to infect host cells. Despite the amino acid sequence differences seen between orthologous T3S proteins of other bacteria, the amino acid sequence of T3S components is highly conserved between chlamydial species, and between serovars of C. trachomatis [15,24,25]. Our BD584 antigen was selected because it is highly conserved between C. trachomatis serovars and C. muridarum. MOMP has been considered the archetype for immunization against Chlamydia infections for the past 30 years [6,26]. However, vaccination with one C. trachomatis serovar of MOMP provides protection against the same serovar but only limited cross-protection across other serovars [26]. We immunized mice with highly conserved T3SS proteins derived from C. trachomatis and challenged with live C. muridarum to demonstrate that these antigens may provide protection between these similar chlamydial species (Fig. 1B). To date, few studies have examined the use of T3S proteins as antigens to vaccinate against Chlamydia.

In other Gram-negative bacteria, there has been considerable success in vaccination strategies using components of the T3SS; specifically, it has been demonstrated that when orthologs of CopB from Shigella spp. are used as antigens, in combination with other T3S proteins, there is significant protection against in vivo challenge with Shigella [12,17–19,27]. Immunization with BD584 represents a novel approach for prevention of chlamydial infection and/or Chlamydia-induced pathology. Experiments are in progress to determine the level of protection afforded by BD584 compared with other chlamydial antigens such as MOMP.

It has been well documented in the literature that a cell-mediated immune response is critical to protecting and clearing a Chlamydia infection. We selected the adjuvant CpG for use with BD584 and immunized mice intranasally in attempts to generate a Th1 polarized immune response at the genital mucosa [28]. To ensure that our immunization strategy was successful, sera from immunized mice were tested for the presence of antigen specific serum IgG. Previously, we have shown that antibodies raised against the translocators, CopB and CopD, can inhibit infection suggesting that antibodies directed towards these proteins block an essential aspect of T3S during infection [15,29]. Serum collected from mice immunized with BD584 reduced infectivity by 77% when compared to control serum. This reduction in infectivity by serum antibodies directed against the translocators suggests that these antibodies are directed against surface exposed epitopes on CopB and CopD. Serum from vaccinated mice was not heat inactivated, and as a result, the observed neutralization could be due in part to antibody dependent complement activation. However, the mechanism of neutralization remains to be elucidated, but is presumably due to the antibodies rendering the T3S inactive and preventing host cell infection. In addition to antibodies, an efficient cell-mediated immune response is essential for protective immunity against chlamydial infection [5]. Our vaccine induced a Th1 polarized immune response as shown by the IgG2c:IgG1 ratios of 10:1. Experiments to further characterize the mechanism of protection are currently underway.

An important characteristic of a Chlamydia vaccine would be the ability to decrease bacterial shedding to reduce transmission and to produce an immune response to prevent Chlamydia-induced immunopathology. Recently, it has been shown that approximately 300 IFUs are required to establish an active Chlamydia infection in mice [30]. During the course of a Chlamydia infection in mice, bacterial shedding occurs for approximately 14–35 days before being cleared from the lower genital tract [6]. In the mouse model of infection, it is believed that pathology in the upper genital tract occurs as a result of an ascending infection from the lower genital tract. To assess the ability of BD584 immunization to reduce bacterial shedding, vaginal swabs were collected and analyzed by qPCR. During the peak of infection, 3–7 days post infection, vaccination with BD584 reduced bacterial shedding by 95% compared to control vaccinated mice. In our study immunized mice shed approximately 90 genome equivalents compared to more than 1500 genome equivalents in the control vaccinated mice on day 5 at the peak of infection. Furthermore, C. muridarum was not detectable in vaginal swabs on day 32 post-infection in the vaccinated mice; whereas, it was detected in control mice. Immunization with BD584 significantly reduced bacterial shedding, at all time points, to <300 genomic copies, which infers <300 IFUs, as PCR detects both live and dead bacteria. Based on the observation that a minimum of 300 IFUs are required to establish an active infection, we speculate that immunization with BD584 may decrease the transmissibility of Chlamydia infections. This data was used to determine the AUC, which is a measure of the total bacterial burden during infection as it reflects both the magnitude of shedding at given time points and the duration of infection. As determined by the AUC analysis, mice intranasally immunized with BD584 and CpG had significantly reduced shedding when compared to control mice. One of the limitations of our study is the use of qPCR as a surrogate for culture to assess shedding.

Since Chlamydia infections causes significant UGT pathology we examined the UGT following Chlamydia challenge to determine whether vaccination could prevent Chlamydia-induced immunopathology. Immunization with BD584 decreased the rate of hydrosalpinx from 80% to 10%, suggesting that BD584 may reduce infertility [31]. The reduction in bacterial shedding, coupled with protection against Chlamydia-induced pathology suggests that BD584 affords a significant degree of protection and could be an effective vaccine for human use. Since the Th1 immune response is required to clear a chlamydial infection and elicit antibodies to prevent infection, the BD584 vaccine likely produced a Th1 polarized immune response, and anti-chlamydial antibodies targeted against the chlamydial T3S proteins. The immune mechanism of protection afforded by BD584 is not known but the combined neutralizing antibodies and cell-mediated immune response...
could be instrumental in reducing bacterial shedding and pathology. We are currently investigating the immune mechanism of protection.

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**Conflict of interest**

The authors declare that they have no conflicting interests.

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