TYPE III SECRETION PROTEINS AS A CHLAMYDIA VACCINE CANDIDATE

A CHIMERIC ANTIGEN CONSISTING OF TYPE III SECRETION PROTEINS AS A CHLAMYDIA VACCINE CANDIDATE

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TITLE: A Chimeric Antigen Consisting of Type III Secretion Proteins as a Chlamydia Vaccine Candidate

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Lay Abstract

Chlamydia is the most common sexually transmitted bacterial infection in the world. The goal of this thesis is to evaluate a novel chlamydia vaccine in a mouse model of genital chlamydia infection. We engineered a fusion protein, BD584, made up of three highly conserved type III secretion (T3S) proteins CopB, CopD, and CT584. We show that vaccination with BD584 generated strong immune responses and protected mice from chlamydia infection and the associated reproductive tract disease. Interestingly, the level of protection afforded by BD584 vaccination is dependent upon the genetic background of the animal. Furthermore, we have identified particular antibody subtypes directed against BD584 as markers of BD584-mediated protective immunity. Lastly, we show that vaccination with BD584 formulated with a clinically safe and effective mucosal adjuvant generates robust immune responses and confers protection against chlamydia in mice. Together, these results provide support for the use of T3S proteins in a chlamydia vaccine.

Abstract

Chlamydia is the most prevalent sexually transmitted bacterial infection in many developed countries, including Canada. Untreated infections in women can lead to a number of complications including pelvic inflammatory disease, tubal factor infertility, and ectopic pregnancy. Public health programs, including screening for at-risk individuals, partner identification, and antibiotic treatment, have had limited success in controlling the rising incidence of chlamydial infections over the past two decades. A chlamydia vaccine that prevents infection and its pathological sequelae is the next essential step to control this persistent public health problem. *Chlamydia spp.* utilize the highly conserved type III secretion (T3S) system as an essential virulence factor for infection and intracellular replication. Here, we evaluated a novel chimeric antigen (BD584) consisting of three T3S proteins from *C. trachomatis* (CopB, CopD, and CT584) as a potential chlamydia vaccine candidate. Intranasal immunization with BD584 elicited strong humoral responses that neutralized infection *in vitro*. Following intravaginal challenge with *C. muridarum*, immunized mice had a 95% reduction in chlamydial shedding and a 87.5% reduction in incidence of upper genital tract pathology compared to control mice. BD584 immunization generated strong cell-mediated and mucosal antibody responses in mice with different genetic backgrounds, and conferred protection against an intravaginal *C. trachomatis* infection in two out of three strains of mice. BD584 formulated with NE01, a mucosal adjuvant known to be safe and effective in humans, was shown to be highly immunogenic and efficacious against *C. trachomatis*

infection in mice. These results suggest that BD584 may represent a promising antigen for use in a chlamydia vaccine.

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First and foremost, I would like to thank Dr. James Mahony for being an extraordinary supervisor and mentor. He has given me the freedom to explore my own academic interests, discover my strengths, and reflect on my weaknesses, but with enough guidance to keep me on the right track. Thank you for valuing my input and always treating me like a future colleague rather than a student.

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Dr. David Bulir, where do I start. You are the smartest and kindest person I know, and you have been the single most influential person in my life over the past few years. Thank you for showing me how to do this thing called science. Thank you for believing in me when I doubted myself. Thank you for being a friend.

Mom and Dad. Now, I know you always want to do something, anything, to help. But when you offer it, I always say no. I hope you know that it is not because I do not

want it, but because I already have from you the best kind of help that any child can ask for. The truth is, knowing that you will always be there for me is what gives me the sustained courage to move forward without fearing what might happen if I fail. It is because of you that I am where I am today. And it is you, not me, who should take credit for what is written in these pages. So from the bottom of my heart, thank you.

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List of Abbreviations

ADCC - Antibody-Dependent Cellular Cytotoxicity

APC - Antigen Presenting Cell

CD - Cluster of Differentiation

cGAS - Cyclic GMP-AMP Synthase

cMLC - Chlamydial Memory Lymphoid Clusters

COMP - Chlamydial Outer Membrane Complex

CPAF - Chlamydia Protease-Like Activity Factor

cSAP - Charge Switching Synthetic Adjuvant Particles

DC - Dendritic Cell

EB - Elementary Body

FPR2 - Formyl Peptide Receptor 2

HPV - Human Papillomavirus

HSP60 - 60 kDa Heat Shock Protein

IDO - Indoleamine 2,3-Dioxygenase

IFN - Interferon

IFU - Inclusion Forming Unit

IL - Interleukin

iLN - Inguinal Lymph Node

IPTG - Isopropyl β-D-1-Thiogalactopyranoside

LGV - Lymphogranuloma Venereum

LPS - Lipopolysaccharide

MHC - Major Histocompatibility Complex

MOMP - Major Outer Membrane Protein

NALT - Nasopharynx-Associated Lymphoid Tissue

NHP - Non Human Primate

NK - Natural Killer

NOD1 - Nucleotide-Binding Oligomerization Domain-Containing 1

NSET - Non-Surgical Embryo Transfer

PAMP - Pathogen-Associated Molecular Patterns

PBMC - Peripheral Blood Mononuclear Cells

PD-L1 - Programmed Death-Ligand 1

pIgR - Polymeric Immunoglobulin Receptor

Pmp - Polymorphic Membrane Protein

PRR - Pattern Recognition Receptors

RB - Reticulate Body

SPG - Sucrose-Phosphate Glutamate

T3S - Type III Secretion

TARP - Translocated Actin-Recruiting Phosphoprotein

TFI - Tubal Factor Infertility

TLR - Toll-Like Receptors

Trm - Tissue Resident Memory

TWAR - Taiwan Acute Respiratory Agent

UGT - Upper Genital Tract

UV-Ct - Ultraviolet Inactivated *C. trachomatis*

VD4 - Variable Domain 4

Declaration of Academic Achievement

All experiments presented in this thesis have been designed by Dr. James Mahony and I. Dr. Ali Ashkar and Dr. Charu Kaushic have contributed to the design of specific experiments in Chapter 3. The BD584 construct was originally designed by Dr. David Bulir. Dr. David Bulir and I have contributed equally to the animal work in Chapter 3, and I performed the immune assays. Sylvia Chong assisted with quantitation of bacteria in Chapter 3. All experiments in Chapters 4 and 5 were performed by me. I received technical help from Dr. Shyamala Ganesan for quantifying cytokines in Chapter 5.

Chapter 1 – Introduction

1.1 The Public Health Problem

Chlamydia is the most prevalent sexually transmitted bacterial infection in many developed countries, including Canada. Globally, the World Health Organization estimates that over 130 million new cases of chlamydia infection occur each year, with the highest incidence occurring in the most impoverished regions of the world where adequate control programs are significantly lacking[1].

Infection can result in acute inflammation characterized by redness, edema, and discharge, resulting in the clinical syndrome of mucopurulent cervicitis in women and non-gonococcal urethritis in men[2]. In women, symptoms include abnormal vaginal discharge, postcoital bleeding, irregular uterine bleeding, and pelvic discomfort. Symptoms in males are typically limited to dysuria and moderate clear-to-whitish discharge. However, up to 90% of women and 50% of men with chlamydia are asymptomatic, and consequently do not have an impetus to seek testing and treatment[2]. Untreated infections in women can lead to a number of complications including pelvic inflammatory disease, tubal factor infertility, and ectopic pregnancy (Figure 1). The estimated costs associated with chlamydia infection in Canada and the United States exceed $50 million and $500 million, respectively[3,4].

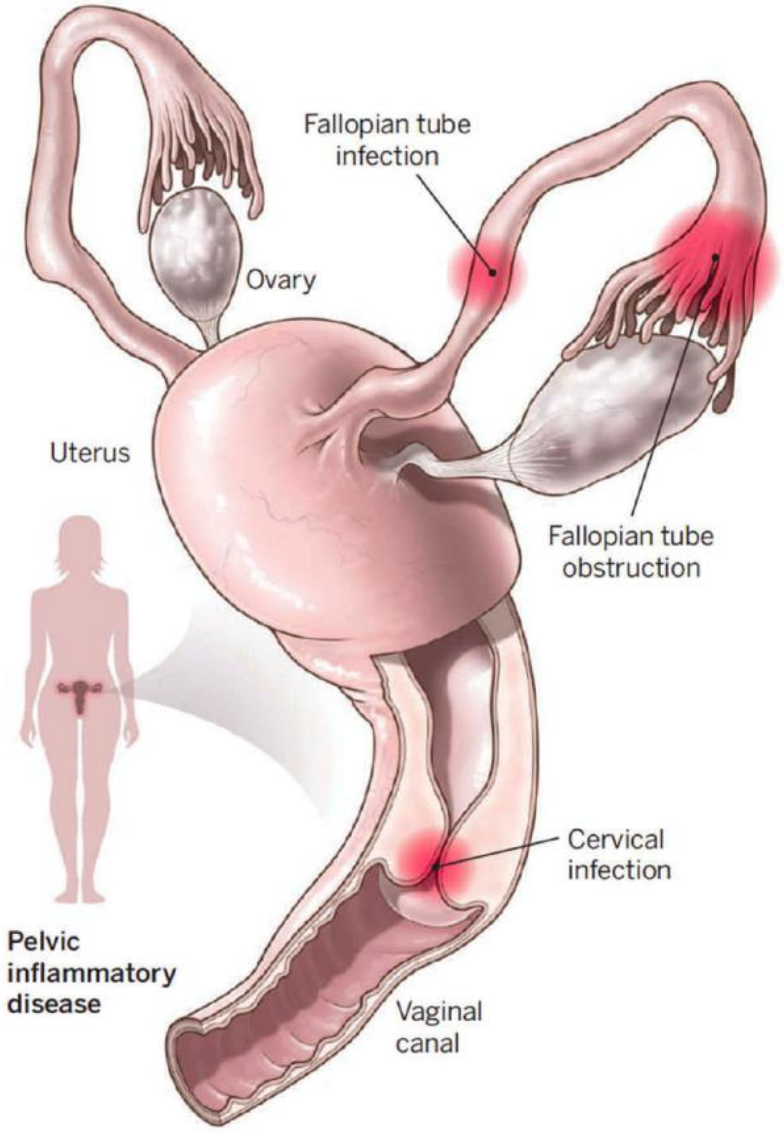


Figure 1. Reproductive sequelae in women as a result of untreated *C. trachomatis* infection. Reproduced with permission from[5].

In affluent countries, public health programs, including screening for at-risk individuals, partner identification, and antibiotic treatment, have had limited success in controlling the rising incidence of chlamydial infections over the past 20 years[6] (Figure 2). It is conceivable that early antibiotic treatment as a result of these public health measures may have blunted the development of protective immunity in individuals. This phenomenon, called the arrested immunity hypothesis, which is supported by epidemiological and experimental evidence[6,7], could have suppressed the development of population-level immunity to chlamydia. A chlamydia vaccine that prevents infection and its pathological sequelae has the potential to significantly reduce the associated health and economic burdens, and is the next essential step to control this persistent public health problem.

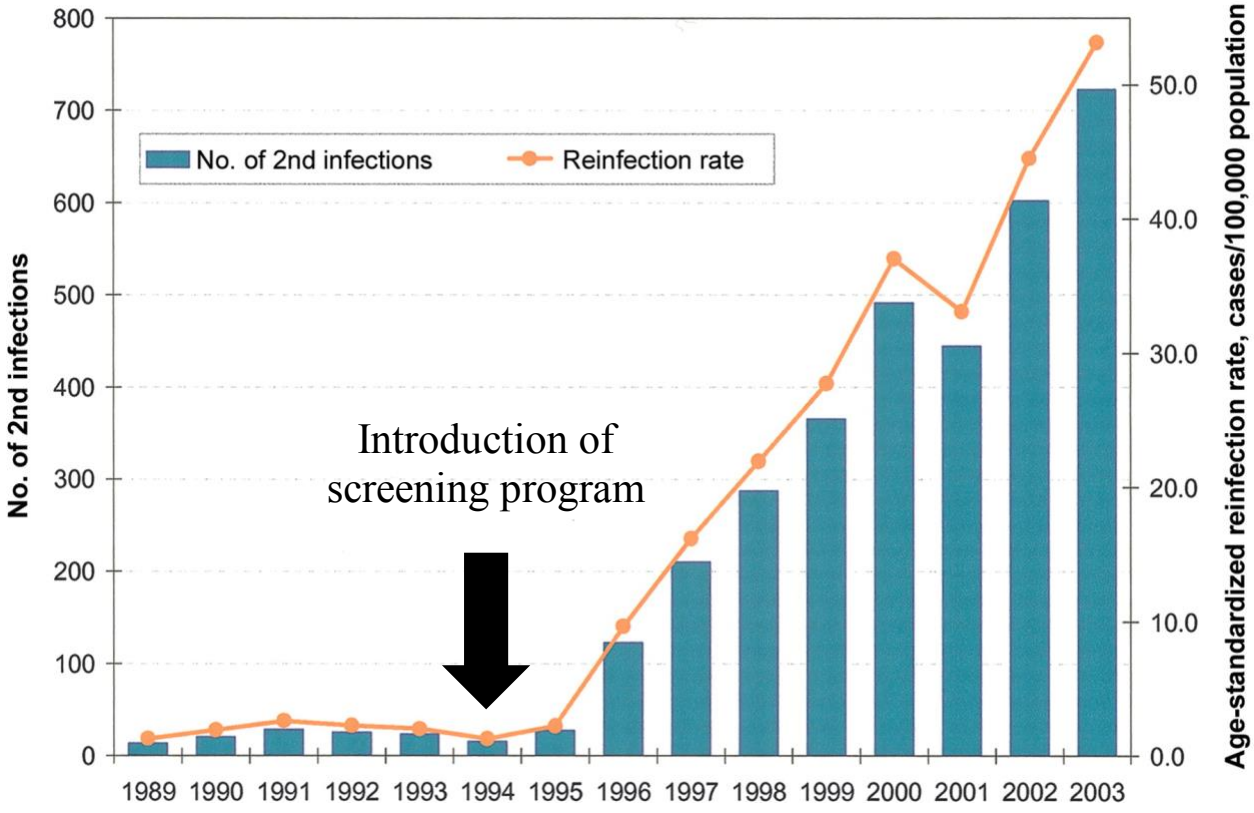


Figure 2. ***C. trachomatis*** **reinfection numbers and rates in Greater Vancouver. 1989-2003. Reproduced with permission from[6].**

1.2 Humanity's Early Efforts to Solve It

The evolutionary arms race between pathogens and hosts have provided each with a set of tools and strategies to outmaneuver the other. As hosts, humans have several at our disposal, including physical barriers like the skin and mucosa, chemical barriers like stomach acid, and biological barriers like our cell-autonomous defense systems[8]. More recently, speaking from an evolutionary time scale, we have acquired the adaptive immune system, which helps us by "remembering" a previous encounter with a pathogen, and responding with greater force and in a more timely fashion the next time we see it[9]. This has worked well for us against many pathogens, including rhinoviruses that cause the common cold. However, for those that cause significant morbidity, humanity has decided that we would rather not see them at all if we do not have to, and understandably so. Moreover, since for some pathogens even that secondary response may not be enough, we would want to make use of the arsenals that nature has given us, but at the same time manipulate them to our advantage.

The first vaccine was against smallpox, and it has been tremendously successful. Perhaps too successful, because we had no failure to learn from. For the next several decades, we hoped that because it worked for smallpox, it would work for everything else, and we have tried our luck with many different pathogens. I use the word "luck" seriously here, because we truly did not understand what we were doing[10]. We were more familiar with the distal cause (some substance isolated from patients with disease) than the proximate cause (immunological memory) of the state of protection from contagious disease. As a result of the lack of understanding of what led to effective immunological

memory, and why some formulations failed, and what that memory consisted of, some vaccines worked (smallpox, polio, measles, mumps, rubella etc.), but many did not (HIV, TB, Chlamydia etc.)[11]. It turns out that artificially engineering a physiological state that nature has optimized for us, and is only achieved by adhering to very specific parameters, can be and has been difficult, and we are only beginning to find out why. In the chlamydia field, part of what prompted us to abandon the trial and error approach and seek understanding is our failed attempts to develop a vaccine against trachoma half a century ago[12].

Trachoma is the world's leading infectious cause of blindness[13]. It is caused by the same species of bacteria that causes the sexually transmitted infection chlamydia. *Chlamydia trachomatis*, the etiologic agent of both of these conditions, was isolated in 1957, and vaccine studies against trachoma began soon after[14]. Formalin-fixed chlamydial elementary bodies (EBs), which we now understand to be the infectious form of the bacteria, were used to vaccinate children. Researchers followed these children for three years, and found that vaccination only conferred partial, strain-specific, and short-lived immunity. Furthermore, when compared to the unvaccinated controls, vaccinated children had a greater risk of developing more severe forms of the disease after exposure to *C. trachomatis*[14,15]. These observations were postulated to be a result of delayed-type hypersensitivity. The same observations were made when similar experiments were carried out in non-human primates[12]. The possibility of deleterious antigens in EBs led researchers away from the use of whole organisms in vaccines and back to animal models for a better understanding of chlamydial immunobiology.

1.3 Going Back to the Drawing Board - Know Your Enemy

1.3.1 Reconnaissance: A Brief History of Discovery

Let us go back another half a century, to 1907, when two German scientists first described members of the *Chlamydiae* phylum. Halberstaedter and von Prowazek took infected conjunctival scrapings from humans and infected orangutans and observed cytoplasmic inclusions via light microscopy in epithelial cells[16]. The causative agent identified at the time was *C. trachomatis*, but was classified as a protozoan named *Chlamydozoa*. *Chlamydia psittaci* was identified as the causative agent of psittacosis, a zoonotic respiratory infection, following an outbreak from 1923-1930, but was misclassified as a "large virus"[16]. It wasn't until the mid 1960's that a researcher named J.W. Moulder provided evidence that *Chlamydiae* were not viruses, but in fact bacteria, by the presence of a cell envelope, RNA and DNA, and the existence of ribosomes via electron microscopy[17,18]. The first specimen of *Chlamydia pneumoniae* (TWAR) was collected from a control subject in a trachoma vaccine study, and was identified as *C. psittaci* until 1989 when Grayston *et al.* identified it as *C. pneumoniae*[19,20].

1.3.2 Target Acquired: *Chlamydia trachomatis*

The term *Chlamydia trachomatis* derives from the Greek words chlamydos ("chlamys" - a coat, which were presumably inclusions formed by the intracellular bacteria that surrounded the nucleus) and trachoma. The human pathogen can be divided

into three biovars based on the type of infection a particular biovar causes, and encompasses 18 serovars, which are classified based on the antigenic properties of the major outer-membrane protein (MOMP). Serovars A, B, Ba, and C cause blinding trachoma, whereas serovars D, Da, E, F, G, H, I, Ia, J, and K are responsible for oculogenital infections, which are infections of the urogenital system in adults, conjunctivitis in adults and children, and pneumonia in children. Serovars L1, L2, L2A, and L3 cause lymphogranuloma venereum (LGV), a primarily sexually transmitted infection of the lymphatics and lymph nodes[21].

1.3.3 Enemy Lifestyle: The *Chlamydia* Developmental Cycle

Chlamydiae have a unique biphasic developmental cycle with morphologically and physiologically distinct forms: the elementary body (EB; which was the form used in early trachoma vaccines) and the reticulate body (RB). The EB, which is infectious, and minimally metabolically active, is responsible for the attachment and invasion of host epithelial cells. Upon entry, chlamydial proteins rapidly modify the newly formed endocytic compartment in order to create a parasitophorous vacuole termed an inclusion. Within the inclusion, the EB differentiates into RB, the metabolically active, replicative, and non-infectious form of the bacterium. The inclusion expands as RBs replicate via binary fission. Following several rounds of division, RBs are triggered to asynchronously differentiate into the infectious EB form, a process that has been recently suggested to be dependent on the size reduction of RBs following rounds of binary fission and

independent of an external signal[22]. Newly formed EBs are then released from the host cell via cell lysis and/or extrusion to infect adjacent cells. In the presence of growth inhibitors, host-derived or pharmaceutical, *C. trachomatis* may acquire a non-replicating, persistent form, which reverts back into infectious forms upon removal of the inhibitor[23] (Figure 3).

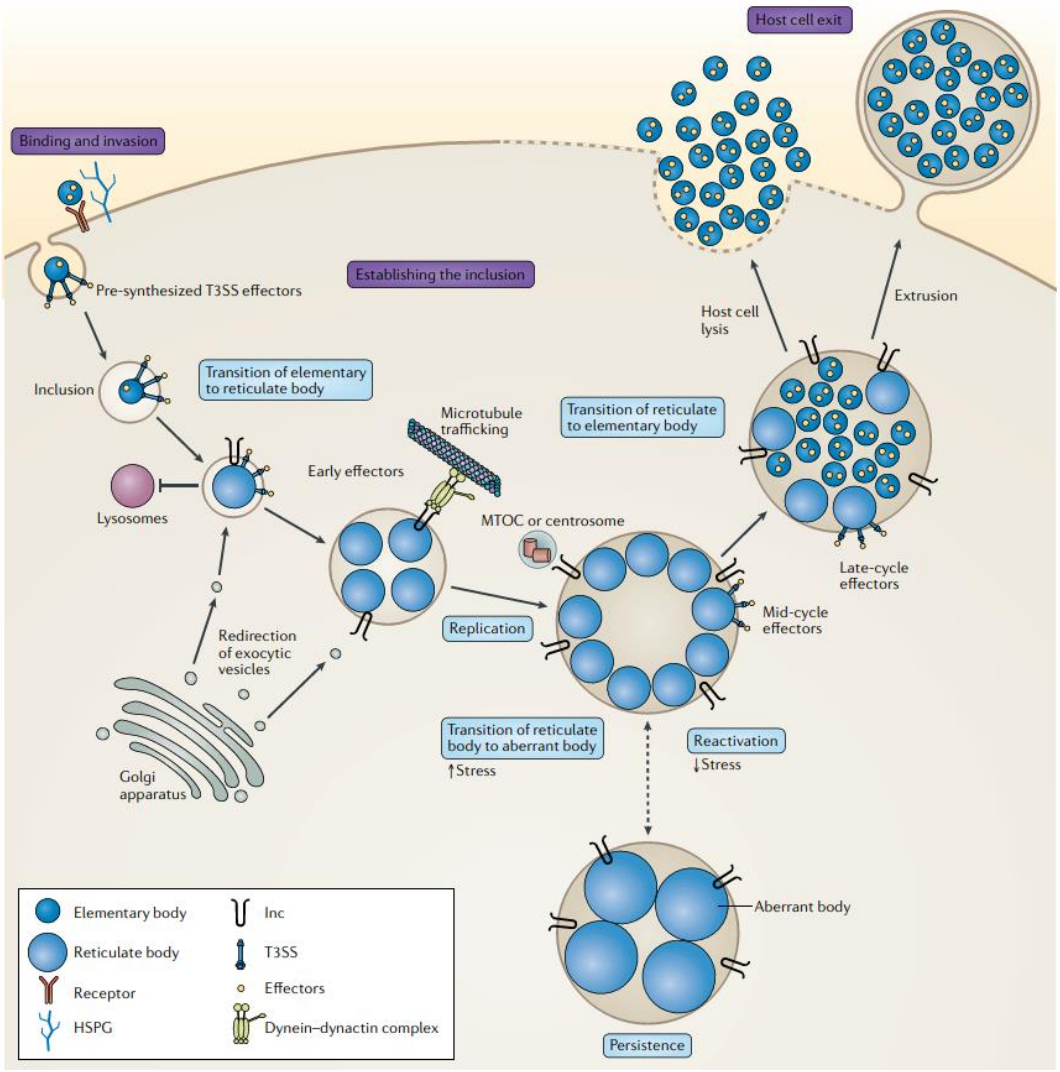


Figure 3. The developmental cycle of *C. trachomatis*. Reproduced with permission from[23].

1.3.4 Trespassing: Adhesion and Invasion

Binding of EBs onto host cells is thought to be a two-step process. It involves an initial reversible interaction between the EB adhesins and heparin sulfate proteoglycans on a host cell, followed by an irreversible binding to a second receptor, of which there are many, including β1-integrin, epidermal growth factor receptor, and receptor tyrosine kinases[24-27]. Several chlamydial proteins have been suggested to mediate invasion, including OmcB, MOMP, and the polymorphic membrane protein (Pmp) family[28]. The absence of a system for targeted inactivation of *Chlamydia* genes has made it difficult to ascertain the extent to which these putative ligands contribute to *Chlamydia* attachment and entry. However, recent advances in the genetic manipulation of *Chlamydia* have made it possible to increase the degree to which we are confident with these proposed interactions[29].

Upon contact with host cells, pre-packaged effectors, of which the translocated actin-recruiting phosphoprotein (TarP) has been the most extensively studied, are injected through the type III secretion (T3S) system to promote cytoskeletal rearrangements that allow for invasion and induce host signaling[30]. Moreover, proteins that make-up the T3S injectisome, including the translocators CopB and CopD, are essential for infection, as neutralization of these proteins by antibodies significantly reduce the number of nascent inclusions[31,32]. Understanding the molecules and processes that lead to attachment and entry could help identify new vaccine targets.

1.3.5 Getting Comfortable: Creating an Intracellular Niche

Newly formed inclusions are transported along microtubules to the microtubule organizing centre (MTOC), which positions the protected bacterial vesicle in close proximity to nutrient rich compartments[33,34]. At the same time, chlamydial survival depends on its ability to inhibit fusion with host cell compartments like lysosomes. Selective fusion is achieved by differentially recruiting proteins from several families of fusion regulators, including RAB GTPases, phosphoinositide lipid kinases, and SNARE proteins[35,36].

Nutrient acquisition by *C. trachomatis* involves incorporation of host cell-derived lipids, including phosphatidylcholine, phosphatidylinositol, sphingomyelin and cholesterol[37]. These lipids are necessary for many processes, including replication, homotypic fusion, and growth and stability of the inclusion membrane, and are acquired via vesicular and non-vesicular pathways[38,39]. Around the mid-cycle stages of infection, *C. trachomatis* induces fragmentation of the Golgi apparatus into mini-stacks that surround the inclusion[40]. However, the role of Golgi fragmentation is unclear, as multiple experimental approaches suggest that it is not required for *C. trachomatis* growth, lipid uptake, or sphingolipid trafficking[41,42]. In parallel with all of these processes, the inclusion body is stabilized by a dynamic scaffold consisting of F-actin and intermediate filaments, which appears to be essential given the highly fragile nature of the inclusion[38,43]. This structure, along with the inclusion itself, largely shields *C. trachomatis* from host immune surveillance, and is therefore a challenge for vaccine development.

1.3.6 To Lyse or Not to Lyse

That is the question.

EBs are released via two mutually exclusive mechanisms: host cell lysis or extrusion. But how *Chlamydia* chooses one over the other is currently unknown. The lytic pathway results in host cell death following, in sequence, the rupture of the inclusion membrane, the nuclear membrane, and the plasma membrane, and has been suggested to be dependent on the chlamydia protease-like activity factor (CPAF)[44,45]. In contrast, extrusion leaves the host cell intact and involves the pinching of the plasma membrane around and the expulsion of the inclusion, a process that requires actin polymerization[44]. Extrusion might benefit *Chlamydia* by shielding it from pre-existing local immune responses, exposing organisms to the extracellular environment only after they have moved to a safer location, or facilitate dissemination by allowing engulfment by macrophages. It represents a newly identified phenomenon that is specific to *Chlamydia*, and has been recently confirmed to occur *in vivo*[46].

Until recently, how *Chlamydia* chooses to pursue either pathway was unclear. Shaw *et al.* recently presented conclusive evidence that the balance of the extrusion and lytic pathways is mediated, at least in part, by CT228[47]. Loss of CT228 expression as a result of targeted chromosomal mutation of CT228 in *C. trachomatis* serovar L2 led to a loss of MYPT1 recruitment to the inclusion membrane and an increase in extrusion *in vitro*. The mutant strain had a delay in clearance of infection and a reduction in the *C. trachomatis* specific systemic humoral response to infection was documented. These observations were expected insofar as it is reasonable to conclude that the extrusion

pathway shields nascent EBs from immune recognition compared to the lytic pathway. However, given that a loss of function in CT228 causes a prolonged infection and suppresses host antibody response, it is unclear how CT228 may act to enhance to evolutionary fitness of *Chlamydia*; the authors have not presented any potential explanations.

1.4 Going Back to the Drawing Board - Know Yourself

The road to [disease] is paved with good intentions.

Not unlike many other pathological outcomes arising from infectious agents, such as pneumonia as a result of influenza infection, chlamydia-induced upper genital tract disease is largely a result of collateral damage from an inflammatory immune response aimed to eliminate the bacteria for protection of the host[48]. In an effort to expel the invading organisms, the body sometimes uses weapons that can unfortunately damage itself. There are two main schools of thought as to how this occurs in chlamydial pathogenesis: the cellular paradigm and the immunological paradigm.

The cellular paradigm posits that it is largely the inflammatory molecules secreted by infected epithelial cells themselves as well as the innate immune system's response to infection that are, over time, driving inflammation, host cell death, and eventually pathology[49]. On the other hand, the immunological paradigm argues that the adaptive immune response, through cytotoxic effectors like perforin and granzyme made by CD8

T cells, is primarily responsible for tissue damage[48]. It is important to note that evidence supporting the cellular paradigm mainly comes from the mouse model, while the immunological paradigm is substantiated by data from guinea pig and primate models, as well as studies in humans. Nonetheless, the two hypotheses are not mutually exclusive, and the true cause of disease is likely a combination of some degree of both. But what is becoming harder to dispute is that a chlamydia vaccine needs to walk a very fine line between conferring protection and exacerbating pathology. A big part of the goal of chlamydia vaccinology has been and will continue to be trying to understand how to do just that.

1.4.1 Raising the Alarm: Recognition of Danger

C. trachomatis normally infects the epithelium in the endocervix of women and the urethra of men[2]. Despite the physical shielding mechanism of the inclusion membrane and the multitude of molecular mechanisms utilized by *Chlamydia* to evade host recognition, epithelial cells can still detect the presence of *Chlamydia* through cell surface receptors, endosomal receptors, and cytosolic sensors. Toll-like receptors (TLRs), such as TLR2 and TLR4, are activated upon binding to *Chlamydia*. TLR2 recognizes peptidoglycan, macrophage inhibitory protein, and plasmid-regulated ligands, while TLR4 is activated upon binding to chlamydial LPS and/or the 60 kDa heat shock protein (HSP60)[50]. Intracellular sensors like cyclic GMP-AMP synthase (cGAS) can sense cytosolic DNA, which eventually leads to the expression of type I interferons (IFNs)[51].

The activation of intracellular peptidoglycan-binding molecule, nucleotide-binding oligomerization domain-containing 1 (NOD1) upon infection with *C. trachomatis* indicates that chlamydial peptidoglycan can access the host cytosol[52]. Activation of these sensors prompts the host cell to release proinflammatory cytokines and chemokines that recruit inflammatory cells, which further produce proinflammatory molecules in a positive feedback cycle[38]. However, this inflammatory response is also responsible for immunopathology via collateral damage and scarring[48].

1.4.2 The First Responders: The Innate Immune Response

Natural killer (NK) cells and neutrophils are the first cells recruited to the site of *C. trachomatis* infection in the female genital tract. Neutrophils have been suggested to play a major role in the reduction of initial chlamydial titers and subsequent spreading of the infection. In support of this notion, mice depleted of neutrophils had a ten-fold greater burden of *Chlamydia muridarum* (the murine-specific species of *Chlamydia*) in the female genital tract than wild type mice[53]. However, both groups of mice were able to resolve infection within the same time frame, suggesting that neutrophils are not necessary for the resolution of infection. Interestingly, evidence from recent studies suggest that *C. trachomatis* may be able to inhibit neutrophil apoptosis[54]. More recently, *C. trachomatis* has been shown to paralyze neutrophils by using CPAF to cleave formyl peptide receptor 2 (FPR2), which recognizes formylated peptides, a pathogen-associated molecular pattern (PAMP)[55].

While neutrophils are generally effective against extracellular bacteria, NK cells are largely responsible for intracellular disturbances, including viral and intracellular bacterial infections, as well as cancer[56]. After *C. trachomatis* stimulation, epithelial cells and dendritic cells (DCs) produce interleukin-18 (IL-18) and IL-12, respectively, which induce NK cells to secrete IFN-γ[57]. Tseng and Rank showed that IFN-γ-producing NK cells are recruited to the site of *C. muridarum* infection within 12-24 h after inoculation. Depletion of NK cells resulted in delayed bacterial clearance and a Th2-polarized immune response, consistent with the role of IFN-γ in directly controlling *Chlamydia* replication and the indirect induction of Th1 immunity through DCs[58].

Upon infection with *C. trachomatis,* macrophages migrate to the site of infection, phagocytose *Chlamydia*, and produce inflammatory cytokines. Compared to epithelial cells, only a small fraction of RBs are detected in macrophages, suggesting that replication of *Chlamydia* inside macrophages is inefficient[59]. The limited ability of *Chlamydia* to proliferate in macrophages is associated with host cell autophagy, in which cells degrade proteins and organelles in the cytoplasm[60]. Furthermore, IFN-γ enhances both autophagy and upregulation of major histocompatibility complex (MHC) class II molecules in macrophages, both of which have been shown to enhance antigen presentation to T cells[60,61]. This may be important during the adaptive immune response to infection where activation of primed T cells then in turn enhance macrophage activity.

1.4.3 Special Weapons And Tactics: The Adaptive Immune Response

In the *C. muridarum* mouse model, adaptive immunity is required for clearance of infection[62]. Specifically, CD4+ T cells alone are sufficient for clearing a primary infection[12]. However, resistance against re-infection is mediated by either CD4+ T cells or B cells (Figure 4). It is important to note that these generalizations apply to only the *C. muridarum* genital tract infection model and only when immunity is generated by a prior *C. muridarum* infection. As discussed below, discrepancies between the relative contributions of the different effectors of the adaptive immune response to the outcome of chlamydial infections can originate from the bacteria used (*C. trachomatis* vs *C. muridarum*) or the animal model used (mouse vs non-human primate).

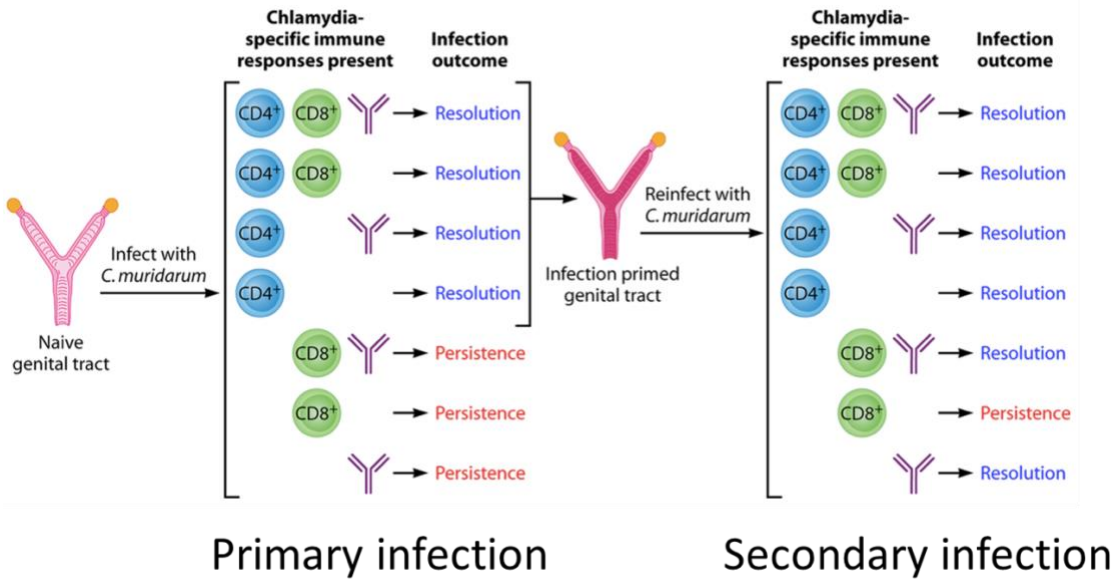


Figure 4. Protective adaptive immune responses from using the *C. muridarum* mouse model of genital tract infection. Reproduced with permission from[12].

4.3.1 - Dendritic Cells

DCs are the primary antigen presenting cells (APCs) and are quintessential in initiating the adaptive immune response. DCs are present in mouse vaginal and cervical mucosae, and are recruited to the site of infection following *Chlamydia* inoculation. Immature DCs are highly phagocytic, while mature DCs are highly efficient at presenting antigens. Antigen presentation to T cells via MHCs activate T cells to initiate a cell mediated and/or humoral immune response. A multitude of studies have demonstrated that DCs are able to activate T cells via MHC class I or class II presentation in chlamydial infection both *in vitro* and *in vivo*[63-65]. Interestingly, studies have shown that DCs incubated with live EBs presented many more peptides on their MHC class II molecules than DCs that were incubated with dead EBs, and that they conferred greater protection against infection when adoptively transferred into naive mice[66].

DCs previously exposed to heat-inactivated *C. trachomatis* EBs were transferred to naïve mice, which were protected against subsequent infection, with the protective response being Th1 mediated[67]. On the other hand, DCs deficient in IL-10 (a Th2-associated cytokine) pulsed with UV-inactivated *C. trachomatis* and adoptively transferred to mice activated primarily Th1 cells[68]. These data are relevant to vaccine development because DCs bridge the innate and adaptive immune systems, and suggest that the type of cytokines and antigens processed by DCs and presented to T cells mediate the Th1/Th2 balance during *Chlamydia* infection.

4.3.2 - CD4 T Cells

Results from animal studies have clearly established that T cells play an essential role in the resolution of *Chlamydia* infection. Specifically, CD4+ T cells that produce IFN-γ likely mediate protection in the *C. muridarum* model of infection. Mice deficient in MHC class II molecules, CD4, IL-12, IFN-γ receptor, and mice depleted of *C. muridarum*-specific CD4+ T cells all have a significantly reduced ability to control infection, suggesting CD4+ T cells are needed for optimal protection against *Chlamydia* infection[64,69-71] Further supporting the crucial role of a Th1 immune response in *Chlamydia* infection, adoptive transfer of *C. muridarum*-specific CD4+ Th1 cells, but not Th2 cells, protected nude mice against *C. muridarum* challenge[72]. Importantly, resolution of infection still occurs in B cell deficient mice depleted of CD8+ T cells, suggesting that CD4+ T cells alone are sufficient for primary protection against *Chlamydia* infection[70].

Characterized by the production of IFN-γ, Th1 cells are the dominant subset of CD4+ T cells associated with *Chlamydia* infection in mice. IFN-γ is essential for the resolution of chlamydial infection, as IFN-γ receptor-deficient mice displayed higher levels of chlamydial shedding and systemic dissemination of infection[71,73,74]. IFN-γ acts by inducing numerous host cell defense pathways such as iNOS and iron deprivation. One of the most appreciated mechanisms by which IFN-γ exerts its anti-chlamydial functions is through the activation of indoleamine 2,3-dioxygenase (IDO), which acts by depleting tryptophan, an essential amino acid without which chlamydial growth would be severely stunted[60]. Interestingly, multifunctional Th1 cells, which secrete other cytokines such as TNF-α along with IFN-γ, are associated with enhanced protection against

chlamydial infection in mice, suggesting that these cytokines work together to enhance protection[75]. This notion is supported by a later study that provided conclusive evidence for a synergistic role of IFN-γ and TNF-α, as well as IFN-γ and IL-17, in inhibition of chlamydial growth[76]. In humans, correlational studies have found a clear association between Th1 responses and protection against chlamydia. EB-induced IFN-γ secretion by PBMCs from *C. trachomatis* infected women correlated with a reduced risk of reinfection[77]. Recently, Bakshi *et al.* found similar associations and confirmed that the source of IFN-γ is CD4+ T cells[78].

In contrast to Th1 cells, Th2 cells are characterized by IL-4 secretion and not considered to be protective against *Chlamydia*. In mouse models, Th2 cytokines, including IL-4, IL-5, IL-10, and IL-13, play insignificant roles in clearing infections, and are associated with exacerbation of pathology, which is thought to be due to their antagonist role towards Th1 responses[79]. However, more recently, Miguel *et al.* found that in *C. trachomatis* infected women, peripheral CD4+ T cells that proliferated upon *ex vivo* stimulation with inactivated EBs predominantly and significantly secreted more IL-4 than IFN-γ, TNF-α, or IL-17[80]. The authors hypothesized that defense against *C. trachomatis* is maintained by Th2 immunity in order to limit immunopathology to reproductive anatomy in women[81]. Supporting a protective role of Type 2 immunity, of which Th2 responses are a subset, the group has recently shown that IL-4 producing eosinophils prevent *C. trachomatis*-induced endometrial damage in mice by stimulating endometrial stromal cell proliferation[82]. However, it is not currently known whether similar processes occur in *C. trachomatis* infected women.

Th17 cells are characterized by their secretion of predominantly IL-17[83]. Though IL-17 does not directly inhibit chlamydial growth, it may indirectly do so by increasing the production of other proinflammatory molecules that are responsible for recruiting protective immune cells. For example, IL-17 promotes Th1 immunity by modulating DC function[84]. As alluded to above, IL-17 may also act synergistically with IFN-γ to control chlamydial replication. However, IL-17 is also associated with Crohn's disease and experimental autoimmune encephalomyelitis, suggesting that Th17 cells may play a role in immunopathology[85,86]. One study found that protection from *C. muridarum* induced UGT pathology correlates with high IFN-γ but low IL-17 T cell responses[87]. Another study also found correlative evidence suggesting that protection against pathology may be determined by a balance of IL-17 signaling[88]. More recently, Andrew *et al.* showed that *C. muridarum* infection and its associated oviduct pathology was less severe in mice deficient in IL-17 than wild type mice[89]. Together, these data suggest that IL-17 may be implicated in *Chlamydia*-induced UGT immunopathology, and that Th17 responses may need to be avoided in a human chlamydia vaccine.

4.3.3 - CD8 T Cells

The role of CD8+ T cells in *Chlamydia* infection has been controversial. While CD8+ T cells migrate to the site of infection, and are capable of destroying cells infected with *Chlamydia*, wild-type and CD8+ T cell knockout mice showed similar rate of clearance of *C. muridarum* following an intravaginal challenge[90,91]. These results are in line with previous evidence demonstrating that CD8+ T cells are not necessary for

clearance of a primary *C. trachomatis* infection[64,92]. Furthermore, depletion of CD4+ T cells, but not CD8+ T cells, from wild type mice that have cleared a primary genital infection compromised protection against reinfection[93]. However, recently, Nogueira *et al.* showed that intranasal immunization with live *C. trachomatis* generated CD8+ T cells that were sufficient for protection against reinfection in the absence of CD4+ T cells[94]. These conflicting results may be a result of differential priming environments in the respiratory and genital tracts. This hypothesis is supported by the observation that compared to DCs in the genital tract, respiratory DCs express lower levels of PD-L1, a negative regulator of CD8+ T cell responses, and that CD8+ T cells confer superior protection in the absence of PD-L1 signaling[93]. Interestingly, it was recently demonstrated that depletion of CD8+ T cells completely abrogated the protective efficacy of a live-attenuated vaccine in a non-human primate (NHP) model of trachoma[95]. These results suggest that CD8+ T cells may play an important role in vaccine-mediated protection against infection.

However, in the *C. muridarum* mouse model, CD8+ T cells have been implicated in infection induced UGT pathology. Following genital *C. muridarum* infection, CD8+ T cell-deficient mice demonstrated less hydrosalpinx when compared to wild type mice[96]. Furthermore, mice deficient in perforin, transport-associated protein 1, and TNF-α also exhibit reduced pathology in infected mice[91,97]. Depletion of CD8+ T cells prior to infection also protects against pathology, and adoptive transfer of TNF-α secreting CD8+ T cells into CD8-deficient mice was sufficient to revert back to the disease phenotype[91]. Therefore, the current available evidence supports the notion that CD8+ T cells can

confer protection against *Chlamydia* infection, but are also likely involved in chlamydial pathogenesis.

4.3.4 - B cells and Antibodies

Early evidence demonstrated that *Chlamydia*-specific antibodies contribute to the protection against *C. trachomatis*[98-100]. However, accumulating evidence supports the notion that B cells may not be essential for clearance of a primary chlamydial infection, but rather play an important role in preventing systemic dissemination of infection as well as protective immunity against reinfection[70,101-103]. In humans, high titres of *C. trachomatis*-specific antibody are not correlated with resolution. In fact, these antibodies are more strongly correlated with increased severity of pathology following infection, such as tubal factor infertility (TFI) in women[104].

Chlamydia-specific serum IgG and IgA are capable of neutralizing the bacteria *in vitro*[105,106]. Mice deficient in the polymeric immunoglobulin receptor (pIgR), which transports secretory IgA from the basolateral side to the luminal side of the epithelium, are significantly more susceptible to infection than wild type mice[107]. This suggests that *Chlamydia*-specific secretory IgA may prevent chlamydial attachment to host cells. On the other hand, IgG-mediated protection against *Chlamydia* may involve antibody-dependent cellular cytotoxicity (ADCC), opsonization, and engulfment by monocytes, as mice deficient in Fc receptors exhibit an increased bacterial burden during infection[108]. Through Fc receptors, *Chlamydia*-specific IgG can also enhance the induction of a Th1 response, and thus indirectly generating protective immunity through T cells.

4.3.5 – Resident Memory T Cells

The importance of a qualitatively and quantitatively appropriate adaptive immune response in anti-chlamydial immunity cannot be over stated. Much of the explanatory narrative on the experimental outcomes and population level observations have been structured based on the Th1/Th2/Th17 framework of cell-mediated adaptive immunity. However, Johnson and Brunham (2016) have suggested that a central paradigm of chlamydial immunity that is based on tissue-resident memory (Trm) T cells and chlamydial memory lymphoid clusters (cMLCs) may have greater explanatory power[109]. They argue that a number of conflicting data that cannot be explained through the cytokine polarization narrative can be comfortably explained using the Trm framework. The most persuasive of them involves a study in 2005, in which Cohen *et al.* showed that EB-induced IL-13 secretion by PBMCs was associated with protection against reinfection[77]. As IL-13 is a prototypical Th2 cytokine, the authors could not reconcile their new data at the time with the prevailing consensus that chlamydial immunity is mediated by Th1 immunity. However, in a Trm model, the presence of both Th1 and Th2 cytokines would not necessarily represent contradictory data. Further substantiating this theory, Johnson *et al.* recently identified a subset of CD4+ T cells that secrete both IFN-γ and IL-13, and have a Trm-like transcriptome[110]. The authors have coined them as "CD4γ13 cells", which when adoptively transferred into naive mice conferred complete protection against oviduct pathology without affecting bacterial clearance. The

emergence of this Trm narrative has generated some discussion among experts in the field[109,111,112].

1.5 Engineering a Solution - *Chlamydia* Vaccinology

1.5.1 Overview of the Mouse Model of Chlamydial Genital Tract Infection

Although the minipig, pig, and NHP models have been used to test vaccine candidates, the vast majority of vaccine studies have been performed using either the *C. muridarum* or *C. trachomatis* mouse models of chlamydial genital tract infection[113]. As vaccine licensure does not require vaccines to have been tested in NHPs or any other animal model in particular, vaccine candidates shown to be effective in the mouse models only do not necessarily face barriers to regulatory approval. Therefore, though NHP studies provide valuable data on mechanisms of vaccine mediated immunity against infection and pathology, and will continue to make important contributions to chlamydial vaccine efforts, mouse models are sufficient for preclinical testing of candidate vaccines.

Chlamydia muridarum, also known as *C. trachomatis* mouse pneumonitis biovar (MoPn), is a natural pathogen of the mouse that causes pneumonitis. Originally isolated from the lungs of mice, intravaginal inoculation of *C. muridarum* causes an acute infection of the lower genital tract[114]. Like *C. trachomatis* infections in women, *C. muridarum* preferentially infects the cervical epithelial cells. Eventually, the infection

ascends to the UGT to cause pathology in the uterine horns and oviducts (analogous to fallopian tubes in women), leading to hydrosalpinx, fibrosis, and infertility (similar sequelae result in women)[115,116]. *C. muridarum* infection in mice is self-limiting and resolves relatively quickly compared to *C. trachomatis* infection in women. Strong immunity against reinfection reliably follows - mice that are re-infected exhibit significantly lower levels of chlamydial shedding (by 2-3 logs) and a lower duration of infection. Furthermore, respiratory tract infection leads to robust protection against both infection and the associated UGT pathology[87]. The nature of infection, with respect to magnitude and duration of shedding, depends to varying degrees on the mouse strain, the dose of inoculation and the age of the mice[117-119]. While not required to establish infection, medroxyprogesterone is often given prior to inoculation of *C. muridarum* to synchronize the estrous cycles of the animals[113]. The most common route of inoculation is intravaginal, while intrauterine or transcervical inoculation, using a non-surgical embryo transfer (NSET) device, have also been used[120].

Chlamydia trachomatis, a natural pathogen exclusively found in humans, does not readily infect mice, and medroxyprogesterone is required to reliably establish infection[121]. Intravaginal inoculation with *C. trachomatis* results in a significantly less robust infection compared to *C. muridarum*, with lower level of bacterial load (by 2-3 logs) and a shorter duration of infection that is often unable to ascend to the UGT[117]. As a result, post-infection sequelae such as hydrosalpinx and infertility typically are not reliably observed[122]. However, recently, it has been shown that transcervical inoculation with *C. trachomatis* offers robust and reliable model to evaluate *C. trachomatis* vaccine

candidates[120]. An above-physiological number of infectious organisms are often required to establish infection, which could overwhelm an otherwise protective immune response generated by a previous infection or vaccination[123]. However, this may be overcome by using clinical isolates of *C. trachomatis* that have not been extensively passaged in the laboratory[124]. Furthermore, the intravaginal *C. trachomatis* model mirrors many aspects of *C. trachomatis* infection in women that the *C. muridarum* model does not, such as the shedding of low numbers of infectious bacteria, minimal inflammation or sign of infection, highly variable pathological outcomes, high rates of re-infection, and the development of an appreciable level of protective immunity only after multiple infections[125-129]. Therefore, while neither model perfectly replicates human infection, both have their place in the study of *Chlamydia* immunobiology and vaccinology. To screen for vaccine candidates that are capable of eliciting protective immunity against pathology, the *C. muridarum* model seems to be more robust in this regard. On the other hand, it is necessary for a human vaccine candidate to demonstrate protective efficacy against the human pathogen *C. trachomatis*, which can be done using the intravaginal or the transcervical *C. trachomatis* murine model.

1.5.2 Intact Organisms

Vaccines consisting of intact organisms, live or dead, have the advantage of containing all of the antigenic components in biologically relevant conformations. Vaccines containing live organisms are particularly effective at inducing robust immunity

largely due to their ability to replicate and thus provide the immune system with persistent exposure to antigens. However, the drawback of live vaccines include the risk of avirulent strains to revert back to pathogenic strains, and the potential for irrelevant or detrimental antigens[130]. In the case of chlamydial vaccines, an additional downside is the laborious process of producing large quantities of organisms needed to vaccinate a sufficiently large number of individuals to be meaningfully effective at the public health level. This is largely due to the fact that *Chlamydia* is an obligate intracellular organism and to date, there has been no successful attempt to grow the bacteria in acellular cultures. Nonetheless, whole-organism chlamydial vaccines have been explored in animals and humans.

Live or inactivated *Chlamydia abortus* vaccines against ovine abortions have been successfully used for many years, suggesting that a human vaccine based on intact chlamydia may be possible[131]. As alluded to in section 1.2, the first human *Chlamydia* vaccine trials were conducted using inactivated *C. trachomatis*. However, these studies found that the vaccines provided only short-term, serovar-specific protection against infection, and that some participants developed more severe disease upon re-exposure to *Chlamydia* than their unvaccinated counter parts[14]. At the time, the investigators hypothesized that the enhanced disease seen among vaccinated individuals may be a result of delayed-type hypersensitivity after infection with *C. trachomatis*[12].

In the 21st century, investigators have used avirulent strains that are cured of the "cryptic" chlamydial plasmid in vaccine studies. These strains have similar growth characteristics compared to wild type strains, but induce limited disease upon infection.

O'Connell *et al.* showed that vaccination with plasmid-deficient *C. muridarum* partially protected mice against repeat challenges with the wild type strain and fully protected against development of UGT pathology[132]. Notably, a similar approach using a plasmidless *C. trachomatis* L2 strain has shown promise in non-human primates[133]. Recently, Stary *et al.* showed that vaccination with whole killed *C. trachomatis* formulated in charge-switching nanoparticles coated with a TLR ligand elicited robust mucosal immunity in mice[134]. However, the safety concerns associated with intact organisms with respect to detrimental antigens and the potential for exacerbating disease has led investigators away from intact organisms. Instead, the focus of *Chlamydia* vaccinology has been on subunit vaccines.

1.5.3 Subunit Antigens

Arguably one of the biggest challenges for researchers aiming to develop a subunit *C. trachomatis* vaccine is to choose the right antigen. For the first few decades immediately after the completion of the early trachoma vaccine trials, MOMP has been the most extensively studied subunit vaccine[135]. However, by the end of the 20th century, researchers had successfully sequenced the entire *C. trachomatis* genome, thus making available to us theoretically every single possible antigen expressed by the pathogen[136]. Recently, new strategies have leveraged this new information to expand the number of potentially protective chlamydial antigens for evaluation.

The first approach is what has been termed "reverse vaccinology" by Rappuoli in 2000[137]. The group had used unbiased bioinformatics analysis of open reading frames of entire bacterial genomes to predict and select antigens largely based on the ability to induce antibody and/or T cell responses during a natural infection. Finco *et al.* in 2011 have identified 120 *C. trachomatis* proteins and assessed them for immunogenicity[138]. A subset of these proteins deemed to induce robust antibody and T cell responses were selected and successfully tested in vaccine efficacy studies. While systematic and unbiased with respect to the selection of an protein-based antigen, a downside of this approach is that potentially protective polysaccharide and lipid based antigens will not be tested at all. Moreover, this strategy skews the process towards the identification of proteins that are highly immunogenic (although not necessarily protective) during natural infection, and proteins that are less immunogenic during natural infection but are highly immunogenic during vaccination (and potentially protective) will be filtered out during selection.

The second approach involves the identification of MHC class I and II bound chlamydial peptides that are directly isolated from *in vitro* infected DCs[139]. Briefly, DCs are generated from mouse bone marrow, infected with *Chlamydia* for 12 hours or more, and lysed. The lysate is then applied to anti-MHC antibody bound affinity columns that select out MHC class I or class II molecules, and peptides in the MHC-peptide complex eluted and analysed using mass spectrometry. The peptides' parent proteins are then cloned and tested for vaccine efficacy. The benefit of this strategy over reverse vaccinology is that its findings are physiologically relevant. However, the downsides are

that only T cell, but not B cell, epitopes are discovered, and that discovered peptides are dependent on the specific MHC of the host. Nonetheless, this approach has been successfully used to discover multiple antigens that induced better or equal protection compared to MOMP[75].

The two approaches combined have been invaluable to chlamydia vaccinology as they have allowed investigators to expand the selection of antigens to be used for vaccine efficacy studies. However, researchers have discovered antigens unaccounted for in these two strategies often in serendipitous ways while studying chlamydial pathogenesis. In this section, I present an overview of the three most promising subunit vaccine candidates against *C. trachomatis*.

5.3.1 MOMP

The chlamydial major outer membrane protein has been the most well-studied and the strongest candidate for a chlamydia vaccine for decades. MOMP has been shown to contribute to attachment by interacting with host cell receptors as well as to the maintenance of structural integrity of the bacterium, since *Chlamydia* has small amounts of peptidoglycan[140,141]. It is a 40 kDa protein that has a trimeric structure[142], constitutes about 60% of the combined mass of outer membrane proteins, comprises of several conserved T cell and B cell epitopes[143,144], and is an immunodominant antigen during natural infection. Pal *et al.* in 1997 showed that vaccination with *C. muridarum* chlamydial outer membrane complex (COMP), a cysteine-cross-linked protein shell containing MOMP, protected mice against *C. muridarum* infection[145]. A later study by

the same group demonstrated that vaccination with purified and refolded MOMP combined with Freund's adjuvant reduced bacterial load upon a *C. muridarum* challenge[144]. Initially, attempts to vaccinate animals using recombinant MOMP (rMOMP) yielded poor results[144,146,147], which suggested that native MOMP (nMOMP) was necessary for optimal protection. The technical difficulty involved in purifying nMOMP has been a significant barrier for moving the antigen through the vaccine pipeline.

However, recently Olsen *et al.* engineered a novel MOMP-based vaccine, Hirep1, consisting of repeating units of the variable domain 4 (VD4) within the MOMP sequence. This region includes a highly conserved and neutralizing B cell epitope (LNPTIAG), which was also discovered previously as a T cell epitope[148]. Antibodies towards this sequence were able to neutralize multiple serovars of *C. trachomatis in vitro*[123]. Vaccination with Hirep1 has been demonstrated to be highly effective at reducing *C. trachomatis* shedding in both mice and minipigs[123,149]. Importantly, adoptive transfer of Hirep1 antibodies into Rag1-KO mice completely prevented *C. trachomatis* infection in half of the animals[150]. This construct has been further developed by fusing it with a cysteine-free, truncated version of rMOMP to generate CTH522, which self-assembles into a quaternary structure[151]. CTH522 is currently the only chlamydia vaccine candidate being evaluated in humans, with results from a phase I clinical trial pending. This approach, by combining conserved regions within variable domains of MOMP from multiple serovars (D,E,F), may have circumvented the challenges associated with

MOMP-based vaccines. This is an exciting avenue of chlamydial vaccine research and development that has brought us a step closer to a human *C. trachomatis* vaccine.

5.3.2 Polymorphic membrane proteins

Polymorphic membrane proteins are unique to *Chlamydiales*[136]. There are nine *pmp* genes in each of *C. trachomatis* and *C. muridarum*, with molecular masses ranging from 100 to 150 kDa. Pmps have been suggested to interact with host membrane proteins to enhance attachment[25], and are likely to be type V secretion autotransporters. Immunogenicity of Pmps have been confirmed in mice, NHPs, and humans[152-154].

Yu *et al.* identified PmpG as the most promising candidate of the Pmps, given that it was able to induce a high number of IFN-γ secreting CD4+ T cells and significantly protected mice against a *C. muridarum* challenge[75]. Interestingly, splenic APCs retained PmpG epitopes on MHC molecules long after infection has cleared, possibly contributing to the antigen's ability to elicit robust immune responses[155]. Compared to MOMP, Pmps exhibit significantly less antigenic diversity, and may elicit more broadly protective immune responses towards multiple serovars[75,156]. This is supported by the observation that antibodies to PmpD was able to neutralize all *C. trachomatis* serovars *in vitro*[156]. Furthermore, vaccination with PmpD from *C. trachomatis* serovar E protected mice against shedding upon challenge with serovar D[157]. Karunakaran *et al.* recently showed that a vaccine containing multiple antigens (Pmps from serovar D, and MOMPs from serovars D, F, and J) protected mice from shedding upon a transcervical *C. trachomatis*

challenge[148]. Therefore, the available evidence suggests that Pmps are strong candidates for a chlamydia vaccine.

5.3.3 CPAF

The chlamydial protease-like activity factor (CPAF) is produced as a zymogen, and becomes activated upon dimerization and autocleavage[158,159]. CPAF degrades intracellular and extracellular host proteins to facilitate inclusion growth and immune evasion. It is highly conserved (99% amino acid similarity) among *C. trachomatis* serovars and is immunodominant during infection in humans[152].

Murthy *et al.* in 2007 showed that intranasal vaccination with rCPAF plus IL-12, a Th1 cytokine, significantly reduced bacterial shedding and UGT pathology upon intravaginal challenge with *C. muridarum*[160]. The same group demonstrated in a later study that vaccination with rCPAF may be able to induce protection against infertility upon repeated challenges with *C. muridarum*[161]. Remarkably, the protection against development of UGT pathology is seen despite the fact that CPAF-mediated protection against chlamydial shedding occurs 1-2 weeks post challenge. This delay may be partly attributable to CPAF being detected only after 24 h of infection. Vaccination with CPAF has also been shown to be effective in a guinea pig model of chlamydial genital tract infection[162]. Recently, Rajeeve *et al.* found that CPAF is used by *C. trachomatis* to suppress the host immune response by cleaving formyl peptide receptor 2 on the surface of neutrophils[55], suggesting that antibodies towards CPAF may enhance neutrophil

activity. However, whether enhanced neutrophil activity is beneficial or detrimental with respect to pathogenesis is unclear.

1.5.4 Adjuvants

One of the greatest mysteries in early immunological research is how we distinguish self from non-self. This avenue of inquiry, which has largely come to an end, has now led us to another puzzling question - how do we distinguish dangerous non-self from innocuous non-self? To differentially respond to proteins in food and proteins in pathogens, the immune system has to have a mechanism by which to recognize foreign molecules as harmless or potentially harmful. The grey areas in between these two categories of molecules can manifest as immunologically driven disease. In the case of the immune system responding to innocuous antigens, we can get allergy; when the immune system fails to respond effectively to harmful sources of antigens, we can get chronic infections.

Among other source of signal, such as route of exposure (e.g. mucosa vs dermis), the immune system has evolved to recognize constituents found in pathogenic microbes that are not products of host biology as signals to contain or eliminate the sources of these constituents, which differ depending on the unique characteristic and lifestyle of the pathogen. In a way, these molecules act as biomarkers that signal to the immune system to mount a tailored response to disrupt the particular strategies employed by that pathogen. This is important, as clearance of different types of pathogens require different types of immune responses. These microbe-associated constituents, called pathogen

associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) in Gram-negative bacteria, are recognized by host pattern recognition receptors (PRRs), such as toll-like receptors (TLRs). When PRRs are activated, the host perceives the presence of a harmful foreign entity, and responds accordingly. In the absence of PRR activation, non-self molecules will be viewed as innocuous, and tolerance ensues. Therefore, for any subunit vaccine consisting of purified biomolecules that lack the ability to activate PRRs, substances with that ability must be added to the formulation to signal to the immune system to respond appropriately.

Although research into adjuvants have been ongoing for over 70 years, only a few adjuvants are licensed for use in humans. Alum (ammonium hydroxide), AS04 (monophosphoryl lipid A [MPL] plus alum), AS03, and MF59 (squalene-based), and liposomes are the only adjuvants that have received regulatory approval for use in human vaccines[163]. Most of these have been used in conjunction with subunit vaccines, including the hepatitis B vaccine and the human papillomavirus vaccine[164,165]. The first adjuvants used in conjunction with chlamydial antigens were alum and other oil in water adjuvants. However, these stimulated a Th2 polarized immune response, which, together with the subsequent finding that Th1 polarized responses were important for chlamydial immunity, led investigators to explore Th1 polarizing adjuvants.

Cheng *et al.* led perhaps one of the most comprehensive investigations into potential adjuvants[166]. Pam_2CKS_4, poly(I-C), MPL, flagellin, imiquimod R837, imidazoquinoline R848, CpG-1826, M-Tri-DAP, and muramyl dipeptide were separately combined with rMOMP to screen for efficacy in a *C. muridarum* respiratory challenge

model in mice. Based on changes in body weight and bacterial load recovered from lungs, it was determined that Pam_2CSK_4 and poly(I-C) were most effective, while CpG-1826 was moderately protective. Using PmpG as a model antigen, Yu *et al.* screened a panel of adjuvants that included dimethyldioctadecylammonium bromide-trehalose 6,6-dibehenate (DDA-TDB), DDA-MPL, DDA-monomycolylglycerol (DDA-MMG), ISCOM, CpG-1826, and Montanide ISA720 plus CpG 1826 using a genital *C. muridarum* challenge model[75]. Vaccination with DDA-MPL or DDA-TDB were found to be most effective as determined by the greatest reduction in bacterial shedding upon a *C. muridarum* challenge. Protection correlated with high frequencies of IFN-γ+TNF-α+CD4+ T cells, supporting the role of multifunctional T cells in anti-chlamydial immunity.

Similarly, Olsen *et al.* have found DDA-TDB, also known as CAF01, to be effective in a *C. trachomatis* genital challenge model[123]. It was combined with CTH522, which, as alluded to above, is the only subunit vaccine candidate currently in clinical trials. Intramuscular vaccination with CTH522/CAF01 generated robust cell-mediated and humoral immune responses that reduced chlamydial shedding in infected mice[123]. DDA is a small cationic molecule that aggregate to form liposomes, which then act as antigen "depots" that promote persistent antigen release and exposure to immune cells. TDB has been shown to interact with monocyte-inducible C-type lectin to activate the FcR-Syk-Card9 pathway in APCs to promote Th1 and Th17 polarized immune responses[167,168]. CAF01 has been successfully used in vaccines against many other

pathogens in preclinical and clinical studies[169,170], and is a promising adjuvant for use in a chlamydia vaccine.

Recently, Stary *et al.* used charge switching synthetic adjuvant particles (cSAPs) to demonstrate the importance of mucosal immunization in generating tissue resident memory T cells that mediate protection against *C. trachomatis* genital tract infection[134]. cSAP consists of poly(D,L-lactic-co-glycolic acid)-b-poly(L-histidine)-b-poly(ethylene glycol) (PLGA-PLH-PEG) and a TLR 7/8 agonist, R848 (resiquimod). PGLA and R848 form the hydrophobic core and PLH and PEG form the hydrophilic surface. At pH 6.5, cSAPs are positively charged on the surface and thus preferentially bind to negatively charged bacteria[171]. Vaccination with UV-inactivated *C. trachomatis* (UV-Ct) formulated with cSAP generated long-lived protection against infection in mice, where as UV-Ct vaccination without cSAP elicited tolerogenic immune responses that led to an increase in susceptibility to infection[134]. Importantly, only intranasal and intrauterine, but not subcutaneous, immunization conferred protection, which was shown to be dependent on the production of *C. trachomatis*-specific resident memory T cells in the genital tract.

1.5.5 Routes of Immunization

In addition to the choice of antigen and the selection of an adjuvant, chlamydia vaccinologists have also pondered on the degree to which the site of antigen/adjuvant delivery influences the outcome of vaccination. Chlamydia is a mucosal infection that is largely contained to the site of initial inoculation, and therefore any chlamydia vaccine

must induce mucosal immunity at the genital tract. However, what constitutes effective chlamydia-specific mucosal immunity and how a vaccine might give rise to such a physiological state is currently under intense investigation.

Traditionally, vaccines are given parenterally, through intramuscular or subcutaneous injections, and generate immunity by eliciting systemic antibody production. However, parenteral immunization is unable to generate sufficient mucosal immunity, and often the best way to generate immunity at a specific site is to administer the vaccine locally. For chlamydia, the vaginal route makes the most sense with respect to eliciting robust immunity at the site of infection. Though vaginal and rectal immunizations have been investigated in HIV vaccine studies, which acted as proof of principle that administering vaccines using these routes can be effective, the bottleneck for implementation is patient acceptance[172,173]. Therefore, alternative means of generating genital immunity have been explored.

Mucosal tissues, like the reproductive tract, have large surface areas that act as physical barriers between the host and potentially pathogenic microbes. Underneath the epithelium lies a network of immune cells and infrastructures, which are part of what is considered to be the common mucosal immune system, in which antigen exposure at one site leads to antigen specific immune responses at remote sites[174]. The sublingual (under the tongue) and intranasal routes of immunization are both capable of generating robust mucosal immunity in the genital tract and have been explored in chlamydia vaccine research.

The sublingual route has been used for delivery of low-molecular weight drugs and immunotherapy for allergies. Underneath the epithelium, CCR7+ DCs present engulfed antigens to T and B cells and sensitize them to respond to CCL28, a chemokine expressed by epithelial cells at a number of sites in the body, including the genital tract[175]. Cuburu *et al.* showed that sublingual immunization with nonreplicating antigens elicit antibody secreting cells and T cells in the genital tract and conferred superior protection against human papillomavirus (HPV) infection compared with systemic immunization[176]. Similarly, O'Meara *et al.* showed that sublingual immunization with MOMP formulated with cholera toxin and CpG reduced chlamydial burden and fibrotic scarring in lungs of mice upon challenge with *C. muridarum*[177]. However, sublingual immunization has not been widely explored by other investigators in the field of chlamydia vaccinology.

Intranasal immunization is in some ways more preferable compared to sublingual immunization. The nasopharynx-associated lymphoid tissue (NALT) is highly vascularized and permeable to antigens, and an analogous structure is absent in sublingual tissue[178]. The presence of esterases, amylases, and phosphatases in saliva also mitigates the potential for reduction in immunogenicity of antigens delivered sublingually[179]. Lymphocytes activated in the NALT express high levels of CCR10, $\alpha_4\beta_1$ and $\alpha_4\beta_7$, which direct these cells to the genital tract with the cognate ligand and receptor interactions[178]. In humans, intranasal immunization, but not intramuscular immunization, elicits secretory IgA in vaginal secretions[180]. Intranasal formulations represent a large portion of needle-free vaccines approved for use in humans or currently undergoing

clinical investigation[181], and have been demonstrated to be effective against *Chlamydia* in a number of animal models[134,162]. However, one concern with toxin-based mucosal adjuvants is that there have been reports of volunteers developing Bell's palsy following immunization, which has been perceived as a barrier to the development of intranasal vaccines[182,183].

1.6 The Gap in the Field

Over the past few decades, a tremendous amount of information have become available to investigators who are working towards a chlamydia vaccine. Advances in biotechnology, such as the advent of multiparameter flow cytometry and the sequencing of the entire chlamydial genome, have been essential in defining the mechanisms by which *C. trachomatis* infections are cleared, the characteristics of a protective anamnestic immune response, and the repertoire of antigens from which vaccine candidates should be selected[184]. From these data, some overarching themes are taking shape. Firstly, mucosal immunization is generally preferable over parenteral routes given its ability to elicit strong and long lasting mucosal immunity. Secondly, subunit vaccines are preferred in most cases since, compared to whole organism approaches, the antigens are well characterized and carefully selected to maximize effectiveness and minimize potential deleterious effects. Thirdly, a Th1 polarized adaptive immune response seems to be protective, while a Th2 response appears to be detrimental. Therefore, the current

available evidence suggests that the ideal chlamydia vaccine will likely be multi-subunit, adjuvanted with a Th1 polarizing adjuvant, and delivered mucosally, which is a good framework on which further knowledge can be built from more focused avenues of inquiry.

The repertoire of antigens selected by the reverse vaccinology and immunoproteomics approaches are, by the nature of these techniques, skewed towards proteins that are highly immunogenic during a natural infection. However, reports have suggested that there is little correlation between a protein's immunodominance during infection and its efficacy as a vaccine[185,186]. Moreover, immunodominant proteins are often under purifying selection, and vaccine induced immune responses towards such proteins may contribute to vaccine escape. Indeed, the 18 *C. trachomatis* serovars are defined based on the antigenic differences in MOMP, one of the most immunodominant proteins during chlamydial infections[152]. It is conceivable that polarizing the immune response towards MOMP would allow *C. trachomatis* to elicit serovar-specific immunity, which would allow heterologous serovars to infect the same host again. On the other hand, subdominant antigens are often highly conserved as a result of lack of immune selection. In many cases, these proteins are functionally important for pathogen invasion, and as such, viruses and bacteria have evolved strategies to direct immune responses away from these antigens[187,188]. Therefore, to generate a comprehensive repertoire of antigens from which we select a few for use in a vaccine, our understanding of the host response to natural infections, particularly with respect to the identity and nature of the

antigens targeted, should be supplemented by an understanding of the mechanisms by which pathogens invade and cause disease.

As an obligate intracellular pathogen with a limited genome size, many virulence factors likely contribute to a complex and highly evolved strategy to manipulate the host cell biology to ensure successful infection. One such factor is the type III secretion (T3S) system, which is highly capable of promoting chlamydial virulence[189] (Figure 5). Genes encoding T3S system proteins were first identified in *Yersinia* spp. and the prototype Ysc-Yop system was subsequently characterized, revealing an intricate bacterial mechanism to subvert eukaryotic host cells[190]. Since then, T3S systems have been identified in numerous pathogenic and non-pathogenic Gram-negative bacteria[191].

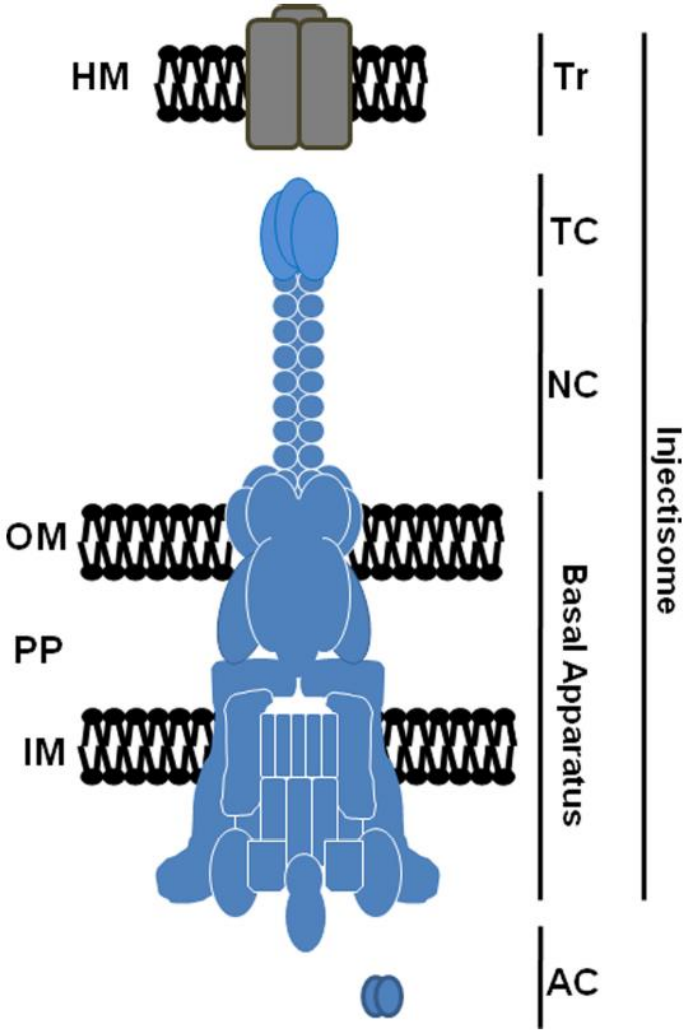


Figure 5. Components of the chlamydial type III secretion system. Reproduced with permission from[189]. HM, Host Membrane; Tr, Translocators; TC, Tip Complex; NC, Needle Complex; OM, Outer Membrane; PP, Periplasm; IM, Inner Membrane.

The T3S system is often referred to as an injectisome or "molecular syringe", through which bacterial effector proteins are directly injected into host cytosol. Essential components of the T3S system include 1) ancillary components in the bacterial cytoplasm providing support to the injectisome, consisting of a set of chaperones and regulatory factors, 2) membrane-associated proteins that form a highly structured core through which effector proteins are secreted, 3) proteins that make up the needle complex and tip complex which bridges the gap between the host and bacterial membranes, and 4) secreted proteins (translocators) required to form "pores" in host membranes through which effector proteins enter the host cytosol[189,192,193] (Figure 5).

The T3S proteins that are surface exposed are of particular interest to vaccinologists as they can be targeted by neutralizing antibodies. There are currently no T3S proteins approved for use as vaccine components. However, as they are absolutely required for virulence and highly conserved within species, T3S proteins have the potential to be excellent candidates for vaccine development. There is optimism surrounding the use of the T3S tip complex and translocators as antigens in the development of vaccines against other pathogenic bacteria. Antibodies to the T3S tip proteins LcrV in *Yersinia spp*. and PcrV in *Pseudomonas aeruginosa* are able to block infection[194,195]. LcrV is a well-characterized *Yersinia* antigen and is currently being tested as a subunit plague vaccine. A broadly protective vaccine consisting of T3S translocator proteins IpaB and IpaD has been demonstrated in *Shigella*[196]. In fact, there has been a great deal of interest in IpaB and IpaD as vaccine candidates recently[196-200], owing to their ability to induce cross-protective immunity in mouse models of *Shigella* infection.

To the best of our knowledge, at the start of the project encompassing this thesis, no *C. trachomatis* surface exposed T3S protein had been tested as a vaccine candidate. This has changed over the last few years, with Koroleva *et al.* reporting that immunization with the T3S needle protein (TC_0037), an ortholog of *C. trachomatis* CdsF, protected mice against *C. muridarum* infection and the associated pathology[201]. However, neither the tip protein nor the translocator proteins have been evaluated as chlamydia vaccine candidates.

Markham *et al.* in 2009 hypothesized that CT584 is the *C. trachomatis* needle tip protein based on its biophysical similarity with known tip proteins from other bacteria[202]. More recently, crystal structures of CT584 and its homologue in *C. pneumoniae* (Cpn0803) have shown that the putative tip protein forms a hexamer in solution consisting of a trimer of dimers[203,204]. Although structural comparison of CT584 with the *Yersinia pestis* tip protein LcrV does not support the contention that CT584 is a needle tip protein, it is possible that the CT584 has retained this functional role despite a lack of structural conservation. Regardless of its biological role, we have shown that immunization with Cpn0803 reduced *C. muridarum* infection and the associated UGT pathology in mice (unpublished data), supporting its candidacy as a chlamydia vaccine antigen. Our lab has previously identified CopB (Cpn0809) and CopD (Cpn0808) from *C. pneumoniae* as the likely chlamydial translocators, based on several lines of evidence including interactions with the needle protein, interactions with their putative chaperones, and the ability of anti-CopB and anti-CopD antibodies to neutralize chlamydial infection *in vitro*[31,32]. Given the successful use of the translocator and tip proteins as vaccines

against other pathogenic bacteria, and the absence of evidence or rationale against the use of analogous proteins in a chlamydia vaccine, there is a strong need to test chlamydial T3S translocator and tip proteins as potential candidates in the search for a human *C. trachomatis* vaccine.

1.7 On the Shoulders of Giants - Objective of Thesis

The objective of my thesis is therefore to evaluate CopB, CopD, and CT584 as a potential chlamydia vaccine candidate. We have designed a chimeric protein – consisting of hydrophilic portions of CopB and CopD, and full length CT584 – termed BD584. **We hypothesize that BD584 immunization will induce strong cell-mediated and humoral immune responses and confer protection against *Chlamydia* in mice.** The following Chapter describes the materials and methodologies used, while Chapters 3, 4, and 5 describe and discuss the results of three studies with the following specific aims, respectively:

1. To design and engineer the chimeric protein BD584 and assess its efficacy as a vaccine antigen in a *C. muridarum* mouse model of genital tract infection;
2. To characterize the immune response to BD584 immunization and evaluate its protective efficacy against *C. trachomatis* genital tract infection in three strains of mice; and

3. To demonstrate, as a proof of principle, that a clinically safe and effective mucosal adjuvant can be successfully used with a chlamydia vaccine.

Chapter 2 – Materials and Methods

2.1 BLASTp

Non-redundant protein sequences were searched with BLASTp using CopB (CT578), CopD (CT579), CT584, or MOMP from *C. trachomatis* serovar D (UW-3/Cx) as query sequences. Using the queries as references, the number of non-identical residues in each identified *C. trachomatis* sequence was calculated, tabulated, and presented as a frequency of non-identical amino acids.

2.2 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 a minimum transmembrane window of 17 and a maximum of 33. Proteins containing predicted transmembrane domains were subjected to Kyte-Doolittle hydrophobicity prediction using a window size of 9.

2.3 Cloning

C. trachomatis (UW-3/Cx) $copB_{1-300}$ and $copD_{1-300}$ were cloned into pDEST17 using the Gateway cloning system according to manufacturer's instructions (Thermo Fisher Scientific, ON, Canada). Briefly, *att*B-containing gBLOCK DNA fragments (Integrated DNA Technologies, ON, Canada) coding for first 100 amino acids of CopB or CopD were cloned, via BP reaction, into pDONR201 donor vectors, which were then used in LR reactions to generate expression vectors pDEST17::$CopB_{1-300}$ and

pDEST17::CopD$_{1\text{-}300}$. Cloning of CT584 in pET-21b and has been described previously[203].

To construct the chimeric BD584 antigen, genomic DNA from *C. trachomatis* serovar D was used as the source for cloning. Using Platinum *pfx* DNA amplification enzymes (Life Technologies), the following PCR products were generated (note: subscript denotes amino acid number): CopB$_{1\text{-}100}$ with a 5' BamHI restriction site and 3' EcoRI restriction site, CopD$_{1\text{-}100}$ with a 5' EcoRI restriction site and a 3' SalI restriction site, full length CT584 with a 5' SalI restriction site and a 3' HindIII restriction site. PCR products were digested with their respective endonuclease (New England Biolabs). The multiple cloning site (MCS) 1 of pET-DUET was restriction digested with BamHI and HindIII (New England Biolabs). Restriction digested CopB$_{1\text{-}100}$, CopD$_{1\text{-}100}$, 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 at McMaster University.

2.4 Protein Expression and Purification

To generate the antigens required in this study pDEST17::CopB$_{1\text{-}300}$, pDEST17::CopD$_{1\text{-}300}$, pET-21b::CT584, pETDuet-1::BD584, and pET32a::MOMP were transformed separately into *Escherichia coli* BL21 (DE3) cells for protein expression and purification.

For expression and purification of CopB, CopD, CT584, and BD584, 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-1-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 (20 mM Tris-HCl pH 7.4, 500 mM KCl, 0.03% lauryldimethylamine oxide [LDAO], 10% glycerol, 10 mM imidazole) for protein purification. 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% Nickel A, 5% Nickel B (20 mM Tris-HCl pH 7.4, 500 mM KCl, 0.03% LDAO, 10% glycerol, 300 mM imidazole), 10% Nickel B, and 15% Nickel B before eluting in 100% Nickel B. The protein was buffer exchanged into PBS using a desalting column and an AKTA FPLC. Purified protein was filter sterilized (0.2 µm) and concentrated using a centrifugal filter (EMD-Millipore), and protein concentration was determined using a BioRad Protein DC Assay with protein standards. Eluted proteins were stored in aliquots at -80˚C and had a purity of > 95%.

For expression and purification of MOMP, 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 IPTG and remained shaking for 4 h at 30˚C. Cells were harvested by centrifugation at 8,000*g* for 5 min and resuspended in 100 mM NaH_2PO_4, 10 mM Tris-Cl, and 6 M GuHCl at pH 8.0 and sonicated. Bacterial lysate was centrifuged at 42,000*g* for 45 min and the supernatant was incubated with Ni-NTA beads (Qiagen, ON, Canada) for 1 hour at room temperature. Beads were washed with 100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, and 0.1% Triton X-114 at pH 7.5 and then with the same buffer without Triton X-114 at pH 6.3 by centrifuging at 1,500*g* for 5 min and discarding the supernatant. MOMP was eluted by incubating beads with 100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M Urea, and 500 mM imidazole at pH 7.4 overnight at room temperature. Eluted MOMP had a purity of > 95% and was buffer exchanged into PBS as described above, filter sterilized (0.2 µm), concentrated, and stored in aliquots at -80°C.

2.5 Size Exclusion Chromatography

BD584 was subjected to size exclusion chromatography in PBS on a Superdex S200 10/300 GL gel filtration column (Amersham Biosciences, Piscataway, New Jersey) at 0.5 mL/min. Size prediction of elution complexes was performed after the column was standardized using a LMW and HMW gel filtration standard kit (GE Life Sciences).

2.6 Chlamydia

C. muridarum (ATCC VR-123) or a clinical isolate of *C. trachomatis* serovar D were propagated in McCoy cells (ATCC CRL-1696) in RPMI 1640 containing 25 mM HEPES, 10% FBS, 2 mM L-glutamine, 50 µg/mL gentamicin, and 1 µg/mL cycloheximide (Infection Media). After 48 hours of infection, *C. trachomatis* was harvested and stored in multiple aliquots in 250 mM sucrose, 10 mM sodium phosphate, 0.5 mM L-glutamic acid (SPG, pH 7.4) at -80˚C.

2.7 Animals

Six to eight week old female C57BL/6, BALB/c, and C3H/HeN mice were purchased from Charles River Laboratory. All experiments involving animals were carried out in accordance with the Canadian Council on Animal Care guidelines and approved by the Animal Research Ethics Board at McMaster University. Every effort was made to minimize animal suffering. Isoflurane and ketamine/xylazine were used during vaccination and infection, respectively. The method of euthanasia was cervical dislocation.

2.8 Vaccination and Challenge

Chapters 3 and 4. For intranasal vaccination, mice were anaesthetized with 5% isoflurane immediately before delivering 20 µg of BD584 and 20 µg of CpG oligodeoxynucleotide 1826 (with a phosphorothioate backbone) (ACGT Corp, ON, Canada) in 20 µL of PBS. Three weeks after the final immunization, mice were pretreated with 2.5 mg of Depo-Provera (Pfizer, ON, Canada) in 100 µl of saline

administered subcutaneously 7 days (Chapter 3) or 10 and 3 days (Chapter 4) prior to challenge. Three weeks after the final immunization, mice were challenged intravaginally with 10^5 IFUs of *C. muridarum* (Chapter 3) or *C. trachomatis* (Chapter 4) in 10 µL of SPG.

Chapter 5. Immediately prior to vaccination, mice were lightly anesthetized with 5% isoflurane. Mice (n=12) were intranasally vaccinated twice, three weeks apart, with 20 µg of BD584 mixed in 20% NE01. Control mice (n=12) were vaccinated with PBS. Three weeks after the second dose, 6 mice in each group were sacrificed to isolate inguinal lymph nodes (iLN) and to collect serum and vaginal secretions for assessment of cell-mediated and humoral immune responses. The remaining 6 mice in each group were challenged with live *C. trachomatis* for evaluation of protection. For challenge, mice were pretreated with 2.5 mg of Depo-Provera (Pfizer, ON, Canada) in 100 µl of saline administered subcutaneously 10 and 3 days prior to intravaginal challenge with 10^5 IFUs of *C. trachomatis* in 10 µL of SPG.

2.9 Collection of Vaginal Washes and Serum

Seven days after the final immunization, vaginal washes were collected for antibody analysis for five consecutive days by pipetting twice with 30 µL of PBS in and out of the vagina to give a total of 60 µL per mouse per day and stored at -70˚C. After samples collection was complete for all five days, vaginal washes were pooled for each mouse, centrifuged at 12,000*g* for 10 min at 4˚C and the supernatant was stored at -70˚C. Blood (submandibular) was collected two weeks after the final immunization, allowed to

clot at room temperature for 30 min, centrifuged at 1,000*g* for 10 min at 4˚C, and serum was stored at -70˚C.

2.10 Lymphocyte Restimulation

Seven days after intranasal priming with BD584/CpG (Chapter 4) or three weeks after the last vaccination with BD584/NE01 (Chapter 5), mouse spleens or inguinal lymph nodes (iLNs) were mechanically disrupted through 40 µm cell strainers (Corning), re-suspended in RPMI 1640 containing 25 mM HEPES, 10% FBS, 2 mM L-glutamine, 50 µM β-mercaptoethanol, 100 IU/mL penicillin, and 100 µg/mL streptomycin (Lymphocyte Media), and seeded into 96 well plates at $5x10^5$ live cells per well. Cells were then supplemented with either Lymphocyte Media alone or Lymphocyte Media containing 10 µg of BD584 and incubated for 96 hours at 37˚C/5% CO_2. Cell culture supernatants were assayed for IFN-γ, IL-17, IL-4, TNF-α, or IL-10 using DuoSet ELISA kits (R&D Systems, MN, USA) according to manufacturer's instructions (Chapter 4) or for IFN-γ, IL-17, IL-5 and TNF-α using luminex (Chapter 5).

2.11 Antibody ELISA

Chapter 3. Serial dilutions of serum samples were made ranging from 1:25 to 1:3200. Approximately 250 ng of BD584 was 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 (PBST). 200 µL of blocking solution (5% BSA in PBST) was added to each well and incubated at room temperature for 1 h. After removal

of the blocking solution 100 µL of the serial dilutions were added to the wells and incubated for 1 h at room temperature while shaking. The serial dilutions were removed, and wells were washed with 200 µL of PBST and incubated for 5 min at room temperature with shaking, and repeated three times. After the final wash was removed, 100 µL of HRP-conjugated goat anti-mouse IgG (Sigma), IgG2c (eBioscience), or IgG1 (eBioscience) secondary antibodies in 5% BSA + PBST 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 manufacturer's' 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 H_2SO_4) 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. Standard curves were generated according to manufacturer's instructions (eBioscience) to determine antibody concentration.

Chapters 3 (for CopB, CopD, CT584, and MOMP only), 4, and 5. NUNC MaxiSorp 96-well plates (Thermo Fisher Scientific, ON, Canada) were incubated with 10 µg/mL of BD584, CopB, CopD, CT584, or MOMP in PBS for one hour. Plates were then blocked with 50 mg/mL of skim-milk in PBST for one hour. Serially diluted serum or vaginal wash samples were added to the wells and incubated for two hours, followed by the addition of HRP-conjugated antibodies specific to mouse IgA, IgG1, IgG2a, IgG2b, IgG2c, IgG3 (Abcam, ON, Canada), or whole IgG (Sigma-Aldrich, ON, Canada) for one hour. The substrate, 3,3',5,5'-tetramethylbenzidine (BD Biosciences, ON, Canada) was

added to each well and incubated for 10-20 min before stopping the reaction with 1 M H_2SO_4. Absorbance at 450 nm (A_{450nm}) was measured on a ELx800 microplate spectrophotometer (BioTek, VT, USA). All steps were performed at room temperature and plates were thoroughly washed with PBST between incubations. The endpoint titer of a sample was defined as the dilution at which the A_{450nm} of the sample was equal to three times the standard deviation of the negative samples.

2.12 *In Vitro* Neutralization

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 via was centrifuged for 1 h at 1500*g* at room temperature. After centrifugation, shell vials were incubated at 37˚C with 5% CO_2 for 48 h. Chlamydial inclusions were stained with the Pathfinder *Chlamydia* detection reagent (BioRad) and visualized with multiple, random fields of view. Percent reduction of infection was calculated compared to a control infection.

2.13 Bacterial Quantification

Chapter 3. Quantitative real-time PCR was used to quantitate 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 to stand at room temperature for 15 min. The tubes were then centrifuged at 14,000*g* 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 *ompA* 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 prove and 5 µL of template. The amplification profile consisted of 95˚C for 10 seconds, and 55˚C for 40 seconds. Standards used for quantitation were from the *ompA* gene cloned into the pGEM-T easy vector system.

Chapters 4 and 5. Vaginal samples were collected from each mouse on days 3, 7, 10, 14, 21, and 28 post challenge using the same swabs used in Chapter 3. Swabs were placed into 0.5 mL of SPG, vortexed with 1 mm Mini-BeadBeater Glass Mill Beads (BioSpec, OK, USA) for 30 seconds, and stored at -70˚C until ready for analysis. Serial dilutions of swab samples were plated onto McCoy cells in 96 well tissue culture plates and centrifuged at 1,500*g* for 1 hour at 25˚c. The inoculum was then replaced with fresh infection media and incubated for 24 h at 37˚C/5%CO_2. Cells were fixed with Fixation Buffer (BD Biosciences, ON, Canada) for 20 min at room temperature, blocked with Perm/Wash Buffer (BD Biosciences, ON, Canada) overnight at 4˚C, and stained with rabbit anti-*Chlamyia* antibody (Genetex, CA, USA) followed with Alexa Fluor 647-conjugated mouse anti-rabbit antibody (Jackson ImmunoResearch Laboratories, PA,

USA) at 37˚C for 1 hour each. *C. trachomatis* inclusions were identified manually under an EVOS FL Cell Imaging System microscope (Thermo Fisher Scientific, ON, Canada). The total number of IFUs per swab was calculated based on the number of IFUs counted per well, accounting for dilution factors, inoculation volumes, and sample volumes.

2.14 Gross Pathology

Genital tracts were harvested for pathological scoring of the oviducts. Scoring was performed as follows: 0 = no hydrosalpinx; 1 = hydrosalpinx invisible to the bare eye; 2 = diameter of hydrosalpinx visible to bare eye but less than that of ovary; 3 = diameter of hydrosalpinx is similar to that of ovary; 4 = diameter of hydrosalpinx is greater than that of ovary.

2.15 Stability of BD584 Mixed with NE01

BD584 was cloned, expressed, and purified as described above, and diluted with either PBS or NT buffer before mixing with NE01. Aliquots of the mixtures were made and stored separately at either 4°C or 25°C for 0, 3, 7, or 30 days. At these indicated time points, samples of each mixture was run on an SDS-PAGE gel and stained with Coomassie Blue.

2.16 Statistical Analysis

GraphPad Prism 6 was used for statistical analysis. Differences were assessed using Student's *t* test for parametric data and Mann-Whitney test for nonparametric data,

one-way ANOVA when comparing more than two groups, or two-way ANOVA when comparing more than one independent variable. Statistical significance is indicated in figures as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

Chapter 3 - The BD584 Antigen and Protection Against *C. muridarum*

3.1 Introduction

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. Despite significant vaccine development efforts over the past three decades, 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.

3.2 Results

3.2.1 CopB, CopD, and CT584 are immune subdominant antigens that are highly conserved in *C. trachomatis.*

A *C. trachomatis* vaccine should be effective against all *C. trachomatis* serovars, and one way to achieve this would be to include antigens that are highly conserved. Therefore, we began our investigation by comparing the degree of amino acid variability in CopB, CopD, and CT584, using MOMP as a reference antigen. As shown in Figure 6A, alignment of known sequences of each protein showed that CopB, CopD, and CT584 have, on average, 0.46, 0.55, and 0.02 non-identical amino acids per 100 residues, respectively, which are significantly fewer compared to MOMP, which averages 4.16 non-identical amino acids per 100 residues. These results show that CopB, CopD, and CT584 are significantly more conserved than MOMP in *C. trachomatis*, which is an advantage for a subunit vaccine for a pathogen with multiple serotypes.

Since antigenic diversity is typically the result of immune selection by the host, we hypothesized that MOMP would be more immunogenic than CopB, CopD, and CT584 during infection. As expected, mice infected with *C. trachomatis* generated significantly higher titres of antibodies to MOMP than to CopB, CopD, or CT584 (Figure 6B). Furthermore, sera from 10/10 mice were positive for MOMP specific antibodies, while only 4/10 had detectable levels of antibodies to CT584, and none had detectable antibodies towards CopB and CopD. Similar results were obtained via Western blot using *C. muridarum* convalescent sera (Figure S1). Together, these results show that CopB,

CopD, and CT584 are more conserved than MOMP in *C. trachomatis*, which may, at least in part, be due to their inability to elicit a robust immune response in the context of an infection.

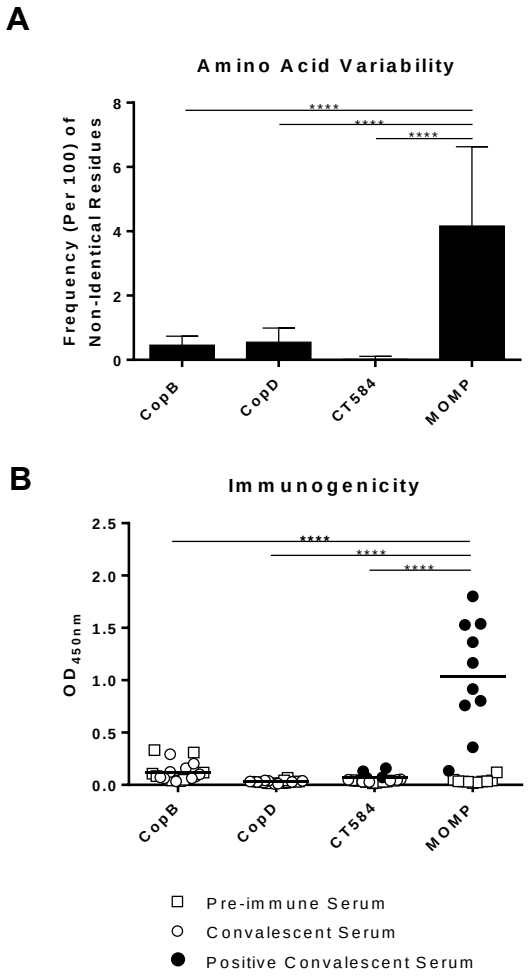


Figure 6. Correlation of antigenic variability and immunogenicity. A. Non-redundant protein sequences were searched with BLASTp using CopB, CopD, CT584, or MOMP from *C. trachomatis* serovar D (UW-3/Cx) as query sequences. The degree of amino acid variability of identified *C. trachomatis* sequences were tabulated and presented as the mean frequency of non-identical residues (per 100 amino acids) with SD depicted. Amino acid variability in CopB, CopD, and CT584 was compared to that in MOMP (reference antigen) using one-way ANOVA followed by Dunn's multiple comparison test. **B.** Female mice (n = 10) were intravaginally infected with 10^5 IFUs of *C. trachomatis* and sera were obtained 90 days post challenge. Sera were diluted 1:200 and the IgG antibody responses to CopB, CopD, CT584, and MOMP were determined by ELISA. Positivity among convalescent sera was predefined as having an A_{450nm} of greater than three times the SD of that of pre-immune sera. Lines represent the mean A_{450nm} of convalescent sera. One-way ANOVA with Dunn's multiple comparison test was used for comparison of the mean OD_{450nm} of convalescent sera among different antigens.

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

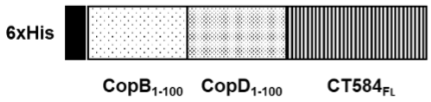
To determine whether the three components of the trivalent BD584 antigen are genetically conserved between *Chlamydia spp*., a bioinformatic analysis was performed to examine the amino acid identity of $CopB_{1\text{-}100}$, $CopD_{1\text{-}100}$, and $CT584_{FL}$ between *C. trachomatis* and *C. muridarum*. BLAST-P analysis revealed that $CopB_{1\text{-}100}$, $CopD_{1\text{-}100}$, and $CT584_{FL}$, were highly conserved between *C. trachomatis* and *C. muridarum*, with 86%, 78%, and 97% amino acid identity, respectively (Figure 7). We have previously shown that antibodies generated against N-terminal peptides of CopB and CopD were capable of neutralizing *Chlamydia spp*. Cloning of $CopB_{1\text{-}100}$, $CopD_{1\text{-}100}$, and $CT584_{FL}$ 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 (Figure 8). Using size exclusion chromatography, BD584 was determined to predominantly exist as a hexamer in solution (Figure S2).

Figure 7. Conserved amino acid sequences of CopB1-100, CopD1-100, and CT584FL orthologs. BLAST-P analysis was performed using the amino acid sequences of $CopB_{1\text{-}100}$, $CopD_{1\text{-}100}$, and $CT584_{FL}$ from *C. trachomatis*. Alignment between *C. trachomatis* and *C. muridarum* orthologs revealed 86%, 78%, and 97% amino acid identity between $CopB_{1\text{-}100}$, $CopD_{1\text{-}100}$, and CT_{584FL}, respectively. Subscripts denotes amino acid number, FL = full length. Figure taken from Bulir *et al.*, 2016.

A

Primer	Sequence
$CopB_{1-100}$(+)	GTTGTTAA**GGATCC**TATGTCCCTTTCATCTTCTTCG
$CopB_{1-100}$(-)	GTTGTTAA**GAATTC**GGATGTCGCATCGGCAGAT
$CopD_{1-100}$(+)	GTTGTTAA**GAATTC**ATGACAACAGGAGTACGTGG
$CopD_{1-100}$(-)	GTTGTTAA**GTCGAC**TGCAGTTGCTGTTTGGCTAG
$CT584_{FL}$(+)	GTTGTTAA**GTCGAC**ATGACGACGAAACCCAAAACT
$CT584_{FL}$(-)	GTTGTTAA**AAGCTT**TTACACAGATTTCGTTAATTCTT

B



C

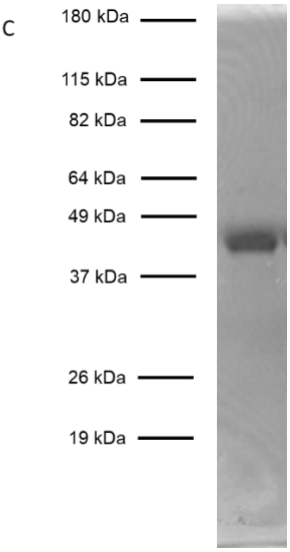


Figure 8. Oligonucleotide primers used to generate recombinant protein BD584. A. BD584 was constructed by amplifying the genes for CopB, CopD and CT584 using genomic DNA from *C. trachomatis* serovar D using the primers indicated. PCR products were digested with their respective endonuclease (in bold), 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. Figure taken from Bulir *et al.*, 2016.

3.2.3 Chlamydial T3S proteins elicit neutralizing antibodies.

Two groups of C57BL/6 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 (Figure 9A). 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. 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 (Figure 9B). 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*. (Figure 10)

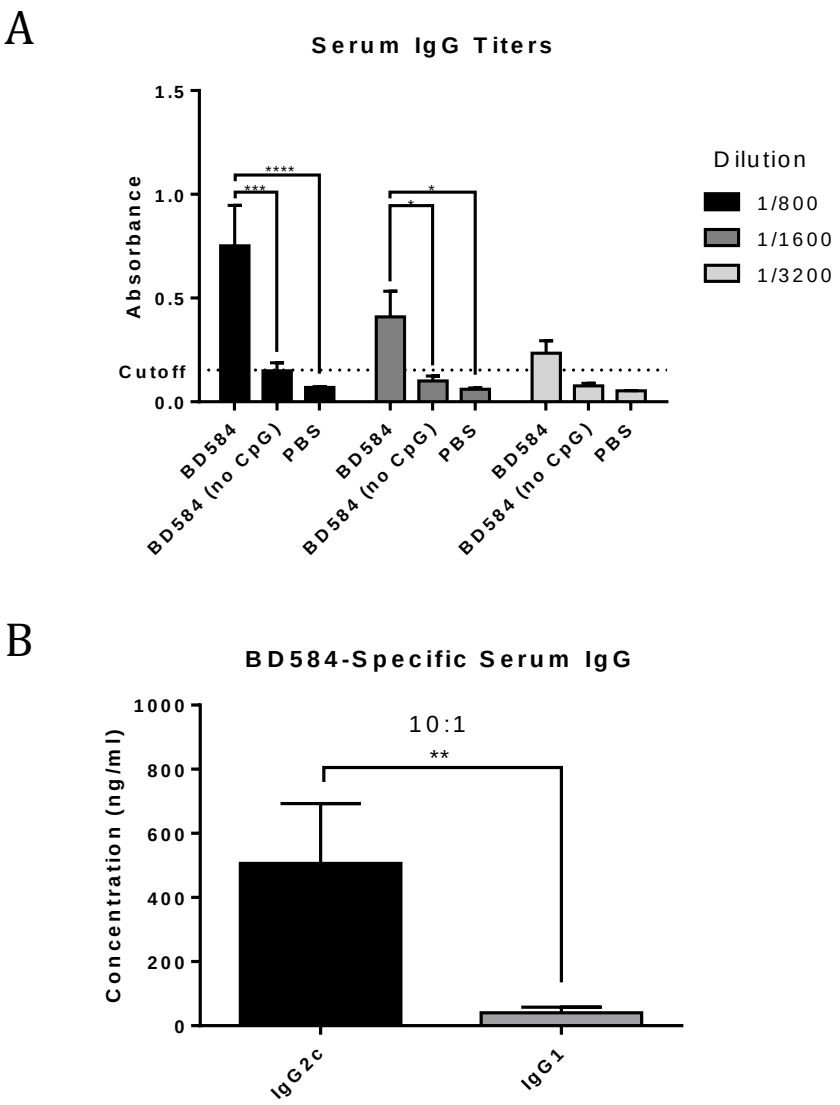


Figure 9. Measurement of serum antibodies to BD584 in immunized mice. A. Serum from vaccinated and control mice were tested by ELISA at serial two-fold dilutions from 1:25 to 1:3200 for specific anti-BD584 antibodies as described in the Methods section. Absorbance for the last three dilutions 1:800, 1:1600, and 1:3200 is shown. Histogram bars represent the mean absorbance for BD584+CpG, BD584 alone, and PBS + SEMs. Cuff off 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. Figure taken from Bulir *et al.*, 2016.

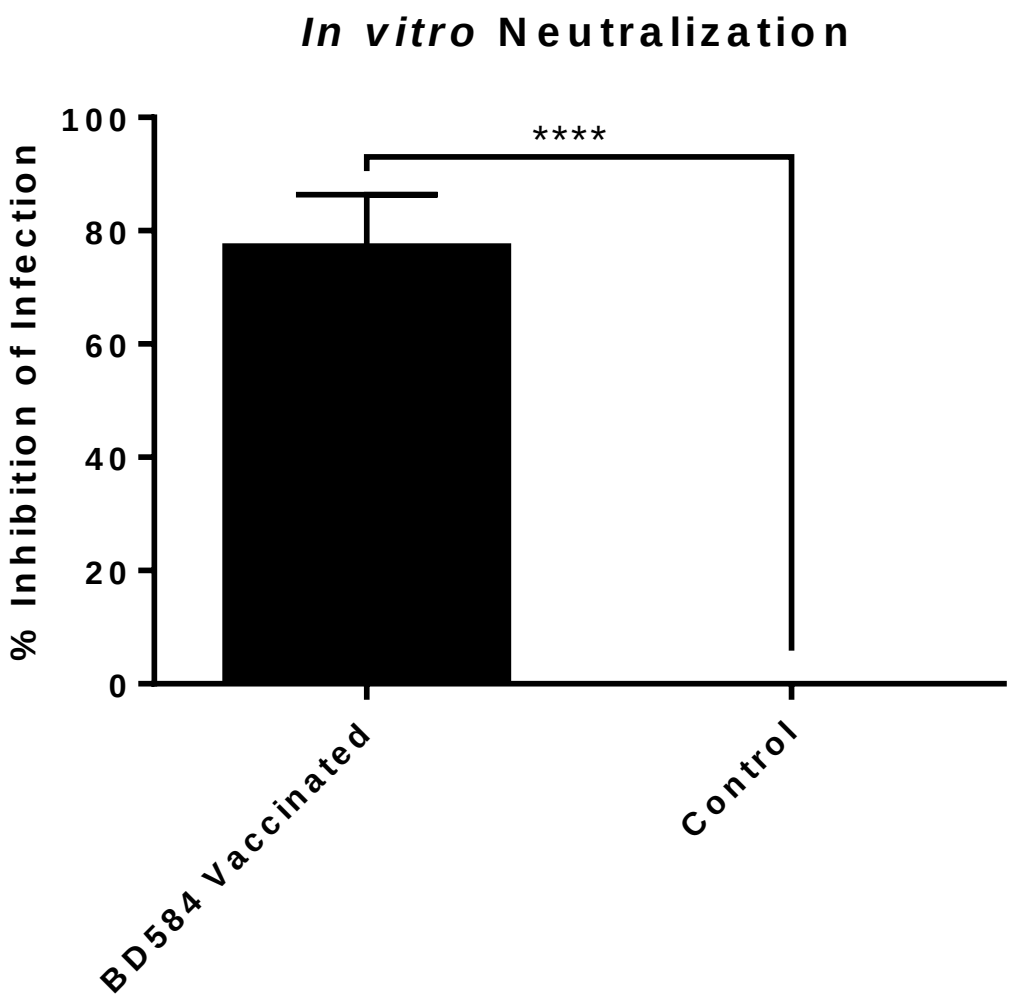


Figure 10. Presence of serum neutralizing antibodies in mice. BD584 vaccinated mice (N=5) and control mice (N=5) were bled, and tested for neutralizing antibodies to *C. muridarum*. Serum (diluted 1:10) was pre-incubated with *C. muridarum* for 1 hour at 37°C and the mixture put on a cell monolayer as described in Materials and Methods. Inclusions were counted at 48 hr. A mean reduction of approximately 77% was observed with serum from the vaccinated group and no reduction was seen with the control group. Figure taken from Bulir *et al.*, 2016.

3.2.4 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) (Figure 11A). Vaccinated mice also cleared the infection sooner than control mice as no *Chlamydia* was detected 32 days post infection in the vaccinated mice (Figure 11A). By calculating the area under the curve (AUC), we determined the total bacterial load during the entire course of infection in each group. AUC analysis further illustrates the reduced infection in mice immunized with BD584 (Figure 11B).

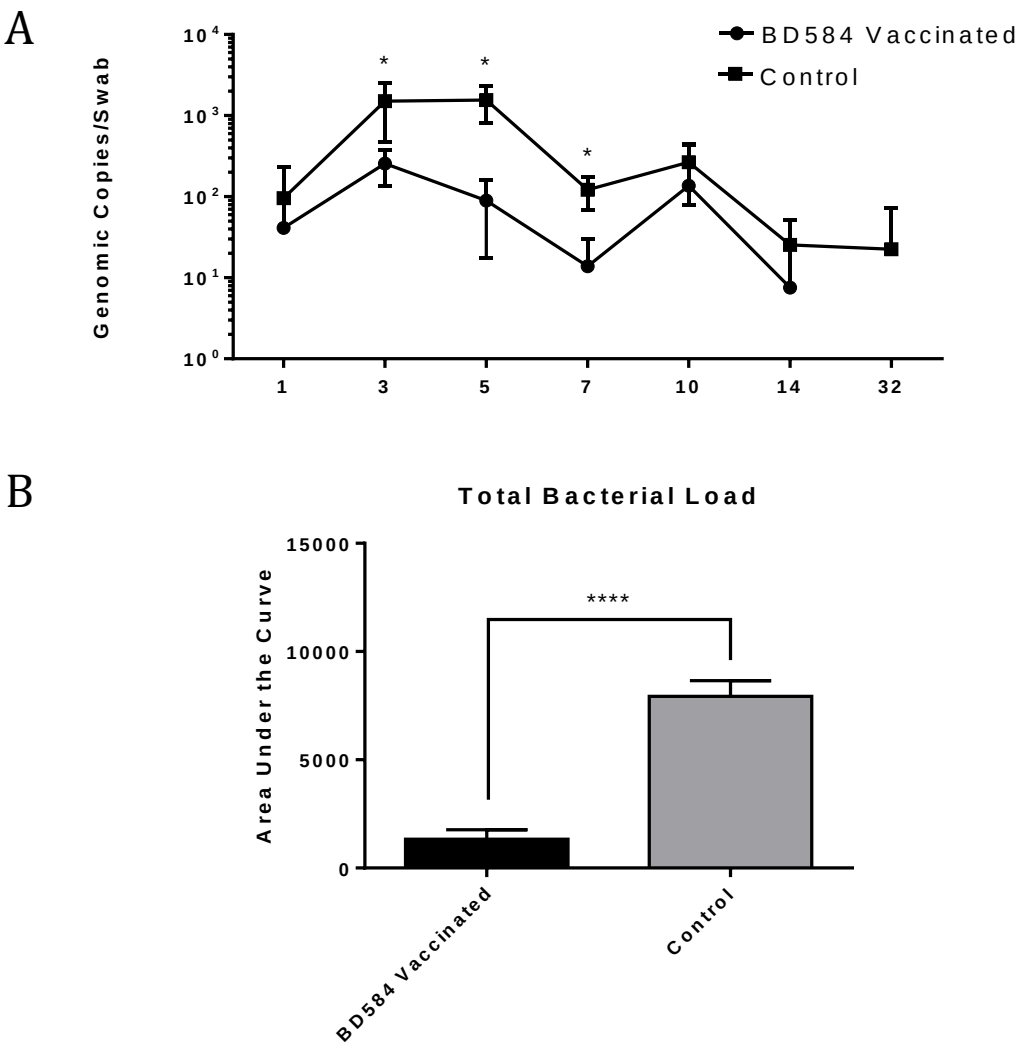


Figure 11. Vaginal shedding of *C. muridarum* in BD584 vaccinated and control mice determined by qPCR. A. Vaginal swabs were collected on days 1, 3, 5, 7, 10, and 14 post infection and *C. muridarum* shedding was determined using qPCR. A 95% reduction in bacterial shedding was observed in the vaccinated group compared to the control group at peak of infection on days 5 and 7. **B.** The total bacterial shedding during the entire course of infection is represented as the area under the curves. Figure taken from Bulir *et al.*, 2016.

3.2.5 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 (Figure 12). Vaccination also reduced the severity of oviduct pathology, but to a lesser degree than the reduction achieved by a prior respiratory *C. muridarum* infection (Figure S4).

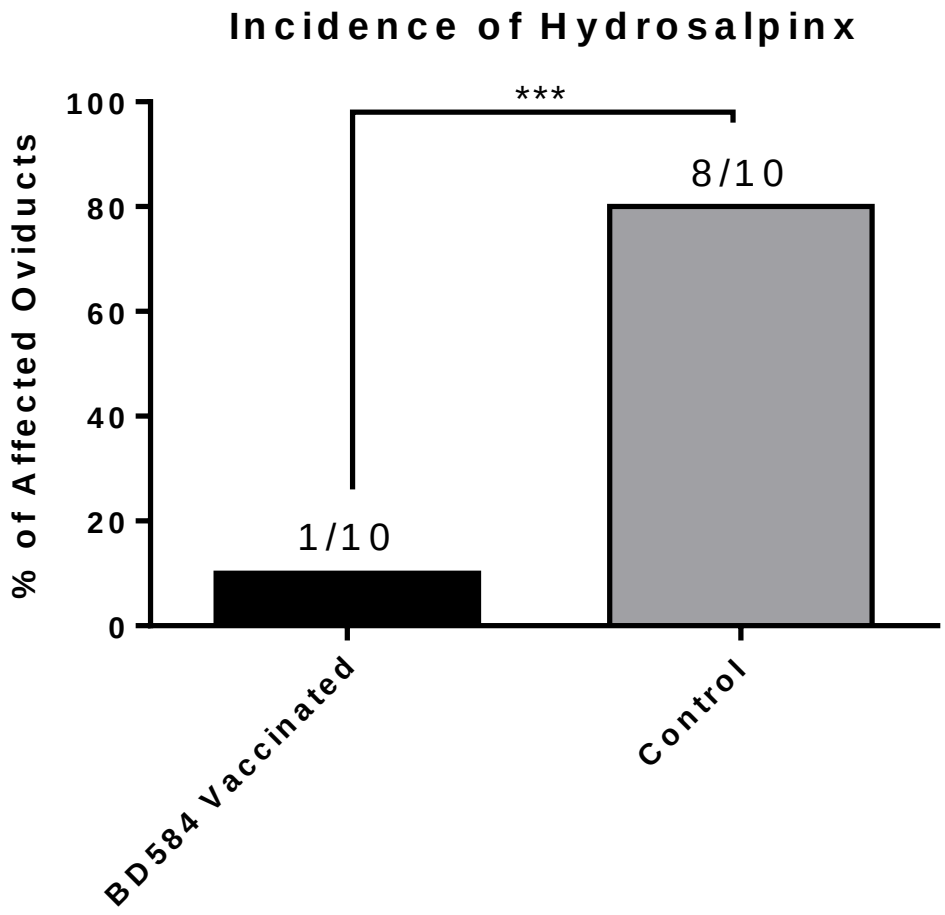


Figure 12. Presence of oviduct pathology (hydrosalpinx) at 35 days post infection. Mice were sacrificed 35 days after intravaginal challenge with *C. muridarum*, and upper genital tracts harvested and analyzed for the presence of hydrosalpinx using a pathology grading scores as described in Materials and Methods. Hydrosalpinx was present in 8/10 ovaries collected from the five unvaccinated mice, compared with only 1/10 ovaries in the vaccinated mice. Fisher's exact test. Figure taken from Bulir *et al.*, 2016.

3.3 Discussion

Despite decades of research on Chlamydia immunobiology, limited success has been achieved in vaccine development[12,205]. 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[194,195,197-200]. 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[206-208].

We show here for the first time that T3SS antigens from *Chlamydia* represent an excellent 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 and *Chlamydia*-induced fallopian tube pathology.

Given that BD584 was cloned from the *C. trachomatis* genome, its ability to afford cross-species protection speaks to its conserved nature and its potential as a broadly protective immunogen for all 18 *C. trachomatis* serovars. Indeed, the T3S components that make up the fusion antigen, CopB, CopD, and CT584, have significantly less antigenic diversity among *C. trachomatis* strains compared to MOMP, which reflects the conservation of these proteins that make up an essential virulence factor required for

cell invasion. Therefore, a vaccine consisting of these proteins may be less susceptible to vaccine escape.

Lack of variability within these bacterial antigens is likely the consequence of minimal selective pressure from the host, which may be the result of their presumably limited ability to induce a protective immune response during natural infection. In support of this notion, we and others have found that individuals and animals infected with *C. trachomatis* frequently developed high antibody titres to MOMP but rarely produce detectable levels of antibodies towards CopB, CopD, or CT584[209]. Thus, while these antigens are certainly capable of eliciting protective immune responses[31,32,210], their limited immunogenicity in the context of an infection, perhaps as part of a newly identified immune evasion mechanism of *Chlamydia*[188], may have shielded them from immune pressure to mutate, providing an excellent opportunity for BD584-mediated 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*[32,211,212]. However, 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[196,197,199,200,213]. 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 components such as MOMP.

It has been well documented in the literature that a cell-mediated immune response is critical to protecting against and clearing a *Chlamydia* infection. Using CpG adjuvant with BD584, we intranasally vaccinated mice in attempts to generate a mucosal Th1 polarized immune response[214]. 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[31,32]. 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 attributed to antibody dependent complement activation. However, the mechanism of neutralization remains to be elucidated, but is presumably due to the antibodies rendering the T3SS inactive and preventing host cell infection. In addition to antibodies, an efficient cell-mediated immune response is essential for protective immunity against chlamydial infection. 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. 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[12]. 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 vaccinated mice. Based on these observations, 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. 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[116]. 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 a 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 T3SS proteins. The precise 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.

Chapter 4 - Efficacy Of BD584 Vaccination Against *C. trachomatis* Infection in Three Strains of Mice.

4.1 Introduction

Previously, our group has shown that C57BL/6 mice immunized with BD584, a fusion protein consisting of the *C. trachomatis* T3S components CopB, CopD, and CT584, were protected against infection and upper genital tract (UGT) pathology upon challenge with the mouse pathogen *C. muridarum*[210]. However, given that significant differences exist between *C. muridarum* and *C. trachomatis* infections[48,113], vaccine-mediated protection against *C. muridarum* does not imply protection against *C. trachomatis*, and it is important to determine the effectiveness of BD584 vaccination against the human pathogen. Furthermore, a more detailed analysis of the cell-mediated and humoral immune response to BD584 vaccination will enhance our understanding of BD584-mediated protective immunity.

Most *Chlamydia* vaccine studies utilizing the murine model have been conducted using a single inbred mouse strain, which is not ideal given the genetic heterogeneity of humans, suggesting the need for vaccine studies using animals with different genetic backgrounds. Moreover, use of animals with genetically determined differences in immune predispositions can also provide insight into host factors that contribute to effective anti-chlamydial immunity. In this study, we used C57BL/6 mice, C3H/HeN, and BALB/c mice to model human genetic heterogeneity and to identify potential correlates of BD584-mediated protection[117,215]. We show here that intranasal immunization with BD584 elicits robust cell-mediated and humoral immune responses in addition to strong mucosal immunity in all three strains of mice and that BD584 immunization confers protection against *C. trachomatis* infection in a strain dependent

way. These results strengthen the rationale for further investigating the use of T3S proteins in a *C. trachomatis* vaccine.

4.2 Results

4.2.1 Intravaginal *C. trachomatis* infection in three strains of mice

To begin our investigation, we selected three strains of mice most commonly used in the field for infection with *C. trachomatis*. As shown in Figure 13, although C57BL/6 mice took the longer to clear, the magnitude and duration of infection in these strains are not significantly different.

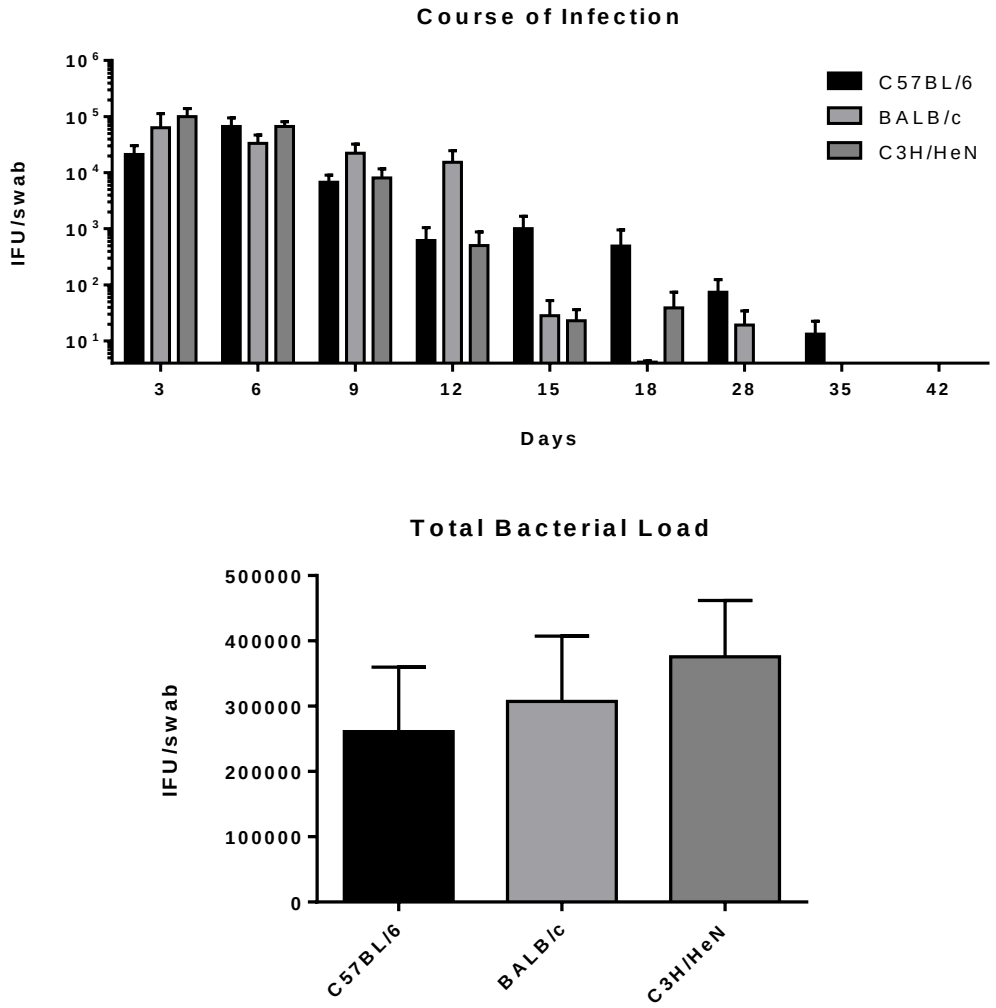


Figure 13. The course of *C. trachomatis* infection following intravaginal challenge in three strains of mice.

C57BL/6, BALB/c, and C3H/HeN mice were infected with 10^5 IFUs of *C. trachomatis* serovar D. Vaginal swabs were obtained on indicated days and titered on McCoy cells to enumerate the number of infectious bacteria shed. Top panel: course of infection. Bottom panel: area under the curve analysis.

Since antibodies contribute to the protection afforded by live infection, we sought to determine whether the antibody responses to intravaginal *C. trachomatis* infection varied between different strains of mice. Upon clearing a primary infection, C3H/HeN mice had significantly lower MOMP-specific serum IgG titers compared to BALB/c mice and minimal antibody responses overall (Figure 14). Serum and vaginal IgA titers were similar between C57BL/6 and C3H/HeN mice. While not statistically significant, C57BL/6 mice had the highest vaginal IgG titers

The type of cell-mediated immunity elicited by means of live infection or vaccination often determines the protective efficacy of an anamnestic response. However, the effect of host genetics on the type of cell-mediated immunity developed following intravaginal *C. trachomatis* infection has not been investigated. After clearing a primary infection, splenocytes from C57BL/6, BALB/c, and C3H/HeN mice produced different cytokines upon re-stimulation with heat-killed EBs. C57BL/6 mice had minimal cell-mediated immune responses compared to BALB/c and C3H/HeN mice (Figure 15). Splenocytes from BALB/c mice produced significantly higher levels of IL-17, characteristic of Th17 immunity, while those from C3H/HeN mice produced significantly higher levels of IL-4, commonly seen in Th2 immunity. Splenocytes from all three strains produced low levels of TNF-α and IL-10. Overall, we show that intravaginal *C. trachomatis* infection elicits adaptive cell-mediated immune responses with distinct cytokine profiles based on host genetics.

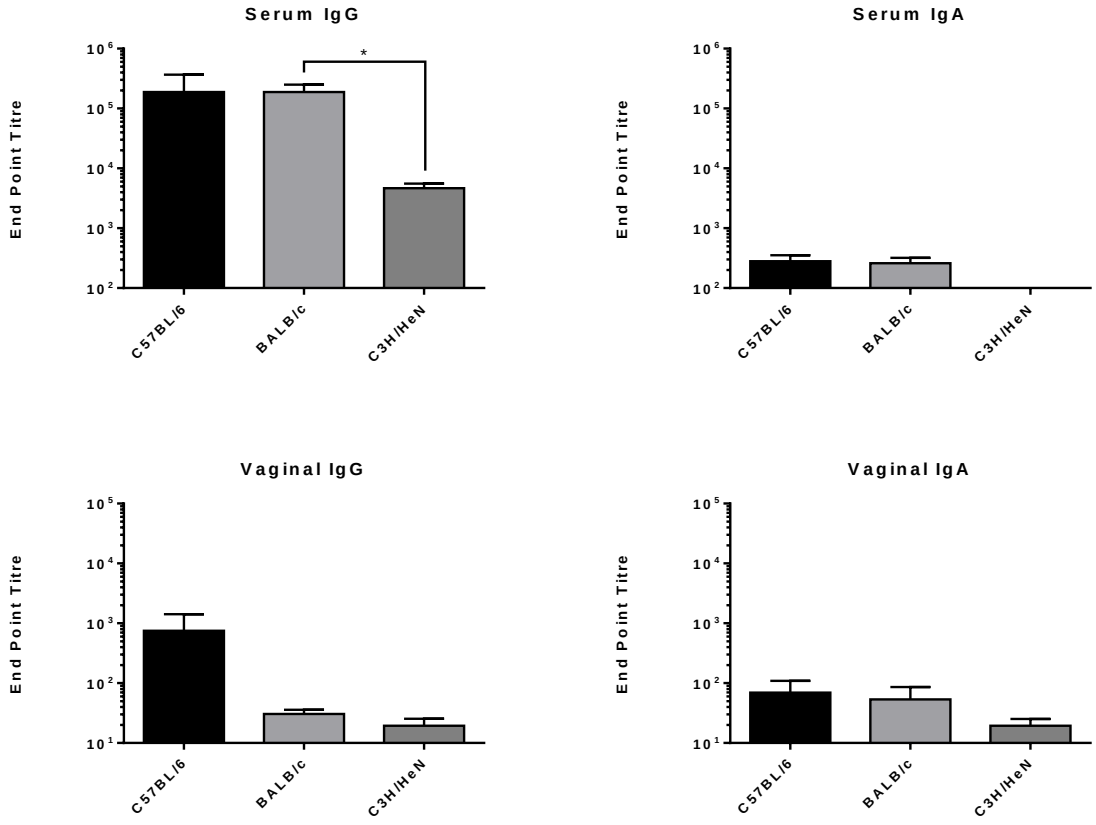


Figure 14. Antibody responses to MOMP in three strains of mice upon clearance of intravaginal *C. trachomatis* infection. Eighty days after clearance of *C. trachomatis* infection, serum and vaginal secretions were obtained as described in Chapter 2. MOMP-specific antibodies were titered using ELISA.

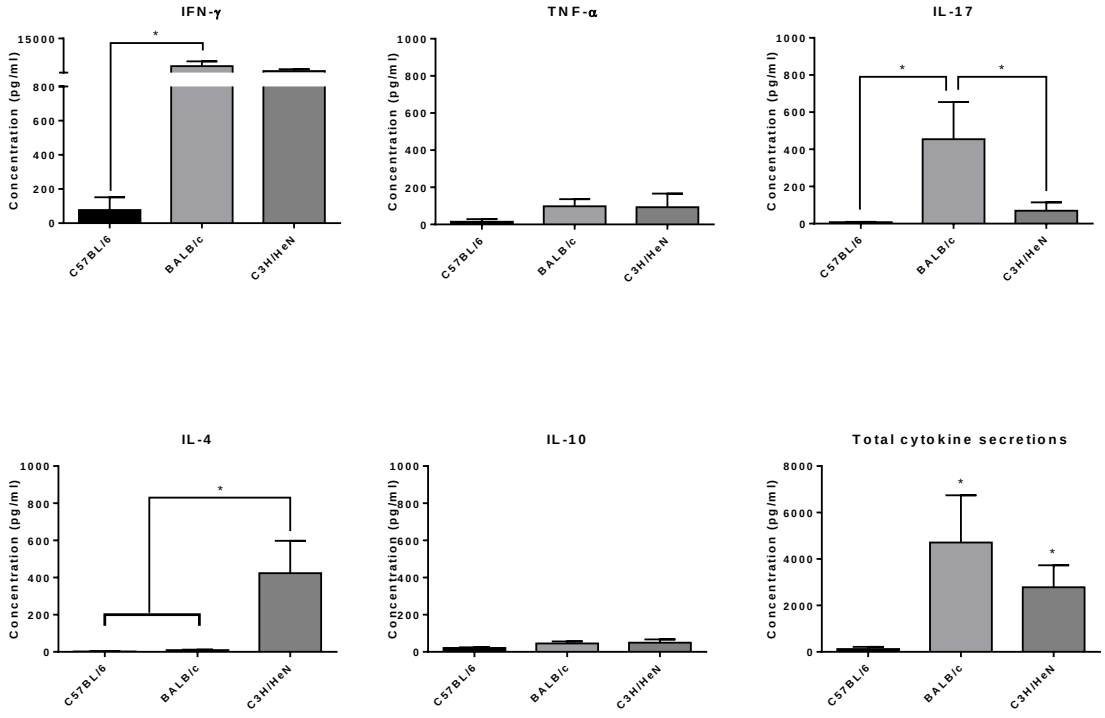


Figure 15. Cell-mediated responses in three strains of mice upon clearance of intravaginal *C. trachomatis* infection. Splenocytes from mice were harvested 90 days post infection, stimulated with heat-killed *C. trachomatis* for 72 hours, and cell culture supernatants were assayed for cytokine production using ELISA.

4.2.2 BD584/CpG vaccination induces robust cell-mediated immune responses

Next, we asked how host genetics might influence immune responses to vaccination. Since cell-mediated immunity has been reported to be essential for protection against *Chlamydia*[48], we examined how host genetics might influence antigen-specific cell-mediated immune responses following vaccination with BD584 and CpG. C57BL/6, BALB/c, and C3H/HeN mice were intranasally vaccinated with BD584/CpG or PBS, and BD584-induced production of cytokines from splenoctyes was analyzed 7 days later. Antigen-specific splenocyte production of IFN-γ and IL-17 in all three strains of mice indicates that a mixed Th1 and Th17 response was induced by intranasal vaccination with BD584/CpG, independent of the genetic background of the animal (Figure 16). No antigen-specific cytokines were produced by splenocytes from mice vaccinated with PBS. As expected with a Th1-polarizing adjuvant, BD584/CpG vaccination did not elicit IL-4 production. Interestingly, the level of BD584-induced production of IFN-γ of was strain dependent, with C3H/HeN mice developing significantly higher IFN-γ responses compared to C57BL/6 and BALB/c mice. However, IFN-γ production induced by the three separate subcomponents of BD584 (CopB, CopD, and CT584) relative to that induced by BD584 were not significantly different between strains (Figure S5A). BD584/CpG vaccinated C3H/HeN mice exhibited a moderately but significantly higher level of antigen-specific TNF-α and IL-10 production compared to C57BL/6 and BALB/c mice (Figure 16). These results suggest that intranasal BD584/CpG vaccination induces a mixed Th1/Th17 antigen-specific cell-mediated

immune response with variable immunogenicity dependent on the animal's genetic background.

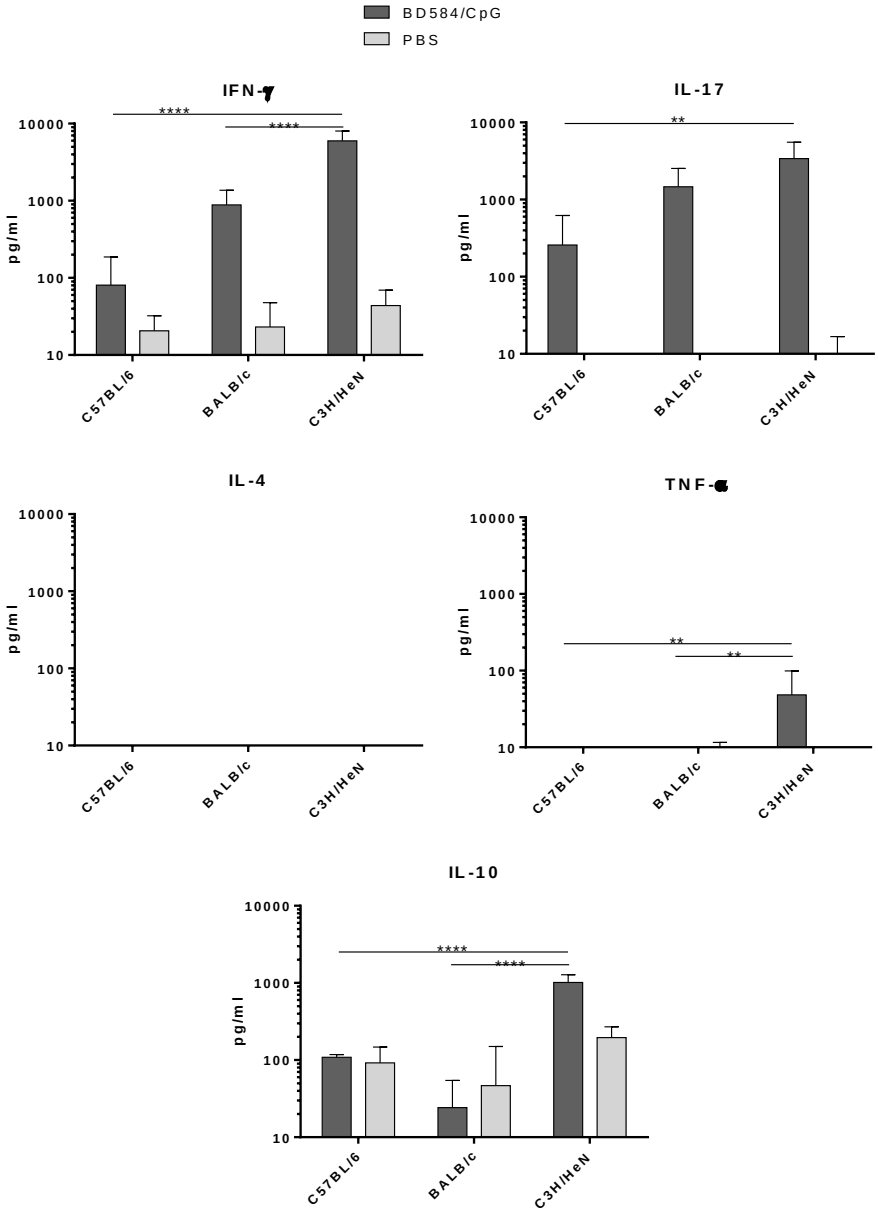


Figure 16. BD584-specific splenocyte cytokine responses following intranasal vaccination with BD584/CpG or PBS. C57BL/6, BALB/c, and C3H/HeN mice were intranasally vaccinated with either BD584/CpG or PBS (n = 5). Seven days later, animals were euthanized, and splenocytes were harvested and stimulated with BD584 for 96 hours. IFN-γ, IL-17, IL-4, TNF-α, and IL-10 were quantitated from culture supernatants using ELISA. Bars and whiskers represent means and SDs, respectively. Statistical differences between different strains of BD584/CpG immunized mice was calculated using two-way ANOVA followed by Tukey's multiple comparisons test.

4.2.3 BD584/CpG vaccination elicits robust systemic and mucosal humoral immune responses

While cell-mediated immunity is essential for protection against *C. trachomatis*, accumulating evidence suggests that a *C. trachomatis* vaccine should also generate strong humoral immunity[216]. Therefore, C57BL/6, BALB/c, and C3H/HeN mice were intranasally vaccinated three times, three weeks apart, and antibody responses were assessed two weeks after the final vaccination. Intranasal vaccination with BD584/CpG elicited robust BD584-specific IgG and IgA responses in both serum and vaginal secretions in all three strains of mice (Figure 17). PBS-vaccinated mice did not generate antigen-specific responses. Interestingly, BD584-specific antibody titres were consistently and significantly lower in C57BL/6 mice compared to BALB/c and C3H/HeN mice. However, CT584-specific serum IgG, when normalized against the level of BD584-specific serum IgG in each mouse, was significantly higher in C57BL/6 mice (Figure S5B). This suggests that the immunogenicity of the CT584 subcomponent relative to that of BD584 is greater in this strain. BD584-specific IgG1, IgG2a/c, IgG2b, and IgG3 titres were lower in C57BL/6 mice compared to BALB/c and C3H/HeN mice (Figure 18). Compared to BALB/c mice, C57BL/6 and C3H/HeN mice exhibited higher IgG2a/c to IgG1 ratios (Figure 19), suggesting a more polarized Th1 response in these strains. Furthermore, C57BL/6 and C3H/HeN mice also had higher BD584-specific vaginal IgG/IgA ratios (Figure 19). Taken together, these results show that BD584/CpG is highly immunogenic in all three strains of mice, and that the subtype of antibodies

associated with antigen-specific Th1 and Th2 responses is at least partially dependent on the animal's genetic background.

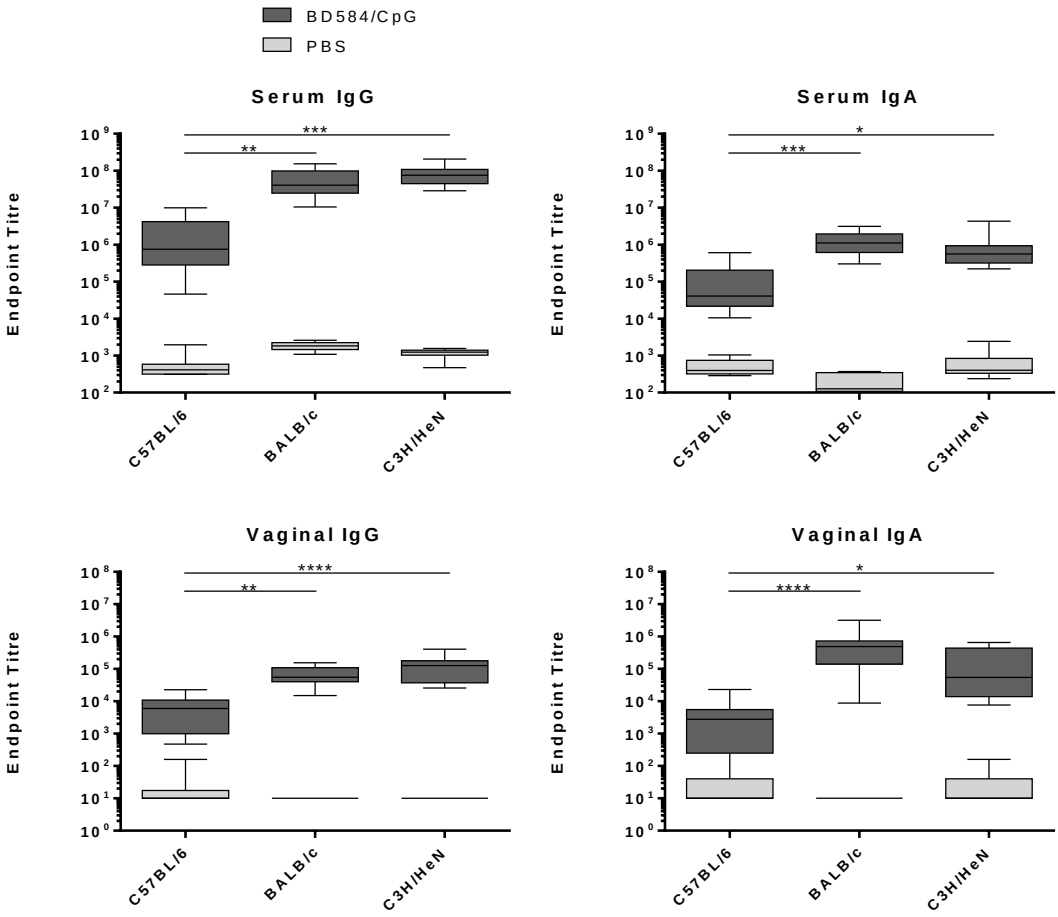


Figure 17. BD584-specific serum and vaginal IgG and IgA in mice following intranasal vaccination with BD584/CpG or PBS. C57BL/6, BALB/c, and C3H/HeN mice were intranasally vaccinated three times, three weeks apart, with either BD584/CpG or PBS (n = 10). One and two weeks after the final vaccination, vaginal washes and blood were collected, respectively, and BD584-specific antibody titers were determined using ELISA. The box indicates 25th and 75th percentiles, the horizontal line within a box indicates the median, and whiskers indicate min to max values. Antibody titers were compared between different strains of BD584/CpG vaccinated mice using one-way ANOVA followed by Dunn's multiple comparisons test.

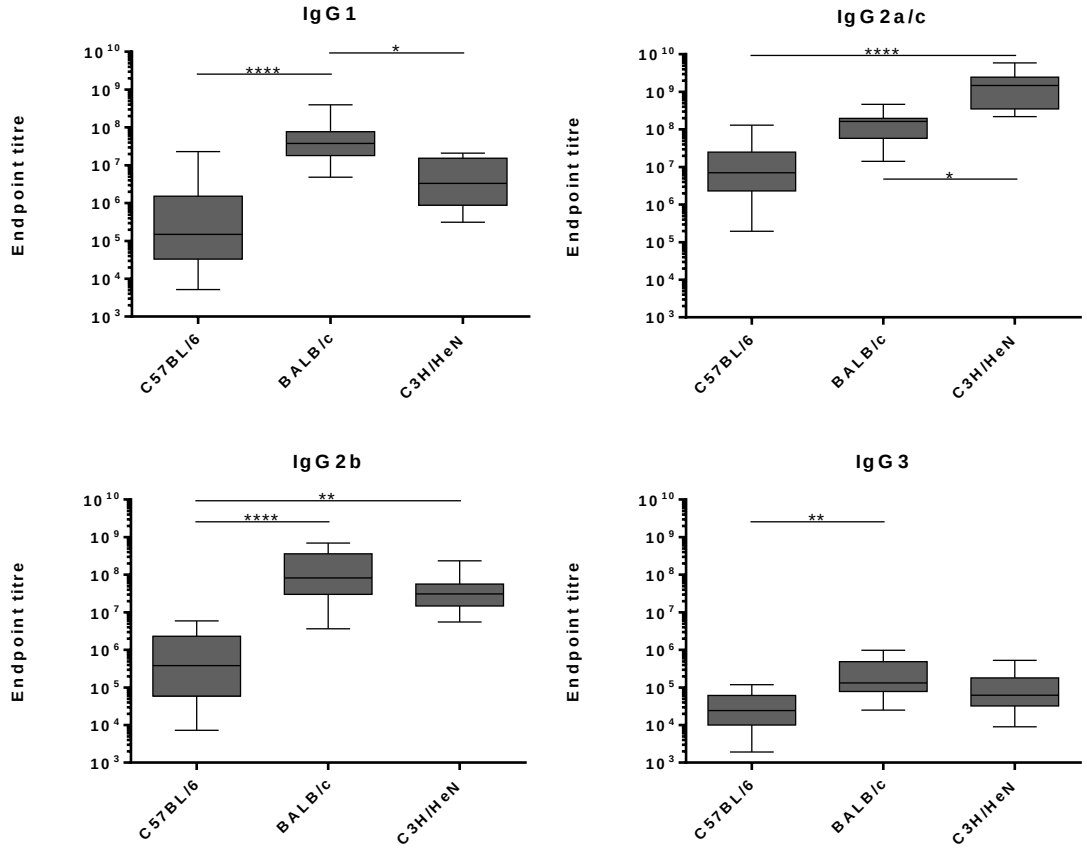


Figure 18. BD584-specific serum IgG isotypes in mice following intranasal vaccination with BD584/CpG. C57BL/6, BALB/c, and C3H/HeN mice were intranasally vaccinated three times, three weeks apart, with either BD584/CpG or PBS (n = 10). One and two weeks after the final vaccination, vaginal washes and blood were collected, respectively, and BD584-specific antibody titers were determined using ELISA. The box indicates 25th and 75th percentiles, the horizontal line within a box indicates the median, and whiskers indicate min to max values. Antibody titers were compared between different strains of BD584/CpG vaccinated mice using one-way ANOVA followed by Dunn's multiple comparisons test.

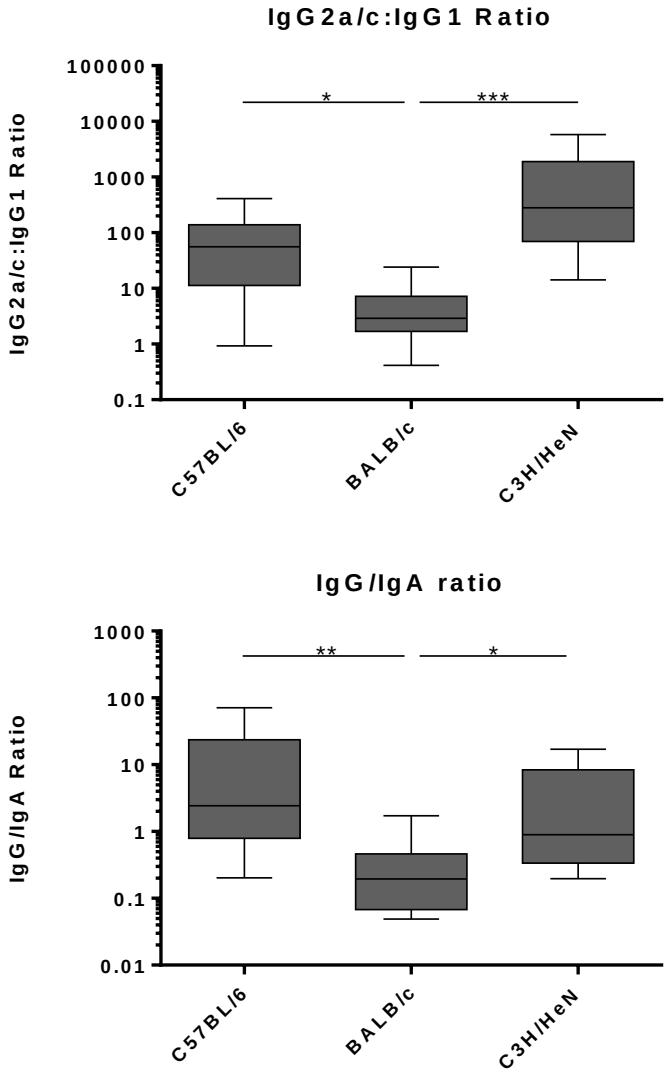


Figure 19. BD584-specific serum IgG2a/c to IgG1 ratio and vaginal IgG/IgA ratio in mice following intranasal vaccination with BD584/CpG. C57BL/6, BALB/c, and C3H/HeN mice were intranasally vaccinated three times, three weeks apart, with either BD584/CpG or PBS (n = 10). One and two weeks after the final vaccination, vaginal washes and blood were collected, respectively, and BD584-specific antibody titers were determined using ELISA. The box indicates 25th and 75th percentiles, the horizontal line within a box indicates the median, and whiskers indicate min to max values. Antibody titers were compared between different strains of BD584/CpG vaccinated mice using one-way ANOVA followed by Dunn's multiple comparisons test.

4.2.4 BD584/CpG immunization reduces bacterial shedding upon intravaginal *C. trachomatis* challenge

Next, we evaluated the ability of BD584/CpG immunization to protect against *C. trachomatis* infection as measured by reduction in bacterial shedding following intravaginal challenge. As shown in Figure 20, C57BL/6 mice immunized with BD584/CpG showed a nearly 100-fold reduction of *C. trachomatis* shedding on days 7 and 10 post challenge compared to the PBS group. Reduction in shedding was seen as early as day 3 post challenge, although the difference at this time point was not statistically significant. Compared to C57BL/6 mice, C3H/HeN mice immunized with BD584/CpG exhibited less reduction in shedding. BD584/CpG immunized C3H/HeN mice shed 4.7- and 1.9-fold less *C. trachomatis* on day 3 and day 7, respectively, compared to the PBS group. An area under the curve (AUC) analysis, which allows for a simultaneous comparison of both the magnitude and duration of infection, showed that BD584/CpG immunization reduced the overall bacterial load by 96% in C57BL/6 mice and 75% in C3H/HeN mice (Figure 21A and 21B). Interestingly, no statistically significant reduction in shedding was observed at any point for BALB/c mice. Overall, these results demonstrate that BD584/CpG vaccination reduces *C. trachomatis* shedding upon challenge in C57BL/6 and C3H/HeN mice but not in BALB/c mice.

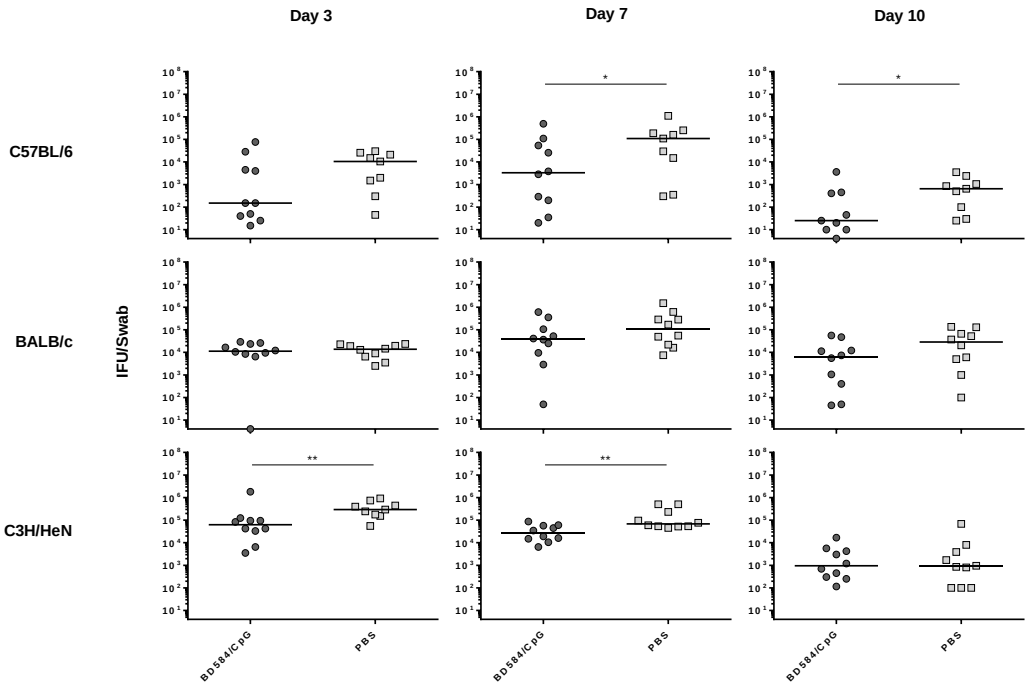


Figure 20. Protective efficacies against *C. trachomatis* genital tract infection in mice vaccinated with BD584/CpG. Three weeks following the final vaccination, mice were challenged intravaginally with 10^5 IFUs of *C. trachomatis*. Vaginal swabs were taken post challenge and *C. trachomatis* shedding was measured by culturing serial dilutions of swab samples on McCoy cells. Statistical difference was calculated using the Mann-Whitney test. Results for C57BL/6, BALB/c, and C3H/HeN mice are represented in the first, second, and third row, respectively. The bar represents the median IFU.

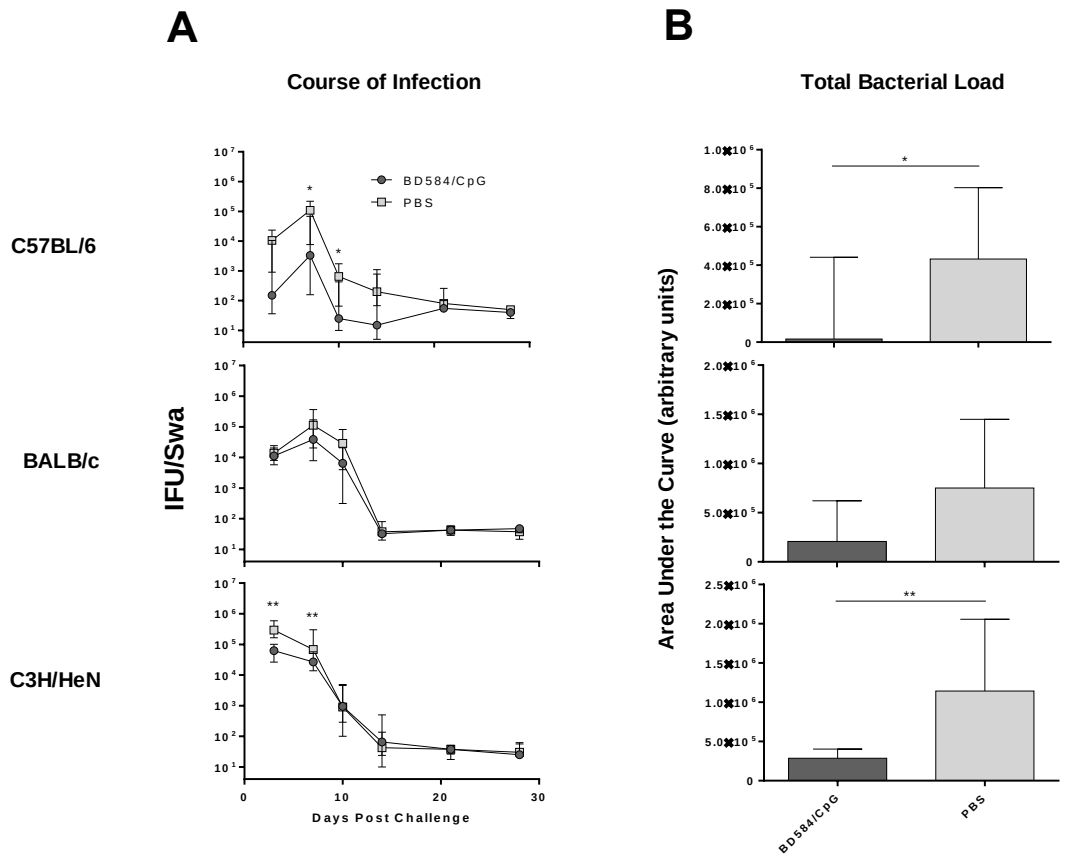


Figure 21. Duration of infection and total bacterial burden in mice vaccinated with BD584/CpG. Three weeks following the final vaccination, mice were challenged intravaginally with 10^5 IFUs of *C. trachomatis*. Vaginal swabs were taken post challenge and *C. trachomatis* shedding was measured by culturing serial dilutions of swab samples on McCoy cells. Statistical difference was calculated using the Mann-Whitney test. Results for C57BL/6, BALB/c, and C3H/HeN mice are represented in the first, second, and third row, respectively. **A.** Course of infection for each group. Individual points represent median IFUs (with interquartile ranges). **B.** Area under the clearance curves. Bars and whiskers represent medians and interquartile ranges, respectively.

4.3 Discussion

Progress towards an effective *Chlamydia* vaccine has been limited partly due to an incomplete understanding of *Chlamydia* immunobiology. Most of what we know about how the host responds to a *Chlamydia* infection comes from the murine intravaginal *C. muridarum* infection model, while the intravaginal *C. trachomatis* infection model in mice has been less utilized[113]. Since the primary goal of a human *Chlamydia* vaccine is to prevent the development of infection-induced pathology, particularly infertility[217], preference in the use of the *C. muridarum* model in certain studies is understandable and defendable as hydrosalpinx, a correlate of infertility, is consistently achieved in that model[116]. However, the *C. trachomatis* model mirrors many aspects of *C. trachomatis* infection in women that the *C. muridarum* model does not, such as the shedding of low numbers of infectious bacteria, minimal inflammation or signs of infection, highly variable pathological outcomes, high rates of reinfection, and the development of an appreciable level of protective immunity only after multiple infections[125,129]. Therefore, while neither model perfectly replicates human infection, both have their place in the study of *Chlamydia* immunobiology.

However, the host response to intravaginal *C. trachomatis* infection in mice has not been extensively characterized. Specifically, it is unknown whether mice with different genetic backgrounds develop cell-mediated adaptive immune responses with distinct cytokine profiles following clearance of infection. The first part of this Chapter has therefore been focused on the genetically determined differences in the type of

cytokines secreted by antigen-specific splenocytes from C57BL/6, BALB/c, and C3H/HeN mice after clearing an intravaginal *C. trachomatis* infection.

In agreement with previous findings using a *C. muridarum* pulmonary infection model, splenocytes from BALB/c mice produced significantly higher IL-17 upon clearance of an intravaginal infection with *C. trachomatis*[218]. While a Th17 response characterized by IL-17 secreting Th17 cells has been implicated in the development of pathology after genital infection with *C. muridarum*[88,89], and possibly *C. trachomatis*[219], Th17 cells also afford protection against infection in the lung model[84]. A novel finding here is that IL-4 production is significantly higher in splenocytes from C3H/HeN mice, suggesting the presence of a Th2 response. Interestingly, *Miguel et al.* showed that individuals infected with *C. trachomatis* predominantly display a Th2 response[80], which has been proposed by the group to have evolved to contain the extent of UGT damage that would otherwise lead to infertility. C3H/HeN mice may therefore represent a better model on which to mimic human genital *C. trachomatis* infection, a hypothesis that could warrant further investigation.

In the second part of this Chapter, we show that vaccination with a novel T3S fusion antigen, BD584, elicits strong cell-mediated and humoral immune responses in animals with different genetic backgrounds and, upon an intravaginal *C. trachomatis* challenge, reduces bacterial shedding in two out of three strains of mice.

To evaluate the ability of BD584 vaccination to confer protection against infection, we immunized three strains of mice with BD584 plus CpG before challenging them intravaginally with *C. trachomatis*. Compared to control mice, BD584/CpG

vaccinated C57BL/6 and C3H/HeN mice had reduced shedding at the peak of infection but similar clearance kinetics. Thus, while BD584/CpG vaccination appears to have no effect on the duration of infection, it can reduce the magnitude of infection, which has the potential to reduce the incidence of disease by limiting transmission of infections. Ideally, however, a vaccine would confer sterilizing immunity, but this is difficult to evaluate in the *C. trachomatis* mouse model since reliable infection requires the use of a high inoculum that could overwhelm an otherwise sterilizing immune response[113].

Previously, we showed that BD584 vaccination reduced *C. muridarum* infection and the associated UGT pathology[210]. In this study, we build on our previous results by showing that BD584 vaccination elicits robust cell-mediated and humoral immune responses and can confer protection against challenge with *C. trachomatis* – the relevant pathogen for a human vaccine. The use of mice with different genetic backgrounds that contribute to the variable outcomes in vaccine efficacy had allowed us to more realistically assess vaccination outcomes in a genetically heterogeneous human population. The disparity of efficacy outcomes and of immune responses in different strains of mice provided us with an opportunity to identify potential correlates of BD584-mediated protection, which is discussed below. Moreover, in our previous study, we measured bacterial load using quantitative PCR, which does not detect live organisms. In the current study, we show that BD584 vaccination reduced shedding of infectious *C. trachomatis*, as quantified by culture, thus providing stronger evidence for vaccine efficacy.

BD584 vaccination induced robust cell-mediated and humoral immune responses

in all three strains of mice, suggesting that the antigen should be immunogenic in a genetically heterogeneous population. Splenocytes from BD584/CpG immunized mice exhibited increased IFN-γ and IL-17 production with no detectable IL-4 production following in vitro BD584 stimulation, suggesting that a mixed Th1 and Th17 response was generated by vaccination. This was expected given that BD584 was delivered intranasally, a route that preferentially generates Th17 responses[220], with a Th1 polarizing adjuvant, CpG[221]. Interestingly, BD584/CpG immunized C57BL/6 mice had the lowest production of IFN-γ and IL-17 among the three strains of immunized mice, but the highest level of protection against *C. trachomatis* shedding, suggesting that BD584-mediated immunity may not be dependent on high IFN-γ and IL-17 production by splenocytes. Similarly, vaccinated C57BL/6 mice had significantly lower levels of BD584-specific IgG and IgA compared to vaccinated BALB/c and C3H/HeN mice, but exhibited the highest level of protection against shedding, suggesting that an increase in the magnitude of BD584-specific humoral responses, at least above a certain level, may not necessarily improve protection against infection. Instead, genetically determined differences in innate and adaptive immunity likely accounted for the difference in vaccine efficacy between the three strains. Increased susceptibility to Chlamydia infection in C3H/HeN mice, compared to C57BL/6 mice, may have masked the protection afforded by BD584/CpG vaccination[117].

However, it is unclear why BALB/c mice were not significantly protected by vaccination. Compared to vaccinated BALB/c mice, vaccinated C57BL/6 and C3H/HeN mice had a significantly higher BD584-specific IgG2a/c to IgG1 ratio, suggesting a more

Th1 polarized response. However, whether higher IgG2a/c directly enhances protection via Fc-dependent mechanisms[108,222], or is merely a surrogate marker for a protective Th1 response[223], or is associated with an unknown mechanism of protection, remains unknown. A high IgG2a/IgG1 ratio may also be functionally similar to a high IgG/IgA ratio; in both cases, the higher ratio may reflect a greater engagement with Fc-dependent effector functions. Importantly, an immune-correlates analysis of an HIV vaccine clinical trial found that higher antigen-specific IgG and lower antigen-specific IgA correlated with protection[224]. The authors suggested that IgA interfered with protective IgG effector functions. Interestingly, in the current study, we also found that the two strains of mice protected by BD584 vaccination exhibited higher BD584-specific IgG/IgA ratios than the unprotected strain (Figure 19). However, conclusive evidence for the role of specific antibody isotypes and subtypes will need to come from mechanistic studies, which are beyond the scope of the current study. Experiments are underway to better understand the mechanisms of BD584-mediated protection.

The ultimate goal of a *C. trachomatis* vaccine is to protect women against UGT pathology. BD584/CpG vaccination significantly reduced the degree of infection-induced uterine horn dilation in C57BL/6 mice 6 months post challenge, but uterine horn diameters were similar between vaccinated and unvaccinated BALB/c and C3H/HeN mice (Figure S6). However, there are limitations to these findings. While oviduct dilation (hydrosalpinx) represents a better measure of UGT pathology and infertility in humans[116], uterine horn dilation is a form of nontubal pathology that has not been shown to be a surrogate marker for tubal infertility[225]. Furthermore, *Chlamydia* immunopathogenesis is

likely different in humans[49], and use of other animal models such as the porcine or non-human primate models, which more closely mimic human reproductive anatomy and physiology, may provide stronger evidence for vaccine protection against UGT pathology[184].

There are strengths and limitations to our experimental design. One advantage of using the intravaginal *C. trachomatis* mouse model is that it mirrors many aspects of *C. trachomatis* infection in women – such as the shedding of low numbers of infectious bacteria, minimal inflammation or signs of infection, high rates of reinfection, and the development of an appreciable level of protective immunity only after multiple infections[125-129]. In addition, intravaginal inoculation of *C. trachomatis* represents the most natural and therefore physiologically relevant route of challenge. A limitation of using *C. trachomatis* is that reliable infection requires the use of a high inoculum that could overwhelm an otherwise protective vaccine-induced immune response. Furthermore, since oviduct dilation is rarely observed in mice after intravaginal *C. trachomatis* infection, we were unable to assess protection against human relevant pathology. However, these aspects of experimental design do not detract from the observed protective efficacy of BD584 vaccination. Rather, given the relatively high inoculum used, it is not unreasonable to suspect that a greater level of protection would be observed if a physiologically relevant dose was used.

In conclusion, we show that immunization with the BD584 fusion antigen consisting of three highly conserved T3S proteins is immunogenic in mice with different genetic backgrounds, elicits robust cell-mediated and humoral immune responses, and

confers protection against *C. trachomatis* infection in two out of three strains of mice tested, strengthening the rationale for exploring the use of T3S proteins in a human *C. trachomatis* vaccine.

Chapter 5 - Vaccination with BD584 Formulated with a Nanoemulsion Mucosal Adjuvant Reduces *C. trachomatis* Shedding in Mice.

5.1 Introduction

In Chapter 3, we showed that intranasal vaccination with a novel chimeric antigen, BD584, consisting of three proteins from the T3S system (CopB, CopD, and CT584) reduces chlamydial shedding and incidence of upper genital tract (UGT) pathology in a *C. muridarum* model of chlamydia genital tract infection[210]. In Chapter 4, we found that BD584 immunization is able to confer protection against a *C. trachomatis* challenge in mice (manuscript in preparation). However, the adjuvant used in both studies was CpG, which is a potent adjuvant but has safety concerns. To move BD584 down the vaccine pipeline, it should be tested with a mucosal adjuvant that is safe and effective in humans.

BlueWillow's vaccine adjuvant technology Nanovax™ (NE01) employs high-energy nanoemulsions that have several key attributes, including the ability to safely enable intranasal administration, generate a balanced cell-mediated immune response, and elicit strong systemic and mucosal immunity[226]. NE01 is an oil-in-water formulation of emulsified soybean oil mixed with surfactants and ethanol, with a size similar to many viruses that infect the respiratory tract[226]. The formulation does not contain any known TLR or other PRR ligands, but has been shown to increase antigen uptake and presentation by DCs, resulting in DC maturation[227,228]. Recently, NE01 has been shown to activate TLR2 and TLR4, and induce cell mediated responses in a MyD88-dependent way, and antibody responses in a MyD88-independent way[229].

Importantly, NE01 induces Th1 and Th17 responses without causing acute inflammation in the respiratory mucosa[226]. NE01 was shown to be safe as an intranasal

seasonal flu vaccine adjuvant in a phase I clinical trial[230]. In fact, it is the only mucosal adjuvant that has been demonstrated to be safe and effective in humans. In this Chapter, we present data on the immunogenicity and efficacy of BD584 adjuvanted with NE01 in a mouse model of *C. trachomatis* genital tract infection.

5.2 Results

5.2.1 Characterization of the BD584/NE01 nano-emulsion

To begin our investigation, we assessed the stability of the BD584/NE01 mixture with BD584 formulated in either PBS or NT buffer and stored at different temperatures (4°C and 25°C) for various durations (0, 3, 7, and 30 days). As shown in Figure 22, the BD584/NE01 mixture is stable at 4°C for 7 days in both PBS and NT buffer, but degrades after one month, as indicated by the increased amounts of lower molecular weight bands. At 25°C, the mixture begins to degrade as rapidly as 3 days, and by day 7, virtually no full length BD584 remains. These data suggest that the BD584/NE01 formulation is stable only for a short period of time and at low ambient temperatures.

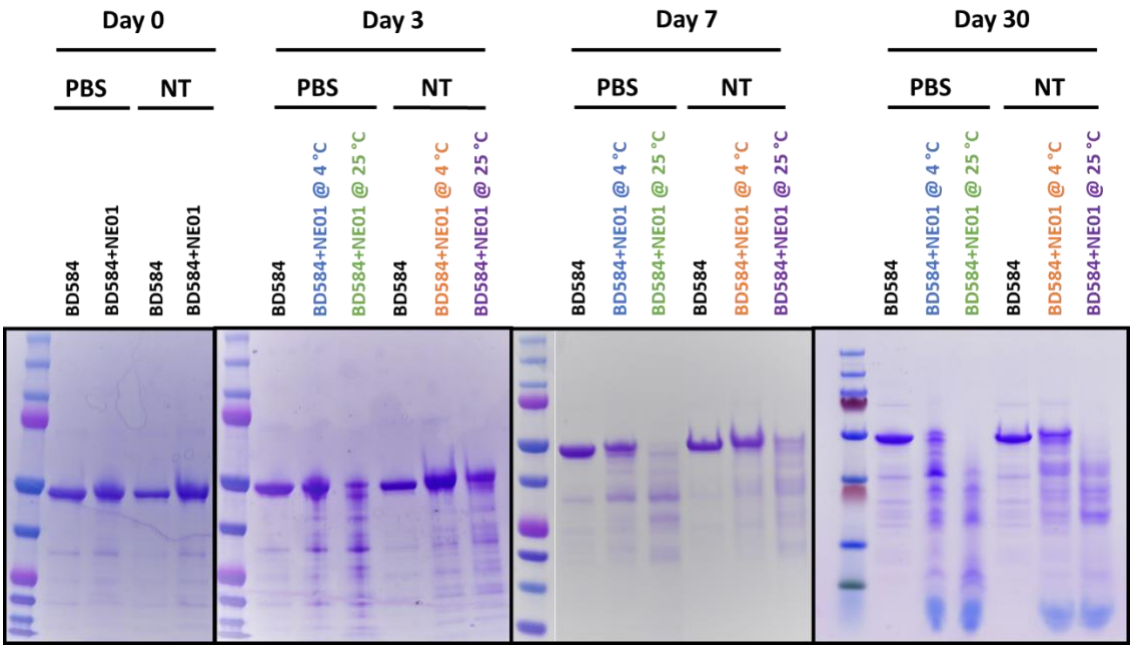


Figure 22. Stability of the BD584/NE01 mixture. BD584 and NE01 were mixed as described in Chapter 2. Stability of different mixtures were assessed at the indicated temperatures and times using SDS-PAGE and Coomassie staining.

5.2.2 Intranasal BD584/NE01 vaccination induced cell-mediated responses in lymph nodes draining the genital tract.

Cell-mediated immune responses are critical for anti-chlamydial immunity[48]. Since the iLNs drain the genital mucosa, the site of *C. trachomatis* infection in women, we assessed the cell-mediated immune responses in the iLNs following intranasal vaccination with BD584/NE01. Three weeks after the second vaccination, BD584-induced TNF-α, IL-5, and IL-17 responses, but not IFN-γ responses, were significantly higher in BD584/NE01 vaccinated mice (Figure 23). These results suggest that intranasal BD584/NE01 vaccination elicits cell-mediated immunity at distal lymph nodes that drain the genital tract and are characterized by predominantly Th2/Th17 responses.

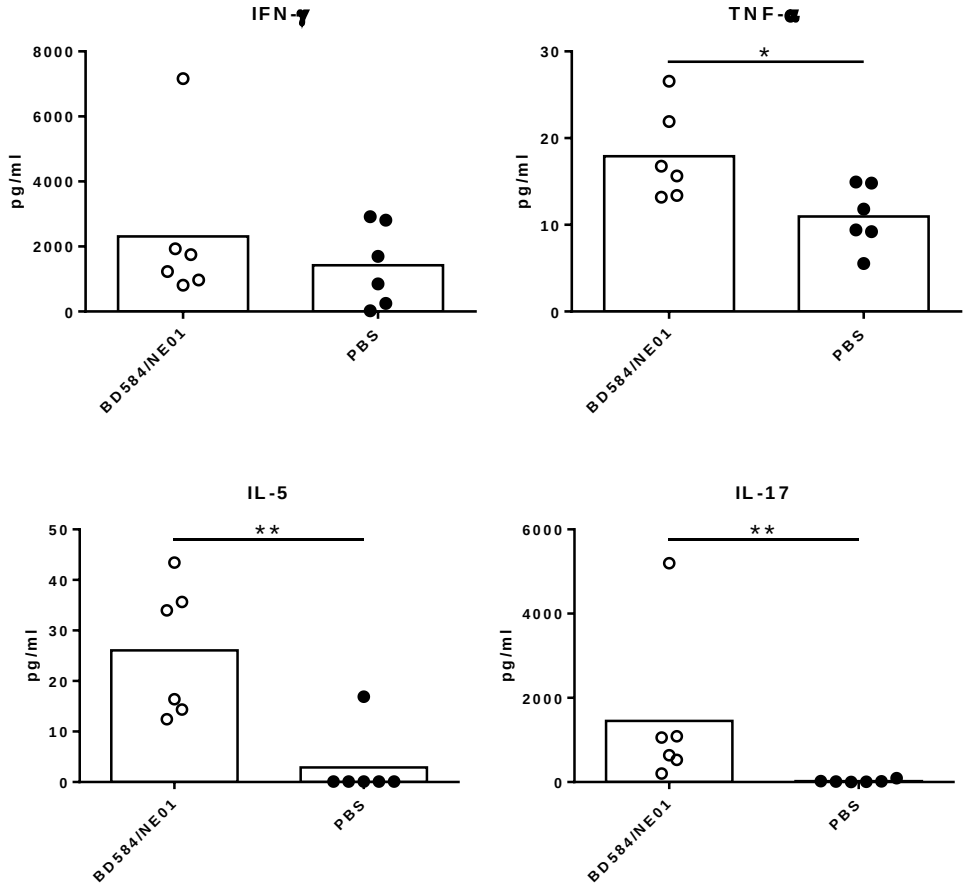


Figure 23. BD584/NE01 vaccination induces cell-mediated immune responses. Female C3H/HeN mice were immunized as described in Chapter 2, sacrificed, and cells from iLNs were stimulated with BD584 to quantify antigen-specific cytokine secretion. Bars indicate the median values and circles represent data from each individual mouse. Mann-Whitney test.

5.2.3 Intranasal BD584/NE01 vaccination induced strong systemic and mucosal antibody responses.

Next, we assessed antibody responses to intranasal BD584/NE01 vaccination. Three weeks after the second dose, BD584-specific serum total IgG and IgA titres were significantly higher in BD584/NE01 vaccinated animals (Figure 24). Interestingly, antibody subtyping revealed that vaccination elicited higher IgG1 responses than IgG2a responses, which is indicative of a Th2 polarized response. Furthermore, BD584/NE01 vaccination elicited robust mucosal humoral responses characterized by high levels of BD584-specific vaginal IgG and IgA (Figure 25). Together, these results suggest that intranasal BD584/NE01 vaccination generates strong systemic and mucosal antibody-mediated immune responses.

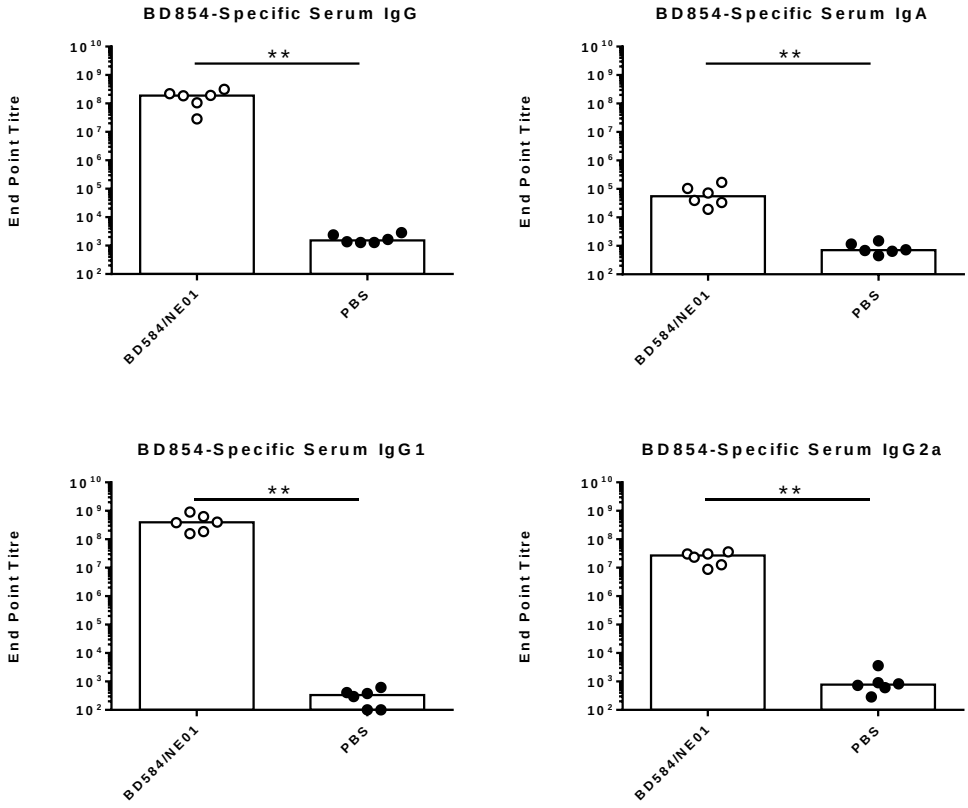


Figure 24. BD584/NE01 vaccination induces systemic antibody responses. Female C3H/HeN mice were immunized as described in Chapter 2 and serum was collected three weeks after the final vaccination. BD584-specific antibody titers were determined using ELISA. Bars indicate the median value and circles represent data from each individual mouse. Mann-Whitney test.

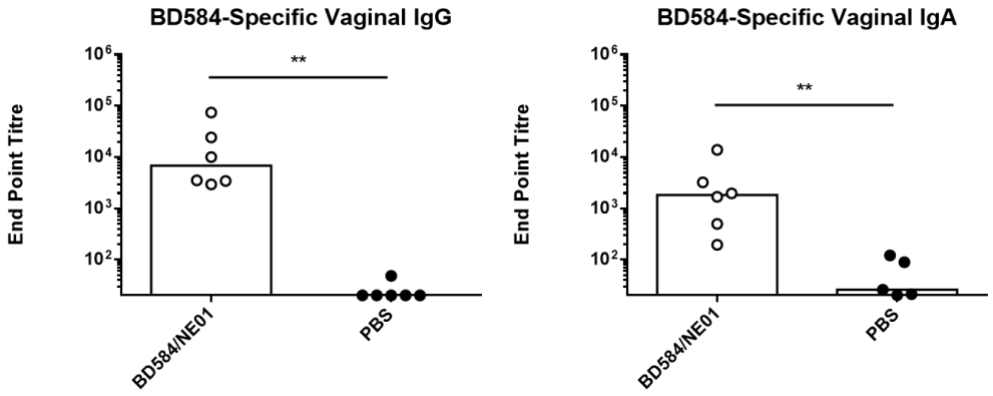


Figure 25. BD584/NE01 vaccination induces vaginal antibody responses. Female C3H/HeN mice were immunized as described in Chapter 2 and vaginal washes was collected three weeks after the final vaccination. BD584-specific antibody titers were determined using ELISA. Bars indicate the median value and circles represent data from each individual mouse. Mann-Whitney test.

5.2.4 Intranasal BD584/NE01 vaccination reduced bacterial shedding upon intravaginal challenge with *C. trachomatis*

To evaluate the ability of BD584/NE01 induced immunity to protect against a chlamydial challenge, we intravaginally infected mice three weeks after the second vaccination with 10^5 IFUs of *C. trachomatis* serovar D. On days three and seven following challenge, BD584/NE01 vaccinated and PBS vaccinated mice displayed similar levels of chlamydial shedding (Figure 26). However, on day 10 following challenge, BD584/NE01 vaccinated mice shed significantly less bacteria than PBS vaccinated mice. This suggests that intranasal BD584/NE01 vaccination reduces chlamydial shedding in mice, albeit only at the end of the course of infection and to a limited extent.

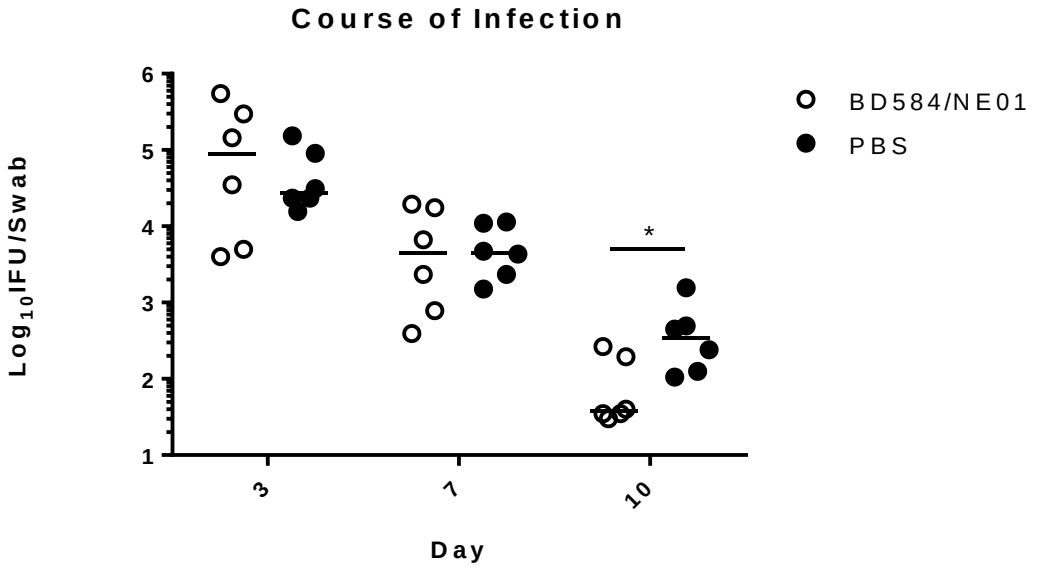


Figure 26. Efficacy of BD584/NE01 vaccination against *C. trachomatis* shedding. Female C3H/HeN mice were immunized and challenged with *C. trachomatis* as described in Chapter 2. Vaginal swabs were taken on days 3, 7, and 10 post challenge and *C. trachomatis* shedding was quantified by culturing serial dilutions of swab samples on McCoy cells. Horizontal lines represent median values of each group and circles represent data from each individual mouse. Mann-Whitney test.

5.3 Discussion

A vaccine, to be widely adopted, should be safe, effective, and practical[231]. Part of what contributes to the latter point is that a vaccine should be formulated in a way that is easily deliverable in the clinical setting in both developed and underdeveloped regions of the world. To this end, we set out to test the stability of the BD584/NE01 formulation at 4°C and 25°C. We found that the mixture is stable at 4°C but only up to 7 days, beyond which the antigen significantly degrades. The formulation is unstable at 25°C, as the antigen rapidly degrades 3 days after mixing. We are now planning to evaluate the storage of the antigen separately from the adjuvant to allow for bedside mixing.

An ideal chlamydia vaccine should generate strong cell-mediated and humoral immunity both systemically and at the site of infection[5]. Intranasal BD584/NE01 vaccination generated strong antigen-specific cell-mediated responses, characterized by Th2 and Th17 cytokines, at distal lymph nodes that drain the genital tract. However, IFN-γ responses were notably absent. This is interesting, since IFN-γ responses were induced when NE01 was combined with other antigens[227,228]. High levels of BD584-specific IgG and IgA were found in systemic and mucosal compartments. In line with the absence of IFN-γ responses, antigen specific IgG1 levels were significantly higher than IgG2a. Although a direct comparison between the efficacies of NE01 and CpG as mucosal adjuvants was not performed, a historical comparison to studies performed in Chapter 4 suggests that BD584 mixed with CpG may induce superior protection against *C. trachomatis* shedding. It may be argued that this provides further credence to the hypothesis that high BD584-specific IgG2a to IgG1 ratios may represent correlates of

BD584-mediated immunity. On the other hand, that BD584/NE01 is protective despite generating low antigen-specific IgG2a to IgG1 ratios may suggest that these isotype ratios represent BD584/CpG-mediated, but not BD584/NE01-mediated, correlates of protection. Further experiments are required to substantiate BD584-specific antibody isotypes as predictors of adjuvant-specific BD584-mediated efficacy.

Animals vaccinated with BD584/NE01 shed significantly less bacteria 10 days after challenge. This is promising as a proof of principle - we show for the first time that vaccination with a subunit antigen mixed with a clinically safe mucosal adjuvant can reduce *C. trachomatis* shedding in an animal model. On the other hand, the degree to which shedding was reduced in vaccinated mice may warrant a re-evaluation of the formulation or the animal model. A limitation of this study is that pathology could not be assessed given the inability of *C. trachomatis* to cause robust UGT pathology in mice[113]. Given that BD584/NE01 vaccination is protective against *C. trachomatis* infection in mice, we are moving back to the *C. muridarum* model to assess the extent to which vaccination with the formulation can protect against UGT pathology. Arguably, this is the more important question, as it relates to the ultimate goal of a chlamydia vaccine - to protect women against UGT disease[231].

Overall, we show that intranasal vaccination with BD584/NE01 elicits robust systemic and mucosal immune responses that reduced chlamydial shedding upon challenge with *C. trachomatis* in mice. To the best of our knowledge, this is the first instance where vaccination with a chlamydial antigen formulated with a mucosal adjuvant known to be safe and effective in clinical trials has elicited protective immunity

against *C. trachomatis*. This study provides sound rationale for further exploring the use of BD584 and NE01 in a chlamydia vaccine.

Chapter 6 – Conclusions

The goal of my PhD research was to evaluate the potential use of T3S proteins in a human chlamydia vaccine. Specifically, we aimed to assess the degree to which immunization with BD584, a chimeric protein consisting of *C. trachomatis* CopB, CopD, and CT584, conferred protective immunity against a *Chlamydia* challenge in a mouse model of chlamydial genital tract infection. We used both the *C. muridarum* and *C. trachomatis* intravaginal challenge models, multiple strains of mice, and two different mucosal adjuvants to test the BD584 antigen. **Taken together, the results presented in this thesis support our hypothesis that BD584 immunization induces robust systemic and mucosal immune responses and confers protection against *Chlamydia* infection and the associated upper genital tract pathology.**

6.1 The mouse model and measures of vaccine efficacy

As with all human diseases, genital chlamydia infection is imperfectly modeled. An additional challenge for studying chlamydia is that humans represent the only natural host for *C. trachomatis*. As such, researchers have modified the typical physiological context in which incident *C. trachomatis* infections occur in women. Alterations include the induction of diestrus using medroxyprogesterone, the use of high chlamydial inoculums to establish infection, and bypassing the cervix in order to inoculate organisms directly into the uterus[120,121]. They are necessary for chlamydia vaccine research and development efforts, but nonetheless limit translatability of animal data. Moreover, the outcome measures used in animal studies cannot be directly extrapolated to humans.

Hydrosalpinx is the best surrogate marker for chlamydia associated infertility in mice, but pathogenesis is likely different than the way in which the modeled human disease, PID, develops[48,49]; vaccine-mediated reduction in incidence or severity of hydrosalpinx may not translate to meaningful outcomes in humans.

The only common element between the model and the human disease is the pathogen itself. However, downstream from the point of inoculation, widely disparate mechanisms are responsible for pathogenesis between mice and humans, and perhaps even between individual humans. Therefore, I argue that the best measures of vaccine efficacy, in the absence of a sufficiently robust animal model, should probe the *distal* and *necessary* cause of disease - the presence of *C. trachomatis* in the genital tract. Absence of pathogen necessarily translates to absence of disease. However, using incidence of infection as a measure of vaccine efficacy would require a large number of animals to minimize the risk of false negative or false positive results, and any outcome would, to a significant degree, depend on the size of the inoculum. A more practical outcome measure would be a reduction in the number of organisms present in the genital tract. Although I have measured UGT changes in this thesis, they are only secondary outcome measures, and the primary outcome measure for BD584-mediated protection against chlamydia is reductions in chlamydial shedding.

6.2 Efficacy

The ultimate goal of a chlamydia vaccine is to reduce the disease burden associated with genital infections in women. Each year, there are approximately 68 million incidence infections in women globally. Given that about 10-15% of infections ascend to the fallopian tubes, of which about 10-15% lead to infertility[1], approximately 1 million new cases of infertility occur annually in the world. Therefore, an effective chlamydia vaccine has the potential to significantly reduce the health burden worldwide.

There are several potential ways for a chlamydia vaccine to do this. First, the vaccine could prevent infection by generating sterilizing immunity; second, it could prevent a lower genital tract infection from spreading to the upper genital tract, where pathogenesis occurs; third, it could control the nature of the immune response such that it does not lead to immunopathology; and lastly, it could reduce duration of infection[231]. Alternatively, a vaccine could also indirectly reduce disease burden by reducing the degree of bacterial shedding, thereby limiting risk of transmission.

We have shown that BD584 vaccination reduces incidence of hydrosalpinx, a strong and reliable correlate of infertility in the mouse model[116], by approximately one log. The mechanism by which this had occurred is unclear. We speculate that since the vaccine reduced vaginal bacterial load by approximately one log, the lower levels of bacteria in the lower genital tract may have translated to fewer numbers of bacteria in the UGT. Presumably the smaller number of bacteria would have prompted a more limited inflammatory response and more contained immunopathology. It is unlikely that the vaccine altered the quality of the immune response to the infection from a pathological

nature to a protective nature. Given the use of CpG and the intranasal route, a predominantly Th1 and Th17 response was generated, both of which are characterized by the production of inflammatory cytokines such as TNF-α and IL-17, which are strongly associated with chlamydial pathogenesis[88,89,91].

Although it was not directly measured, BD584 vaccination has the potential to significantly reduce transmission of genital chlamydial infections. Given that a minimum number of chlamydial organisms are required to establish infection[232], a one-log reduction in the bacterial load may translate to reduced risk of transmission. From a public health perspective, reduced transmission is a meaningful goal for a chlamydia vaccine.

6.3 Immune correlates of BD584-mediated protection

Ideally, all those at risk of being infected would receive the vaccine. Realistically, only a portion of the population, likely those who are candidates for the HPV vaccine, would be vaccinated. Even if everyone who should be vaccinated is vaccinated, not all of them will develop immunity. This disparity is of course partly attributable to biological variability. In this thesis, we have shown that part of this variability is attributable to host genetics. We showed that the genetic background of mice played a large role in determining whether, and the degree to which, vaccination conferred protection against infection. Given these observations, a few questions naturally follow. What are the

genetic determinants of vaccine mediated protection? What is the mechanism of vaccine mediated protection? What are the immune correlates of vaccine mediated protection?

The answers to all of these questions have implications for chlamydia vaccinology. But when moving a vaccine from preclinical to clinical testing, which underlies our current research efforts, perhaps the most relevant question is the last one. How can we know, with a reasonable level of confidence, whether a vaccine will work without directly measuring efficacy (the ethics and logistics of which pose significant challenges)?

The available evidence strongly suggests that antigen-specific IFN-γ-secreting CD4+ T cells represent a correlate of immunity against *Chlamydia*[48]. But it is important to note that most of this evidence come from studies utilizing natural infection as the means of generating *Chlamydia* specific immunity. Since a human chlamydia vaccine is not yet available, identification of immune correlates of protection in human studies so far have also all been in the context of natural immunity[129]. In animal studies, much of the data supporting IFN-γ-secreting CD4+ T cells as immune correlates have come from studies investigating immunity generated from a natural *C. muridarum* infection[12,233]. Therefore, it is likely that the available evidence is biased towards a marker of infection-induced immunity, and markers of vaccine-induced immunity may have been overlooked. To be clear - I am not disputing that IFN-γ-secreting CD4+ T cells is *a* correlate of protection, I am suggesting that it may not be the *only* one. Furthermore, it is likely that the correlate of protection depends on the antigen used. For instance, antibodies against an intracellular protein would have no functional consequence, and would be unlikely to

serve as a correlate of protection for that antigen. On the other hand, polysaccharide or lipid antigens would not be loaded onto MHC molecules; CD4+ T cell responses towards these antigens would not be generated, and therefore would not be a correlate of immunity.

In this thesis, we have identified BD584-specific IgG2a/IgG1 ratio, and BD584-specific IgG/IgA ratio, as correlates of BD584-mediated protection against *C. trachomatis* infection. Compared to IgG1, IgG2a has greater effector functions including complement-dependent cytotoxicity[234]. Similarly, IgG has greater effector functions than IgA. Assuming similar affinity, the greater avidity of IgG2a and IgG over IgG1 and IgA in binding to components of BD584 on the surface of *Chlamydia* could have led to greater bacterial killing. This is not the first time competitive binding between antibody subtypes and isotypes have been implicated as immune correlates of protection. In an immune-correlates analysis of an HIV vaccine trial, it was found that higher antigen-specific IgG and lower antigen-specific IgA were associated with protection[224]. The authors suggested that IgA may have interfered with protective IgG effector functions. If high BD584-specific IgG2a/IgG1 or IgG/IgA ratio as an immune correlate of protection is translatable into humans, it would provide an efficient way to assess the potential of BD584 vaccination prior to efficacy studies.

6.4 Formulation with a novel mucosal adjuvant

Ideally, a chlamydia vaccine would be administered at a mucosal surface. Delivery through the nasal route reliably generates strong immune responses at distal mucosal sites including the genital tract. However, there are currently no mucosal adjuvants licensed for use in humans. BlueWillow's Nanovax™ NE01 has been shown to be safe and effective in humans, and represent a strong candidate as a mucosal adjuvant for a chlamydia vaccine. We have shown that BD584 administered with NE01 induces systemic and mucosal immune responses with strong cell mediated responses at lymph nodes draining the genital tract and robust humoral responses in the genital tract itself. Vaccination with the BD584/NE01 formulation significantly reduced *C. trachomatis* shedding upon intravaginal challenge. Importantly, this study represents a proof of principle that a mucosal adjuvant demonstrated to be safe and effective in humans can be applied to a chlamydia vaccine. We are continuing to work with BlueWillow towards a mucosal subunit vaccine against chlamydia.

6.5 Immune-subdominant proteins as vaccine antigens

This thesis highlights the idea that proteins that are immune subdominant during natural infection can be effective as vaccines. Typically, the choice of antigen for a subunit vaccine has relied upon studies that identify immunodominant antigens during infection. Part of the idea is that since we are trying to mimic the immunity generated by

a natural infection, we should look at which microbial proteins our immune system predominantly responds to during infection. However, while this strategy can work for pathogens against which natural immunity is effective, it is not clear that this should be the approach for pathogens that generate suboptimal immunity.

Individuals can and often do have multiple chlamydia infections in their lifetime, suggesting that primary infection does not elicit protective memory responses[129]. In animal models, it has been experimentally demonstrated that complete immunity against *C. trachomatis* requires multiple infections[235]. These observations suggest that natural immunity against chlamydia is suboptimal, which gives credence to the idea that a chlamydia vaccine should generate "unnatural" immunity[187]. To this end, in addition to researching immunodominant antigens, it may be wise to test antigens that the immune system does not typically respond to during a natural infection, as it may be a strategy for chlamydia to divert immune responses towards unprotective antigens[188]. Results from this thesis demonstrate that immune subdominant antigens, including CopB, CopD, and CT584, are highly efficacious against chlamydia, decreasing bacterial load at the peak of shedding by up to 100-fold. Worth noting is that these proteins make up an essential virulence factor, the disruption of which has been shown to significantly reduce infection rates[31,32,201]. This highlights the need to study pathogen-host interactions to identify vaccine targets.

6.6 Next steps

The work reported here addresses some important questions about chlamydia vaccinology but at the same time reveals a few avenues for further inquiry. First, it would be important to identify the specific component(s) of BD584 that contribute(s) to protection. This could be achieved by immunizing with CopB, CopD, CT584, or a combination thereof, and challenging animals with *C. trachomatis*. Analysis of antibody responses using overlapping BD584 epitopes could identify immunogenic regions within BD584 that potentially contribute to humoral immunity. BD584 sequences that contribute to cell-mediated immunity could be identified using similar strategies. The benefit of identifying the sequences that are and are not needed for protection would allow us to optimize the design and production of the vaccine, for instance, by designing an antigen containing repeats of protective epitopes to enhance immunogenicity.

Second, it would be useful to know which component(s) of BD584-specific adaptive immunity contribute(s) to protection. B-cell deficient mice, T-cell depletion, and adoptive transfer of immune serum and/or T cell populations could be used to identify components of adaptive immunity that are necessary and/or sufficient for BD584-mediated protection. Furthermore, the notion that high BD584-specific IgG:IgA or IgG2a:IgG1 ratio may contribute to BD584-mediated anti-chlamydial immunity should be tested. This would add to the existing body of evidence on the components of adaptive immunity that contribute to anti-chlamydial immunity and could provide support to the hypothesis that mechanisms of vaccine-mediated immunity may be different from mechanisms of infection-generated immunity. Efforts on vaccine delivery strategies and

adjuvant design may benefit from being no longer limited to the generation of IFN-γ secreting CD4+ T cells as the only end goal.

In parallel with the above aims, our ultimate goal is to move our vaccine into clinical trials. To this end, we are continuing our work with BlueWillow to further investigate the use of BD584 and Nanovax^{TM} formulations and planning a collaboration with investigators who are using the pig model to test chlamydia vaccines.

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Appendix

1. Detailed Methods Used to Obtain Novel Materials Used in Thesis

1.1 Purification of BD584, CopB, CopD, and CT584

Equipment:

Akta HPLC

Sonicator

Centrifuge

Buffers:

NiA

20 mM Tris-HCl pH 7.4 (4°C)

500 mM KCl

0.03% LDAO

0.02% BME (add fresh every time)

10 mM imidazole

10% glycerol

NiB

20 mM Tris-HCl pH 7.4 (4°C)

500 mM KCl

0.03% LDAO

0.02% BME (add fresh every time)

300 mM imidazole

10% glycerol

PBS

Procedure:

1. Expression.
    1. Set up 125 ml of overnight culture in LB + 100 µg/ml ampicillin from glycerol stock. Autoclave 3L of 2X LB and keep warm overnight.
    2. Transfer 20 ml of overnight culture into each of the 6 L cultures (made from 3L of 2X LB). Add ampicillin to working concentration of 100 µg/ml.
    3. Grow until the culture reaches an OD600 of ~0.6 (~2.5 h). Induce with 0.2 mM IPTG for 3 hours at 37 ˚C.
2. Protein extraction. Keep cold at all times from this point on.
    1. Pellet bacteria at 8,000 g for 5 min, remove supernatant.

2. Resuspend bacterial pellet in 25 ml NiA with 2 EDTA-free protease inhibitor tablets (Thermo-Fisher).
3. Sonicate at setting 5, 2.5 min.
4. Pellet insoluble fractions at 42,000 g for 45 min.
5. Filter the supernatant through a 0.2 µm filter and transfer filtered lysate to superloop.

3. Purification.
    1. Prepare HisTrap column by stripping with 15 ml 0.05 M EDTA pH 8, washing with 15 ml H2O, and charging with 15 ml 100 mM NiCl.
    2. Equilibrate column by running at least 5 ml of NiA through it on the FPLC.
    3. Once UV levels off, inject lysate.
    4. After binding the lysate to the His column, load again with NiA until UV levels off.
    5. Wash with 5% NiB.
    6. Wash with 10% NiB.
    7. Wash with 15% NiB.
    8. Elute with 100% NiB.
    9. Transfer eluate back to superloop.
4. Buffer exchange.
    1. Load at least 60 ml of PBS onto HiTrap desalting column 26/10
    2. Inject purified protein into the column

3. After injection completes, load PBS again.
4. Collect eluate, beginning at ~12 ml after beginning injection.
    1. Collect until UV levels off or conductance rises.

5. Storage.
    1. Filter sterilize purified protein with 0.2 µm filter.
    2. Concentrate down to less than 1 ml using Millipore by centrifuging at 3,000 RPM at 4 ˚C. (~40 min)
    3. Pipette up and down to homogenize concentrated solution and transfer solution to a 50 ml falcon tube.
    4. Pellet any precipitates by centrifuging the tube at 3,000 RPM (benchtop centrifuge) for 5 min at 4 ˚C.
    5. Measure protein concentration in the supernatant via Bradford Assay, and aliquot supernatant for storage at -80 ˚C.
6. Assess purity.
    1. Run an SDS-PAGE gel, coomassie, and western blot to assess purity and confirm purification of His-BD584.

1.2 Purification of *C. muridarum* and *C. trachomatis* rMOMP

Materials:

Qiagen Superflow Ni-NTA beads

Lysis Buffer:

100 mM NaH_2PO_4

10 mM TrisCl

6 M GuHCl

pH 8

Wash Buffer 1:

100 mM NaH_2PO_4

10 mM TrisCl

8 M Urea

0.1% Triton X-114

pH 7.5

Wash Buffer 2:

100 mM NaH_2PO_4

10 mM TrisCl

8 M Urea

pH 6.3

Elution Buffer:

100 mM NaH_2PO_4

10 mM TrisCl

8 M Urea

500 mM imidazole

pH 7.4

Refolding Buffer:

1X PBS

Procedure:

1. Set up 45 ml O/N of pET30a-CmrMOMP or pET32a-CtrMOMP in BL21 (DE3) with 50 µg/ml Kanamycin or 100 µg/ml ampicillin
2. Next day, inocluate 40 ml of O/N culture in 2 L of autoclaved LB supplemented with the appropriate antibiotic

3. Grow until OD600 reaches 0.4-0.8 then induce with 0.1 mM IPTG 4h or O/N at 30˚C
4. After 4 hours, or the next day, spin down bacteria at 8000 g for 5 min and resuspend in 25 ml of Lysis Buffer and sonicate for 2 min on setting 5
5. Spin down lysate at 19000 g for 45 min and incubate supernatant with 2 ml of Ni-NTA beads* for 1 hour at RT nutating
    1. *To prepare beads:
        1. Take 4 ml of bead solution and spin down to get 2 ml of beads
        2. Strip with 2x with 1 column volume of 0.05 M Na_2EDTA
        3. Wash 2x with 1 column volume of ddH_2O
        4. Charge 2x with 1 column volume of 100 mM Ni_2Cl
        5. Equilibrate 2x with 1 column volume of Lysis Buffer
6. Spin down beads at 2700 RPM (tabletop), remove supernatant and incubate with 10 ml WB1 for 15 min
7. Spin down beads at 2700 RPM, remove supernatant and incubate with with 10 ml WB2 for 15 min
8. Spin down beads at 2700 RPM, remove supernatant and incubate with 10 ml Elution Buffer overnight
9. Spin down beads at 2700 RPM, take supernatant and buffer exchange into PBS at 4˚C.
10. Aliquot and store at -80˚C.

1.4 Gene Constructs

gBLOCK: CopB$_{1-100}$ (gene fragment coding for the first 100 amino acids of CopB)

GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGATTACGATATCCCAACGACC

GAAAACCTGTATTTTCAGGGCATGAGCCTGAGCAGTTCTTCCTCATCGGATAG

TAGCAACCTGAAAAACGTGCTGAGCCAGGTGATTGCGAGCACCCCGCAGGGC

GTGCCGAACGCGGATAAACTGACCGATAACCAGGTGAAACAGGTGCAGCAG

ACCCGCCAGAACCGCGATGATCTGAGCATGGAAAGCGATGTGGCGGTGGCG

GGCACCGCGGGCAAAGATCGCGCGGCGAGCGCGAGCCAGATTGAAGGCCAG

GAACTGATTGAACAGCAGGGCCTGGCGGCGGGCAAAGAAACCGCGAGCGCG

GATGCGACCAGCTAAGACCCAGCTTTCTTGTACAAAGTGGTCCCC

Purple: CopB$_{1-100}$

Green: Gateway att sites

Red: Stop codon

gBLOCK: CopD$_{1-100}$ (gene fragment coding for the first 100 amino acids of CopD)

GGGGACAAGTTTGTACAAAAAAGCAGGCTTAGATTACGATATCCCAACGACC

GAAAACCTGTATTTTCAGGGCATGACCACCGGCGTGCGCGGCGATAACGCGC

CGGATCCGAGCCTGCTGGCGCAGCTGACCCAGAACGCGAACAGCGCGAGCG

CGGCGAGCACCGGCAAAAACGGCCAGGTGGCGGGCGCGAAACAGGAAAACG

TGGATGCGAGCTTTGAAGATCTGCTGCAGGATGCGCAGGGCACCGGCGGCAG

CAAAAAAGCGACCGCGAACCAGACCAGCAAAAGCGGCAAAAGCGAAAAAG

CGCAGGCGAGCAGCGGCACCAGCACCACCACCAGCGTGGCGCAGGCGAGCC

AGACCGCGACCGCGTAAGACCCAGCTTTCTTGTACAAAGTGGTCCCC

Purple: CopD$_{1\text{-}100}$

Green: Gateway att sites

Red: Stop codon

2. Supplementary Figures

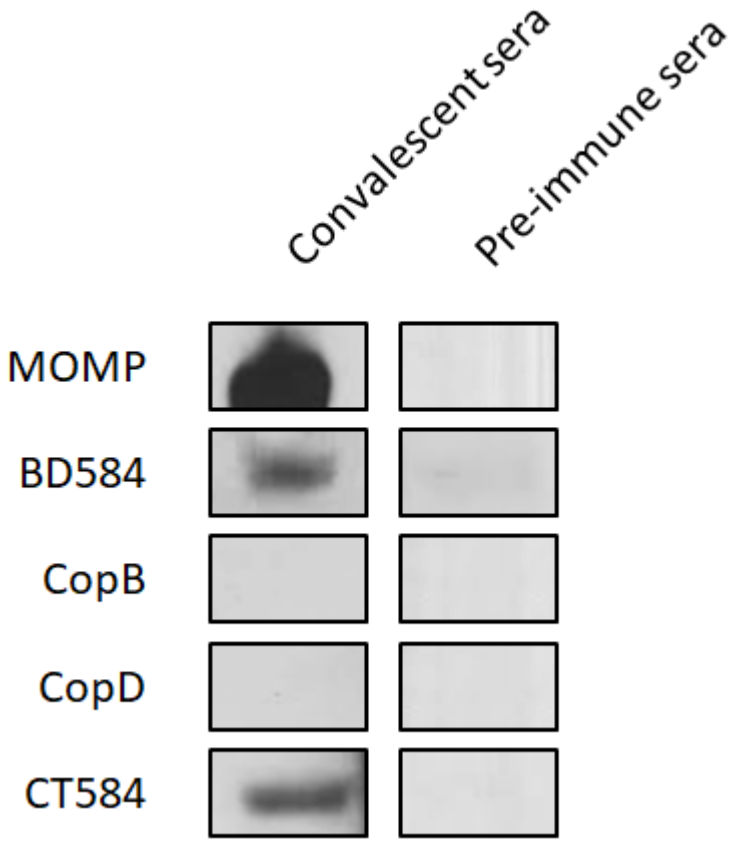

Figure S1. Antibodies elicited by MOMP and T3S components following a natural genital *C. muridarum* infection. Using Western blot, purified chlamydial antigens were probed with sera from mice that have resolved a *C. muridarum* infection or with pre-immune sera.

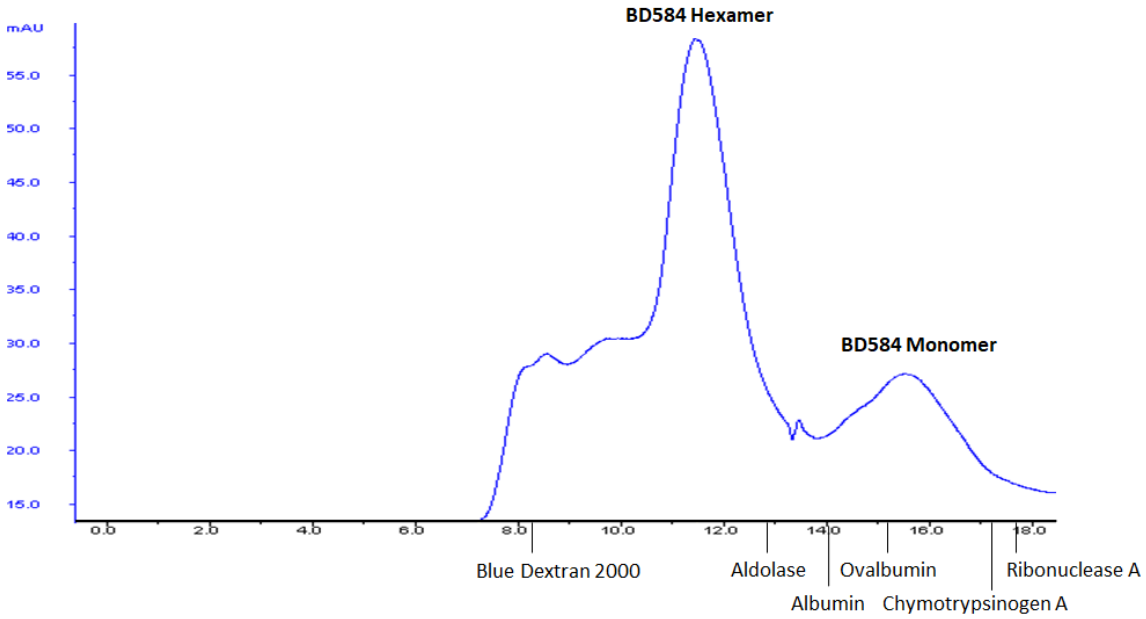

Figure S2. Size exclusion chromatography of BD584. BD584 was expressed, purified, and subject to size exclusion chromatography as described in Chapter 2. mAU, milli-absorbance units.

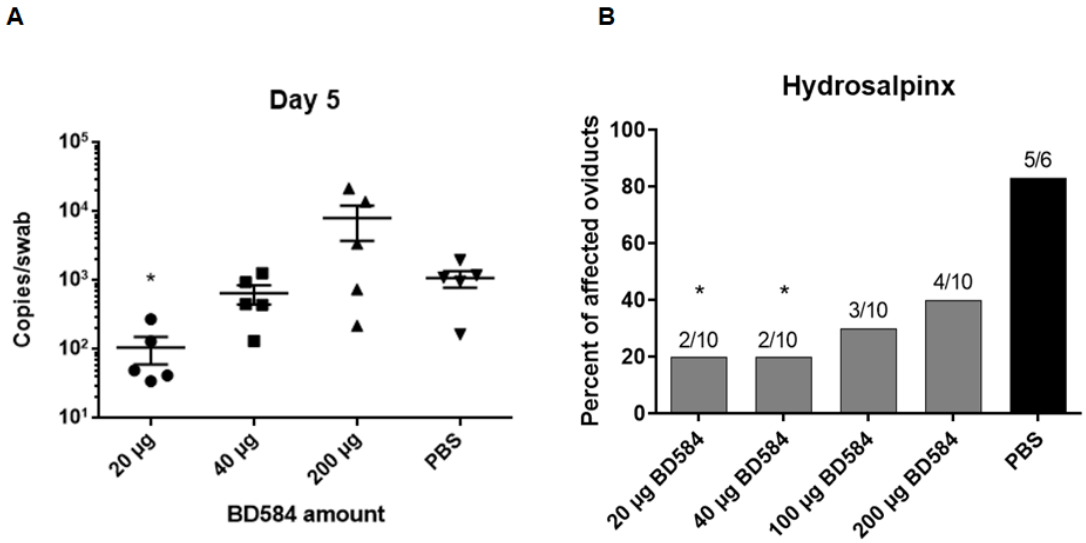

Figure S3. *C. muridarum* shedding and oviduct pathology in mice vaccinated with different doses of BD584 formulated with CpG. Three weeks following the final vaccination, mice were challenged intravaginally with 10^5 IFUs of *C. muridarum*. A. Vaginal swabs were taken on day 5 post challenge and *C. muridarum* shedding was measured by culturing serial dilutions of swab samples on McCoy cells. Mann-Whitney test compared to PBS. B. Mice were sacrificed 35 days after intravaginal challenge with *C. muridarum*, and upper genital tracts harvested and analyzed for the presence of hydrosalpinx. Fisher's exact test compared to PBS.

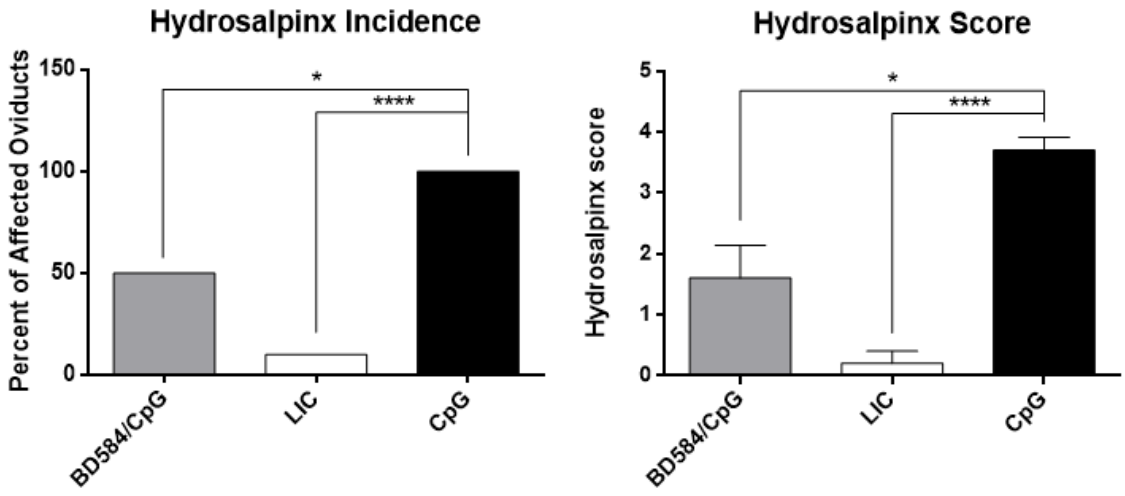

Figure S4. Protection against *C. muridarum*-induced oviduct changes in mice intranasally vaccinated with BD584/CpG, 10^3 IFUs of live *C. muridarum* (LIC), or CpG. Mice were sacrificed 35 days after intravaginal challenge with *C. muridarum*, and upper genital tracts harvested and analyzed for the incidence and severity of hydrosalpinx using a pathology grading scores as described in Chapter 2. The incidence and severity of hydrosalpinx between groups were compared using Fisher's exact test and one-way ANOVA with Dunn's multiple comparisons test, respectively. LIC, live infection control (respiratory *C. muridarum* infection).

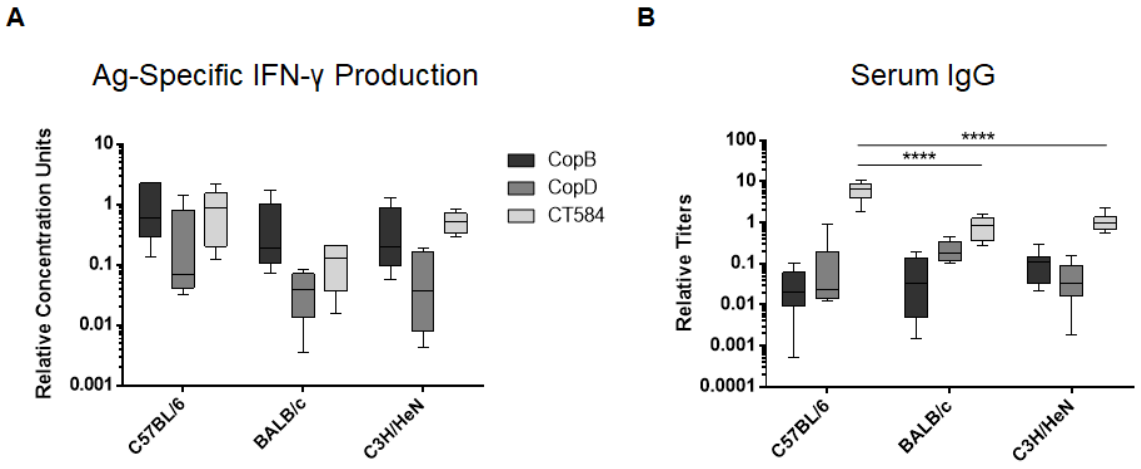

Figure S5. CopB-, CopD-, and CT584-specific cell-mediated and humoral responses following intranasal vaccination with BD584. A. Mice (n=5) were intranasally vaccinated with BD584/CpG. Seven days later, animals were euthanized, and splenocytes were harvested and stimulated with CopB, CopD, CT584, or BD584 for 96 hours. IFN-γ in culture supernatants were quantified by ELISA. **B.** Mice (n = 10) were intranasally vaccinated three times, three weeks apart, with either BD584/CpG or PBS. Two weeks after the final vaccination, blood was collected and antigen-specific antibody titers were determined using ELISA. Antigen specific cytokine and antibody responses were normalized against those specific to BD584. The box indicates 25th and 75th percentiles, the horizontal line within a box indicates the median, and whiskers indicate min to max values. Cytokines and antibody titers were compared between different strains of BD584/CpG vaccinated mice using one-way ANOVA followed by Dunn's multiple comparisons test.

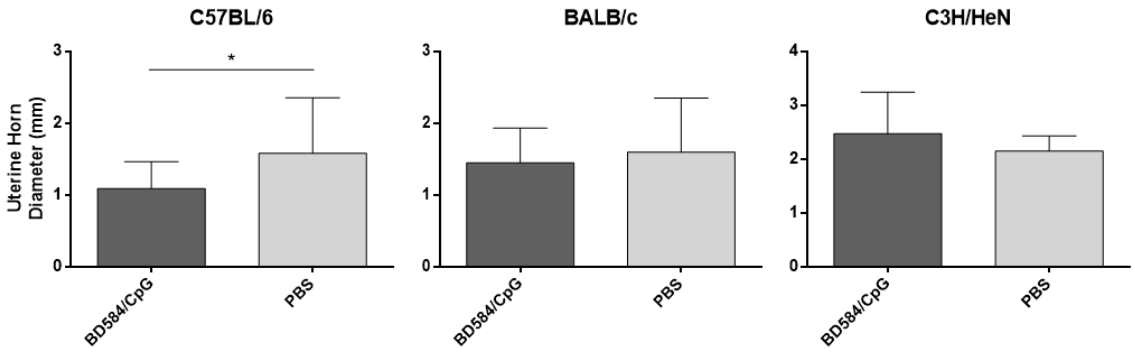

Figure S6. Protection against *C. trachomatis*-induced UGT changes in mice vaccinated with BD584/CpG. Six months after *C. trachomatis* challenge, mice were euthanized, UGTs were collected, and cross-sectional diameter of each uterine horn was measured. Bars and whiskers represent means and SDs, respectively. Statistical difference was calculated using Student's *t* test.

3. Published Journal Articles